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SUPPLEMENT ARTICLE

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Retention, stability, iron bioavailability and sensory evaluation of extruded rice fortified with iron, folic acid and vitamin B_{12}

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Abstract

Fortification of rice with micronutrients using extrusion technology is considered a sustainable strategy to prevent nutritional deficiencies in general population. The objective of the present study is to assess the retention, stability and iron bioavailability from indigenously developed triple fortified rice (iron, folic acid and vitamin B_{12}) during rinsing and different cooking methods. Further, we also assessed the acceptability of fortified rice in adult human volunteers. The retention of iron during rinsing with excess water was ≥90%, whereas folic acid and vitamin B_{12} levels were reduced by ~25% during rinsing. Watertight cooking of rice (in electric cooker or on flame) had no additional effect on the nutrient levels as compared with rinsed rice, implying their stability during cooking. However, cooking with excess water followed by decanting led to loss of 45% iron and ≥70% folic acid and vitamin B_{12}. The dialyzable iron and ferritin synthesis in Caco-2 cells was significantly ($P < .01$) higher from fortified rice compared with unfortified rice. In addition, inclusion of ascorbic acid significantly ($P < .01$) increased the iron bioavailability from the fortified rice. Triangle tests in adult human subjects revealed that there are no significant sensory differences among fortified and unfortified rice. Further, fortified rice consumption appears to bridge the gaps in dietary iron intake deficits in children and women of reproductive age. These results suggest that the iron-, folic acid- and vitamin B_{12}-fortified rice has higher retention and stability of fortified nutrients and is acceptable for consumption in adult human volunteers.

KEYWORDS

bioavailability, folic acid, fortified rice, iron, sensory properties, vitamin B_{12}

1 | INTRODUCTION

Anaemia is a serious public health concern among the developing countries, including India. Pregnant, lactating women and children are particularly vulnerable groups due to their higher nutrient demand. According to the National Family Health Survey (NFHS, 2016), 70% of children (6–59 months), 55% of women (15–49 years) and 24% of men (15–49 years) are anaemic in India. Although aetiology of anaemia could be multi-factorial, poor density and bioavailability of iron from staple food crops are reported to be a major contributing factor (Nair & Iyengar, 2009). Further, studies conducted in preschool/school children and pregnant women indicated very high prevalence of

anaemia with concurrent iron deficiency and deficiencies of other micronutrients such as folic acid, vitamin B_{12} and vitamin A (Laxmaiah et al., 2012; Nair et al., 2016; Sivakumar et al., 2006). A recent study among urban adults also found suboptimal intakes of micronutrients and high prevalence of subclinical deficiencies among apparently healthy urban adults (Shalini et al., 2018). Although targeted therapeutic supplementation is being practised, owing to its poor compliance, food fortification is considered an alternate long-term food-based strategy to prevent and control micronutrient deficiencies at population level (Hurrell, 2002; WHO, 2006).

Fortification is often more cost-effective than other strategies, especially if the technology and distribution systems already exist. Fortification of staple foods, such as wheat flour with one or more micronutrients, is now a widely accepted strategy and is adopted in many countries (Allen, 2006). Nevertheless, rice is a staple food for more than 3 billion people worldwide, particularly among southern and eastern parts of peninsular India (Muthayya et al., 2012). In general, rice is milled, polished and consumed as whole grain and thus is not readily amenable for fortification. Dusting and spray-coating of polished rice with micronutrient powders has been attempted but with limited success due to loss of nutrients during typical rinsing and cooking procedures (Kyritsi, Tzia, & Karathanos, 2011; Steiger, Muller-Fischer, Cori, & Conde-Petit, 2014). Therefore, development of technologies for rice fortification with substantial nutrient retention, stability, bioavailability and acceptability in target groups is essential. Further, rigorous testing of the efficacy of fortified rice in improving the nutritional status of the target groups in the context of ongoing food security/food fortification programmes is needed to guide and inform policy.

Recent technological improvements enabled the fortification of rice with minerals and vitamins wherein rice flour fortified with either single or multiple nutrients is extruded in to artificial-rice kernels appearing similar in size and shape to that of natural rice grains (Mishra, Mishra, & Rao, 2012; Steiger et al., 2014). These fortified rice kernels, in turn, are blended with normal rice at fixed proportions to achieve mandatory fortification levels set by the regulatory agencies. During the past decade, iron-fortified ultra rice was shown to improve the iron status in Indian school children when tested as part of mid-day-meal programme (Moretti et al., 2006; Radhika et al., 2011). Further, studies in other Asian, South American and African countries also successfully demonstrated the efficacy of fortified rice in improving the iron status (Angeles-Agdeppa, Capanzana, Barba, Florentino, & Takanashi, 2008; Beinner, Velasquez-Melendez, Pessoa, & Greiner, 2010; Hotz et al., 2008; Losso et al., 2017). However, ensuring effective delivery of fortified rice via food security programmes in a country like India requires indigenous production technologies/facilities.

In the present study, we report the retention and stability of iron, folic acid and vitamin B_{12} during rinsing and cooking of indigenously developed triple-fortified rice. Further, we also report the bioavailability of iron using coupled *in vitro* digestion/Caco-2 cell model and acceptability of fortified rice in adult human volunteers. Further, we also computed the potential contribution of fortified rice to the dietary iron intakes of children, adolescents and women of reproductive age (WRA) in Andhra Pradesh, a predominantly rice-consuming state of southern India.

2 | MATERIALS AND METHODS

2.1 | Materials

Folic acid, vitamin B_{12}, 1-hexanesulfonic acid sodium salt, triethylamine (TEA), glacial acetic acid, porcine pepsin, pancreatin and porcine bile extract were purchased from Sigma Chemical Co. (Bangalore, India). Iron standards (1 mg/mL), ultra-pure nitric acid (HNO_3) and hydrogen peroxide (H_2O_2) were procured from Merck, Germany. A 12–14-kDa molecular weight cut-off dialysis membrane (Spectra/Por-7 dialysis tubing) was procured from Spectrum laboratories, Europe. Acetonitrile (HPLC grade) and sodium hydroxide analytical grade were procured from Rankem India Ltd.

2.2 | Production of fortified rice kernels

The extruded rice kernels were produced by a hot extrusion technology. The target nutrients, citric acid (0.5%), maltodextrin (0.5%) and other stabilizing ingredients were added to the rice flour; dough was prepared through a pre-conditioning process and extruded in a Twin-Screw Extruder machine in to rice-shaped kernels by the manufacturer (M/s. Suvarnabhoomi Enterprise Pvt Ltd., Namakkal Dist, Tamilnadu, India). The extruded rice kernels contained 1200 mg iron as micronized ferric pyrophosphate (MFPP, mean of particle size of ~3.5), 13 mg folic acid and 100 μg vitamin B_{12}/100 g.

2.3 | Fortification

Fortification was done at a ratio of 1:100 (for testing the fortification levels, acceptability and iron bioavailability) and at 1:10 (to aid in testing the retention and stability of folic acid and vitamin B_{12}). Briefly, either 22.5 or 24.75 kg of normal rice in a steel ribbon blender (fabricated locally) was mixed with 2.5 kg (1:10 ratio) or 0.25 kg (1:100 ratio) of fortified extruded rice kernels, respectively, for 3 min. The fortified rice was divided in to six equal portions and stored separately in air-tight plastic containers. The levels of nutrients in fortified rice were estimated as described below.

2.4 | Rinsing of rice

Three independent portions of fortified rice (0.5 kg each) were soaked in 1 L milli-Q water, swirled and left for 15 min. The water was then discarded followed by two quick rinsing cycles with 1 L water. The rinsed rice was then lyophilized to dryness and powdered in a kitchen blender, and the nutrient content of which was estimated in duplicates as described below.

2.5 | Cooking of rice

Independent portions of fortified rice (0.5 kg each) were rinsed thrice as described above, suspended in 1 L of deionized water and cooked with each of the following three methods in triplicates: (i) in an electric cooker; (ii) in a pressure cooker; and (iii) cooked with excess water (1.5 L) until the rice was cooked 80%, decanted the excess water and further cooked until done. The cooked rice was then made to paste, lyophilized to dryness and powdered in a kitchen blender, and the nutrient content was measured in duplicates as described below.

2.6 | Determination of iron

Iron content in powdered unfortified, premix, fortified (1:10 and 1:100 ratio) raw, rinsed or cooked rice samples were estimated as described previously (Palika et al., 2013). Briefly, powdered rice fractions (0.5 g) were weighed into digestion vessels, followed by addition of 4.5 mL of 0.1 N HCl to each vessel; 2 mL of 65% HNO_3 and 1 mL 33% H_2O_2 were added to facilitate the digestion. The digestion vessels were sealed and subjected to microwave digestion (MARS XPRESS, CEM Corporation, USA). After cooling, the vessel contents were filtered, and the iron content in the digest was estimated by atomic absorption spectrometry (Shimadzu AA7000, Japan).

2.7 | Extraction and HPLC analysis of folic acid

A total of 10 g of powdered rice fractions were mixed with 100 mL of 0.1 M phosphate buffer (pH 6.0) in a conical flask. The solution was incubated in a sonicator bath for 45 min (Branson, USA). The suspension was centrifuged at 10,000 rpm for 15 min at 4°C and clarified by filtration through 0.22 μm syringe filters. The folic acid content of this filtrate was analysed by HPLC as described previously (C. M. M. Silveira, Della Lucia, Pirozi, Montini, & Pinheiro-Sant'Ana, 2016). Briefly, reverse phase chromatography of folic acid was performed on a C-18 column (Thermo-Hypersil ODS, 5 μ, 250 × 4.6 mm) connected to a HPLC equipped with an online UV monitor and controlled by the Chromequest software (Thermo scientific, USA). The column was pre-equilibriated and eluted with water–methanol (80:20) mobile phase (supplemented with 5 mM hexanesulfonic acid sodium salt, 1% glacial acetic acid and 0.1% tri ethyl amine pH 5.0) at a flow rate of 1 mL/min while monitoring the flow at 282 nm. Folic acid in the sample was identified by the retention time and quantified by comparing peak area with authentic standard. The recovery of folic acid from spiked powdered rice samples using above extraction method was always found to be 90–103%. A representative HPLC chromatogram of folic acid analysis is given in Figure S1A in the Supporting Information.

2.8 | Extraction and HPLC analysis of vitamin B_{12}

A total of 15 g of powdered rice fractions were suspended in 100 mL milli-Q water in a conical flask. The solution was agitated in a sonicator bath for 45 min (Branson, USA), centrifuged at 10, 000 rpm for 15 min at 4°C and filtered through 0.22 μm syringe filters. The vitamin B_{12} in the filtrate was concentrated by solid phase extraction using Strata C-18-E (55 μm, 70 A) columns, pre-equilibrated with 2% acetonitrile. The bound vitamin B_{12} was eluted from the column using 50% ACN and concentrated in a centrifugal vacuum evaporator (Concentrator *Plus*, Eppendorf, Germany), reconstituted in 250 μL of mobile phase and analysed by HPLC as described previously (Heudi, Kilinc, Fontannaz, & Marley, 2006). Briefly, reverse phase chromatography of vitamin B_{12} was performed on a C-18 column (Thermo-Hypersil ODS, 5 μ, 250 × 4.6 mm) connected to a HPLC equipped with an online UV monitor and controlled by the Chromequest software (Thermo scientific, USA). A 100-μL aliquot of concentrated extract or 25 ng of standard B_{12} was loaded on to column. The column was pre-equilibrated and eluted with water–methanol (78:22) mobile phase at a flow rate of 1 mL/min while monitoring flow at 361 nm. Vitamin B_{12} in the sample was identified by the retention time and quantified by comparing peak area with authentic standard. The recovery of vitamin B_{12} from spiked powdered rice samples using above extraction method was always found to be 87–98%. A representative HPLC chromatogram of vitamin B_{12} analysis is given in Figure S1B.

2.9 | Assessment of *in vitro* dialyzability

The simulated gastrointestinal digestion was performed as described previously (Argyri, Birba, Miller, Komaitis, & Kapsokefalou, 2009) with minor modifications. Powdered rice samples (0.625 g) were hydrated with 10 mL of normal saline in 50 mL tubes for 30 min. The pH of samples was adjusted to 2.0 with 2 N HCl and final volume was made up to 12.5 mL with normal saline. A total of 2 mL aliquot of this digesta was transferred to six-well plates in triplicates. A 0.1 mL pepsin solution (40 mg/mL in 0.1 N HCl) was added to each well, covered and incubated for 2 h at ambient temperature on an orbital shaker. At the end of this incubation, a Transwell insert fitted with a 12–14-KDa molecular weight cut-off dialysis membrane (Spectra/Por-7 dialysis tubing, Spectrum laboratories, Europe) was housed in individual wells of 6-well plate, thus creating an apical and basolateral chamber. The apical chamber was filled with 2 mL PIPES buffer (pH 6.5; the buffer diffused through the membrane and raised the pH of the samples to 6.5). After 30 min, 0.5 mL of a pancreatin (2 mg/mL) and bile salt (12 mg/mL) mixture in 0.1 M $NaHCO_3$ was added to each well, and incubated for further 2 h. Aliquots of the dialysate from the apical chambers were collected, and the iron content was estimated immediately using the Atomic Absorption Spectrophotometer (Shimadzu AA-7000, Japan).

2.10 | Ferritin expression in Caco-2 cells

The coupled *in vitro* digestion/Caco-2 cell model was used for measuring the ferritin induction as described previously (Pullakhandam, Nair,

Pamini, & Punjal, 2011). Briefly, either 1 mL saline (reagent blank) or 1 g of rice samples were subjected to *in vitro* gastric and intestinal digestion in the presence and absence of 250 μmol/L ascorbic acid (freshly prepared in 0.1 N HCl), followed by feeding the digesta to differentiated Caco-2 cells for a period of 24 h. At the end of incubation, the cells were washed and lysed. A human ferritin sandwich ELISA kit (Calbiotech, USA) was used for ferritin estimation in cell lysates, as per the manufacturer's instructions. The colour intensity was measured using an ELISA plate reader (BioTek, Powerwave HT-1).

2.11 | Sensory evaluation

Informed written consent from the respondents was taken before conducting the sensory study. A sample size of 84 respondents has been calculated considering an α = 0.05 significance level, β = 0.10 (90% power) and a P_D = 0.25 (the chance of detecting a difference is less than 25%).

The triangle tests (Meilgaard, Vance Civille, & Thomas Carr, 1999) were performed among apparently healthy adult men and women (n = 84; mean age = 30.4, 54% men and 46% women) to test if they could distinguish the fortified rice from unfortified rice both in the uncooked and cooked form, evaluated separately. Equal quantities of fortified and unfortified rice were prepared simultaneously using traditional recipe (rice to water ratio of 1:2), in identical electric cookers. The sensory test was conducted immediately thereafter. The study was conducted at 11:00 a.m. in a dedicated facility under uniform lighting conditions. The three rice samples (30 g each), of which two were identical and one different were served in polyethylene cups, identified by a three-digit random code, simultaneously, to the respondents. The samples were presented in a randomized block design, i.e. the six possible order combinations were randomized across the respondents and presented for sensory evaluation. The respondents were asked to evaluate the samples from left to right and identify which one among the three samples differed from the other two and also describe how it differed based on sensory properties. The uncooked and cooked rice was tested in separate sessions. Each session included 12 respondents. The respondents were blinded and were informed only about the test procedures at the beginning of the session, and specific information about the type/identity of rice was revealed only after the study was completed.

2.12 | Calculation of potential contribution of fortified rice to the dietary iron intakes in rice eating population

To understand the potential contribution of the fortified rice to the dietary iron intakes among different age groups, the habitual iron intake data was summed with additional iron contributed by fortified rice considering the rice intake data of Andhra Pradesh (NNMB, 2012), a predominantly rice consuming south Indian state. The percentage iron intake with respect to EAR and RDA (Ghosh et al., 2019; Ghosh, Sinha, Thomas, Sachdev, & Kurpad, 2019) was then computed to reflect the potential differences due to fortified rice. The current regulation of iron content in fortified rice in India ranges from 28 to 42.5 mg/kg rice when ferric pyrophosphate is used as the iron fortificant (https://ffrc.fssai.gov.in/commodity?commodity=fortified-rice). Therefore, a level of 30 mg/kg iron was considered for the purpose calculations.

2.13 | Statistics

The rinsing and cooking of rice by each method was carried out thrice on independent day, and target nutrients were analysed in duplicates for each sample to generate six observations. The in vitro dialyzability and ferritin induction were analysed in triplicate and in two independent experiments. The mean and standard deviation (SD) were computed using Microsoft Excel (2007). Unpaired t tests were performed to compare the mean differences in iron content, dialyzable iron content. One-way ANOVA followed by Tukey's post hoc test was performed to compare the means (Version 19, SPSS Inc, Chicago, US). The sensory data was analysed using the binomial test with an expected probability of correct identification of 1/3 in the triangle test. The differences were considered significant at $P < .05$.

3 | RESULTS

3.1 | Nutrient content of extruded rice premix, fortified and unfortified rice

The representative images of the premix (extruded rice kernels) and fortified rice are shown in Figures S2. The mean (±SD) iron (1200 ± 43.2 mg/100 g), folic acid (12700 ± 16.7 μg/100 g) and vitamin B_{12} (94 ± 4.75 μg/100 g) content in premix were at expected levels (Table 1). In the unfortified rice, iron content was 0.27 ± 0.03 mg/100 g, whereas folic acid and B_{12} were undetectable using the HPLC method. The iron and folic acid content of fortified rice (1:100) were 12.1 ± 0.84 mg/100 g and 121 ±1.01 μg/100 g, respectively. However, the B_{12} levels remained undetectable even in the fortified rice (1:100), possibly due to very low levels of this nutrient in the sample (~1 μg/100 g).

3.2 | Sensory evaluation of fortified rice in adult human volunteers

In a sensory panel of 84 adult volunteers, 24 and 33 respondents correctly identified the odd rice sample in the uncooked and cooked form, respectively (Table S1), which is less than the critical number of correct responses required (n_c = 36) to detect a significant difference at 5% level of significance. This indicates that the fortified and unfortified rice were sufficiently similar, both in the uncooked and cooked form.

TABLE 1 Iron, folic acid and vitamin B_{12} content of fortified rice premix, fortified and unfortified rice

Nutrient	Premix	Fortified rice (1:100 ratio)	Fortified rice (1: 10 ratio)	Unfortified rice	FSSAI fortification standards[$]
Iron (mg/100 g ± SD)[#]	1200 ± 43.2	12.1 ± 0.84[#]	136 ± 9.8	0.27 ± 0.03	2.8–4.25
Folic acid (μg/100 g ± SD)	12,700 ± 16.7	121 ± 1.01	1443 ± 117	ND	75–125
Vitamin B_{12} (μg/100 g ± SD)	94 ± 4.75	ND*	10.2 ± 0.7	ND	0.75–1.25

[#]Iron fortification was kept at 120 mg/kg levels purposefully to facilitate further clinical trial to provide 12 and 18 mg iron/day with 100 and 150 g ration of rice/day.
*ND; not detectable.
[$]FSSAI draft guidelines on rice fortification, 2016.

3.3 | Retention and stability of nutrients during rinsing and cooking

Due to methodological limitations the B_{12} levels in fortified rice (1:100 ratio of blending) were not detectable. Therefore, retention and stability analysis were performed using 1:10 fortification ratio to aid in analysis. The iron (136 ± 9.8 mg/100 g), folic acid (1443 ± 117 μg/100 g) and vitamin B_{12} (10.2 ± 0.7 μg/100 g) levels of fortified rice are also at the expected fortification levels (Table 1). The mean (±SD) iron content of rinsed fortified rice (122 ± 12.5 mg/100 g) was similar to the fortified rice. However, folic acid and vitamin B_{12} content significantly ($P < .05$) reduced by ~25% during rinsing of fortified rice (Figure 1A-C). Interestingly, cooking of rice in electric or pressure cooker led to small additional decline in folic acid (~10–16%) but not vitamin B_{12} compared with rinsed rice, but the differences were not significant. Cooking of fortified rice in excess water followed by decanting led to significant loss of iron (45%), folic acid (75%) and vitamin B_{12} (71%). Thus the cumulative retention and stability of nutrients during rinsing and cooking in an electric or pressure cooker were 100% for iron and ~70% for folic acid and vitamin B_{12}.

3.4 | Bioavailability of iron from fortified rice

The percentage dialyzable iron from unfortified rice (16.66% ± 1.0) is significantly higher compared with fortified rice (4.54% ± 0.28) (Table 2). However, the absolute dialyzable iron content from the fortified rice (0.55 ± 0.06 mg/100 g) was found to be much higher than that of unfortified rice (0.045 ± 0.006 mg/100 g). The Caco-2 cell

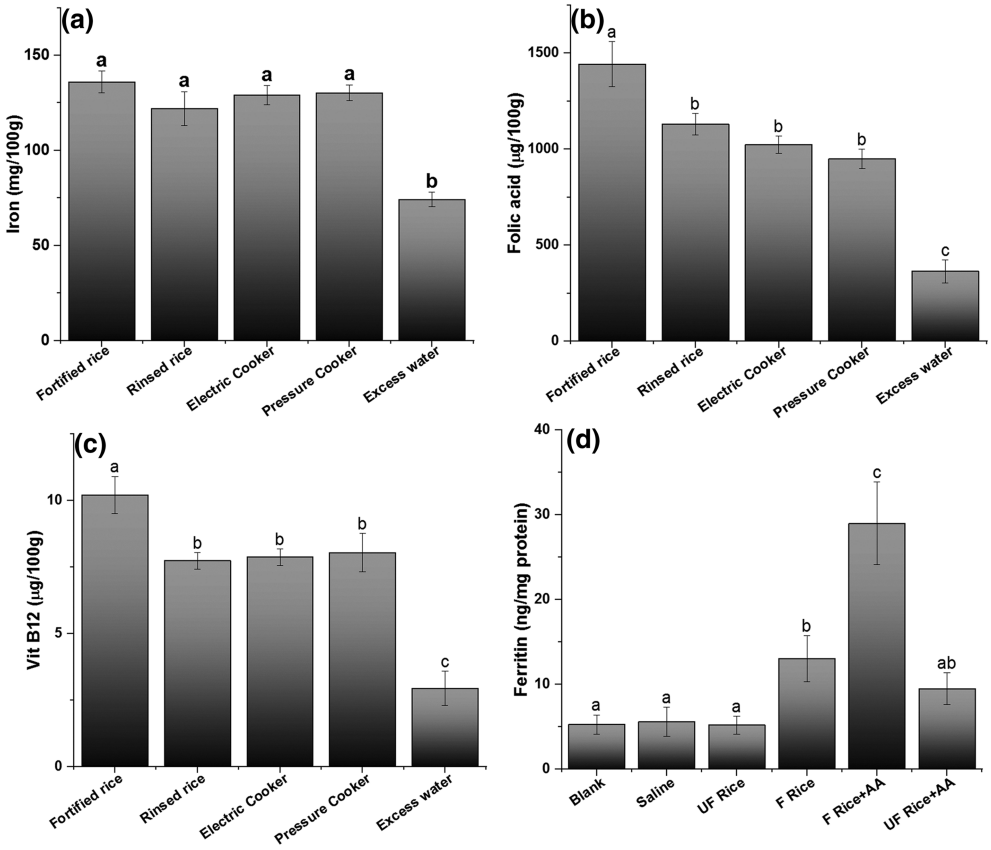

FIGURE 1 Retention of nutrients during rinsing and cooking of fortified rice and Caco-2 cell ferritin formation: Fortified rice (1:10 ratio of blending) rinsed with excess water followed by 3 different cooking methods followed by measurement of iron (A), folic acid (B) and vitamin B12 (C) as described in methods. The ferritin content of differentiated Caco-2 cells (D) unexposed (Blank-MEM) and exposed to saline (reagent blank), unfortified (UF) and fortified (F; 1:100 ratio of blending) rice digests in the absence and presence of ascorbic acid (AA; 250 μmol/L). The bars represent mean ± SD of cell ferritin (ng/mg cell protein), and the bars that do not share a common superscript differ significantly ($P < .05$)

TABLE 2 *In vitro* dialyzability of iron from fortified rice[a]

Nutrient	Iron (mg/100 g ± SD)	Dialyzable iron (mg/100 g ± SD)	Dialyzability (% ± SD)
Fortified rice	12.4 ± 0.84*	0.55 ± 0.06*	4.54 ± 0.28
Unfortified Rice	0.27 ± 0.03	0.045 ± 0.006	16.66 ± 1.0*

[a]Fortification was done at 1:100 ratio of premix to that of rice.
*The asterisks indicate significant difference ($P < .01$) between fortified and unfortified rice by unpaired *t* tests.

ferritin levels exposed to fortified rice digesta was significantly higher ($P < .01$) compared with blank (untreated cells), saline (reagent blank) or unfortified rice (Figure 1D). Further, ferritin response with unfortified rice remained similar to that of either blank or saline control. Inclusion of ascorbic acid induced ferritin synthesis from both fortified and unfortified rice digests compared with its absence, but it was significant ($P < .01$) only from fortified rice.

3.5 | Contribution of fortified rice to the dietary iron intakes

The median habitual dietary intake of iron and additional iron intake due to consumption of fortified rice among 1–17-year-old children and women of reproductive age (WRA) are presented in Table 3. The dietary iron intakes are grossly deficient with respect to recently computed EAR (range: 26–63%, mean ± SD; 42.44% ± 9.7) and RDA (range: 13–47%; mean ± SD: 27.2% ± 9.98) among all age groups among the rural population of Andhra Pradesh. But additional iron intake due to fortified rice, computed considering the median rice intakes across age groups, substantially improved the iron intakes with respect to EAR (range: 58–126%; mean ± SD 96.07% ± 17.19) and RDA (range: 34.7–93%; mean ± SD 62.2% ± 18.7), respectively, among all age groups.

4 | DISCUSSION

Periodic surveys in India demonstrated very high prevalence of anaemia among Indians, more so among children and women of reproductive age (NFHS 2-3 and DLHS). Similarly, studies in infants, children and apparently healthy adult population have indeed demonstrated high prevalence of anaemia and multiple micronutrient deficiencies among Indian population, particularly that of iron, folic acid and vitamin B_{12} as well as other nutrients (Nair et al., 2016; Shalini et al., 2018; Sivakumar et al., 2006). The dietary intake assessments also indicated gross inadequacy of both macro (except carbohydrate) and micronutrients among Indian population (NNMB, 2002). In the context of high prevalence of deficiencies and estimated inadequacies in dietary intakes, improving the intakes through food fortification has been suggested as a cost effective food based strategy to control micronutrient malnutrition. Because the fortification is done centrally at controlled and predefined nutrient levels, the outreach of these technologies for safe and sustainable delivery of micronutrients on a daily basis is very high.

The Food Safety Standards Authority of India (FSSAI, 2016) suggested fortification of milk and edible oil with fat soluble vitamins (A and D) and wheat flour and rice with iron, folic acid and vitamin B_{12}, apart from other nutrients. Extruded rice fortified with iron and other micronutrients is considered an ideal vehicle for fortification of rice, and iron-fortified rice has been shown to improve the iron status among different physiological groups across the world, including in Indian children. The operational feasibility of fortified rice supply through already ongoing public distribution systems and school lunch programmes makes it a promising public health strategy. However, assessing the retention, stability, bioavailability and acceptability of fortified rice is required for ensuring their intended beneficial outcomes when implemented in public health programmes. The results of the present study demonstrate that fortification of rice with extruded rice kernels improves its micronutrient content to the desired levels and that the retention of nutrients is ≥70% during rinsing and cooking of rice either in an electric or pressure cooking. However, boiling of rice in excess water followed by decanting of starch led to 50% loss of iron and >70% loss of B vitamins.

The extruded rice kernels resembled that of natural rice grains in shape and size but are opaque in nature compared with transparent natural polished rice grains. However, when blended with natural rice, the fortified rice is indistinguishable either in the uncooked or cooked form by adult volunteers. The iron, folic acid and vitamin B_{12} levels of the fortified rice (1:100 or 1:10 ratio of blending) were at the desired levels of fortification and in conformity with draft FSSAI regulation (FSSAI, 2016), except that iron fortification was deliberately set at 120 mg/kg levels to facilitate delivery of 12 and 18 mg iron/child/day, through a single meal to primary (6–11 years; 100 g rice/day ration) and high (12–15 years; 150 g rice/day ration) school children participating in mid-day-meal programme (Radhika et al., 2011). Also the same ration is expected to provide 1 RDA of both folic acid and vitamin B_{12} through a single meal (RDA, 2010).

Rice purchased or supplied through public distribution programmes in India is usually rinsed with excess water to remove particulate matter or husk before it is boiled with water either in an electric cooker or in pressure cooker over flame. In some households, particularly in rural settings, rice is boiled with excess water followed by decanting the starch. Therefore, retention and stability of fortified nutrients during rinsing and different cooking methods needs to be studied. The iron content of uncooked, rinsed or cooked (watertight cooking in electric or pressure cooker) rice are similar, implying high retention of iron. Similar to these results, previous study with iron fortified ultra rice also found no differences in iron content of uncooked or cooked rice (Radhika et al., 2011). Further, 80–100% retention of iron and zinc was observed during soaking, rinsing and cooking of extruded fortified rice produced by different manufacturers (Wieringa et al., 2014). Similarly, very high retention of iron from extruded rice fortified with different iron compounds or polymer coated rice was reported either during rinsing or cooking (Losso et al., 2017; Moretti,

TABLE 3 Contribution of iron from fortified rice to the total dietary intake of iron in rural population of Andhra Pradesh

	Age (years)	Sex	§Dietary Iron intake (mg/day)	¥Iron intake from diet as percent of		§Median intake of rice (g/day)	‡Iron intake from fortified rice (mg/day)	Total iron intake (Diet + fortified rice) (mg/day)	Total iron intake (%)	
				EAR	RDA				EAR	RDA
Children (boys + girls)	1	BOTH	1.80 (1.09,3.45)	32.13	24.65	48.40 (25.7,79.0)	1.45	3.25	58.06	44.54
	2	BOTH	3.30(2.08,4.99)	50.80	38.85	94.35 (65.0,130.0)	2.83	6.13	94.35	72.15
	3	BOTH	4.49(2.78,6.58)	63.24	47.26	129.90 (92.1,187.7)	3.90	8.39	118.13	88.28
	4	BOTH	4.43 (2.78,7.39)	58.25	42.57	132.70 (93.7,177.0)	3.98	8.41	110.63	80.85
	5	BOTH	3.68 (2.81,5.61)	44.94	32.61	133.30(92.8,181.0)	4.00	7.68	93.71	68.00
	6	BOTH	4.70(3.21,7.38)	52.82	37.91	175.10(127.0,227.7)	5.25	9.95	111.84	80.27
	7	BOTH	4.50 (3.25,6.93)	47.36	33.58	192.35 (141.2,243.0)	5.77	10.27	108.11	76.64
	8	BOTH	4.48 (3.38,6.90)	43.93	30.69	190.95 (122.7,264.2)	5.73	10.21	100.09	69.92
	9	BOTH	4.73 (3.65,6.78)	43.04	29.59	214.40(153.4,281.6)	6.43	11.17	101.51	69.79
Adolescents	10	BOYS	6.60 (4.23,9.41)	61.07	45.17	233.85 (170.4,359.9)	7.02	13.61	126.03	93.22
	11	BOYS	5.53 (3.83,9.48)	48.08	35.22	234.40 (129.0,332.1)	7.03	12.56	109.23	80.01
	12	BOYS	6.13 (4.38,9.23)	48.69	35.46	318.50 (247.0,396.1)	9.56	15.69	124.52	90.69
	13	BOYS	5.78 (4.36,7.63)	41.91	30.12	276.10 (187.5,368.1)	8.28	14.07	101.93	73.26
	14	BOYS	7.09 (4.74,9.40)	45.75	32.53	317.10 (211.6,414.4)	9.51	16.60	107.12	76.16
	15	BOYS	5.47 (4.57,11.4)	32.57	22.99	248.60 (195.0,350.5)	7.46	12.93	76.96	54.32
	16	BOYS	6.21 (4.47,7.93)	34.87	24.53	353.80 (206.8, 489.4)	10.61	16.82	94.50	66.49
	17	BOYS	7.05 (4.84,11.0)	38.33	26.71	344.30 (228.3,493.1)	10.33	17.38	94.46	65.84
	10	GIRLS	5.51 (3.36,8.47)	35.80	17.07	226.40 (150.7,306.6)	6.79	12.30	79.90	38.09
	11	GIRLS	5.30 (4.53,7.91)	33.35	16.07	285.45 (197.5,361.5)	8.56	13.87	87.21	42.02
	12	GIRLS	5.81 (4.20,8.08)	35.43	17.19	252.60 (193.5,332.8)	7.58	13.39	81.64	39.61
	13	GIRLS	4.47 (3.51,7.85)	26.45	12.96	250.70 (158.0,337.4)	7.52	11.99	70.95	34.76
	14	GIRLS	6.79 (4.65,9.44)	39.02	19.23	305.00 (200.0,377.5)	9.15	15.94	91.60	45.15
	15	GIRLS	6.06 (4.08,9.50)	33.86	16.88	276.40 (199.0,365.3)	8.29	14.35	80.18	39.98
	16	GIRLS	6.22 (4.85,9.32)	34.20	17.05	303.40 (261.4,405.5)	9.10	15.33	84.21	41.99
	17	GIRLS	5.48 (4.27,7.88)	29.63	14.86	283.00 (193.3,344.5)	8.49	13.97	75.52	37.86
WRA	18–49	Female	7.22 (5.17,9.96)	48.13	20.63	337.00 (249.3,444.5)	10.11	17.33	115.53	49.51

¥EAR and RDA values for WRA, children and adolescents were adopted from Ghosh, Sinha, Thomas, et al., 2019; Ghosh, Sinha, Shivakumar, et al., 2019.

Abbreviations: EAR, estimate average requirement; RDA, recommended dietary allowance; WRA, non pregnant, non lactating women of reproductive age.

§Values are median (P25, P75) author's computation from (NNMB, 2012).

‡Considering 30mg iron/kg of fortified rice.

Lee, Zimmermann, Nuessli, & Hurrell, 2005; Peil, Barrett, Rha, & Langer, 1982; Wieringa et al., 2014). These results together suggest that iron retention and stability is very high during rinsing and water tight cooking of rice.

Although the extruded rice kernels appear opaque and are easily distinguishable from natural rice, blending of this rice with natural rice did not result in apparent sensory difference between the fortified and unfortified rice when tested among adult subjects. Previous organoleptic studies conducted in India also reported that the iron-fortified rice is similar to the unfortified rice in terms of sensory properties and is well accepted by school children (Moretti et al., 2005; Moretti et al., 2006; Radhika et al., 2011). Studies from Vietnam and Cambodia using rice fortified with multiple micronutrients, namely, iron, zinc and vitamins A, B_1, B_3, B_6, B_9 and B_{12} reported higher acceptability by children and women, though perceptible sensory differences were observed compared with unfortified rice (Khanh Van et al., 2014). Together these evidences indicate that fortified rice is well accepted and can be used interchangeably.

Rinsing of fortified rice led to ~25% reduction in folic acid and vitamin B_{12} levels, unlike that of iron. It is possible that either leakage of surface nutrients or disintegration of extruded rice kernels during rinsing might lead to such losses. However, the unaltered iron content due to rinsing implies leakage of surface B vitamins. Cooking in an electric or pressure cooker resulted in a small but insignificant loss of folic acid but not vitamin B_{12} compared with rinsed rice, implying that cooking has no additional impact on vitamins. Similar to these results, substantial retention of folic acid (74%) and vitamin B_{12} (89%) from fortified rice produced by different manufacturers has been reported during rinsing and cooking. Further, >75% retention of folic acid has been demonstrated during variety of cooking methods (C. M. Silveira et al., 2017). Together this evidence indicates that folic acid and vitamin B_{12}, although significantly lost during rinsing of rice with excess water, cooking has no additional impact on their stability. However, boiling of rice with excess water followed by decanting led to significant loss of all the three nutrients. Direct boiling of the extruded rice kernels in water also led to disintegration of almost 60% grains forming mushy paste, thus explaining the loss of nutrients.

Bioavailability of minerals, particularly that of iron, is influenced by its content, type of iron compound, oxidation state and other dietary constituents apart from physiological status of the host. Measuring the dialyzable iron content or induction of ferritin synthesis in Caco-2 cells after simulated *in vitro* digestion of test foods was considered an ideal approach to measure the bioavailability of iron in test foods (Argyri et al., 2009; Glahn, Lee, Yeung, Goldman, & Miller, 1998). Moreover, iron bioavailability in Caco-2 cells paralleled the observations in human studies (Au & Reddy, 2000). We have also demonstrated that absorption ratios of zinc from high and low phytate wheat flour are similar to that of humans (Sreenivasulu, Raghu, Ravinder, & Nair, 2008). Therefore, we have measured both dialyzability and ferritin induction in Caco-2 cells from fortified and unfortified rice. The dialyzable iron content and induction of ferritin in Caco-2 cells was significantly higher from fortified rice as compared with unfortified rice, consistent with the higher iron content of the latter. Further, inclusion of ascorbic acid, a known enhancer of iron absorption, increased the ferritin content of Caco-2 cells exposed to both fortified and unfortified rice. However, the extent of increase in the ferritin content was higher with fortified rice compared with unfortified rice. These results together suggest that the absolute amount of bioavailable iron is higher from fortified rice, owing to its higher content of iron. Further, the fortified iron bioavailability could be improved by dietary modification to include with vitamin C enriched fruits or vegetables.

Rice and wheat are major staple foods in India, and their fortification with iron, folic acid and vitamin B_{12} is being considered to improve the dietary intakes with an aim to reduce the prevalence of anaemia. The dietary iron intakes among children and WRA of rural Andhra Pradesh population was grossly inadequate with respect to both estimated EAR and RDA and that additional iron intake through fortified rice substantially improved the intakes with respect to both of the above metrics. The fact that we used a lowest iron fortification standard (30 mg/kg rice), for computation of intakes, the actual contribution could be somewhat higher depending on the levels of iron in the fortified rice. These results suggest that consumption of fortified rice substantially reduces the dietary deficits of iron among populations.

5 | CONCLUSIONS

These results suggest that it is possible to achieve target nutrient levels through fortification of rice by blending with extruded rice kernels without changing sensory properties of the rice. Further, the bioavailable iron content from fortified rice is higher compared with unfortified rice, and it can be further improved by inclusion of vegetables and fruits. Further, the losses of B vitamins observed during the rinsing of rice can be adequately compensated by adding overages to the premixes. However, poor retention of nutrients during cooking with excess water followed by decanting, warrants educating the target population and school kitchen personnel on desired cooking methods to be adopted to reduce the nutrient losses. The computed additional dietary iron intakes due to fortified rice appear to bridge the gaps in intake deficits with respect to dietary requirement metrics.

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CONTRIBUTIONS

YWJ and RP performed laboratory studies; RM and SB performed sensory evaluation studies; NBK performed statistical analysis. Raghu and LT designed and supervised the study. All authors contributed in writing the manuscript and approved the final version.

ETHICAL APPROVALS AND REGISTRATION

The study is approved by the Institutional Ethical Committee of the ICMR-National Institute of Nutrition, Hyderabad, India (IEC Protocol Number: 10/I/2017), and is registered with the Clinical Trials Registry, India (CTRI Trial Registration Number: CTRI/2017/11/010655).

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SUPPORTING INFORMATION

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