Development of a Fortified Juice Beverage: Assessment of Iron Bioavailability Using an Established Caco-2 Cell Bioassay

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Abstract

Poor iron bioavailability is a contributing factor to the high prevalence of iron deficiency anemia in India, and food fortification with bioavailable iron is one strategy to help address the problem. Validated in vitro methods to measure iron (Fe) bioavailability are useful tools that can be leveraged to estimate the bioavailability of Fe in foods and beverages. In the present study, the objective was to determine if 15% of the Indian Recommended Dietary Allowance (RDA) of Fe as Micronized Iron Pyrophosphate (MIP) in 200 mL (i.e. 3.2 mg Fe per 200 mL) of a fortified mixed Fruit and Vegetable Juice Beverage (FJB) provides bioavailable Fe. To test this objective, various amounts of MIP were added to the FJB with 3 different levels of ascorbic acid (i.e. vitamin C; a known enhancer of Fe bioavailability and an essential nutrient). Vitamin A (retinyl acetate) at a level of 90 µg/200 mL was also added to all samples, except for some controls. The results demonstrated that 15% RDA of Fe (as MIP) in a 200 mL serving of juice fortified with equimolar ascorbic acid resulted in good Fe bioavailability for this form of Fe. Overall, the study demonstrated that a FJB product could be developed to be an excellent source of dietary Fe.

Keywords: Fortified juice beverage, iron bioavailability, in vitro, Caco-2 cell bioassay, iron fortification

Abbreviations: RDA= recommended dietary allowance; FJB= fortified juice beverage; MIP = micronized iron pyrophosphate, NaFeEDTA = sodium iron ethylenediaminetetracetic acid; FWB= fortified water beverage; FBC= fortified breakfast cereal; AA= ascorbic acid; vitamin C

Introduction

Iron Deficiency Anemia (IDA) is the most prevalent micronutrient deficiency, affecting up to 2 billion people globally including more than half of all women of reproductive age (15–49 years) in India [1, 2]. Despite three decades of national supplementation programs iron deficiency continues to be a grim public health problem in India existing across income quintiles, age groups and gender (24 percent men) [3]. Multiple factors including inadequate dietary intake and low bioavailability of iron have contributed to the high incidence of IDA. The Indian government considers food fortification an effective means to address micronutrient deficiencies. The Food Safety Standards Authority of India published draft standards for food fortification in 2016 and supports voluntary fortification of wheat flour and rice via state-wide initiatives [4]. These initiatives are well aligned with the global recommendations of the World Health Assembly (WHA). However, WHA also highlights that "a diet containing adequate amounts of bioavailable iron should underpin all efforts for prevention and control of anemia" [5]. Selecting and testing an appropriate food vehicle and fortificant for iron bioavailability are important considerations to help improve nutritional status and reduce the prevalence of IDA.

Iron (Fe) can be present in the human diet in either the heme or non-heme forms. Heme Fe is from hemoglobin, most commonly found in red meats, and is seldom used as a food fortificant due to cost, processing issues, shelf life, color and cultural issues. Non-heme iron is present in plant foods that contain inhibitors of Fe absorption such as phytic acid, oxalic acid and certain polyphenols. Heme Fe is considered to be highly available relative to non-heme Fe as it is not affected by these inhibitors. In general, it is believed that individuals who consume diets with moderate amounts of heme Fe seldom experience Fe deficiency; whereas those consuming diets lacking in heme Fe are more at risk for Fe deficiency [6].The high prevalence of iron deficiency in India may be partially attributed to iron-poor vegetarian diets that include consumption of green leafy vegetables, fruits and pulses that do not significantly impact anemia status [7]. Non-heme forms of Fe are the most common form of fortificants used in fortifying food products. In recent years, many forms of non-heme Fe have been developed and utilized in a broad range of food and beverage products and these fortificants exhibit considerable variance in bioavailability depending on the nature of the food product. In

general, reduced or elemental Fe (Fe0) tends to have the lowest bioavailability, whereas forms such as Ferrous Sulphate (FeSO4), Sodium Iron EDTA (NaFeEDTA), ferric pyrophosphate, and encapsulated forms are similar in bioavailability with occasional differences due to unique interactions with the food matrix [8]. Indeed the choice of Fe form is often determined more by organoleptic interactions with a given food, while simultaneously balancing the nutritional label, focusing on content with little concern as to bioavailability [9].

The nutritional quality (i.e. bioavailability) of Fe in any given food product or meals determined by the interactive effects of the intrinsic form(s) of the Fe in the food matrix, the form of the fortified Fe, the amount of the fortified Fe, and the overall solubility of the Fe as it interacts with components of the food or meal both before and during digestion. For many food products, Fe bioavailability is often not assessed as the producer is not aware of the potential benefits of doing so, or is simply willing to assume that the formulation will provide significant absorbable Fe based on content alone.

The primary objective of the present study was to utilize an established Caco-2 cell bioassay for Fe bioavailability to assure that a Fortified Mixed Fruit And Vegetable Juice Beverage (FJB) containing Micronized Iron Pyrophosphate (MIP) (15% Indian RDA), Ascorbic Acid (AA) (15% Indian RDA) and vitamin A (15% Indian RDA as retinyl acetate), also a nutrient of public health concern in India, per 200 ml serving would deliver substantial absorbable Fe [8]. In addition, the study was designed to

1) Determine the proper level of ascorbic acid needed to maintain the Fe bioavailability in FJB;

2) Explore if additional Fe would increase the amount of bioavailable Fe in FJB;

3) Compare the bioavailability of MIP to sodium iron EDTA (NaFeEDTA) in FJB;

4) Compare Fe bioavailability from the FJB when consumed in the presence of spinach.

Spinach is often mistakenly assumed to be a rich source of Fe by some Indian consumers; therefore, we measured Fe content and assessed Fe bioavailability from spinach alone and in combination with the FJB to demonstrate this nutrition myth. The study also assessed Fe bioavailability in two other commercial Fe fortified products, a Fe Fortified Water Beverage (FWB) and a Fe Fortified Breakfast Cereal (FBC), available in the Indian market.

Materials and Methods

Sample Source and Preparation

The unfortified juice was prepared at PepsiCo India and shipped frozen to Cornell University. Samples were prepared immediately prior to the experiment, at Cornell University as per the amounts listed in Table 1. Both Fe and AA stock solutions were prepared fresh immediately prior to the experiment. MIP and NaFeEDTA were supplied by DSM Nutritional Products,

Mumbai, India. Vitamin A in the form of retinyl acetate was supplied by Sigma-Aldrich (CAS Number 127-47-9). Ascorbic acid was supplied by Sigma (CAS Number 50-81-7).

Aside from the juice formulations, a FWB product (Sample 17) and FBC (Sample 18) were tested. Fresh baby spinach was purchased from a local grocery store in the United States and 2 g (wet weight) was included in samples 19-22. Fe content for these foods is shown in Table 1.

Iron Content Analysis

Fe content of all samples was conducted via inductively coupled plasma emission spectroscopy (ICP-ES). To do so, 0.5 ml liquid or 0.5 g solid samples were dried down and then treated with 3.0 mL of 60:40 HNO_3 and $HClO_4$ mixture into a Pyrex glass tube and left for overnight to destroy organic matter. The mixture was then heated to 120°C for two hours and 0.25 mL of 40 µg/g yttrium added as an internal standard to compensate for any drift during the subsequent inductively coupled plasma atomic emission spectrometer (ICP-AES) analysis. The temperature of the heating block was then raised to 145ºC for 2 h. Then, the temperature of the heating block was raised to 190°C for ten minutes and then turned off. The cooled samples in the tubes were then diluted to 20 mL, vortexed and transferred into auto sample tubes to analyze via ICP-AES. The model of the ICP used was a Thermo iCAP 6500 series (Thermo Jarrell Ash Corp., Franklin, MA, USA).

Fe Bioavailability Analysis

An established Caco-2 cell bioassay was utilized to provide measurement of relative Fe bioavailability between samples [10]. This model uses Caco-2 cell ferritin formation as a marker of Fe uptake. Caco-2 cells are an established human intestinal cell line that is commonly used to study intestinal absorption of nutrients. In these cells, the intracellular Fe storage protein known as ferritin forms in direct proportion to cell Fe uptake. Measurement of this protein provides a sensitive and accurate measurement of cell Fe uptake, negating the needs for more expensive methods such as isotopic labelling.

When conducting a bioassay such as the one used in this study, it is important to utilize quality controls to ensure that a valid run of the bioassay was performed. As in all runs of this model system in the Glahn lab, the quality controls are known as baseline, Fe, Fe + AA, and a dehulled red lentil. The baseline sample is merely all of the solutions used in the bioassay, including the digestive enzymes. For the Fe control, 56 µg of $FeCl_3$ (1 µmole) is added. For the Fe + AA, 56 µg $FeCl_3$ and 3.50 mg AA (20 µmoles) are added. The lentil sample contains 55 µg Fe/g sample dry weight. As per the experimental samples, 3 independent replicates were run for quality controls. In the present study, the Caco-2 cell ferritin values (mean ± standard deviation) for these controls were: Baseline, 3.3 ± 0.4; Fe, 27.0 ± 4.8; Fe +AA, 501.1 ± 21.7; lentil, 13.0 ± 1.1. Units were ng ferritin per mg cell protein. Baseline values are expected to be low, i.e. ≤ 5, which indicates that minimal contamination of Fe was present in the system. The Fe sample

Table 1: Description of the samples used for the experiment

Sample	% RDA Fe	% RDA AA	Molar Ratio AA:Fe	Sample Description[1]	Fe (mg)	AA (mg)	Vit. A (μg)
1	0	0.0	NA	Juice (unfortified)	0	0	0
2	15	0.0	NA	FJB (Fe as MIP)	3.2	0	0
3	15	15.0	0.60:1	FJB (Fe as MIP) + AA	3.2	6	0
4	15	15.0	0.60:1	FJB (Fe as MIP) + AA+ Vit. A	3.2	6	90
5	17	32.5	0.87:1	FJB (Fe as MIP) + AA + Vit. A	3.6	13	90
6	20	35.0	1.05:1	FJB (Fe as MIP) + AA + Vit. A	4.2	14	90
7	15	37.5	1.5:1	FJB (Fe as MIP) + AA + Vit. A	3.2	15	90
8	20	65.0%	1.96:1	FJB (Fe as MIP) + AA + Vit. A	4.2	26	90
9	15	50.0	2:01	FJB (Fe as MIP) + AA + Vit. A	3.2	20	90
10	15	65.0	2.6:1	FJB (Fe as MIP) + AA + Vit. A	3.2	26	90
11	15	82.5	3.3:1	FJB (Fe as MIP) + AA + Vit. A	3.2	33	90
12	15	100.0	3.96:1	FJB (Fe as MIP) + AA + Vit. A	3.2	40	90
13	15	125.0	4.95:1	FJB (Fe as MIP) + AA + Vit. A	3.2	50	90
14	15	0	NA	FJB (Fe as NaFeEDTA) + Vit. A	3.2	0	90
15	15	15.0	0.60:1	FJB (Fe as NaFeEDTA) + AA + Vit. A	3.2	6	90
16	15	25.0	1:01	FJB (Fe as NaFeEDTA) + AA + Vit. A	3.2	10	90
17	16	NA	NA	FWB	3.36	0	0
18	25	25.0	NA	FBC	5.25	10	30
19	0	NA	NA	Spinach alone (serving size = 100 g wet basis)	NA	NA	NA
20	15	50.0	2:01	FJB (Fe as MIP) + Vit. A + AA+ spinach	3.2	20	90
21	15	37.5	NA	FJB (Fe as MIP) + Vit. A + AA + spinach	3.2	15	90
22	0	50.0	NA	Juice (no iron) + Vit. A + AA + spinach	NA	20	90

[1]NA = not applicable

Fortified Juice Beverage (FJB)

Fortified Water Beverage (FWB)

Fortified Breakfast Cereal (FBC)

Ascorbic acid (AA) and Vitamin A (Vit. A)

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ferritin value was within the range of 23-90 ng/mg cell protein, and as expected the AA should enhance the ferritin formation demonstrating the responsiveness of the cells to highly available Fe^{+2} created by the 20:1 molar ratio of AA added to the FeCl3. The value of Fe + AA control is usually in the range of 250 – 550 ng ferritin/mg cell protein. The lentil sample is used to represent a solid food sample, and is normally in the range of 6-20 ng ferritin/mg cell protein. It is important to note that this system, like most bioassays, has a range of responsiveness that can vary from one run to the next. However, as is also true of most bioassays, the positive and negative controls exhibit responsiveness in parallel thus making the bioassay useful as it defines relative differences. This bioassay can accommodate up to 22 samples plus the quality controls; thus, all samples can be directly compared in this study as they were analyzed on the same run of the bioassay.

Caco-2 cell growth is also important to monitor when using this bioassay. Healthy Caco-2 cell monolayers, grown under the culture conditions of this model, should yield 2.5–3.0 mg cell protein per 9.6 cm2 well at 14 days post seeding. In the present study, the Caco-2 protein levels were 2.6 ± 0.1 mg cell protein per well, indicating consistent and healthy growth across the multi-well plates.

Statistical Analysis

Data were analyzed using the software package GraphPad Prism (GraphPad Software, San Diego, CA). Data were analyzed using analysis of variance incorporating normalization of variance, if needed, and Tukey's post test to determine significant differences ($P < 0.05$) between groups. Unless noted otherwise values are expressed as mean ± 1 standard deviation; n = 3 independent replications.
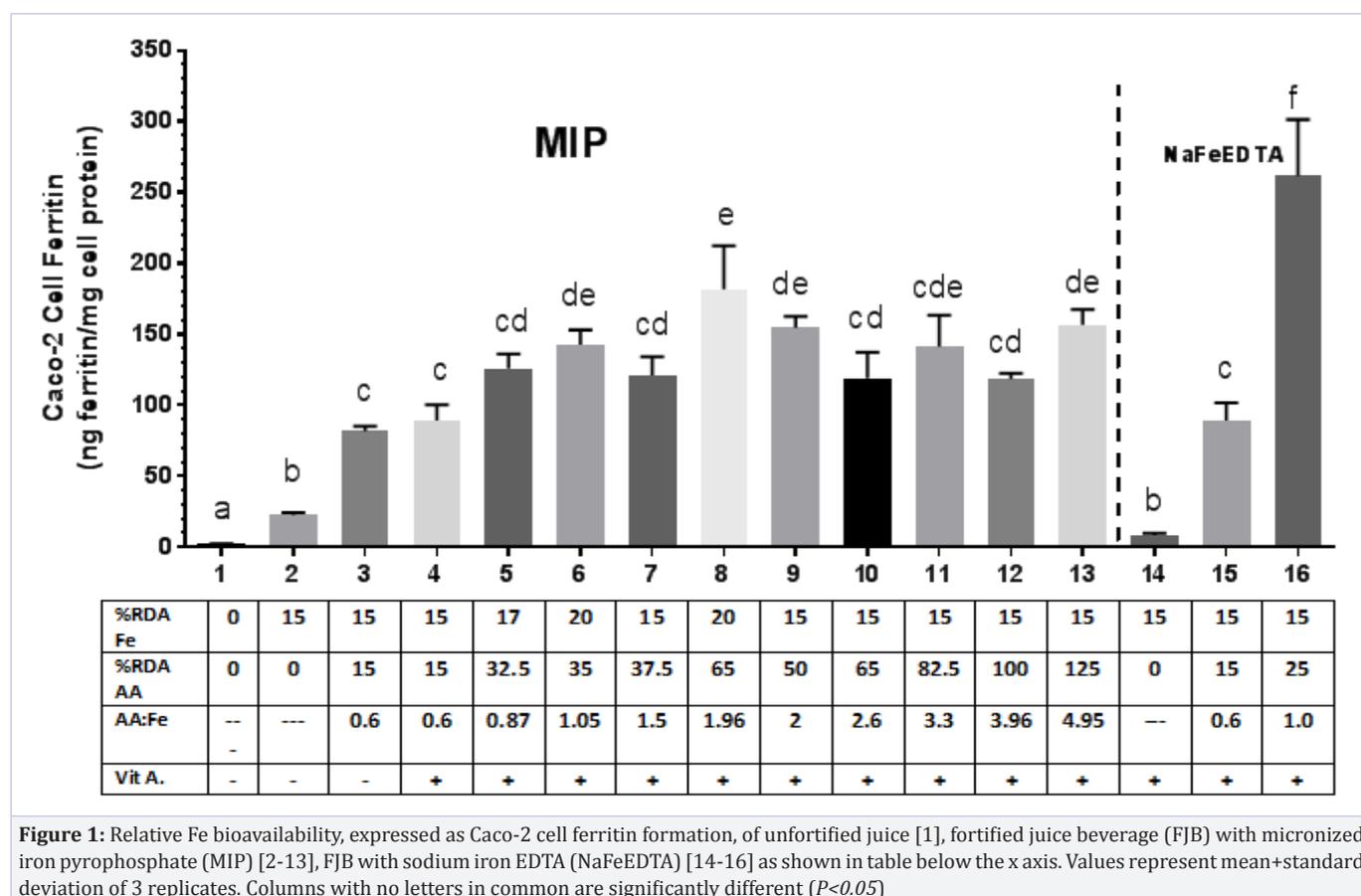
Results

The FJB used in the present study contained only 1.1 µg intrinsic Fe per ml; thus, essentially all of the Fe in the experimental sample came from the fortified Fe. The unfortified juice exhibited no difference from the baseline control, thus indicating no significant intrinsic bioavailable Fe in the juice.

The Fe content of the spinach was 10.3 µg Fe per g wet weight. The FWB contained 17.7 µg Fe per ml equivalent to 3.54 mg per 200 mL serving.

The FBC contained 227.6 µg Fe per g dry weight, equivalent to 6.8 mg Fe per serving size of 30g.

As only 3 levels of Fe were used in the study (i.e. 15, 17 and

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
%RDA Fe	0	15	15	15	17	20	15	20	15	15	15	15	15	15	15	15
%RDA AA	0	0	15	15	32.5	35	37.5	65	50	65	82.5	100	125	0	15	25
AA:Fe	---	---	0.6	0.6	0.87	1.05	1.5	1.96	2	2.6	3.3	3.96	4.95	---	0.6	1.0
Vit A.	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+

Figure 1: Relative Fe bioavailability, expressed as Caco-2 cell ferritin formation, of unfortified juice [1], fortified juice beverage (FJB) with micronized iron pyrophosphate (MIP) [2-13], FJB with sodium iron EDTA (NaFeEDTA) [14-16] as shown in table below the x axis. Values represent mean+standard deviation of 3 replicates. Columns with no letters in common are significantly different (*P<0.05*)

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20% Indian RDA), only a small effect of Fe content on higher Fe uptake was possible in the present study. Such effect was likely part of the increase in ferritin formation observed across samples 3-8 Figure 1. However, across this same range of samples the AA levels were also increasing.

AA is known to enhance Fe uptake, and this effect is first observed in comparison of samples 2 versus 3 Figure 1. As additional amounts of AA were added in samples 4 -13, a maximal effect of added AA becomes evident as no additional increase in ferritin formation occurs.

In the FJB matrix, bioavailability of the MIP was similar to NaFeEDTA when Fe and AA content was the same, as evident in comparison of samples 3 versus 15 Figure 1. However, increased AA with NaFeEDTA resulted in the highest ferritin formation observed with the FJB formulations (sample 16, Figure 1).

The Caco-2 cell ferritin values from the FWB and FBC were 35.3 ± 1.9 and 169.7 ± 10.7, respectively Figure 2.

Fresh spinach has relatively low Fe content and low Fe bioavailability, exhibiting a ferritin value of 2.0 ± 0.1, statistically equal to baseline Figure 2. The presence of spinach decreased Fe uptake when added to the juice (samples 7 and 9 versus 20 and 21).
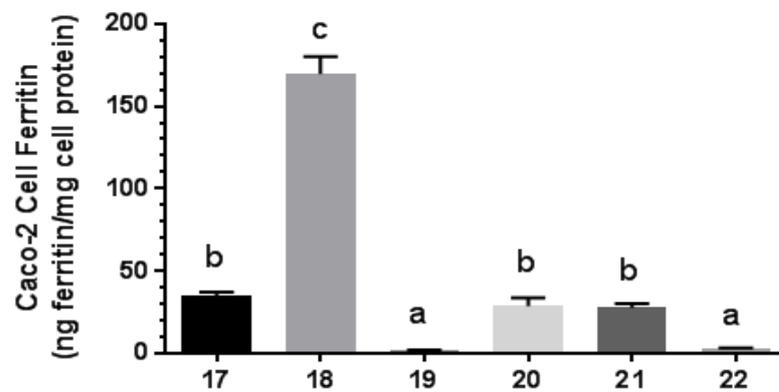
%RDA Fe	16	25	0	15	15	0
%RDA AA	0	25	NA	50	37.5	50
AA:Fe	NA	0.6:1	NA	2:1	1.5	NA
Vit A.	NA	NA	NA	+	+	+

Figure 2: Relative Fe bioavailability, expressed as Caco-2 cell ferritin formation, of the fortified water beverage (FWB) [17], the fortified breakfast cereal (FBC) [18], fresh spinach [19], spinach plus the fortified juice beverage (FJB) with varying AA and Fe (in the form of MIP) [20, 21], and spinach plus juice (with no iron) [22] as shown in table below the x axis. Values represent mean +standard deviation of 3 replicates. Columns with no letters in common are significantly different (*P<0.05*).

Discussion

The present study exemplifies how this validated Caco-2 bioassay can be used to optimize a FJB to improve Fe bioavailability [11].

The results demonstrate that the 15% RDA of Fe generates a relatively strong Caco-2 cell ferritin formation. This indicates that the FJB should be an good source of Fe fortification. If consumed with other foods high in inhibitory factors, such as spinach, then the bioavailability of the Fe could likely decrease Figure 2. Components in the spinach matrix, such as oxalic acid may have contributed to the decrease. It is also possible that the simple addition of solid plant matter contributed to the effect. Fe can bind quite easily to solid background material and become unavailable for uptake.

To fully understand the Fe bioavailability results, one must be familiar with the mechanism of action of AA on non-heme Fe. AA reduces Fe^{+3} (ferric) to Fe^{+2} (ferrous) without complexing the Fe. Fe^{+2} is soluble at near neutral pH and thus is free to interact with the Fe uptake transporter, and thus be taken up by the intestinal epithelial cell, the first step in Fe absorption, and the primary factor in Fe bioavailability. For this Fe uptake to occur, AA must be present in the reduced form in relatively equal or slightly higher molar ratio than the Fe. AA also counteracts the presence of inhibitory compounds present in the food matrix or diet, such phytic acid, citric acid or polyphenols which can complex Fe and compete with the uptake transporter for the available Fe - depending on their concentration and the AA levels. It is the net sum of the above effects that ultimately controls Fe bioavailability.

Given that the food product matrix is liquid, the Fe bioavailability is primarily a function of the amount of added Fe, the solubility of the form of the Fe in the FJB matrix (ie. MIP vs.

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NaFeEDTA), the relative amount of AA, and the relative amounts of other factors in the FJB such as citric acid, polyphenols or other unknowns. Although not analyzed in this study, phytic acid is unlikely to be present in any significant amount in the FJB. Polyphenols were not measured in this study, but given the results, inhibitory polyphenols do not appear to be present in any significant amounts [12, 13].

Overall fortifying with 15% RDA of Fe and with a molar AA:Fe ratio equal to 1 is good option for the following reasons:

Although adding 20% RDA of Fe resulted in more absorbable iron, this amount may be infeasible as it could affect the organoleptic quality of the juice. Also, with more Fe, additional AA would need to be added to maintain the benefit over the shelf life of the product, and this may be a challenge in India given the regulatory restrictions which limit addition of AA to ≤40 mg per serving [15].

Shelf life is an important consideration with addition of AA, as factors such as time, temperature, pH and oxygen can play a role in oxidation of AA and thus reduce levels of AA and hence Fe bioavailability [16]. Thus, sufficient overage of AA must be added to maintain the molar AA:Fe level at a level greater than or equal to 1. In the present study the AA was added to the samples immediately before the start of the experiment; hence there was little time for AA degradation to occur. Of note, the intrinsic AA level in the unfortified juice sample was not measured but the FJB is not expected to deliver any significant amount of intrinsic AA.

The NaFeEDTA form of Fe yielded a similar Fe bioavailability relative to the MIP form when the AA:Fe ratio was 0.6; but when the AA:Fe ratio was increased to 1.0 the Fe uptake from the NaFeEDTA formulation was significantly higher, even more than when additional Fe and AA was added to the MIP formulation (Figure 1; samples 8 versus 16). Using this form of Fe may be infeasible due to cost and/or potential formulation issues. One should also consider that the MIP form adds substantial bioavailable Fe to the FJB, thus it is a strong nutritional product for delivering more Fe based on the fortificant levels explored in the present study.

Finally, in regards to the FBC and FWB, given the cereal is solid and the FJB and FWB are liquids, relative comparisons should only be useful based on the amount of Fe present in the in vitro digestion. For the FBC, the Fe bioavailability was quite high, relatively equal to the highest values observed with the FJB formulations. However, the FBC also had Fe added at a concentration of 25% RDA, substantially more than the FJB formulations. The lower Fe bioavailability from the FWB may be partially attributed to it not containing any AA.

In conclusion, the present study clearly defines a target formulation of 15% RDA Fe with a molar AA:Fe ratio ≥1 that enhances Fe bioavailability, and suggests the FJB maybe an appropriate vehicle to deliver bioavailable Fe to the Indian population where prevalence of Fe deficiency is a significant public health concern.

Conflict of Interest and Funding Disclosure

Ethical Approval: NA

Clinical Trial registration: NA

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