Original Communication

Use of White Beans instead of Red Beans May Improve Iron Bioavailability from a Tanzanian Complementary Food Mixture

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Abstract: In the study presented, an in vitro digestion/Caco-2 cell culture model was used to assess the amount of bioavailable iron from a modified Tanzanian complementary food formulation. The main objective of the study was to determine whether a change from red beans to white beans in the complementary food recipe would improve iron bioavailability from the mixture, as recent studies had indicated that iron bioavailability in white beans is significantly higher compared to that in the colored beans. The white beans had a significantly higher ($p<0.0001$) amount of ferritin formation (13.54 ng/mg) when compared to all other porridge ingredients including the red beans (2.3 ng/mg), and it is plausible that the complementary food formulated with the white beans may be superior to that formulated with the red beans, with reference to iron bioavailability. The results are important as they suggest that substitution of complementary food ingredients with high anti-nutrient concentrations with those that have lower anti-nutrient concentrations may improve iron bioavailability from complementary food home-recipes.

Key words: Tanzanian complementary food; iron bioavailability, iron; in vitro digestion/Caco-2 model

Introduction

Inadequate intake of bioavailable iron among African children is a consequence of a dependence on monotonous cereal-based complementary foods that are rich in inhibitors of iron absorption [1,2]. Traditionally, plant-based complementary foods are prepared from high-extraction cereal flours like sorghum and finger millet that are rich in phytic acid and polyphenolic compounds. These compounds are known to complex with iron and prevent its absorption. Hence other approaches that involve germina-

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tion and/or fermentation have been used to improve iron bioavailability from these foods [3,4].

Eliminating phytic acid from foods has been the primary strategy of increasing iron availability in plant-based complementary foods. This is because its structure gives it the ability to bind essential minerals, such as iron and zinc, resulting in lower absorption of these elements [5]. Also, the anti-nutritional component of phytic acid is found in many complementary food ingredients such as cereals, seeds, and beans. Thus, processes that result in the enzymatic and/or non-enzymatic hydrolysis of phytic acid from cereal and legume ingredients used in the preparation of complementary foods have been employed so as to improve iron bioavailability in such foods that are consumed by infants in developing countries [6,7].

In a recent study by Mbithi-Mwikya *et al.* [8], a common African complementary food composed of finger millet, kidney beans, peanuts, and mango was formulated and processed to reduce phytic acid. This was done by germination and fermentation of the finger millet and kidney beans. As reported, phytates present in the mixture were reduced by 81% and tannins to undetectable limits. A follow-up to this study was a double-blind, randomized, placebo-controlled trial reported by Mamiro *et al.* [9], which was conducted in Kilosa district, Tanzania with infants 6 to 12 months old. In this study, no effect on iron status was observed between the processed complementary food and the unprocessed food even though processing reduced phytates by 34% and improved iron solubility by 19%. The lack of improvement in iron status for infants who received the processed complementary food was attributed to the low iron content of the complementary food and partly to residual phytates.

The efforts by Mbithi-Mwikya *et al.* [8] highlight the opportunities available for designing a low-cost complementary diet for resource-poor settings using locally available foods. But it is vital that the iron bioavailability potential of these foods be improved. Therefore, the question of how to make these foods suitable for the complementary feeding period, with reference to iron adequacy while minimizing costs, still remains. The goal of this study was therefore to analyze whether a low-cost dietary modification would further enhance iron bioavailability from the complementary food. Recent studies on white and colored beans such as that documented by Hu *et al.* [10] indicate that iron bioavailability from white beans is significantly higher compared to that of colored beans. Therefore based on these observations, our objective was to determine if replacing the red beans in the complementary food formulation with white beans would improve iron bioavailability from the complementary food as assessed in the Caco-2 cell model. In addition, we also assessed whether using a different strain of bacteria for the fermentation process would further improve iron bioavailability from the complementary foods.

Materials and Methods

Food Samples

Finger millet (*Eleusine coracana*) and peanuts (*Arachis hypogoea*) were purchased from a supermarket in Kenya and light-red kidney beans (*Phaseolus vulgaris*), white navy beans (*Phaseolus vulgaris*), and mangos (*Mangifera indica*) were purchased from a grocery shop in Ithaca, New York.

Formulation and processing of the ingredients of the complementary food

The complementary food was formulated and the ingredients prepared as described previously by Mbithi-Mwikya *et al.* [8]. For the fermentation process, in addition to the *Lactobacillus* fermentation reported by Mbithi-Mwikya *et al.* [8], *Bifidobacteria* fermentation was also carried out as a separate treatment.

The different complementary food samples and treatments are summarized in Table I. The proportion of iron (in percentage) contributed by each ingredient to the unprocessed complementary food recipe is also shown in Table I. In the case of beans, the proportion of iron contributed by white beans is shown in parenthesis next to the proportion contributed by red beans. Because sample A and B did not undergo any germination or fermentation, they are sometimes referred to in the text as unprocessed while the rest of the samples are referred to as processed.

Table II gives more detailed differences between the samples. Beans type column refers to the color of the beans used in the formulation of the complementary foods as reported by Mbithi-Mwikya *et al.* [8], and treatment refers to the germination and fermentation processes employed to promote hydrolysis of phytate in the finger millet and beans used in the complementary food mixture. The difference between recipes was the beans type used – either red

Table I: Summary of the complementary food recipe tested in Experiment 2

Complementary food ingredients and the proportion of iron they contribute to the porridge recipe				
CF Samples	Finger millet	Beans	Peanuts	Mango
A,B (g)	13.00	4.40	1.60	1.00
C,D,E,F,G,H,J,K (g)	13.04	3.82	1.60	1.54
Iron proportion (%)	75.17	20.39 [19.32]	4.39	0.42

Note: CF samples = Complementary food samples coded A-K for ease of reference

Table II: Summary of the differences and similarities between the complementary food samples tested in Experiment 2

CF	Beans type		*Treatment*
A	RED		*NONE*
B		WHITE	*NONE*
C	RED		*Germination and L. amylovorous fermentation*
D		WHITE	*Germination and L. amylovorous fermentation*
E	RED		*Germination L. salivarius fermentation*
F		WHITE	*Germination L. salivarius fermentation*
G	RED		*Germination B. breve fermentation*
H		WHITE	*Germination B. breve fermentation*
J	RED		*Germination B. infantis fermentation*
K		*WHITE*	*Germination B. infantis fermentation*

Note: CF= Complementary food samples coded A-K for ease of reference

or white and recipe pairs are similar in the type of treatment they undergo as shown on Table II. For example, recipes A and B are different based on the type of beans in the complementary food but similar because they both do not undergo germination or fermentation.

Experiment 1

The purpose of this experiment was to compare iron bioavailability from the individual ingredients that would be used to formulate the complementary food. Therefore, 1.0 g dry weight of lyophilized and ground samples of each ingredient was compared via the *in vitro* model for amount of bioavailable iron.

Experiment 2

The purpose of this experiment was to determine the effects of the "processing" methods – germination and fermentation -on the amount of bioavailable iron from the processed and unprocessed complementary foods. These food samples contain the mixture of complementary food ingredients (i. e. finger millet, beans, peanuts, and mangos) defined in Table I. Therefore, 20 g of the dry flour mixture was cooked in 200 mL of water for 20 minutes at 100 °C and the resulting porridges assessed for amount of bioavailable iron.

Mineral analysis

Mineral analysis of the samples was conducted by inductively coupled plasma-emission spectroscopy (ICAP; ICAP model 61E Trace Analyzer; Thermo Jarrell Ash Corporation, Waltham MA). 0.3 g dry sample was weighed into borosilicate glass test tubes and chemically digested using 1 mL of 100 % HNO_3 at 120 °C. After the samples were completely dry, a further 1 mL of 100 % HNO_3 was added at 150 °C until the residue was light brown to yellow in color. Then 1 mL of HNO_3:$HClO_4$ at 1:1 volume ratio was added and the temperature increased to 180 °C for 2 hours, and then to 240 °C until the digested samples were dry. Samples were later resuspended in 5 % (v/v)

HNO_3 before analysis on the inductively coupled plasma-emission spectrometer (ICP) [11–13].

Phytate analysis

Phytate was analyzed using acidic extraction of the dry samples, followed by liquid chromatography [14]. Samples were analyzed with a Dionex Liquid Chromatograph System (Dionex Corp., Sunnyvale, CA) using PO_4 and phytate standards (IP_5 and IP_6) dissolved in 0.125% (v/v) H_2SO_4. The results (IP_6 only) are expressed as μmole of phytate per g (DW).

Chemicals, enzymes and hormones

Unless otherwise stated, all chemicals, enzymes, and hormones were purchased from Sigma Chemicals Co. (St. Louis, MO).

Cell Culture

Caco-2 cells were obtained from the American Type Culture Collection (Rockville, MD) at passage 17 and used in experiments at passage 25–33. Cells were seeded at a density of 50,000 cells/cm^2 in collagen-treated, six-well plates (Costar Corp., Cambridge, MA). The cells were grown in Dulbecco's Modified Eagle Medium (GIBCO, Grand Island, NY) with 10% v/v fetal calf serum (GIBCO), 25 mmol/L N-(2-Hydroxyethyl) piperazine-N'-2-ethane sulfonic acid (HEPES), and 1% antibiotic antimycotic solution (GIBCO). The cells were maintained at 37 °C in an incubator with a 5% CO_2/95% air atmosphere at constant humidity, and the medium was changed every 2 days. The cells were used in the iron uptake experiments at 13 days post-seeding.

In Vitro Digestion

The preparation of the digestion solutions – pepsin, pancreatin, and bile extract – and the *in vitro* digestion was performed as previously published [15]. Exactly 1.0 g of dry samples or 1.0 mL of liquid samples was used for each sample digestion.

Harvesting

Harvesting of the cell monolayers was performed as previously published [15].

Cell Protein Analysis

Caco-2 cell protein was measured from samples that had been solubilized in 0.5 mol/L NaOH, using a semimicro adaptation of the Bio-Rad DC protein assay kit (Bio-Rad Laboratories, Hercules, CA). A 25 μL sample of the sonicated Caco-2 cell monolayer, harvested in 2 mL of water, was used for each protein measurement expressed in mg.

Ferritin Analysis

A one-stage, two-site immunoradiometric assay was used to measure Caco-2 cell ferritin content (FER-Iron II Ferritin Assay, RAMCO Laboratories, Houston, TX). A 10 μL sample of the sonicated Caco-2 cell monolayer, harvested in 2 mL of water, was used for each ferritin measurement expressed per unit cell protein (ng ferritin/mg cell protein).

All glassware used in the sample preparation and analyses was acid-washed.

Statistics

Statistical analyses of the data were performed using the software package GraphPad Prism v4 (GraphPad Software, San Diego, CA) and *JMP* v6.0 (SAS Institute Inc, Cary NC). Means were considered to be significantly different if p values were ≤ 0.05.

Results and Discussion

Iron and phytate concentration

The measured iron concentration, phytate concentration (IP_6 only), and phytate:iron molar ratio of the complementary food ingredients are summarized in Table III. As seen in Table III, finger millet had the highest iron concentration, which may explain why finger millet forms the bulk of the complementary food recipe. Also, as seen in Table III, the red and white beans had very similar iron concentrations, followed by peanuts and mango. With regard to phytate concentration, peanuts had the highest

Table III: Iron concentration, phytate concentration, and phytate: iron molar ratio of the dry ingredients used in the formulation of the complementary foods

Porridge ingredient	Iron (μg/g)* Mean ± SD	IP_6 (μmoles/g)* Mean ± SD	Molar ratio (IP_6)/Iron
Finger millet	74.53± 3.77	5.93 ± 0.45	4.40:1
Red beans	59.73± 1.81	11.51± 0.50	10.80:1
White beans	56.61± 1.64	11.61 ± 0.44	11.50:1
Mango	5.37± 0.12	0.32 ± 0.06	3.30:1
Peanuts	35.36± 2.12	11.99 ± 0.56	18.90:1

* $n = 3$ for iron and phytate
SD = Standard Deviation

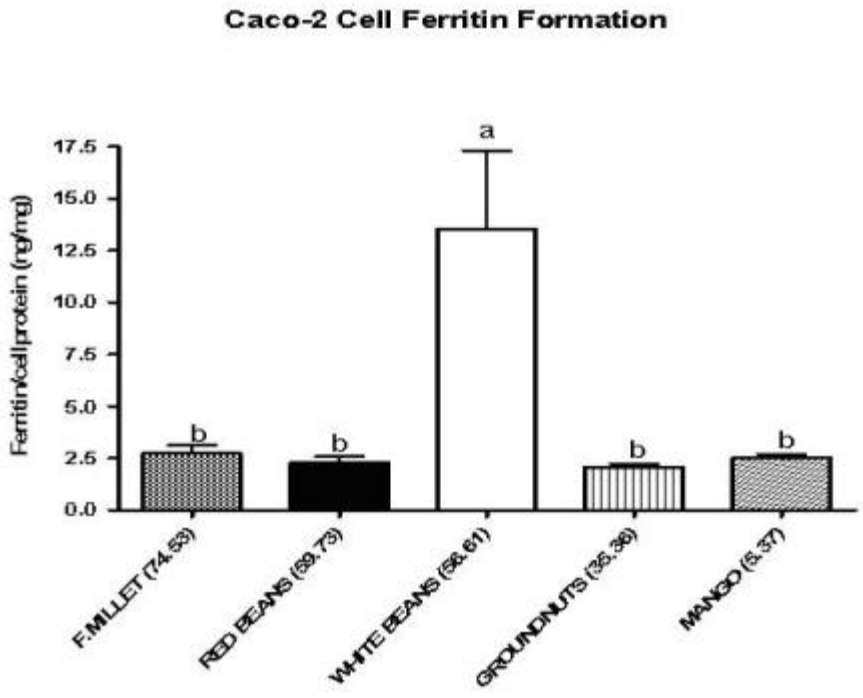

Figure 1: Experiment 1: Caco-2 cell ferritin formation in response to lyophilized, ground samples of individual ingredients used to formulate the complementary food mixture. Ferritin formation in the cells is a measure of iron uptake and thus an index of bioavailability. 1 g (DW) of each sample was analyzed in the model and the resulting mean ferritin values were log-transformed to better approximate a normal distribution. Mean comparison for all pairs as seen above, was done using Tukey-Kramer HSD. Bar values (mean ± SEM, n = 6) with no letter in common are statistically different ($p<0.05$). On the x-axis, values in parentheses indicate the concentration of iron (μg/g) in each sample.

values, followed by white beans, red beans, finger millet, then mango.

Experiment 1

Figure 1 shows ferritin formation in cells treated with 1.0 g of dry complementary food ingredients. It is clear from the graph that white beans had a significantly higher amount of bioavailable iron when compared to all the other ingredients ($p<0.0001$). It is important to note that the iron content and phytate concentration of the red and white beans were very similar, yet there was a big difference in the amount of bioavailable iron from these beans. As explained by Hu *et al.* [10] this difference could be attributed to a lack of certain polyphenols such as kaempferol and quercitrin (which are strong inhibitors of iron bioavailability) in the seed coat of the white beans. Also, although finger millet had the highest iron concentration compared to the other complementary food ingredients, it had very low amounts of bioavailable iron, which can also be attributed to phytate and polyphenols [16].

Table IV: Summary of two-way ANOVA test for significant difference between type of beans, treatment types, and their interaction

Source	Degrees of Freedom	Sum of Squares	F Ratio	p-value
beans	1	11.217029	37.9431	<.0001
treatment	4	3.021125	2.5548	0.0502
beans *treatment	4	1.060401	0.8967	0.4730

Note: Statistical Analysis done using *JMP* v6.0.3

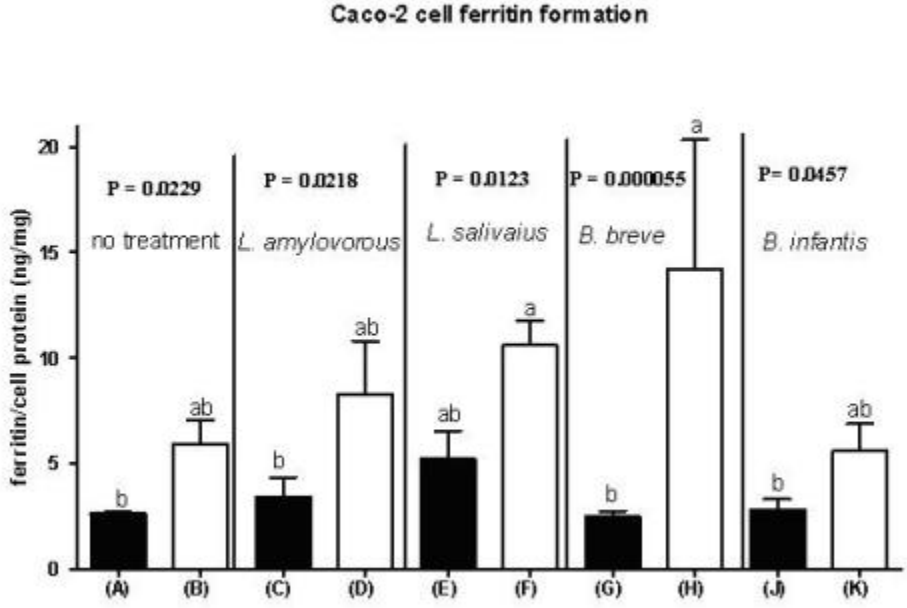

Figure 2: Experiment 2: Amount of bioavailable iron in complementary food samples (A-K) assessed using the Caco-2 cell model. Black bars represent red beans samples and white bars, white beans samples. Type of microbe used for fermentation for each pair of samples is shown above the bars. One mL of each porridge was analyzed in the model and the resulting mean ferritin values were log-transformed to better approximate a normal distribution. Ferritin formation in the cells is an index of iron bioavailability. Mean comparison, and comparisons for all pairs were done using Tukey-Kramer HSD. Paired-contrast p-values are also reported. Bar values (mean ± SEM, n = 6) with no letter in common are statistically different ($p<0.05$).

Experiment 2

Figure 2 shows the results of a one-way ANOVA and mean comparison for all pairs done using the Tukey-Kramer Honestly Significant Difference (HSD) test. As seen from the graph, the porridge recipes F (white beans, *L. salivarius*) and H (white beans, *B. breve*) were significantly different ($p<0.0001$) from the following porridge recipes- A (red beans, no treatment), C (red beans, *L. amylovorus*), G (red beans, *B. breve*), and J (red beans, *B. infantis*).

A two-way analysis of variance (see Table IV) was carried out to determine if there were statistically significant differences between the beans, the treatments, and the interaction between the beans and treatments. Overall, it is clear that the amount of bioavailable iron is significantly different between the beans ($p<0.0001$), but not between the treatments ($p=0.0502$) or the interaction of the two ($p=0.4730$). Hence, most of the differences between the porridges are due to the type of beans (red or white).

The paired-contrasts in Figure 2 further expound on the significant differences between the complementary food samples with similar treatments but formulated using different type of beans – the red beans or white beans. As shown in Figure 2, the amount of bioavailable iron from the complementary food samples formulated with the white beans was consistently higher compared to those that had the

red beans. The highest statistically significant difference occurred between the complementary food mixture samples G (red beans, *B. breve*) and H (white beans, *B. breve*) ($p<0.0001$). This suggests that using *B. breve* for the fermentation process when preparing the complementary food ingredients may further improve iron bioavailability from the complementary food recipe formulated by Mbithi-Mwikya *et al.* [8].

Based on the results, we can conclude that the type of beans – either red or white- accounted for most of the significant differences in the amount of bioavailable iron between the porridges, with the white-bean porridges having higher amounts of bioavailable iron compared to the red-bean porridges. These results would be consistent with the *in vitro* studies by Hu *et al.* [10] that compared iron bioavailability between the white and colored beans and showed that the white beans contained higher levels of bioavailable iron mainly because of lower inherent polyphenols such as kaempferol. Thus in addition to fermentation and germination of the finger millet and beans, a change from red to white beans in the complementary food recipe formulated by Mbithi-Mwikya *et al.* [8] may further enhance iron bioavailability from the recipe.

In addition, it is also important to note that finger millet, which constitutes about 65 % of the complementary food recipe, has relatively high iron content but low amounts of bioavailable iron mainly due to both phytate and polyphenols [16]. Increasing its iron bioavailability potential will further increase the amount of bioavailable iron from the complementary food recipe. Therefore, future studies would do well to profile the polyphenolic compounds in finger millet that may influence iron bioavailability, and to look for additional ways to reduce or eliminate their effect on iron bioavailability in the food matrix. Studies on processes (in addition to germination and fermentation) that can further reduce phytates should also be encouraged.

The most important question in this analysis is whether the differences in the amount of bioavailable iron seen between the red and white beans as formulated in this complementary food mixture would result in physiological differences or benefits in infants. As highlighted by the data presented in this paper and in retrospect to the article by Mamiro *et al.* [9], a human trial would be very useful as it could reveal the extent to which the complementary food formulated with the white beans is physiologically superior to that formulated with the red beans, with reference to iron bioavailability.

Conclusion

Simple technologies aimed at improving the nutritional quality of complementary foods have recently been popularized in many low-resource areas in developing countries, and widely used to reduce or eliminate anti-nutrients such as phytate and polyphenols present in cereals and legumes used to prepare infant foods [17]. The advantage of processes such as germination and fermentation is that they can be adapted to local conditions and easily practiced at the household level [18]. However, in some cases, degradation of phytic acid and/or polyphenols alone does not result in improved iron bioavailability as is evident in the study by Mamiro *et al.* [9]. Thus, other approaches such as the substitution of ingredients with high anti-nutrient concentration with those that have lower anti-nutrient concentration may help improve iron bioavailability from plant-based complementary foods. As shown by the data in this experiment, substituting red beans with white beans in the recipes resulted in significantly higher amounts of bioavailable iron *in vitro*. That said, strategies to improve the bioavailability of iron in complementary foods consumed in developing countries would probably be sufficient to overcome the deficits if additionally iron-rich foods and/or iron promoters such as ascorbic acid are incorporated into home recipes, or if other forms of iron fortification are employed [19].

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