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Vitamins and Minerals

Bioavailable Iron and Vitamin A in Newly Formulated, Extruded Corn, Soybean, Sorghum, and Cowpea Fortified-Blended Foods in the In Vitro Digestion/Caco-2 Cell Model

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Abstract

Background: Fortified-blended foods (FBFs), particularly corn-soybean blend (CSB), are food aid products distributed in developing countries. The US Agency for International Development food aid quality review recommended developing extruded FBFs with the use of alternative commodities such as sorghum.

Objective: The objective of the study was to determine bioavailable iron and vitamin A content from newly developed extruded corn, soybean, sorghum, and cowpea FBFs compared with the nonextruded traditional food aid FBFs, corn-soy blend 13 (CSB13) and corn-soy blend plus (CSB+).

Methods: Eleven extruded FBFs—sorghum-cowpea ($n = 7$), sorghum-soy ($n = 3$), and corn-soy ($n = 1$)—along with 2 nonextruded FBFs—CSB13 and CSB+, and Cerelac (Nestlé), a commercially available fortified infant food, were prepared. Bioavailable iron and vitamin A contents were assessed by using the in vitro digestion/Caco-2 cell model. Dry FBFs, aqueous fractions, and Caco-2 cell pellet vitamin A contents were analyzed by HPLC. Dry FBF and aqueous fraction iron contents were measured by atomic absorptiometry, and bioavailable iron was assessed by measuring Caco-2 ferritin contents via ELISA.

Results: Iron and vitamin A concentrations in Cerelac and dry FBFs ranged from 8.0 to 31.8 mg/100 g and 0.3 to 1.67 mg/100 g, respectively. All of the extruded FBFs contained 4- to 7-fold significantly higher ($P < 0.05$) aqueous fraction iron concentrations compared with CSB13 and CSB+. However, there were no significant differences in Caco-2 cell ferritin and vitamin A concentrations between extruded FBFs, nonextruded FBFs, and or the basal salt solution negative control.

Conclusion: Results support the theory that the consumption of newly developed extruded sorghum-cowpea, sorghum-soy, and corn-soy FBFs would result in iron and vitamin A concentrations comparable to traditional nonextruded CSB13 and CSB+ FBFs.
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Introduction

Fortified-blended foods (FBFs) are porridge mixes composed of cereals and legumes that have been milled and fortified with vitamins and minerals. FBFs are major food aid products for young children, women, and other vulnerable groups in developing countries. Historically, corn-soy blend (CSB) has been the most widely distributed FBF in a majority of the food aid–receiving countries (1). The US Agency for International Development (USAID) Food Aid Quality Review (FAQR) recommended developing novel FBFs using cereals that are both culturally and nutritionally acceptable in Africa. It also recommended sorghum as an alternative to corn or wheat and suggested other legumes could be paired with it as alternatives to soy (2). One logical legume to investigate is cowpea, because Africa is the world's leading producer of cowpea (95%)

Keywords: fortified-blended food, corn-soy blend plus, micronutrient bioavailability, iron, vitamin A, whey protein concentrate, food aid, Title II foods, in vitro digestion/Caco-2 cell model

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Abbreviations used: AAS, atomic absorption spectrophotometer; BHT, butylated hydroxytoluene; CSB, corn-soy blend; CSB+, corn-soy blend plus; FAQR, Food Aid Quality Review; FBF, fortified-blended food; NaFeEDTA, sodium iron EDTA.

in addition to sorghum (41%) (3). Both sorghum and cowpea are drought-tolerant, sustainable, and non–genetically modified grains, which is preferred by some food aid–recipient nations.

Sorghum and cowpea are rich in iron and are complementary proteins (4, 5); however, sorghum and cowpea also contain the antinutritional factors phytates and tannins, which impair iron bioavailability (6–9). Extrusion is a food-processing technique that cooks food with the use of high temperature under high pressure in combination with moisture and mechanical shear (10). The desirable effects of this cost-effective method are that it decreases viscosity; increases palatability, starch, and protein digestibility; and reduces antinutritional factor contents, thereby potentially improving iron bioavailability (11, 12).

Extruded novel sorghum-cowpea, sorghum-soy, and corn-soy FBFs were developed based on the USAID FAQR recommendations (2) and USDA commodity requirements (13, 14) as part of the Micronutrient Fortified Food Aid Pilot Project (15). Traditional, nonextruded FBFs, CSB13 and ACB plus (CSB+), were procured to use as comparisons for the newly developed FBFs. The purpose of this study was to assess bioavailable iron and vitamin A concentrations of newly developed extruded sorghum-cowpea blend, sorghum-soy blend, and CSB14 FBFs compared with traditional nonextruded FBFs, CSB13 and CSB+, in the in vitro digestion/Caco-2 cell model. These micronutrient deficiencies were chosen because they are a substantial public health issue for many women and children throughout the world (16). The in vitro digestion/Caco-2 model was used because it is a widely used, inexpensive model to study the bioavailability of nutrients from foods and supplements (17–21). It has been successfully used to screen for iron bioavailability of a variety of complementary foods (22), lentils (23), wheat (24), cassava (25), and supplemented food stuffs (26). We use the term "bioavailable" to describe the amount of compound in the Caco-2 cells after they have been treated with aqueous fractions produced by in vitro digestion. To the best of our knowledge, this is the first study to use this model to assess both bioavailable iron and vitamin A.

Methods

Chemicals

Unless stated otherwise, all reagents were purchased from Sigma-Aldrich or Fisher Scientific. Double deionized water was used for porridge preparation, in vitro digestion, reagent preparation, and vitamin A extraction. To prevent iron contamination, glassware used in the sample preparation, in vitro digestion, and iron analysis was acid washed by soaking in 5% nitric acid solution for no less than 12 h and rinsing with double deionized water before use. Acetonitrile, methanol, chloroform, hexane, and ethanol were HPLC grade.

FBF formulations

Extruded sorghum-cowpea ($n = 7$), sorghum-soy ($n = 3$), and corn-soy ($n = 1$) were formulated on the basis of FAQR requirements (2). Two white (variety 1, Fontanelle 4575; variety 2, 738Y) and 1 red (217X Burgundy) sorghum varieties, whole or decorticated, were used in producing extruded sorghum-cowpea FBFs, and cowpea flour was sourced commercially (**Tables 1** and **2**). Extruded sorghum-soy FBFs contained white sorghum variety 1 (Fontanelle 4575), whole or decorticated, with low-fat (1.85%), medium-fat (6.94%), or full-fat (16.93%) soy. Extruded

TABLE 1 Composition of extruded FBFs[1]

Ingredient	SCB, % ($n = 7$)	SSB, % ($n = 3$)[2]	CSB14, % ($n = 1$)
Sorghum flour	24.7	47.6	—
Cowpea flour	38.6	—	—
Corn flour	—	—	48.1
Soy flour	—	15.7	15.2
Sugar	15.0	15.0	15.0
WPC 80	9.5	9.5	9.5
Vegetable oil	9.0	9.0	9.0
Vitamin and mineral premix	3.2	3.2	3.1

[1]CSB14, corn-soy blend 14; FBF, fortified-blended food; SCB, sorghum-cowpea blend; SSB, sorghum-soy blend; WPC, whey protein concentrate.
[2]For extruded SSB (full-fat soy), WPC 80 was increased from 9.5% to 13% and vegetable oil was decreased from 9% to 5.5%.

CSB14 was formulated with degermed corn with medium-fat soy. The other FBF components—sugar, oil, whey protein concentrate with 80% protein content, and vitamin-mineral premix—were added after extrusion to prevent destruction of micronutrients. Both nonextruded FBFs, CSB13 and CSB+, were purchased from Bunge Milling. The major difference between these 2 CSBs is that CSB+ is a more-recently released CSB, with heat-processed corn and soy and improved micronutrient formulation (27). Cerelac (Nestlé), a commercially available fortified infant food, was purchased from a local store and included as a reference control as has been done previously (22). It is worth noting that the iron and vitamin A fortificants differed between Cerelac and extruded and nonextruded FBFs. Extruded FBFs contained ferrous sulfate ($FeSO_4$) and sodium iron EDTA (NaFeEDTA), CSB+ contained ferrous fumarate and NaFeEDTA, and CSB13 and Cerelac contained only ferrous fumarate (**Table 3**). Extruded and nonextruded FBFs contained retinyl palmitate, whereas Cerelac contained retinyl acetate. All of the FBFs were stored at −20°C in zip-lock bags covered with aluminum foil.

FBF porridge preparation

Twenty grams of dry FBF or Cerelac was slowly added to 80 g of boiling water in a beaker on a hot plate and stirred vigorously for 2 min, removed from the hot plate, and stirred for another minute. Nonextruded CSB13 and CSB+ were prepared in a similar manner, although 11.75

TABLE 2 Composition of nonextruded FBFs and Cerelac[1]

Ingredient	Nonextruded FBFs, %		Cerelac ingredients[2]
	CSB13	CSB+	
Cornmeal	69.5	—	Wheat flour
Soy flour, defatted	21.9	—	Fat-free milk
Soybean oil, refined	5.5	—	Sugar
Minerals	3.0	—	Milk fat
Vitamin antioxidant premix	0.1	—	Corn oil
Corn (white or yellow)	—	78.5	Palm oil
Whole soybeans	—	20.0	Calcium carbonate
Vitamin/mineral	—	0.2	Sodium phosphate
Tricalcium phosphate	—	1.2	Bifidus cultures
Potassium chloride	—	0.2	Vitamin/mineral

[1]CSB+, corn-soy blend plus; CSB13, corn-soy blend 13; FBF, fortified-blended food.
[2]For Cerelac (Nestlé), ingredient percentages were not available; ingredients are listed in the order on the label.

TABLE 3 FBFs and Cerelac macronutrient and micronutrient compositions (100 g)[1]

Nutrient	Extruded FBFs SCB/SSB/CSB14	Nonextruded FBFs CSB13	Nonextruded FBFs CSB+	Cerelac[2]
Energy, kcal	380	386	380	400
Protein, g	17	16	14	13
Fat, g	8	9	6	10
Vitamin A[3], mg	0.49	0.82	1.04	0.34
Thiamin (vitamin B-1), mg	0.65	0.61	0.20	0.28
Riboflavin (vitamin B-2), mg	0.93	0.48	1.4	0.8
Niacin (vitamin B-3), mg	9.1	6.3	8.0	2.4
Pantothenic acid (vitamin B-5), mg	3.7	3.2	1.6	1.3
Vitamin B-6, mg	0.8	0.5	1.0	0.3
Folic acid (vitamin B-9), mg	0.09	0.25	0.11	0.05
Vitamin B-12, mg	0.002	0.001	0.002	0.001
Vitamin D, mg	0.03	0.005	0.01	0.004
Vitamin E, mg	13.2	1.0	8.3	3.0
Vitamin K, mg	0.03	0.001	0.03	0.03
Vitamin C, mg	40.0	40.0	90.0	16.0
Calcium, mg	279.1	650.0	452.0	427.0
Total iron (Fe), mg	13.0	10.6	6.5	5.3
$FeSO_4$, mg	11.0	0.0	4.0	0.0
NaFeEDTA, mg	2.0	0.0	2.5	0.0
Ferrous fumarate, mg	0.0	10.6	4.0	5.3
Iodine, mg	0.23	0.0	0.04	0.03
Phosphorus, mg	291.0	522.0	290.0	427.0
Potassium, mg	163.2	563.0	140.0	NA
Zinc, mg	5.5	5.9	5.0	2.1

[1]CSB, corn-soy blend; CSB+, corn-soy blend plus; FBF, fortified-blended food; NA, not available; NaFeEDTA, sodium iron EDTA; SCB, sorghum-cowpea blend; SSB, sorghum-soy blend.
[2]For Cerelac (Nestlé), macronutrient and micronutrient values were calculated on the basis of the percentage Daily Value information provided on the label, and daily values for children aged <4 y were provided by reference 28.
[3]Extruded and nonextruded FBFs contained vitamin A in the form of retinyl palmitate, whereas Cerelac contained retinyl acetate.

and 13.79 g dry FBF, respectively, were used and they were cooked for 10 min on a hot plate following the preparation instructions for CSB+ (13, 14). Porridges were then covered with aluminum foil and kept in a water bath at 37°C for 10 min to prevent skin formation. Porridges were then weighed, and water lost during preparation was added back in to bring the final weight to 100 g. Porridges were transferred to 50-mL polypropylene tubes, covered in aluminum foil, blanketed with nitrogen, sealed, and stored at −80°C. Porridges were prepared in duplicate on 2 different days (4 replicates). Later, these replicates were used for in vitro digestion/Caco-2 cell experiments.

In vitro digestion

Porridge aliquots were subjected to in vitro digestion as described previously (29). Ten milliliters of basal salt solution (120 mmol NaCl/L, 5 mmol KCl/L, and 6 mmol $CaCl_2$/L) was added to 2.5 g of thawed FBF in a beaker, homogenized with a laboratory homogenizer for 2 min, and then mixed on a magnetic stir plate for 5 min. Ten-milliliter aliquots of homogenized FBFs were then subjected to 3 continuous in vitro digestion phases: 10-min oral, 1-h gastric, and 2-h small intestine.

Oral digestion. Saliva solution containing 0.9 mg KCl, 0.89 mg $NaPO_4$, 0.57 mg $NaSO_4$, 0.3 mg NaCl, and 1.69 mg $NaHCO_3$/mL deionized water was prepared and used for all experiments. Ten milliliters of homogenized FBF solution and 8 mL of freshly prepared artificial saliva [uric acid (0.015 mg/mL), urea (0.2 mg/mL), mucin (0.025 mg/mL), and α–amylase (10.55 mg/mL) dissolved in saliva solution] were added to a 50-mL conical tube. Tubes were mixed well, blanketed with nitrogen, sealed with parafilm, and incubated placing horizontally in a shaking water bath at 37°C at 85 rpm for 10 min in the dark.

Gastric digestion. After oral digestion, the digesta pH was decreased to 2.5 ± 0.1 by slowly adding 1 M HCl and then 2 mL of freshly made pepsin solution (40 mg/mL in 100 mM HCl) was added. The final volume was then adjusted to 40 mL with the basal salt solution, blanketed with nitrogen, sealed with parafilm, and incubated in a shaking water bath at 37°C at 85 rpm for 1 h in the dark.

Small intestinal digestion. The gastric phase was terminated by increasing the digesta pH to 6.0 ± 0.1 with 1 M $NaHCO_3$ and placing the tubes on ice. Two milliliters of pancreatin (10 mg/mL) and lipase (5 mg/mL) solution (both in 100 mM $NaHCO_3$) was then added along with 3 mL of bile extract (40 mg/mL 100 mM $NaHCO_3$), and the digesta pH was adjusted to 6.5 ± 0.1 with 1 M NaOH. The final volume was adjusted to 50 mL with the basal salt solution, blanketed with nitrogen, sealed with parafilm, and incubated in a shaking water bath at 37°C at 85 rpm for 2 h in the dark.

Isolation of aqueous fraction from digesta

After small-intestine digestion, 10-mL digesta aliquots were transferred to 15-mL polypropylene tubes and centrifuged at 5000 × *g* for 45 min at 5°C. Supernatants were collected by puncturing the side of the tube with an 18-gauge needle and 10-mL syringe without disturbing the pellet.

Supernatants were filtered by using 0.22-μm syringe filters (SLGP 033 RS; Millipore), and fresh aqueous fractions were used to treat the Caco-2 cells. Aqueous fractions aliquots were blanketed with nitrogen and stored at –80°C for later analysis (29).

Dry porridge blend and aqueous fraction iron determination

Dry porridge blend and aqueous fraction iron contents were analyzed with the use of an atomic absorption spectrophotometer (AAS). Dry porridge blend iron contents were analyzed (American Association of Cereal Chemists 40–70.01, 1999) by the American Institute of Baking International Analytical Services (Manhattan, Kansas). Briefly, 10 g of sample was taken in ashing vessels and dried to ash overnight at 500°C in a muffle furnace. Residue was dissolved in 10 mL of concentrated hydrochloric acid, boiled, and evaporated to near dryness on a hot plate. The resulting residue was redissolved in 20 mL of 2 N HCl, filtered, and diluted to 100 mL with water. Iron concentrations were then measured on an AAS. Aqueous fraction iron concentrations, filtered samples, were directly measured on an AAS (AAnalyst 100; Perkin Elmer).

Caco-2 cell cultures

Caco-2 cells (ATCC HTB37) purchased from American Type Culture Collection were used in the experiment at passages 32 and 33. The cells were maintained at 37°C in an incubator with 5% CO_2/95% humidity, and media were changed every other day. Caco-2 cells were initially cultured in growth-enhanced treated T-75 flasks (TP 90076; Midsci) in the presence of DMEM (Gibco), supplemented with 15% FBS (Atlanta Biologicals), 1% L-glutamine, 1% nonessential amino acids, 1% antibiotic/antimycotic (penicillin/streptomycin) solution, and 0.2% amphotericin B (29). Confluent cells were subcultured by incubating with 5 mL of 0.25% trypsin-EDTA solution for 5 min, which was then inactivated by adding 10 mL of 15% DMEM. After trypsinization, cell suspensions were collected into 50-mL conical tubes and centrifuged at 129 × *g* for 5 min at room temperature, the supernatant media was discarded, and the cell pellet was collected. After resuspending the cell pellet and counting with a hemocytometer, cells were seeded at 50,000 cells/cm^2 in tissue culture–treated 6-well plates (Corning, Inc.). After being seeded at day 0, the cells usually became confluent 4–5 d later, at which point they were switched from media containing 15% FBS to 7.5% FBS to slow growth. Cells were used in the iron and vitamin A bioavailability experiments 14 d postseeding (30, 31).

Aqueous fraction Caco-2 treatment. On day 13, 1 d before the experiment, Caco-2 monolayers were provided fresh media. On day 14, media were removed before treating the cells with 0.25 mL fresh aqueous fraction and 1.75 mL DMEM for iron or 0.5 mL fresh aqueous fraction and 1.5 mL DMEM for vitamin A, which were then incubated for 12 (32, 33) or 4 (25, 26, 34) h, respectively. Samples were randomly assigned to wells; Cerelac was used as a reference control on each plate. A negative control was prepared with 0.25 mL of basal salt solution containing no iron and 1.75 mL DMEM. A ferrous sulfate (FeSo4) positive control was prepared with basal salt solution to provide 0.1 μg Fe/well or 0.2 μg Fe/well. These iron concentrations were selected to match with the estimated iron concentration in the digested FBF aliquots that were added to the Caco-2 cells. In vitro digestion and Caco-2 cell culture experiments were completed in duplicate on different days using different cells passages.

Caco-2 ferritin and protein determination. After incubation, treatments were removed and cells were washed with 2 mL of ice-cold 2× PBS. Caco-2 monolayers were lysed by adding 350 μL of mammalian protein extraction reagent/well (Thermo Fisher Scientific) (35) and incubated in 6-well plates for 10 min on a plate shaker at 120 rpm. Caco-2 monolayers were scraped with a cell scraper (Fisher Scientific), collected into microcentrifuge tubes, sonicated for 3 min, and centrifuged at 14,000 × *g* for 10 min at room temperature. Cell lysate supernatants were transferred to microcentrifuge tubes and stored at –20°C for ferritin and protein determination, which was completed within 24 h (21, 35, 36). Ten microliters of cell lysate solutions was used for determining ferritin concentrations (nanograms per milliliter) by using ELISA (Spectro Ferritin kit, S-22; Ramco Laboratories, Inc.), as done previously (23, 36). Twenty-five microliters of cell lysate solutions was used for measuring protein concentrations with the use of Pierce bicinchoninic acid protein assay kits. Ferritin content (nanograms per milligram of cell protein) was calculated as a ratio of cell ferritin (nanograms per milliliter) to cell protein (milligrams per milliliter) (36).

Harvesting Caco-2 monolayers for vitamin A assessment. After incubation, treatments were removed and cells were washed with 2 mL of ice-cold 2× PBS followed by 2 mL of ice-cold 2 g albumin/L in PBS. After discarding the washing solutions, Caco-2 monolayers were removed with the use of a cell scraper (Fisher Scientific) and collected into amber-colored microcentrifuge tubes using 1 mL ice-cold PBS. This process was repeated 2 additional times using 0.5 mL of ice-cold PBS, for a total of 2 mL PBS collected into the same microcentrifuge tubes and centrifuged at 327 × *g* for 45 min at 5°C. Supernatant PBS was discarded by carefully inverting the microcentrifuge tubes for 20 s. Tubes with cell pellets were then blanketed with nitrogen and stored at –80°C for vitamin A analysis (34).

Extraction of vitamin A in dry porridge, aqueous fraction, and Caco-2 cells. Vitamin A was extracted from dry porridge, aqueous fractions, and Caco-2 cell pellets as described previously (37), with modifications. For dry porridge, ∼1 g of dry porridge in a 50-mL screw-cap glass tube was homogenized in 4 mL of deionized water before adding 10 mL of ethanol with 0.1% butylated hydroxytoluene (BHT) and 4 mL of super-saturated potassium hydroxide (KOH) solution. Samples were mixed on a vortex and incubated in a water bath at 70°C for 30 min, mixing on a vortex every 10 min. The tubes were placed on ice, and 6 mL of deionized distilled water was added. The samples were initially extracted with 10 mL of hexane and then twice with 5 mL of hexane. Tubes were mixed on a vortex each time hexane was added and left on ice to allow layer separation. The top hexane layer was collected into a clean glass tube with a Pasteur pipette, completely dried in a Vacufuge (model 5301; Eppendorf North America), and reconstituted in 400 μL of mobile phase.

For aqueous fractions, 8 mL of the thawed aqueous fraction was extracted with 10 mL of ethanol with 0.1% BHT and 4 mL of super-saturated KOH. The rest of the extraction procedure was the same as described for dry porridge. Finally, reconstituted (in 400 μL mobile phase) aqueous fractions were syringe filtered with the use of Whatman

TABLE 4 Dry FBFs, Cerelac, and aqueous fraction iron concentrations[1]

	Cereal	Cereal type	Legume	Dry FBF (mg/100 g)	Aqueous fraction[2] (μg/10 mL)
Sorghum-cowpea blends					
1	White sorghum 1	Whole	Cowpea	19.5	0.93 ± 0.02[a]
2	White sorghum 1	Decorticated	Cowpea	18.6	1.03 ± 0.06[a]
3	White sorghum 1	Decorticated-C	Cowpea	15.1	0.66 ± 0.11[a,c]
4	White sorghum 2	Whole	Cowpea	19.5	0.68 ± 0.17[a,c]
5	White sorghum 2	Decorticated	Cowpea	15.9	0.82 ± 0.03[a,c]
6	Red sorghum	Whole	Cowpea	19.1	0.80 ± 0.08[a,c]
7	Red sorghum	Decorticated	Cowpea	15.3	0.62 ± 0.18[a,c]
Sorghum-soy blends					
8	White sorghum 1	Whole	Low-fat soy	23.4	0.95 ± 0.06[a]
9	White sorghum 1	Decorticated	Medium-fat soy	15.6	0.85 ± 0.05[a,c]
10	White sorghum 1	Whole	Full-fat soy	20.7	0.92 ± 0.03[a]
Corn-soy blends					
11	CSB14	Degermed corn-C	Medium-fat soy	15.6	0.75 ± 0.09[a,c]
12	CSB13	Cornmeal	Defatted soy flour	31.8	0.17 ± 0.03[b]
13	CSB+	Whole corn	Whole soy	8.0	0.14 ± 0.03[b]
Cerelac					
14	Wheat	Wheat flour	NA	11.4	0.42 ± 0.01[b,c]

[1]Values are means ± SEMs unless otherwise indicated. CSB13 and CSB+ are nonextruded FBFs; all other blends are extruded FBFs. White decorticated sorghum and degermed corn flour were sourced commercially and expected to have coarse particle size. Fat percentages in low-fat soy, medium-fat soy, and full-fat soy are 1.85%, 6.94%, and 16.93%, respectively. Within a column, means without a common superscript letter differ ($P < 0.05$). C, commercial; CSB, corn-soy blend; CSB+, corn-soy blend plus; FBF, fortified-blended food; NA, not applicable.
[2]Aqueous fractions ($n = 2$) from 2 different in vitro digestion experiments (4 replicates).

polyvinylidene difluoride filters with 0.45-μm pore size (catalog no. 6779-1304; GE Healthcare Biosciences) before placing in HPLC vials.

Cell pellets were weighed, then 2 mL of ethanol with 0.1% BHT and 1 mL of super-saturated KOH were added and incubated at 70°C for 30 min, mixing on a vortex every 10 min (26). The tubes were kept on ice and 1 mL of deionized distilled water was added. Samples were extracted with 5 mL of hexane 3 times and the hexane layers were dried in the manner similar to dry porridges. Dried hexane layers were reconstituted in 400 μL of mobile phase.

HPLC analysis. All of the samples were analyzed on the same day they were extracted. A Shimadzu HPLC system consisting of a DGU-20A3 built-in degasser, an LC-20AB solvent delivery pump, an SIL-20ACHT auto-sampler, a CTO-20AC column holding oven, a CBM-20A communicator module, and an SPD-M20A Photodiode Array Detector with an Agilent Eclipse XDB 5-mm C18 (250 mm × 4.6 mm) analytical column at 25°C was used for analysis. A mobile phase of methanol:acetonitrile:chloroform (47:47:6; vol:vol:vol) at a flow rate of 1.0 mL/min was used; detection was at 325 nm (38) and data were analyzed by using LC solution software. An external retinyl acetate (catalog no. 1716002; USP) standard curve was used for quantification, with its concentration determined by using a spectrophotometer (Jenway 6305; Bibby Scientific US).

Statistical analysis

Data were analyzed with the use of 1-factor ANOVA with Tukey's test on SAS 9.3 (SAS Institute, Inc.), with $P < 0.05$ considered significant. Natural logs were used to transform data that did not meet the model assumptions.

Results

Iron concentration in dry FBF and aqueous fraction

Dry FBF iron concentrations ranged from 8.0 to 31.8 mg/100 g (**Table 4**). Extruded dry FBF iron concentrations (15.1–23.4 mg/100 g) were higher than CSB+ (8.0 mg/100 g) but lower than CSB13 (31.8 mg/100 g). Cerelac's iron concentration (11.0 mg/100 g) was lower than all of the FBFs, except for CSB+. Among the sorghum-containing FBFs, iron concentrations were higher in whole sorghum than in the decorticated FBFs.

Aqueous fractions iron concentrations ranged from 0.14 to 1.03 μg/mL (Table 4). All of the extruded FBFs had significantly higher aqueous fraction iron concentrations compared with CSB13 and CSB+. There were no significant differences between extruded FBFs or between CSB13, CSB+, and Cerelac aqueous fraction concentrations. Cerelac aqueous fraction iron concentrations (0.42 μg/mL) were significantly lower compared with whole white sorghum 1 cowpea, decorticated white sorghum 1 cowpea, whole sorghum-soy (low-fat), and whole sorghum-soy (full-fat) extruded FBFs.

Ferritin concentration in Caco-2 cells treated with aqueous fractions

There were no significant differences between FBF aqueous fraction and basal salt solution (negative control) treatment ferritin concentrations (**Table 5**). Among the extruded FBFs, CSB14 had the highest ferritin concentration (6.78 ng/mg) and whole sorghum-soy (full-fat) had the lowest ferritin concentration (4.10 ng/mg). The $FeSO_4$ positive control (0.2 μg Fe/well) treatment resulted in significantly higher ferritin concentrations than Cerelac, the negative control, and all FBFs, except for corn-soy (medium-fat) and CSB+. There was a dose-dependent increase in ferritin concentrations in response to $FeSO_4$ treatment.

TABLE 5 Caco-2 cell ferritin concentrations after aqueous fraction treatment[1]

	Cereal	Cereal type	Legume	Ferritin (ng/mg)
Sorghum-cowpea blends				
1	White sorghum 1	Whole	Cowpea	6.21 ± 1.68[a]
2	White sorghum 1	Decorticated	Cowpea	4.66 ± 0.05[a]
3	White sorghum 1	Decorticated-C	Cowpea	4.74 ± 0.05[a]
4	White sorghum 2	Whole	Cowpea	6.52 ± 1.83[a]
5	White sorghum 2	Decorticated	Cowpea	5.47 ± 2.05[a]
6	Red sorghum	Whole	Cowpea	5.24 ± 1.35[a]
7	Red sorghum	Decorticated	Cowpea	4.13 ± 1.01[a]
Sorghum-soy blends				
8	White sorghum 1	Whole	Low-fat soy	6.51 ± 2.17[a]
9	White sorghum 1	Decorticated	Medium-fat soy	5.97 ± 3.06[a]
10	White sorghum 1	Whole	Full-fat soy	4.10 ± 1.22[a]
Corn-soy blends				
11	CSB14	Degermed corn-C	Medium-fat soy	6.78 ± 0.64[a,b]
12	CSB13	Cornmeal	Defatted soy flour	4.72 ± 0.73[a]
13	CSB+	Whole corn	Whole soy	7.39 ± 1.76[a,b]
Cerelac				
14	Wheat	Wheat flour	NA	4.89 ± 0.53[a]
Controls				
Basal salt solution (negative control)				4.75 ± 1.04[a]
$FeSO_4$ (0.1 μg Fe/well) (positive control)				15.87 ± 5.73[a,b]
$FeSO_4$ (0.2 μg Fe/well) (positive control)				29.65 ± 3.26[b]

[1]Values are means ± SEMs; $n = 2$. CSB13 and CSB+ are nonextruded FBFs; all other blends are extruded FBFs. White decorticated sorghum and degermed corn flour were sourced commercially and expected to have coarse particle size. Fat percentages in low-fat soy, medium-fat soy, and full-fat soy are 1.85%, 6.94%, and 16.93%, respectively. Within a column, means without a common superscript letter differ ($P < 0.05$). C, commercial; CSB, corn-soy blend; CSB+, corn-soy blend plus; FBF, fortified-blended food; NA, not applicable.

Vitamin A concentration in dry FBFs and aqueous fraction

Vitamin A concentrations of dry FBFs ranged from 0.54 to 1.67 mg/100 g (**Table 6**). CSB+ (1.67 mg/100 g) and Cerelac (0.3 mg vitamin A/100 g) had the highest and lowest vitamin A concentrations, respectively. Whole sorghum FBFs had slightly higher vitamin A concentrations than their corresponding decorticated sorghum FBFs. Aqueous fraction vitamin A concentrations of FBFs and Cerelac were similar and not significantly different. However, in general, sorghum-cowpea FBFs contained higher concentrations (50.8–80.1 ng/mL) than sorghum-soy and corn-soy FBFs (33.0–49.1 ng/mL).

Vitamin A concentration in Caco-2 cells treated with aqueous fraction

Caco-2 cell pellet vitamin A concentrations were not significantly different, after aqueous fraction treatment, between extruded and nonextruded FBFs and Cerelac (**Table 7**). The interesting trend was that red sorghum concentrations were lower than those in white sorghum. Sorghum-soy FBFs with full-fat and medium-fat soy had nonsignificantly higher vitamin A concentrations than low-fat sorghum-soy FBFs. The vitamin A concentrations in all of the FBFs and Cerelac were higher than the negative control concentrations of 2.09 μg/g, with the exception of red sorghum FBFs, white whole sorghum-soy (low-fat soy), CSB13, and CSB14.

Discussion

Seven of the extruded FBFs and 1 nonextruded FBF showed ferritin responses that were higher than negative control levels, but none were significantly higher (Table 5). It should be noted that previous studies conducted with a similar food matrix also did not observe increases above the negative control ferritin response. In a study comparing standard and iron-biofortified black beans that used the in vitro digestion/Caco-2 model, ferritin response for black bean varieties was significantly lower than the negative control (39). In another study comparing red and white beans with the use of the in vitro digestion/Caco-2 model, all sample ferritin responses were at, or significantly below, the negative control (40). It should also be noted that the same study found that white bean consumption resulted in significant improvements in iron status compared with red bean consumption in pigs (40). Rats fed dry extruded sorghum-cowpea, extruded sorghum-soy, and extruded corn-soy (CSB14) FBFs or CSB+ showed no significant difference in hemoglobin and liver iron concentrations, consistent with our Caco-2 results reported here (41).

The amount of iron added to Caco-2 cells in each well ranged from 0.11 to 0.25 μg/well, which was similar to the range (0.05–0.39 μg/well) found in different rice varieties that also did not significantly increase ferritin concentrations (20). However, by adding ascorbic acid, the iron bioavailability of the rice sample significantly increased beyond the negative control levels (20). It should be noted that extruded FBFs are fortified with 40 mg ascorbic acid/100 g, and CSB13 and CSB+ are fortified with 40 mg and 90 mg ascorbic acid/100 g, respectively (Table 3). Some studies have achieved significant ferritin response by increasing the food sample quantity. One study increased the sample quantity from 0.5 g to 1 g and then to 3 g due to a low ferritin response with lower amounts (<3 g) of food (24). However, their unleavened bread flour samples contained very low iron concentrations (0.67–4.67 mg/100 g) compared with our FBF samples (8.0–31.8 mg Fe/100 g). This

TABLE 6 Dry FBF, Cerelac, and aqueous fraction vitamin A concentrations[1]

	Cereal	Cereal type	Legume	Dry FBF (mg/100 g)	Aqueous fraction[2] (ng/mL)
Sorghum-cowpea blends					
1	White sorghum 1	Whole	Cowpea	0.75	60.8 ± 10.0
2	White sorghum 1	Decorticated	Cowpea	0.70	69.6 ± 15.9
3	White sorghum 1	Decorticated-C	Cowpea	0.54	50.8 ± 5.0
4	White sorghum 2	Whole	Cowpea	0.71	52.7 ± 7.7
5	White sorghum 2	Decorticated	Cowpea	0.56	80.1 ± 15.4
6	Red sorghum	Whole	Cowpea	0.76	77.6 ± 2.8
7	Red sorghum	Decorticated	Cowpea	0.54	67.4 ± 27.7
Sorghum-soy blends					
8	White sorghum 1	Whole	Low-fat soy	0.72	48.0 ± 4.0
9	White sorghum 1	Decorticated	Medium-fat soy	0.59	44.8 ± 10.9
10	White sorghum 1	Whole	Full-fat soy	0.75	49.1 ± 0.8
Corn-soy blends					
11	CSB14	Degermed corn-C	Medium-fat soy	0.56	45.9 ± 12.8
12	CSB13	Cornmeal	Defatted soy flour	1.16	33.0 ± 0.4
13	CSB+	Whole corn	Whole soy	1.67	41.5 ± 28.2
Cerelac					
14	Wheat	Wheat flour	NA	0.30	55.6 ± 25.6

[1]Values are means ± SEMs unless otherwise indicated; $n = 2$. CSB13 and CSB+ are nonextruded FBFs; all other blends are extruded FBFs. White decorticated sorghum and degermed corn flour were sourced commercially and expected to have coarse particle size. Fat percentages in low-fat soy, medium-fat soy, and full-fat soy are 1.85%, 6.94%, and 16.93%, respectively. C, commercial; CSB, corn-soy blend; CSB+, corn-soy blend plus; FBF, fortified-blended food; NA, not applicable.
[2]There were no significant differences.

experiment used 2 g porridge, because >2 g resulted in a thick sample that was poorly digested.

The $FeSO_4$ positive control (0.2 μg Fe/well) ferritin concentrations were significantly higher than FBFs, Cerelac, and the negative control, except for CSB+ and CSB14. This positive control has been used previously (42), although at a 25 μg Fe/well treatment, which is >100 times higher than the concentration used in this study. However, comparison of our positive control ferritin response with the previous study was not possible because the results were presented as percentage of relative bioavailability compared with the $FeSO_4$. Aqueous fraction iron concentrations used to treat the Caco-2 cells were not correlated to ferritin synthesis, as has been found previously (20, 21, 36).

TABLE 7 Caco-2 cell vitamin A concentrations after aqueous fraction treatment[1]

	Cereal	Cereal type	Legume	Vitamin A[2] (μg/g cells)
Sorghum-soy blends				
1	White sorghum 1	Whole	Cowpea	2.59 ± 0.35
2	White sorghum 1	Decorticated	Cowpea	2.52 ± 0.13
3	White sorghum 1	Decorticated-C	Cowpea	2.34 ± 0.15
4	White sorghum 2	Whole	Cowpea	2.23 ± 0.12
5	White sorghum 2	Decorticated	Cowpea	2.29 ± 0.15
6	Red sorghum	Whole	Cowpea	1.89 ± 0.52
7	Red sorghum	Decorticated	Cowpea	1.99 ± 0.14
Sorghum-soy blends				
8	White sorghum 1	Whole	Low-fat soy	1.96 ± 0.11
9	White sorghum 1	Decorticated	Medium-fat soy	2.13 ± 0.01
10	White sorghum 1	Whole	Full-fat soy	2.09 ± 0.69
Corn-soy blends				
11	CSB14	Degermed corn-C	Medium-fat soy	1.96 ± 0.01
12	CSB13[3]	Cornmeal	Defatted soy flour	2.03
13	CSB+[3]	Whole corn	Whole soy	2.43
Cerelac				
14	Wheat	Wheat flour	NA	2.24 ± 0.10
Control				
Basal salt solution (negative control)				2.09 ± 0.21

[1]Values are means ± SEMs unless otherwise indicated; $n = 2$. CSB13 and CSB+ are nonextruded FBFs; all other blends are extruded FBFs. White decorticated sorghum and degermed corn flour were sourced commercially and expected to have coarse particle size. Fat percentages in low-fat soy, medium-fat soy, and full-fat soy are 1.85%, 6.94%, and 16.93%, respectively. C, commercial; CSB, corn-soy blend; CSB+, corn-soy blend plus; FBF, fortified-blended food; NA, not applicable.
[2]There were no significant differences.
[3]$n = 1$ due to sample loss.

Iron analysis showed differences between whole and decorticated dry FBF iron concentrations. All of the whole sorghum-cowpea FBFs and the whole sorghum-soy FBFs had higher iron concentrations thantheir corresponding decorticated FBFs (Table 4). Because iron is distributed in different regions of the grain, including the outer layers, it is not surprising that decortication or dehulling results in lower iron concentrations (43, 44). CSB13 had ~77% higher (31.8 mg/100 g) and CSB+ had ~56% lower (8.0 mg/100 g) dry FBF iron concentrations compared with the extruded FBFs (~18.0 mg/100 g). USDA commodity requirements (13, 14) require that CSB13 contain 14.7–30.0 mg Fe/100 g and that CSB+ contain 9.0–21.0 mg Fe/100 g. Thus, iron concentrations in CSB13 were slightly higher than the upper limit, whereas CSB+ iron concentrations were slightly below the lower limit set by the USDA.

All extruded FBFs contained 250-640% significantly higher aqueous fraction iron concentrations compared with CSB13 and CSB+ (Table 4). One of the reasons for the low aqueous fraction iron concentration in CSB13 and CSB+ may be that extruded FBFs were cooked with the use of 20% solids, whereas CSB13 and CSB+ porridges were cooked with the use of 11.75% and 13.79% solids, per their instructions (13, 14). There was no difference in aqueous fraction iron concentrations between CSB13 and CSB+ despite 4-fold higher iron concentrations in dry CSB13 FBFs. CSB+ is an improved formulation of CSB13, with an enhanced nutrient profile and with ingredients partially cooked through dry roasting (14). This heat processing may partially explain why CSB+ aqueous fraction iron concentratons were similar to CSB13; previous studies observed a 16–32% increase in iron availability with roasting and malting (45). Another reason for the improved iron availability may have been that CSB+ is fortified with both ferrous fumarate and NaFeEDTA. The latter iron fortificant chelates with native iron in the diet and protects it from binding to antinutritional factors (46–48).

There were no significant differences between Caco-2 cell vitamin A concentrations (Table 7). Rats fed dry extruded sorghum-cowpea, extruded sorghum-soy, and extruded corn-soy (CSB14) FBFs or CSB+ showed no significant difference in serum retinol concentrations, which is consistent with our Caco-2 results reported here (41). There are limited data on in vitro vitamin A bioavailability. Although there are many studies on carotenoid bioavailability (25, 34), to the best of our knowledge only 1 study has examined vitamin A bioavailability with the use of the Caco-2 cell model (26). Vitamin A content in the current study ranged from 0.3 mg/100 g in Cerelac to 1.67 mg/100 g in CSB+ (Table 6). Vitamin A content in Cerelac matched its labeled content; however, CSB+ and CSB13 had 61% and 41% higher vitamin A concentrations, respectively, than the required amounts (Table 3). Aqueous fraction vitamin A concentrations were not significantly different (Table 6). Interestingly, nonextruded CSB13 and CSB+ that contained the highest dry FBF vitamin A concentrations had the lowest aqueous fraction concentrations compared with extruded FBFs and Cerelac. It is possible that the longer cooking time for preparing these porridges may have affected the vitamin A concentrations (26). Another reason for the lower vitamin A aqueous fraction concentrations of CSB+ may be that it contains lower fat contents than the newly formulated FBFs.

Limitations

The major limitation of this study is that we did not find significant differences between the negative control and our treatments. Because, to our knowledge, this is the first study looking at both bioavailable iron and vitamin A concentrations, we used a method developed for assessing carotenoid bioavailability (25, 29, 34, 49) and modified it to estimate both iron and vitamin A simultaneously. Because this methodology is new, it has not been validated against human bioavailability data. However, it is notable that the lack of differences in iron and vitamin A outcomes between extruded FBFs is consistent with a rat study that fed these FBFs (41). The use of inserts with dialysis membranes in a 2-compartment arrangement, as has been done previously (18–21, 23, 50), may have resulted in different outcomes. However, it should be noted that there are previous studies that used the same 2-compartment methodology that also did not find significant changes compared with negative controls (39, 40). Another possibility might have been adding exogenous iron to the food samples to help improve the ferritin response. Assessing cell differentiation by measuring the brush border enzyme activity (51) rather than using cells at 14 d postseeding like was used in this study may have also improved outcomes. Overall, the lack of method standardization and responsiveness to treatment can be viewed as limitations of the in vitro digestion/Caco-2 model. Further studies are recommended with the use of animals and humans to determine the iron and vitamin A bioavailability of new extruded FBFs and traditional nonextruded FBFs.

Conclusions

To the best of our knowledge, this is the first study to measure both bioavailable iron and vitamin A with the use of an in vitro digestion/Caco-2 cell model. Although the lack of difference between the negative control and treatments should be noted, our results suggest that the consumption of newly developed extruded sorghum-cowpea, sorghum-soy, and corn-soy FBFs will result in iron and vitamin A status comparable to traditional nonextruded CSB13 and CSB+ FBFs.

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