The Journal of Nutrition
Nutritional Epidemiology

Iron Bioavailability from Infant Cereals Containing Whole Grains and Pulses: A Stable Isotope Study in Malawian Children

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ABSTRACT

Background: Compared with infant cereals based on refined grains, an infant cereal containing whole grains (WGs) and pulses with adequate amounts of ascorbic acid to protect against absorption inhibitors could be a healthier source of well-absorbed iron. However, iron absorption from such cereals is uncertain.

Objective: We measured iron bioavailability from ferrous fumarate (Fefum) added to commercial infant cereals containing *1*) refined wheat flour (reference meal), *2*) WG wheat and lentil flour (WG-wheat-lentil), *3*) WG wheat and chickpea flour (WG-wheat-chickpeas), and *4*) WG oat flour (WG-oat) and from ferrous bisglycinate (FeBG) added to the same oat-based cereal (WG-oat-FeBG).

Methods: In a prospective, single-blinded randomized crossover study, 6- to 14-mo-old Malawian children ($n = 30$) consumed 25-g servings of all 5 test meals containing 2.25 mg stable isotope-labeled iron and 13.5 mg ascorbic acid. Fractional iron absorption (FIA) was assessed by erythrocyte incorporation of isotopes after 14 d. Comparisons were made using linear mixed models.

Results: Seventy percent of the children were anemic and 67% were iron deficient. Geometric mean FIA percentages (–SD, +SD) from the cereals were as follows: *1*) refined wheat, 12.1 (4.8, 30.6); *2*) WG-wheat-lentil, 15.8 (6.6, 37.6); *3*) WG-wheat-chickpeas, 12.8 (5.5, 29.8); and *4*) WG-oat, 9.2 (3.9, 21.5) and 7.4 (2.9, 18.9) from WG-oat-FeBG. Meal predicted FIA ($P \leq 0.001$), whereas in pairwise comparisons, only WG-oat-FeBG was significantly different compared with the refined wheat meal ($P = 0.02$). In addition, FIAs from WG-wheat-lentil and WG-wheat-chickpeas were significantly higher than from WG-oat ($P = 0.002$ and $P = 0.04$, respectively) and WG-oat-FeBG ($P < 0.001$ and $P = 0.004$, respectively).

Conclusion: In Malawian children, when given with ascorbic acid at a molar ratio of 2:1, iron bioavailability from Fefum-fortified infant cereals containing WG wheat and pulses is ≈13−15%, whereas that from FeBG- and Fefum-fortified infant cereals based on WG oats is ≈7−9%. *J Nutr* 2022;152:826–834.

Keywords: iron, bioavailability, ferrous fumarate, ferrous bisglycinate, infant cereals, infants, Malawi

Introduction

Iron-deficiency anemia (IDA) during early childhood is common and may result in irreversible cognitive and developmental deficits later in life (1, 2). In Malawi, prevalence of iron deficiency (ID) among preschool children is 43% (3). Infant cereals are among the first foods introduced during complementary feeding, and iron fortification of infant cereals can provide additional iron during this vulnerable period (1, 4–6).

Formulation of the cereals used for infant foods can have major effects on nutrient profile and iron bioavailability. In whole-grain (WG) cereals, the endosperm, bran, and germ are present, whereas refined cereals contain only the endosperm (7). WG cereal flours are generally preferred to refined cereal flours for infant cereal production due to multiple health benefits (8) and may have beneficial effects on glucose and insulin metabolism in children (9). However, although the bran and germ contain the highest proportions of fibers, vitamins, and minerals, they also can contain high amounts of phytic acid (PA) and polyphenols, the 2 main inhibitors of iron absorption (10, 11).

Legumes and their flours are an alternative protein source to dairy in complementary and ready-to-use therapeutic foods in resource-limited settings (12–14). They can also contain phytates, polyphenols, and some proteins that could add to the

Manuscript received May 19, 2021. Initial review completed June 29, 2021. Revision accepted November 30, 2021.
First published online December 27, 2021; doi: https://doi.org/10.1093/jn/nxab406.

inhibitory effect from WGs (11, 15–17). To our knowledge, except for soya (18, 19), the effect of the addition of different types of pulse flours to infant cereals on iron absorption has never been investigated in young children. Using *in vitro* simulated digestion coupled with the Caco-2 cell model (20), the effect of the addition of different legumes (i.e., red and yellow lentils, yellow peas and chickpeas, and wheat WG flours) on the iron bioaccessibility from complete infant cereals was evaluated. The results of the recipes containing lentils (red and yellow) suggested some improvement of the iron uptake by the cells, whereas the recipe containing chickpeas was found to be the most impactful compared with that from infant cereals containing refined wheat flour (M Sabatier, unpublished results). Furthermore, the impact may also be influenced by the type of WG cereal flour used. For instance, in adults, the lowest iron absorption was observed from porridge from oat flour, which also had the highest percentage of native PA compared with wheat, rice, or maize flour porridge (21).

The choice of iron fortificant plays an important role on iron absorption. Ferrous fumarate (Fefum) is the recommended iron fortificant for cereal-based complementary foods because of its high bioavailability and organoleptic stability (1), but its absorption may be lower in a cereal formulation consisting of WG and pulses. To increase iron bioavailability in high-phytate food matrices, the addition of absorption enhancers such as ascorbic acid (AA) or the use of iron chelates as fortificants is recommended (1, 11). Addition of AA can counteract the inhibitory effects of PA and some polyphenols on iron absorption (1, 22); in the presence of AA, a PA:iron (Fe) molar ratio of <6:1 has a negligible effect on iron absorption (11).

Ferrous bisglycinate (FeBG) is an iron–amino acid chelate that may have higher bioavailability than ferrous sulfate ($FeSO_4$) in inhibitory food matrices and dairy products (1, 23). However, its higher absorption in comparison to other iron salts in young children has been poorly investigated. In addition, it appears that depending on its level of dissociation in the gut lumen, the iron absorption from this complex may be affected by enhancers and inhibitors in the meal matrix (24–26). Thus, whether the use of FeBG overcomes the inhibitory effects of the matrix of cereals containing WG and its advantage over the use of Fefum is uncertain.

Therefore, the co-primary objectives of this study were to measure iron bioavailability from Fefum added to AA-fortified infant cereals containing: *1*) a mixture of WG wheat and lentil flour, *2*) a mixture of WG wheat and chickpea flour, and *3*) WG oat flour and compare them with a refined-grain wheat flour–based meal (the reference meal). The co-secondary objectives were to measure iron bioavailability from Fefum and FeBG added to the infant cereal containing WG oat flour and AA and compare them with the reference meal. We hypothesized that: *1*) iron bioavailability from the cereals containing WGs and pulses would be similar to the reference cereal, despite their higher content of inhibitory compounds because of the presence of AA; and *2*) iron bioavailability from the WG oat-based cereal containing Fefum would be lower than the reference cereal, whereas iron bioavailablity of the same cereal containing FeBG would be comparable or superior to the reference cereal.

Methods

Study site and population

This study was conducted in Zomba, a rural town in southern Malawi. We recruited caregiver–child pairs from the pediatric outpatient clinic at Zilindo Health Center and performed the study at the Training and Research Unit of Excellence (TRUE) located at Zomba Central Hospital between January and May 2019. We obtained informed written consent or, if the caregiver was illiterate, informed oral consent with a thumbprint in the presence of a literate witness. Inclusion criteria for the children were: *1*) age 6–14 mo; *2*) no history of major disease; *3*) no acute illness; *4*) hemoglobin (Hb) >70 g/L; *5*) not underweight or wasted [weight-for-age *z* score (WAZ) and weight-for-length *z* score (WLZ) >−2]; *6*) caregiver of legal age of consent; *7*) anticipated residence in the area for the duration of the study; *8*) expected to consume the entire 25-g serving during test cereal feeding as assessed during adaptation; *9*) completed 3 doses of preventive treatment of malaria with dihydroartemisinin piperaquine; and *10*) completed 1 dose of anthelmintic treatment with albendazole.

Ethical procedures

Ethical review committees of the ETH Zurich and the College of Medicine, University of Malawi approved the study. We registered the study at clinicaltrials.gov as NCT03754543.

Study design

This was a single-blind, prospective crossover trial as described in main study procedures below (**Figure 1**). In all infants, we measured iron bioavailability from 4 infant cereals: *1*) Fefum added to an infant cereal containing AA based on a mixture of WG wheat and lentil flour (WG-wheat-lentil-Fefum); *2*) Fefum added to an infant cereal containing AA based on a mixture of WG wheat and chickpea flour (WG-wheat-chickpeas-Fefum); *3*) Fefum added to an infant cereal containing AA based on refined-grain wheat flour (refined wheat-Fefum) (reference meal), and *4*) Fefum added to an infant cereal containing AA based on WG oats (WG-oat-Fefum). Finally, the same oat-based cereal formulation was used to evaluate the absorption from FeBG (WG-oat-FeBG).

Screening and adaptation

During screening, we collected a finger-prick blood sample for determination of Hb and C-reactive protein (CRP). Using a digital scale (Seca), we calculated child body weight by measuring the body weight of the mother alone and when holding the child to the nearest 0.01 kg. We measured child length using a rigid measurement board to the nearest 0.5 cm. We assessed demographics, health status, and feeding habits of the children using a standardized questionnaire. To ensure complete consumption of the labeled 25-g test meal during the study period, children had to consume 25 g of cereal fortified with AA and 2.25 mg Fefum reconstituted with 90 mL of water daily for 5 d as an adaptation. The children consumed the first, fourth, and fifth meals at the research unit under supervision of the study team. After completion of the meals, we rinsed the feeding bowls 2 times with 5 mL of water and the children consumed the rinse water. For the remaining 2 meals, we provided caregivers with 2 sachets of cereal and a graduated measuring cup and showed them how to prepare the cereal at home. Children able

Source of support: Société des Produits Nestlé, S.A. Nestlé was involved in the design of the study, production of the test cereals, interpretation of results, and preparation of the manuscript but not the execution of the study in the field or the statistical analysis.
Author disclosures: MS and NPH are employees of Société des Produits Nestlé S.A., the sponsor of the trial. GM is a fellow supported by the Consortium for Advanced Research Training in Africa (CARTA). All other authors report no conflicts of interest.
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Abbreviations used: AA, ascorbic acid; AGP, α-1-acid glycoprotein; CRP, C-reactive protein; Fe, iron; FeBG, ferrous bisglycinate; Fefum, ferrous fumarate; $FeSO_4$, ferrous sulfate; FIA, fractional iron absorption; Hb, hemoglobin; ID, iron deficiency; LAZ, length-for-age *z* score; PA, phytic acid; PF, plasma ferritin; PHep, plasma hepcidin; sTfR, soluble transferrin receptor; WAZ, weight-for-age *z* score; WG, whole grain; WLZ, weight-for-length *z* score.

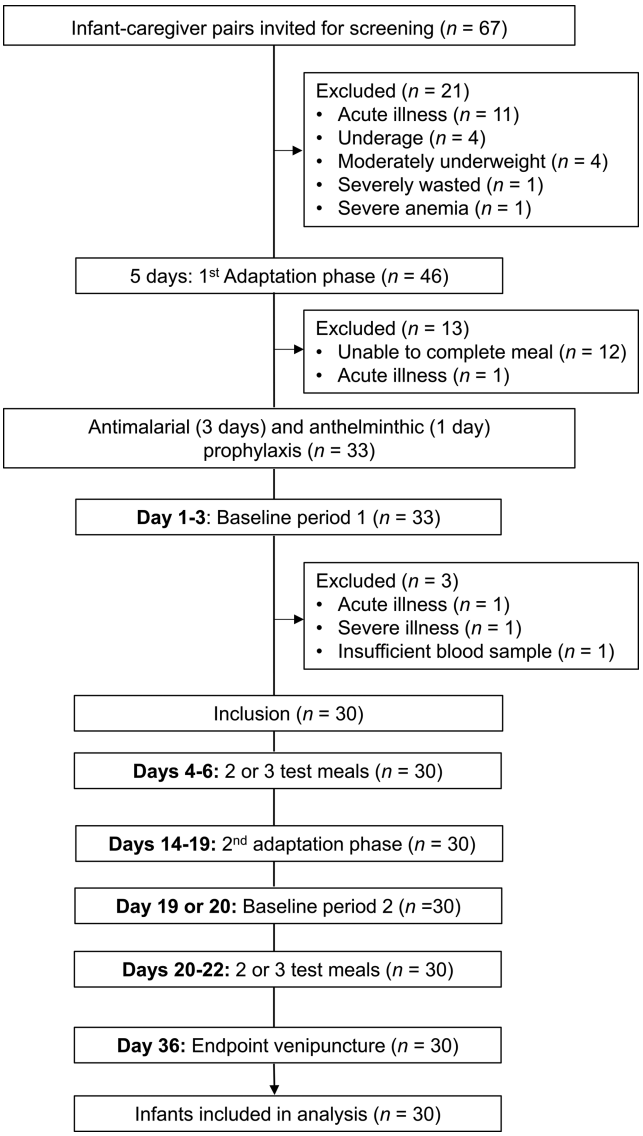

FIGURE 1 Study design and participant flowchart.

to consume and complete the serving during adaptation were included into the main study.

Antimalarial and anthelmintic prophylaxis

After a successful adaptation, the children received the antimalarial prophylaxis P-Alaxin (Bliss GVS Pharma) once daily over 3 consecutive days. We calculated the dose based on body weight (0.5 tablet/d for 5–9.9 kg and 1 tablet/d for 10–19.9 kg). They also received anthelmintic prophylaxis as a single dose of the albendazole oral suspension (ALwo; Leben Laboratories PVT Limited).

Randomization and main study procedures

Randomization of the order of test cereals for each infant was done using R statistical programming environment (R 3.6.0 software; R Core Team). The children were randomly assigned to a sequence of test meals. Each sequence of meals was designed to start with 2 or 3 test cereals consumed on consecutive days in 2 study periods separated by 14 d. The sequence also ensured that test cereals with the same iron label were not consumed in the same period.

Main study procedures were as follows (Figure 1). At study day 1 (baseline period 1), we collected a blood sample by venipuncture for determination of Hb, plasma ferritin (PF), soluble transferrin receptor (sTfR), plasma hepcidin (PHep), CRP, and α-1-acid glycoprotein (AGP). We calculated child body weight and measured child length as described above. One to 3 d later, we invited the caregiver–child pairs for the first test meal. Caregivers were advised to feed the children nothing except breast milk after the previous evening meal and not to feed any breast milk to the infant during the 3 h before the test meal. Fourteen days after the last test cereal, we collected whole blood by venipuncture or heel prick for iron isotopic analysis and for the repeat measurement of Hb, sTfR, PF, CRP, and AGP (after the first study period only). We calculated child body weight and measured child length as described above.

Five days prior to the endpoint measurements and blood draw of study period 1, the children went through a second adaptation phase, as described above to mimic study period 1. The blood and anthropometric assessments done at the end of study period 1 formed the baseline for study period 2. One to 3 d later, the children consumed the second round of test meals, and 14 d after the last meal, a final blood sample was collected for iron isotopic analysis and for the repeat measurement of Hb.

All 5 test meals were consumed between 06:30 and 09:00. They consisted of 25 g of milk-based cereal flour (with and without legumes flour) (Société des Produits, Nestlé S.A.), reconstituted with 90 mL warm bottled water (Aqua Pure purified water, still; Bowler Beverage Company Ltd.). The iron isotopes and AA were added to the test cereals just before feeding. All test cereals contained 2.25 mg labeled iron: *1*) WG-wheat-lentils was fortified with 2.25 mg of iron as ^{54}Fe-labeled Fefum; *2*) WG-wheat-chickpeas was fortified with 2.25 mg of iron as ^{57}Fe-labeled Fefum; *3*) refined wheat (reference meal) was fortified with 2.25 mg of iron as ^{54}Fe-labeled Fefum; *4*) WG-oat was fortified with 2.25 mg of iron as ^{57}Fe-labeled Fefum; and *5*) WG-oat-FeBG was fortified with 2.25 mg of iron as ^{58}Fe-labeled FeBG. Then, 100 μL of freshly dissolved AA solution containing 13.5 mg AA was added after addition of iron isotope to achieve a molar ratio of 2:1 relative to labeled iron. Members of the study team fed the test cereals to the children using a bowl and spoon. We rinsed all utensils with 10 mL water, and the children consumed the rinse water. After feeding, we observed the children at the study site for 2 h, and during this time, intake of foods and/or fluids was not allowed.

Infant cereal

The infant cereals were produced according to the specifications for a commercial product at the Nestlé Product Technology Center - Orbe. They were composed primarily of white wheat flour (70% extraction), skim milk powder, and the addition of either dehulled red lentil flour or dehulled chickpea flour with WG wheat flour or WG oat flour. The flours were mechanically milled by the suppliers. For the lentil and chickpea flour, split lentil cotyledons or sound and dry red lentils (Crimson) and sound, healthy, dry, and clean yellow chickpeas (Kabuli), respectively, were used. For both the refined and whole-wheat flour, Berdun and/or Marius and/or Rimbaud wheat was used, but only the endosperm and ash content were present in the former, whereas the latter had all 3 fractions (bran, germ, and endosperm). WG oat (mixture of Ivory and Husky) was heat treated for stabilization prior to milling and contained the 3 fractions. The aqueous slurry was cooked by steam injection and roller-dried. The skim milk powder and the whole milk added in the oat recipe were then dry-mixed with the roller-dried cereal base. They all contained *Bifidobacterium lactis* at a level of 4.3×10^7 CFU per serving to support gut health and immunity as currently added in commercial products. For the purpose of this study, no iron or AA was added to the test cereals during the production process. The addition of labeled iron fortificant and AA was done as previously described. The nutritional composition was analyzed and the microbiologic safety was ensured before the product was released from the Product Technology Center. Quantities of flour and milk in the test cereals as well as their nutritional composition are shown in **Table 1**.

Stable isotope labels

^{54}Fe- and ^{57}Fe-labeled Fefum and ^{58}Fe-labeled FeBG were prepared from ^{54}Fe-, ^{57}Fe-, and ^{58}Fe-enriched elemental iron (99.4%, 95.55%,

TABLE 1 Energy, nutrient, flour, and milk composition of test cereals per 25-g serving as consumed by the participating Malawian children, without added labeled iron and ascorbic acid

	Cereal type			
Nutritional composition	WG-wheat-red lentils	WG-wheat-chickpeas	Refined wheat (reference)	WG-oat
Energy, kcal	109	107	103	108
Protein, g	3.6	3.7	3.5	4.2
Total fat, g	3.2	2.8	2.0	2.9
Carbohydrates (g)	16.4	17.8	16.7	16.4
Sugar, g	5.1	5.1	6.9	3.7
Total dietary fiber, g	2.6	2.4	1.0	2.3
Total cholesterol, mg	1.2	1.1	1.7	4.3
Calcium, mg	142	142	76	85
Iron, μg	370	330	170	340
Vitamin C, μg	500	500	560	635
Phytic acid, mg	60.9	59.7	20.5	50.0
Polyphenol,[1] mg	25.9	27.2	23.5	34.8
Flours and milk				
Refined wheat flour, g	8.0	8.0	13.5	8.3
Whole-grain flour, g	4	4	0	8
Pulses flour, g	2	2	0	0
Skimmed milk, g	4.3	4.5	6.5	5.0
Whole milk 26% fat, g	0	0	0	2.5

[1] As Gallic acid equivalents. 2.25 mg of labeled iron and 13.5 mg of ascorbic acid were added to each test cereal prior to feeding after reconstitution of the infant cereals in warm water. WG, whole grain.

and 99.89% isotopic enrichment, respectively; Chemgas) by Dr. Paul Lohmann GmbH (Emmerthal). We analyzed the labeled iron compounds for iron isotopic composition and the tracer iron concentration via isotope-dilution mass spectrometry as outlined in the analytic methods.

Laboratory analyses

At screening, we measured Hb and CRP using the QuikRead go CRP + Hb kit (Orion diagnostica Oy). For the subsequent visits, venous or heel stick blood samples were drawn into heparin- and EDTA-coated tubes. Hb, hematocrit, and total RBC count were measured immediately after blood draw using Sysmex Automated Hematology analyzer (XP-300; Sysmex Corporation). Whole blood and heparin plasma aliquots were stored at –20°C until further analysis at ETH Zurich. Iron and inflammation status (PF, sTfR, CRP, AGP) were measured using a multiplex immunoassay (27), and PHep was measured using a commercially available ELISA (DRG Instruments GmbH). Anemia was defined as Hb <110 g/L (28). ID was defined as PF <12 μg/L and/or sTfR >8.3 mg/L (27), and IDA was defined as Hb <110 g/L (28) and PF <12 μg/L and/or sTfR concentration >8.3 mg/L (27). CRP or AGP concentrations >5 mg/L and >1 g/L, respectively, were defined as indicating inflammation (27). Whole blood samples were mineralized in duplicate with the use of nitric acid and microwave-assisted digestion (TurboWave; MLS), followed by separation of the iron from the blood matrix via anion-exchange chromatography and a subsequent precipitation step with ammonium hydroxide (29). We measured iron isotope ratios by using an inductively coupled plasma mass spectrometer (Neptune; Thermo Finnigan) equipped with a multicollector system for simultaneous iron beam detection (29).

Composition of the test meals

Mineral analyses of the test cereals was done in triplicate. We measured phytate concentration in the 4 cereals using a modification of the method of Makower (30) in which iron was replaced by cerium in the precipitation step; after the mineralization of the precipitate, inorganic phosphate was determined colorimetrically (31) and converted into phytate concentrations. We measured iron and calcium concentrations in the cereals by inductively coupled plasma mass spectrometry after mineralization as described above. We determined total polyphenol concentrations (as gallic acid equivalents) in the cereals spectrophotometrically after extraction in a mixture of water and acetone, according to a modification of the Folin–Ciocalteu method.

Calculation of iron absorption

We calculated the amounts of ^{54}Fe, ^{57}Fe, and ^{58}Fe isotopic labels in blood 14 d after administration of the last test meal in each study period based on the shift in iron-isotopic ratios and the estimated amount of iron circulating in the body. Circulating iron was calculated based on Hb concentrations and blood volume that was estimated from weight and measured Hb at the time of blood collection, assuming 65 mL/kg body weight (32). The calculations were based on principles of isotope dilution and took into account that iron isotopic labels are not monoisotopic, using the methods described by Turnlund et al. (33) and Cercamondi et al. (34). For the calculation of iron absorption, we assumed a 75% incorporation of the absorbed iron based on a previous study that measured erythrocyte isotope incorporation from intravenously infused ^{58}Fe in Ghanaian children (35).

Sample size calculation

The aim of the trial was to measure the FIA with a certain precision as indicated by the standard error. Therefore, the study was powered for precision rather than based on a hypothesized effect size. With a sample size of 20 children, a relative bioavailability could be estimated with a standard error of $0.44/20^{1/2} = 0.1$. The 0.44 SD was derived using data from a bioavailability study investigating iron bioavailability from a novel iron fortificant consisting of ferric iron complexed with phytic acid and hydrolyzed corn protein (36). With an estimated dropout rate of 20%, sample size was increased to 24 children. Furthermore, due to an anticipated 45% prevalence of elevated CRP in the study population and an expected reduction to 30% after the preventive medications that would be administered to each child, we recruited an additional 6 (30% × 20) children, bringing the total sample size to 30 children.

TABLE 2 Anthropometric measurements and iron and inflammation indices of the participating Malawian children at baseline of periods 1 and 2[1]

Characteristic	Baseline	
	Period 1	Period 2
Total (male/female), *n*	30 (16/14)	
Age, mo	10.2 ± 2.2	10.7 ± 2.1
Body length, cm	69.7 ± 4.7	70.4 ± 4.4
Body weight, kg	8.3 (7.2, 9.7)	8.6 (7.4, 10.1)
Weight-for-age, *z* score	−0.50 ± 1.08	−0.34 ± 1.14
Weight-for-length, *z* score	0.25 ± 1.06	0.42 ± 1.11
Length-for-age, *z* score	−1.20 ± 1.26	−1.20 ± 1.22
Hemoglobin, g/L	106 ± 9	106 ± 8
Hematocrit, %	33.2 ± 2.1[2]	33.9 ± 1.9[3]
Total RBCs, $10^6/\mu$L	5.0 ± 0.4[2]	5.1 ± 0.4[3]
Plasma ferritin, μg/L	18.1 (7.6, 43.1)	15.6 (5.9, 41.3)[4]
Plasma ferritin adjusted,[5] μg/L	14.4 (6.4, 32.1)	14.4 (5.5, 37.8)[4]
Soluble transferrin receptor, mg/L	9.1 (6.9, 12)	10.2 (7.8, 13.4)[4]
Anemia, *n* (%)	21 (70.0)	20 (66.7)
Iron deficiency, *n* (%)	20 (66.7)	24 (82.7)[4]
Iron-deficiency anemia, *n* (%)	13 (43.3)	17 (58.6)[4]
CRP, mg/L	0.7 (0.2, 2.9)	0.8 (0.2, 3.8)[4]
Elevated CRP, *n* (%)	3 (30.0)	4 (13.8)[4]
AGP, g/L	0.8 ± 0.3	0.8 ± 0.3[4]
Elevated AGP, *n* (%)	8 (26.7)	8 (27.6)[4]
Inflammation, *n* (%)	9 (30.0)	9 (31.0)[4]
Plasma hepcidin, ng/mL	3.3 (0.9, 12.4)	3.1 (0.8, 11.0)[4]

[1] Values are presented as mean ± SD or geometric mean (−SD, +SD) unless otherwise indicated. AGP, α-1-acid glycoprotein; CRP, C-reactive protein.
[2] $n = 24$.
[3] $n = 26$.
[4] $n = 29$.
[5] Adjusted for inflammation (37).

Data and statistical analysis

We calculated WAZ, WLZ, and length-for-age *z* score (LAZ) using WHO Anthro software (version 3.2.2; WHO). Due to insufficient whole blood, 10 children did not have hematocrit and total RBC measurements at baseline for study periods 1 and 2. At baseline for study period 2, 1 child had insufficient plasma to measure PF, sTfR, CRP, AGP and PHep.

We adjusted PF for inflammation. For the 2 baseline samples of the 2 study periods, the children were grouped into incubation (geometric mean PF concentration: 21.3 μg/L and 26.3 μg/L), early convalescence (geometric mean PF concentration: 29.4 μg/L and 16.7 μg/L), late convalescence (geometric mean PF concentration: 29.5 μg/L and 17.3 μg/L), and reference groups (geometric mean PF concentration: 14.4 μg/L). Using the ratio of the geometric mean of the reference group to incubation, early convalescence and late convalescence (37) resulted in the following correction factors for baseline of study periods 1 and 2: incubation, 0.68 and 0.55; early convalescence, 0.49 and 0.87; and late convalescence, 0.49 and 0.88. We analyzed biochemical and anthropometric data and iron absorption using SPSS (IBM SPSS Software, Version 24; SPSS, Inc.) and GraphPad Prism 8 (GraphPad Software). Normality was assessed using the Shapiro–Wilk and Kolmogorov–Smirnov tests and visualization of q–q plots. If data were not normally distributed, natural log (ln) transformation was applied before further analysis. From WG-oat-FeBG, 1 FIA value was defined as an outlier by data inspection and sensitivity analysis test. Descriptive statistics at baseline of study periods 1 and 2 are shown in Table 2. Values in the text and tables are presented as means ± SDs for normally distributed data and as geometric means (–1 SD, +1 SD) for normally distributed data after ln transformation. To determine predictors of FIA, the anthropometric, iron, and inflammation status values of the respective baseline measurement were used.

Using a random intercept linear mixed-effect model analysis, we assessed the effect of meal and study period on FIA. Meal and period were defined as fixed effects. Participants were defined as random intercept effects using a variance component structure matrix. We included the study period in our models on FIA to correct for potential period effect related to when the stable isotope was administered (i.e., period 1 or 2). Data were corrected for multiple comparison using Bonferroni correction. Log (ln) transformed data were used if data were not normally distributed, and the model was validated by visualizing q–q plots of the residuals. For each FIA value, we included as covariates the corresponding WAZ, LAZ, WLZ, ferrtin, sTfR, PHep, CRP, and AGP values as measures of iron, inflammation- and anthropometric markers. For all tests performed, P values < 0.05 were considered statistically significant.

Results

Participant characteristics

We screened 67 children, and 30 were enrolled and completed the study as shown in Figure 1. The mean ± SD age at enrollment was 10.2 ± 2.2 mo. For all the children, we included absorption values for all the cereals except WG-oat-FeBG, in which we included values for 29 children. Analysis of iron status and inflammation parameters measured at baseline of period 2 included 29 children. Anthropometric, iron, and inflammation indices at baseline of periods 1 and 2 are reported in Table 2. At both baselines, 70% and 67% of the children had anemia, 67% and 83% had ID, and 9 children had inflammation.

Test meal composition

The mean native iron, calcium, PA, and polyphenol concentrations per serving of each test cereal are shown in Table 1. The molar ratio of added AA:Fe was 2:1 for all the cereals, and the PA:Fe molar ratio was similar and highest in the WG wheat cereals containing lentil flour (1.97:1) and chickpea flour (1.96:1), as shown in Table 3.

Iron bioavailability

The geometric mean FIA percentages (−SD, +SD) from the 4 cereals fortified with Fefum were as follows: WG-lentil, 15.8 (6.6, 37.6); WG-chickpeas, 12.8 (5.5, 29.8); refined wheat (reference), 12.1 (4.8, 30.6); and WG-oat, 9.2 (3.9, 21.5) and 7.4 (2.9, 18.9) from the WG-oat when fortified with FeBG, as shown in Figure 2.

In a linear mixed-model analysis including meal and period as fixed effects and WAZ, LAZ, WLZ, ferrtin, sTfR, PHep, CRP, and AGP as covariates, meal ($P < 0.001$) had a significant effect on FIA but not the period in which it was consumed ($P = 0.63$). Furthermore, only CRP ($\beta = 0.14$; $P = 0.02$), PHep ($\beta = –0.34$; $P < 0.001$), LAZ ($\beta = –1.06$; $P = 0.04$), and WAZ ($\beta = 1.96$; $P = 0.03$) significantly predicted FIA but not WLZ ($P = 0.05$), ferritin ($P = 0.65$), sTfR ($P = 0.94$), or AGP ($P = 0.67$). Pairwise comparisons showed that FIA from only the WG-oat-FeBG cereal was significantly different from the reference meal ($P = 0.02$). However, FIAs from WG-lentils and WG-chickpeas were significantly higher than from WG-oat-Fefum ($P = 0.002$ and $P = 0.04$, respectively) and WG-oat-FeBG ($P < 0.001$ and $P = 0.004$, respectively).

Contribution to the daily requirements for absorbed iron

Using the measured iron absorption (% FIA) and the total iron content of tested products, the quantity of absorbed iron

TABLE 3 Total AA and Fe content, AA:Fe and PA:Fe molar ratio, FIA, amount of absorbed iron, and percentage of daily requirement for absorbed iron met per serving (25g dry powder) of each cereal consumed by the participating Malawian children[1]

Characteristic	Total AA, mg	Total Fe,[2] mg	AA:Fe	PA:Fe	FIA, %	Absorbed,[3] mg	Percent of requirement[4] met
WG-lentil + ^{54}Fefum	14.0	2.6	1.7	2.0	15.8	0.4	57.5
WG-chickpeas + ^{57}Fefum	14.0	2.6	1.7	2.0	12.8	0.3	45.8
Refined Wheat + ^{54}Fefum	14.1	2.4	1.8	0.7	12.1	0.3	40.7
WG-oat + ^{57}Fefum	14.1	2.6	1.7	1.7	9.2	0.2	33.1
WG-oat + ^{58}FeBG	14.1	2.6	1.7	1.7	7.4[5]	0.2	26.5

[1] AA, ascorbic acid; Fe, iron; FeBG, ferrous bisglycinate; Fefum, ferrous fumarate; FIA, fractional iron absorption; PA, phytic acid; WG, whole grain.
[2] Sum of native and labeled iron (2.25 mg).
[3] Calculated using the total quantity of iron present per serving.
[4] Based on a daily requirement of 0.72 mg/d (6).
[5] $n = 29$.

from each serving of cereal was calculated as well as the corresponding contribution (%) to the total requirement for iron absorbed (i.e., a median of 0.72 mg/d in children aged 6–12 mo) (6). Each cereal serving provided the following amounts of absorbed iron that corresponded to the respective percentage of daily requirement met: WG-lentils, 0.41 mg (57.5%); WG-chickpeas, 0.33 mg (45.8%); reference, 0.29 mg (40.7%); WG-oat, 0.24 mg (33.1%); and WG-oat-FeBG, 0.19 mg (26.5%), as shown in Table 3.

Discussion

Our main study findings are that in mostly iron-deficient Malawian children: *1*) iron bioavailability from Fefum consumed in AA-fortified WG wheat-based cereals containing lentil and chickpea flour were comparable to refined wheat cereal, *2*) iron bioavailability from FeBG-fortified WG oat-based cereal was significantly lower than from the refined wheat cereal fortified with Fefum, and *3*) iron bioavailability from FeBG and Fefum consumed in WG oat-based cereals were comparable but

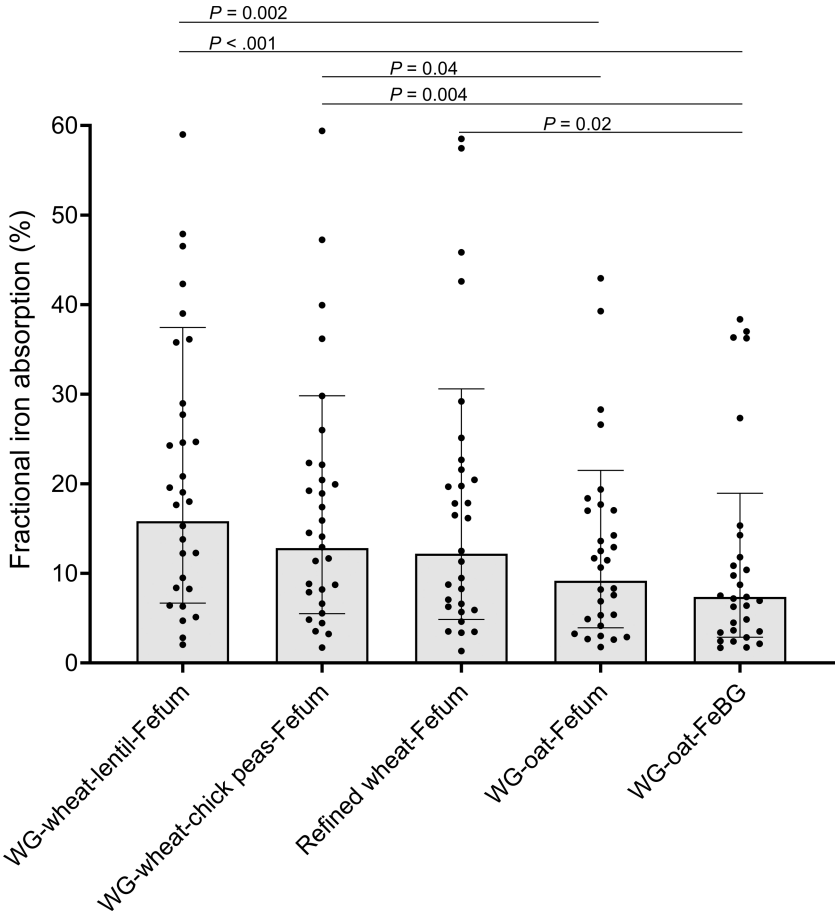

FIGURE 2 Fractional iron absorption (%) in Malawian children ($n = 30$) from iron as Fefum in: *1*) WG wheat cereal containing lentil flour (WG-wheat-lentil-Fefum), *2*) WG wheat cereal containing chickpea flour (WG-wheat-chickpeas-Fefum), *3*) refined wheat flour (refined wheat-Fefum), and *4*) predominantly WG oat cereal (WG-oat-Fefum) and from the same oat-based cereal with iron added as FeBG (WG-oat-FeBG). Values are individual data points, the bars show the geometric means, and the whiskers extend from –SD to +SD. For WG-oat-FeBG, $n = 29$. FeBG, ferrous bisglycinate; Fefum, ferrous fumarate; WG, whole grain.

significantly lower than that from the Fefum consumed in WG wheat lentil- and chickpea-based cereals.

In African children, ID is most prevalent during the first year of life (38). We reported a baseline anemia prevalence of 70%, similar to a previous Malawian study that reported a prevalence of 80% in 6- to 11-mo-old infants (39) and studies conducted in other settings (40, 41). Plasma hepcidin plays a major role in systemic iron metabolism and is downregulated when the body's demand for iron is high (42). In our study, PHep was a significant negative predictor of FIA. Our findings are consistent with stable iron isotope studies conducted in Kenyan infants (43, 44) and Gambian preschool children (45) that also found hepcidin is a significant predictor of fractional iron absorption.

High iron bioavailability from iron-fortified infant cereals is important, as they are often among the first foods that are introduced during complementary feeding. Only few studies have evaluated bioavailability of iron from infant cereals similar to the ones tested in the present study in young children (19, 46). In iron-deficient infants, bioavailability of iron as $FeSO_4$ from refined wheat-based infant cereal (similar to the reference meal in this study) was similar without and with dephytinization (8.7% compared with 8.5%, respectively) (46), possibly due to the low initial molar ratio of PA:Fe and the simultaneous addition of AA at a molar ratio of 2:1 relative to iron that anyway overcame the effects of PA (46). Furthermore, studies in infants (19) and young children (47) have investigated bioavailability of 2.5 mg iron from wheat- and soy flour–based infant cereals with added AA at a molar ratio of 3.2:1 relative to iron. In mostly non-anemic and non–iron-deficient, healthy Polish infants, iron absorption from Fefum was higher than from ferric pyrophosphate (4.1% compared with 1.3%) (19). In iron-deficient and anemic Bangladeshi children, iron absorption was consistently 3 to 4 times higher from $FeSO_4$ than Fefum in both healthy (15.6% compared with 5.4%) and *Helicobacter pylori*–infected children, before (19.7% compared with 5.3%) and after (22.5% compared with 6.4%) treatment (47). We reported a 2-3-fold higher geometric mean FIA from Fefum in the refined wheat cereal compared with Fefum measured in the Polish infants and the uninfected Bangladeshi children (19, 47). This may be due to differences in age-related regulation of iron absorption (48), iron and inflammation status of the study population, erythrocyte incorporation rate used (90% compared with 75%) to calculate iron absorption (which may result in an underestimation of iron absorption) (48, 49), and/or differences in meal matrix i.e., soy infant cereals in the previous studies (19, 47) had a higher molar ratio of PA:Fe and possibly conglycinin protein that is reported to decrease iron absorption (17).

In the present study, fractional iron absorption from WG-wheat-lentils and WG-wheat-chickpeas cereal did not differ significantly from the reference meal, despite the fact that these 2 cereals contained about 3 times the amount of PA and almost 2 times the amount of calcium compared with the reference meal. A sharp decline in iron absorption is expected when the molar ratio of PA:Fe in a food product without AA is found to be >1:1 or 0.4:1 (11). In addition, concentration of calcium per serving of the WG-wheat-lentils and WG-wheat-chickpeas cereals was within the range of calcium concentrations that inversely correlated with iron absorption when added to bread rolls (50). Therefore, our result suggests that the added AA:Fe molar ratio of 2:1 was enough to overcome the inhibitory effects of phytate and polyphenols, as well as that from calcium present in the WG wheat and the pulses recipes. This is also in agreement with previous observations showing that in the presence of AA, a meal matrix with a PA:Fe ratio <6:1 has low impact on Fe absorption (11, 46). In addition, the concentrations of casein and whey proteins, compounds in milk known to inhibit iron absorption, but to a lesser extent than the other nutrients (11, 51), were also lower in the WG-wheat-lentils and WG-wheat-chickpeas cereals.

Although not significantly different, our findings show a 24% lower bioavailability of Fefum from the WG-oat recipe compared with the refined wheat flour reference meal. The WG oat-based cereals contained the highest amount as well as different profiles of protein and polyphenols, thus contributing to an overall higher level of inhibitors than in the other recipes. This would be in agreement with a study investigating bioavailability of iron from oat- and wheat-based porridge in adults (21). In addition, *in vitro* studies performed with yellow beans and using the Caco-2 cell model suggest that some polyphenols, which may also be present in lentils, could promote iron absorption (52). However, to the best of our knowledge, this has not been demonstrated in human studies. On the other hand, it cannot be excluded that the dietary fibers present in the whole grains and the pulses promoted iron absorption, especially for the WG wheat lentil-based cereal.

FeBG has been reported to be an efficient fortificant in inhibitory meal matrices (23), and studies have shown that its absorption compares favorably with $FeSO_4$ (24, 26). It has been proposed that the presence of ionic and covalent bonds in the amino acid chelate and its high stability constant reduce interactions with inhibitory ligands in the gut, thereby improving iron absorption from the compound (53). In adult men, iron bioavailability from FeBG was 4–6 times higher than $FeSO_4$ (without AA) when both compounds were consumed in 1 or separate whole-maize porridge meals (24). To our knowledge, only 1 study has reported iron absorption from FeBG-fortified food in young children. In 9-mo-old, iron-replete children, iron bioavailability from FeBG (9%) was similar to $FeSO_4$ (9.9%) when consumed in the same vegetable puree but lower when consumed from an inhibitory WG cereal (PA:Fe: 2.2:1; no added AA, 5.2% and 3.8%, respectively) (26). Thus, in non–iron-deficient young children, iron absorption from FeBG is impaired by inhibitory nutrients in food. However, the nonsignificant 1.4-fold higher absorption from FeBG than $FeSO_4$ suggests that it could be a better choice for the fortification of such a matrix. Two studies have compared the efficacy of FeBG and $FeSO_4$ provided as iron supplements in infants (54, 55). In a retrospective study with preterm infants, supplementation with FeBG at a dose of 0.75 mg/kg/d demonstrated an efficacy comparable to iron sulfate at a dose of 3 mg/kg/d (55). In anemic infants who received 5 mg/kg of Fe daily for 28 d as either FeBG or $FeSO_4$, both groups had significant hemoglobin increases (54).

Our findings show a nonsignificant difference in bioavailability of iron from FeBG (7.4%) compared with Fefum (9.2%) when consumed in WG-oat cereal, as well as a significant 39% lower iron bioavailability from FeBG compared with Fefum in the noninhibitory reference meal (12.1%). Therefore, in agreement with the previous study with fortified food in infants (26), iron absorption from FeBG appears to be affected by dietary iron inhibitors. Furthermore, our findings suggest that when both compounds are coingested with AA, FeBG provides no nutritional benefit over Fefum. The slightly lower absorption could be explained by a lower dissociation of iron from the chelate with the gastric pH of young children, thus preventing

the absorption-enhancing effect of AA. Consistent with studies indicating that absorption of FeBG is regulated by iron status (24), in our study, PHep was a significant negative predictor of iron absorption from FeBG.

Our study has several strengths: *1*) we included 6- to 14-mo-old children living in a low-income setting, a target group for complementary food fortification strategies; *2*) we administered multiple stable iron isotope labels to compare FIA from 5 cereals, thereby allowing for the discrimination of meal-specific effects, and *3*) each child consumed all the cereals, allowing for within-subject comparisons. A limitation of our study is the intrasubject variability demonstrated by the reported FIA values that were 1.3 times higher in the meals consumed in the second period compared with the first period. Although we do not report a significant period effect, this increase may partly be attributed to weight gain and the subsequent increase in blood volume over the course of the study. In addition, the grains and pulses used in this study should not be assumed to reflect all possible pulse varieties, since *in vitro* studies suggest that certain varieties of lentils and chickpeas may modulate iron absorption in different ways (56–58).

In summary, although whole grains and pulses may be rich in antinutritional factors, our results suggest that when added to infant cereals fortified with Fefum and AA, iron remains well absorbed and would represent a healthier and significant source to rapidly growing infants.

Acknowledgments

We thank the caregivers who consented to their children's participation in the study and the entire study team at TRUE Centre, College of Medicine, University of Malawi. We also thank R. Jongstra (ETH Zurich) for her support during fieldwork and C. Netland (ETH Zurich) for her contribution to field and laboratory work, together with C. Zeder, A. Krzystek, T. Christ (ETH Zurich, Switzerland), and J. Erhardt (Willstaett, Germany) for supporting the laboratory analysis. We also thank J. T. Foman, D. Grathwohl, C. Tudorica, G. Fiore, and V. Cernyskov (Nestlé Research, Switzerland) for assistance with study operational support and test cereal production.

The authors' contributions were as follows—MAU, DM, MS, NPH, and MNM: designed the study; MAU, GM, and MNM: conducted the study; MAU, CZ, and NUS: analyzed the data and performed the statistical analyses; MAU, GM, NUS, DM, CZ, KP, MS, NPH, MBZ, and MNM: participated in data interpretation and edited the manuscript; MAU and MBZ: wrote the first draft of the manuscript; and all authors: read and approved the final manuscript.

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