The effects of iron fortification on the gut microbiota in African children: a randomized controlled trial in Côte d’Ivoire

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ABSTRACT

Background: Iron is essential for the growth and virulence of many pathogenic enterobacteria, whereas beneficial barrier bacteria, such as lactobacilli, do not require iron. Thus, increasing colonic iron could select gut microbiota for humans that are unfavorable to the host.

Objective: The objective was to determine the effect of iron fortification on gut microbiota and gut inflammation in African children.

Design: In a 6-mo, randomized, double-blind, controlled trial, 6–14-y-old Ivorian children (n = 139) received iron-fortified biscuits, which contained 20 mg Fe/d, 4 times/wk as electrolytic iron or nonfortified biscuits. We measured changes in hemoglobin concentrations, inflammation, iron status, helminths, diarrhea, fecal calprotectin concentrations, and microbiota diversity and composition (n = 60) and the prevalence of selected enteropathogens.

Results: At baseline, there were greater numbers of fecal enterobacteria than of lactobacilli and bifidobacteria (P < 0.02). Iron fortification was ineffective; there were no differences in iron status, anemia, or hookworm prevalence at 6 mo. The fecal microbiota was modified by iron fortification as shown by a significant increase in profile dissimilarity (P < 0.0001) in the iron group as compared with the control group. There was a significant increase in the number of enterobacteria (P < 0.005) and a decrease in lactobacilli (P < 0.0001) in the iron group after 6 mo. In the iron group, there was an increase in the mean fecal calprotectin concentration (P < 0.01), which is a marker of gut inflammation, that correlated with the increase in fecal enterobacteria (P < 0.05).

Conclusions: Anemic African children carry an unfavorable ratio of fecal enterobacteria to bifidobacteria and lactobacilli, which is increased by iron fortification. Thus, iron fortification in this population produces a potentially more pathogenic gut microbiota profile, and this profile is associated with increased gut inflammation. This trial was registered at controlled-trials.com as ISRCTN21782274. Am J Clin Nutr 2010;92:1406–15.

INTRODUCTION

Iron fortification can be an effective strategy to control iron-deficiency anemia in developing countries (1, 2), and the food-stuffs most often used for mass fortification are cereal flours. Worldwide, the most commonly used fortificants for flours are elemental iron powders such as hydrogen-reduced iron or electrolytic iron despite their low bioavailability (absorption of these poorly soluble forms of iron is often as low as <2–3%) (1). Flour-fortification programs are in place or in the planning stages in 78 countries (3), including in one-quarter of the population of sub-Saharan Africa (4). Côte d’Ivoire has mandated the addition of electrolytic iron to wheat flour (decree 025 issued on 18 January 2007). Low absorption of iron fortificants results in >90% of the iron passing unabsorbed into the colon. Most iron in the human body is tightly bound to various proteins that limit iron supply to potential pathogens, and during infection, iron supply is sharply reduced in the extracellular compartment (5). But there is no similar system for the sequestration of dietary iron in the gut lumen. Iron is a growth-limiting nutrient for many gut bacteria, and multiple strains vigorously compete for unabsorbed dietary iron in the colon because colonization depends on the ability of the bacteria to acquire iron and other essential growth nutrients (6).

Although iron is an essential nutrient for most of the gut microbiota, some beneficial barrier bacteria, such as lactobacilli, play an important role in the prevention of colonization by enteric pathogens but do not require iron (7). In contrast, for most enteric gram-negative bacteria (eg, Salmonella, Shigella, or pathogenic Escherichia coli), iron acquisition plays an essential role in the virulence and colonization of most pathogenic strains (8). In animal studies, increasing doses of iron produced a linear increase in diarrhea incidence and coliform number, and the ratios

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2 The sponsors of the study played no role in the design of the trial, analyses of data, or preparation of the manuscript.

3 Supported by the Medicor Foundation (Vaduz, Liechtenstein), the Swiss National Science Foundation (Bern, Switzerland), the Swiss Foundation for Research in Nutrition (Zurich, Switzerland), and the Swiss Federal Institute of Technology (ETH) Zurich (Zurich, Switzerland). Escherichia coli O157H715 was provided by Roger Stephan.

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Received April 29, 2010. Accepted for publication September 24, 2010. First published online October 20, 2010, doi: 10.3945/ajcn.110.004564.
of bifidobacteria and lactobacilli to enterobacteria were modified (9). These ratios can be an index of gut health because higher ratios provide greater resistance to infection (10).

Therefore, an increase in unabsorbed dietary iron in humans through fortification or supplementation could modify the colonic microbiota equilibrium and favor the growth of pathogenic strains over barrier strains. This would be an important adverse effect because diarrheal disease is the cause of death of ~1 in 6 children <5-10 year old in sub-Saharan Africa (11). A recent systematic review suggested that iron supplementation was associated with a small increase in diarrheal disease (12). A World Health Organization (WHO) Consultation, which interpreted the Pemba study in which untargeted iron supplementation increased mortality in children (13), cautioned that it is unclear whether risks of iron are specific to malaria or whether they apply to other infections, including sepsis from enteric bacteria (14). Thus, our study objective was to determine the effect of a poorly absorbed iron fortificant on gut microbiota, gut helminthes, and gut inflammation in African children in an area with high rates of diarrheal disease. Specifically, our hypotheses were that 1) iron fortification would increase the ratio of fecal enterobacteria to bifidobacteria and/or lactobacilli, 2) favor the colonization by potentially pathogenic strains, and 3) increase gut inflammation.

SUBJECTS AND METHODS

Study design

This study was nested within a larger 2 × 2 × 2 intervention trial that tested the interactions of the intermittent treatment of malaria, antihelminthic treatment, and iron fortification (15). The study population consisted of 6–14-year-old school children in rural central Côte d’Ivoire with a high infectious disease burden (16). The main dietary staples in the study region are rice and yam. The usual wet season is from March to November. The study was done during one school year from November 2006 to July 2007. For the effect of iron on hemoglobin concentrations, with the assumption of a mean (±SD) hemoglobin concentration of 117 ± 12 g/L (17), to detect a hemoglobin concentration increase of 8 g/L, 65 children were needed in each group to achieve a power of 90% at a 5% level of significance. For the microbiota substudy, we estimated 30 subjects per group would be adequate for comparisons of the dominant bacteria on the basis of previous studies (18, 19). Ethical approval was given by the Swiss Federal Institute of Technology (ETH) Zurich (Zurich, Switzerland) and the Ministry of Health in Côte d’Ivoire. Written informed consent was obtained from parents or legal guardians of children who participated in the study. The larger trial in which this study was nested was registered at controlled-trials.com as ISRCTN21782274. Dietary iron intake was assessed by 3-d weighed food records at the midpoint of the intervention in 24 households and compared with recommended dietary intakes (20).

Inclusion criteria were, for girls, nonpregnant (self-reported) and, for boys and girls, no major chronic illnesses, no use of iron-containing supplements, and anticipated local residence for the study duration. Children were randomly assigned to either the iron-fortification group or the control, no-iron group. The iron group received 2 fortified biscuits (Midor AG, Meilen, Switzerland) [electrolytic Fe (A-131; Dr Lohmann GmbH, Emmerthal, Germany), 20 mg Fe/d per child] 4 times/wk; the control group consumed identical but unfortified biscuits. The biscuits were made from low-extraction wheat flour and contained (per 100 g) 454 kcal, 76 g carbohydrate (16.5 g sucrose), 1.5 g total fiber, 14 g fat, and 6.3 g protein. The iron content of biscuits was confirmed at the Swiss Federal Institute of Technology Zurich by using atomic absorption spectrometry (Spectra AA-50; Varian, Palo Alto, CA).

Active and passive case detections for diarrhea and other gastrointestinal illness were carried out throughout the study. For active detection, teachers administered a health questionnaire monthly with a 2-d recall period. If an illness was reported, the child was examined by the study physician. Diarrhea was defined as ≥3 loose, watery stools in a day (21). For the passive reporting, parents and guardians were encouraged to refer children free of charge to the local health center as soon as they presented a symptom or illness.

Blood and stool variables were assessed at baseline and after 6 mo. Hemoglobin concentrations were measured in whole blood with an AcT8 Counter (Beckman Coulter; Krefeld, Germany) on the day of blood sampling. Plasma was divided into aliquots, transported frozen, and stored at −25°C. Zinc protoporphyrin (ZPP) concentrations were measured on washed red blood cells with a hematofluorometer (Aviv Biomedical; Lakewood, NJ) ≤7 d after sampling. Plasma ferritin (PF) and C-reactive protein (CRP) concentrations were measured with an automated chemiluminescent immunoasay system (IMMULITE; Diagnostic Products Corporation, Los Angeles, CA). α1-Acid glycoprotein (AGP) concentrations were measured by immunoturbidimetry (Cobas Mira; Roche Diagnostics, Rotkreuz, Switzerland). Soluble transferrin receptor (TfR) concentrations were measured by using an automated immunonephelometric assay (Cobas Integra 800; Roche Diagnostics). Anemia was defined according to the WHO (22); iron deficiency was defined as a PF concentration <30 μg/L or TfR concentration >8.2 mg/L and ZPP concentration >40 μmol/mol heme (22, 23). Systemic inflammation was defined as an AGP concentration >1.2g/L or CRP concentration >10 mg/L.

A single, fresh, morning stool sample was collected at baseline and after 6 mo. Two Kato-Katz thick smears (41.7 mg) were prepared from each stool sample according to standard protocols (24), and hookworm eggs were counted microscopically. The slides were reexamined, and the number of eggs of Ascaris lumbricoides, Schistosoma mansoni, and Trichuris trichiura were counted. For the conversion to eggs per gram of feces, a multiplication factor of 24 was used. The remaining stool was divided into aliquots and stored at −70°C until further analyses. Fecal calprotectin concentrations were measured by using an immunoassay (European SpA, Trieste, Italy) and expressed as micrograms per gram.

Gut microbiota

Stratified by sex, 30 children were randomly selected from each group for the gut microbiota analyses. None of the children had unusual diet habits or received antibiotics in the 3 mo before the baseline stool collection; during the trial, the number and days of antibiotics prescribed did not differ between the 2 groups (P = 0.942); no child included in the gut microbiota analyses was
given antibiotics in the 3 wk before the endpoint assessment. Fecal samples were collected from 0900 to 1130 at the school; children were given prelabeled beakers with lids and were asked to provide a stool sample. The beakers were immediately placed in an ice chest with cooling elements and taken to the local laboratory at the end of each morning. Stool samples were split into aliquots, and the aliquot for gut microbiota analysis was frozen immediately at $-30^\circ$C until analyses.

DNA extraction

Total bacterial DNA was extracted from fecal samples (200 mg feces) with a Fast DNA SPIN kit (MP Biomedicals, Illkirch, France) according to the manufacturer’s instructions. DNA concentrations were measured with a NanoDrop ND-1000 Spectrophotometer (Witec AG, Littau, Switzerland) at a wavelength of 260 nm, and samples were stored at $-24^\circ$C until further analysis.

Analyses of gut microbiota by polymerase chain reaction and temporal temperature gradient electrophoresis analyses

Primers HDA1-GC and HDA2 were used to amplify the variable regions 2 and 3 of the bacterial 16S ribosomal RNA (rRNA) genes (Table 1) and investigate the whole bacterial diversity. A GC-rich sequence (5' CCC CCC CCC CCC GCC CCC CCC GCC CCC GCC CCC GCC C 3') was added to the 5' end of the reverse primer. Reaction tubes contained 1 $\mu$L fecal DNA, 25 $\mu$L polymerase chain reaction (PCR) Master Mix (2x) (Fermentas), and 0.4 $\mu$L of each primer in a final volume of 50 $\mu$L. One microliter of fecal DNA was used for standardization because preliminary tests showed only a small variability in DNA concentrations extracted from 1 $\mu$L fecal DNA (in a concentration range of 100–300 ng/$\mu$L) and that this variability did not affect the temperature gradient electrophoresis (TGGE) profiles (see supplemental Figure 1 under “Supplemental data” in the online issue). PCR amplifications were performed by using the following conditions: an initial DNA denaturation and enzyme activation at 94°C for 4 min, 30 cycles consisting of denaturation (30 s at 94°C), annealing (30 s at 58°C), elongation (1 min at 68°C), and a final elongation at 68°C for 7 min. PCR product concentrations and sizes were estimated by using 2% agarose gel electrophoresis that contained ethidium bromide (0.1 ng ethidium bromide/mL), in 1× TBE. The Dcode universal mutation detection system (Bio-Rad Laboratories, Reinach, Switzerland) was used for the sequence-specific separation of amplicons. These amplicons were loaded into a 1-mm polyacrylamide gel that consisted of 9% (vol:vol) polyacrylamide (vol:vol, acrylamide-bisacrylamide, 37.5: 1) and 8 mol urea/L with 1.5 × TAE as the electrophoresis buffer. A prerun of 15 min at a constant voltage of 20 V preceded a run at 65 V. The temperature of the gel system was programmed to increase by 0.3°C/h from 66°C to 70°C. In addition, similarly obtained PCR products from known bacterial strains were loaded to allow standardization of the band migration and gel curvature between different gels. This ladder consisted of DNA of the following organisms listed in migration order: Bacteroides thetaiotaomicron [Deutsche Sammlung von Mikroorganismen (DSM) 2079; http://www.dsmz.de/], Lactobacillus acidophilus (DSM 20079), Roseburia intestinalis (DSM 14610), E. coli (American Type Culture Collection 25288; http://www.atcc.org/), and Bifidobacterium longum (DSM 20219).

The investigation of the Lactobacillus community was performed by 2 successive nested PCRs (26); the first was performed with the primers Bact-0011f and Lab-0677r (Table 1). The reaction mixtures (50 $\mu$L) consisted of 25 $\mu$L PCR Master Mix (2x) (Fermentas), 1 $\mu$mol primer Bact-0011f/L, 1 $\mu$mol primer Lab-0677r/L, and $\approx$1 $\mu$L bacterial DNA. The PCR products, which were used as template for the second PCR, were firstly purified with a QIAquick PCR Purification Kit (Qiagen AG, Basel, Switzerland). To prevent the amplification of residual genomic DNA or low-yield aspecific amplicons formed in the first PCR, purified samples were diluted to a concentration of 15 ng/$\mu$L. The second nested PCR was performed with the universal primers Bact-0124-GCF and Univ-0515r. The reaction mixtures (50 $\mu$L) consisted of 25 $\mu$L PCR Master Mix (2x) (Fermentas), 1 $\mu$mol primer Bact-0124-GC/L, 1 $\mu$mol primer Univ-0515r/L, and 1$\mu$L of the previously amplified eluted DNA samples. PCR product concentrations and sizes were estimated by using 2% agarose gel electrophoresis that contained ethidium bromide (0.1 ng ethidium bromide/mL) in 1× TBE. The Dcode universal mutation detection system was also used for the sequence-specific separation of amplicons. These amplicons were loaded in a 1-mm polyacrylamide gel that consisted of 8% (vol:vol) polyacrylamide (vol:vol, acrylamide-bisacrylamide, 37.5: 1) and 7 mol urea/L with 1.5 × TAE as the electrophoresis buffer. A prerun of 15 min at a constant voltage of 20 V preceded a run at 76 V. The temperature of the gel system was programmed to increase by 0.2°C/h from 64°C to 70°C.

Gels were stained with ethidium bromide solution (2.5 mg ethidium bromide/mL; Fluka, Buchs, Switzerland) for 45 min, rinsed for 30 min, and scanned under ultraviolet light (Alphalager system; Alpha Innotech Corporation, San Leandro, CA). Gel patterns were analyzed with GelCompar II software (Applied

| TABLE 1 | Primers used for polymerase chain reaction and temporal temperature gradient electrophoresis analyses of gut microbiota |
|-----------------|-----------------|-----------------|
| Target group    | Primer and sequence (5’–3’) | Reference |
| All bacteria    | HDA1-GC, GACTCTTAGGCGGCCGACAG1 | (25) |
|                 | HDA2, GTATTACCGCCGTCTGGCCA | (25) |
| Lactobacillus spp. | Bact-0011f, AGAGTTTGATCTTACGAGCAG | (26) |
|                 | Lab-0677r, CACCGCTACACAGGAG | (26) |
|                 | Bact-0124-GCF, GGACGGTTACAGACAG1 | (26) |
|                 | Univ-0515r, ATCGTATTCGCGGTACG | (26) |

1 GC clamp: CGC CGG GGG CGC GCC CCC GGC GGG CGG GGG CCA CCG GGG G.
Maths NV, Sint-Martens-Latem, Belgium). Each band was identified and normalized on the basis of the migration of the marker. Baseline and endpoint samples from the same volunteer were always comigrated side by side for accurate comparisons.

### Analyses of gut microbiota by quantitative real-time PCR

PCR amplification and detection were performed with an ABI PRISM 7500-PCR sequence-detection system (Applied Biosystems, Zug, Switzerland). Each reaction mixture of 25 µL was composed of 2× SYBR Green PCR Master Mix (Applied Biosystems), with each of the specific primers at a concentration of 0.20 µM, and 1 µL template DNA diluted to DNA concentrations of 5 or 0.5 ng/µL depending on the targeted bacterial group. The quantitative real-time PCR conditions were kept at the presettings of the ABI PRISM 7500-PCR sequence-detection system (Applied Biosystems) with an initial heating step of 2 min at 50°C and a denaturation step of 10 min at 95°C followed by 40 amplification cycles at 95°C for 15 s and 60°C for 1 min. The fluorescent products were detected during the second step of each cycle. A melting-curve analysis was performed after amplification to evaluate the specificity of the primers and the quality of the PCR. Primers used in this study are listed in Table 2. They were synthesized and purified by Microsynth (Microsynth, Balderschwang, Switzerland). Amplified 16S rRNA genes from the following bacteria served as standard templates: pLME21 plasmid that contained *Bifidobacterium lactis* 16S ribosomal DNA (rDNA) for universal primers (34), *B. thetaotaomicron* (DSM 2079) for *Bacteroides* spp. primers, *Bacteoides lactis* (DSM 20081) for *Lactobacillus/Leuconostoc/Pediococcus* spp., *E. coli* O157H45 (Laboratory of Food Biotechnology culture collection, www.bt.ltw.ethz.ch/) for *Enterobacteriaceae* primers, and *Salmonella* Typhimurium N15 (Laboratory of Food Biotechnology culture collection) for *Salmonella* primers. For each primer pair, a standard curve was calculated in the same run and used for the calculation of the number of 16S rDNA or gene copies detected in each sample.

Because the copy number of 16S rDNA genes varies according to the species considered, the data for *Bacteroides*, *Lactobacillus*, and *Enterobacteriaceae* enumeration were normalized by using the different mean copy numbers of 4.63, 5.54 and 7.0, respectively, referenced in the Ribosomal RNA Database (35, 36). Normalization was not necessary for *Bifidobacterium* species enumeration because they possess a single copy of the *sfp* gene used as the target (37). Therefore, results were expressed in cell numbers standardized by dividing absolute copy numbers obtained by the mean copy number for that bacterial group.

### Detection of bacterial gut pathogens by PCR

Pathogenic strains of *E. coli*, such as enteropathogenic *E. coli* (EPEC), enteraggregative *E. coli* (EAEC), Shiga toxin-producing *E. coli* (STEC), and enteroinvasive *E. coli* (EIEC) as well as *Shigella* spp. were detected by using the primers of Aranda et al (38) for multiplex PCRs (Table 2). Each PCR mixture of 25 µL contained PCR Master Mix (2×) (Fermentas), 1 µmol/L of each primer, and 1 µL DNA. PCRs were carried out with an initial heating step for 3 min at 95°C and 35 amplification cycles of 95°C for 30 s, 58°C (for EPEC and EAEC primers) or 50°C (for EIEC and STEC primers) for 30 s, and 72°C for 1 min followed by a final elongation step at 72°C for 7 min. PCR products were visualized by separation in a 2% agarose gel and stained in ethidium bromide. Amplified 16S rRNA genes from the following bacteria served as positive controls: *E. coli* O157H45 (Laboratory of Food Biotechnology culture collection) for EPEC, EAEC, STEC, and EIEC detection and *Shigella flexneri* (American Type Culture Collection 29903) for *Shigella* primers.

### TABLE 2

Primer used for quantitative real-time polymerase chain reaction (PCR) and multiplex PCR analyses of gut microbiota

<table>
<thead>
<tr>
<th>Target group</th>
<th>Primer and sequence (5′–3′)</th>
<th>Method used</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>All bacteria</td>
<td>Eub338B, ACTCTTACGGGAGGCAGCACGAG</td>
<td>Real-time PCR</td>
<td>(27)</td>
</tr>
<tr>
<td>Bacteroides spp.</td>
<td>Eub518R, ATACACGGGCTGTGCGG</td>
<td>Real-time PCR</td>
<td>(27)</td>
</tr>
<tr>
<td><em>Bifidobacterium</em> spp.</td>
<td>Bac303F, GAAGGTCCCCCACATTG</td>
<td>Real-time PCR</td>
<td>(27)</td>
</tr>
<tr>
<td>Lactobacillus/Leuconostoc/Pediococcus spp.</td>
<td>Bfr-Fmrev, CGCKACTTGGCTGGTTCAG</td>
<td>Real-time PCR</td>
<td>(27)</td>
</tr>
<tr>
<td>Enterobacteriaceae</td>
<td>xfp-fw, ATCTTCCGACCAGBAYGAGAG</td>
<td>Real-time PCR</td>
<td>(27)</td>
</tr>
<tr>
<td>Salmonella spp.</td>
<td>xfp-rv, CGATVAGCGAAAGCAGG</td>
<td>Real-time PCR</td>
<td>(27)</td>
</tr>
<tr>
<td>Enteropathogenic <em>E. coli</em></td>
<td>F_Lacto 05, AGCACGTAGGAACTCACTCA</td>
<td>Real-time PCR</td>
<td>(30)</td>
</tr>
<tr>
<td>Enteraggregative <em>E. coli</em></td>
<td>R_Lacto 04, CGCCACTGTGTCCTCACCATATA</td>
<td>Real-time PCR</td>
<td>(30)</td>
</tr>
<tr>
<td>Shigella spp.</td>
<td>Eco1457F, CATTGAGTACTCCGCAAGAAGA</td>
<td>Real-time PCR</td>
<td>(31)</td>
</tr>
<tr>
<td>Enteroinvasive <em>E. coli</em> and <em>Shigella</em> spp.</td>
<td>Eco1652R, CTCTACGAGACTCAAGCTTG</td>
<td>Real-time PCR</td>
<td>(31)</td>
</tr>
<tr>
<td>Enteroinvasive <em>E. coli</em> and <em>Shigella</em> spp.</td>
<td>InvA 139, GTGAAATATTCGCCACGTCCGCA</td>
<td>Real-time PCR</td>
<td>(32)</td>
</tr>
</tbody>
</table>

1. *E. coli*, *Escherichia coli*. 

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Statistical analyses

Data analyses were done with SPSS software (version 16.0; SPSS Inc, Chicago, IL) and Instat 3.0 GraphPad (Graph Pad Software Inc, La Jolla, CA). Analyses were done by using a per protocol approach. Nonnormally distributed variables were transformed by using appropriate algorithms. To compare groups, 1- or 2-factor analyses of variance were used, with post hoc t tests. Because the lactobacilli numbers in the 2 groups were significantly different at baseline, we analyzed these data with analysis of covariance by using the baseline values as covariates. Rate ratios of gastrointestinal illness episodes were calculated with Poisson regression (95% CI) relative to the control group. Linear regression was used to determine associations between variables. Repeated-measures analysis by using general linear models was used to compare changes in the binary variables during the intervention. Comparisons of TGGE profiles were performed using the Dice similarity coefficient (Dsc) analysis based entirely on the results of band classification. Dsc values were compared on the basis of the presence or absence of bands. The Dice coefficient was defined as follows:

\[
\text{Dsc} = \frac{2j}{(a+b)} \times 100
\]

where \( j \) is the number of common bands between samples A and B, and \( a \) and \( b \) are the total number of bands in samples A and B, respectively. The distance between 2 TGGE profiles was then calculated as

\[
\text{Distance} = 100 - \text{Dsc}
\]

This coefficient ranged from 0 (no common bands) to 1 (identical band patterns) (19). Significance was set at \( P < 0.05 \).

RESULTS

At baseline, 74 children were assigned to the iron group, and 73 children were assigned to the control group. During the study, there were 4 dropouts in the iron group (2 children dropped out because of relocation out of the study area, one child developed diabetes, and one child refused to consume the biscuits) and 4 dropouts in the control group (3 children dropped out because of relocation out of the study area, and one child developed splenomegaly); thus, 70 and 69 children completed the study. There were no significant differences in baseline biomedical characteristics between iron and control groups (Table 3). In subjects who participated in the gut microbiota study (\( n = 30 \) in each group), there were no significant differences in the variables shown in Table 3 between the 2 groups at baseline (data not shown) with the exception of PF without inflammation, the median (range) concentrations of which were 107 (22–270) and 61 (27–173) \( \mu \)g/L in the subgroups of the control and iron groups, respectively.

At baseline in all children, 54% of children were infected with helminths (of these, 93% of helminths were hookworm, 4% of helminths were \( T. \) trichiura, and 3% of helminthes were \( A. \) lumbricoides). The geometric mean intensity of hookworm was 108.8 eggs/g feces (95% CI: 81.2, 145.7 eggs/g feces); no child exceeded the WHO cutoff values for light infection. The hookworm prevalence and egg burden increased in both groups during the intervention without significant differences between groups (Table 3). At the endpoint, there were no \( T. \) trichiura or \( A. \) lumbricoides detected in any of the subjects in either group.

Estimated daily mean (±SD) iron intakes before the introduction of the iron fortificant were 12.2 ± 3.8 mg Fe for children <10 y of age, 14.5 ± 3.5 mg Fe for boys >10 y of age, and 13.7 ± 2.9 mg for girls >10 y of age. Assuming a 10% dietary iron bioavailability, this translates into 138%, 94%, and 44% of the requirements for absorbed iron (20). During the trial, the mean (±SD) number of biscuits consumed was 165 ± 21 out of 200 biscuits offered (83% compliance), and compliance between groups was comparable (\( P = 0.79 \)). The iron content of the fortified biscuits was 9.6 ± 0.3 mg Fe. Thus, the total additional iron consumed by children who received fortified biscuits was 1585 ± 217 mg electrolytic Fe, which corresponded to 8.8 ± 1.2 mg fortificant Fe/d; therefore, the iron intervention increased the overall dietary iron intake by ≈60–70%.

The iron fortificant was very poorly absorbed; anemia and iron status worsened during the 6-mo study period, and there was no difference after 6 mo in the indicators of marrow iron supply (TIR and ZPP) or storage iron (PF) (Table 3). Compliance was >94% for monitoring of self-reported illness via the monthly questionnaire. There was no significant difference in gastrointestinal illness between the groups: the rate ratios (95% CIs) of diarrhea, constipation, and vomiting in the iron group relative to the control were 1.0 (0.6, 1.4), 0.8 (0.5, 1.1), and 1.4 (0.8, 2.5), respectively.

Gut microbiota

Iron fortification modified the gut microbiota: in the TGGE profiles of PCR amplicons (V2–V3 region) from the fecal samples, iron fortification caused an increase in bacterial dissimilarity compared with the control group (32.3 ± 12.5% compared with 15.0 ± 7.5%; \( P < 0.0001 \)) (Figure 1, A and B), which showed a higher variation rate in bacterial diversity after iron fortification. However, the mean number of bands in the TGGE profiles that compared iron and control groups was not significantly different at 6 mo (Figure 1). In the analyses using \( Lactobacillus \) group-specific primers, there was no significant change in the presence of \( Lactobacillus \) species with iron fortification. Although each volunteer in the iron group showed a microbiota profile at 6 mo that strongly differed from that at baseline (see Figure 2 under “Supplemental data” in the online issue for examples), a specific band pattern related to iron fortification was not detected.

At baseline in all 60 children, there were significantly greater mean (±SD) cell numbers (log cell number/g feces) of enterobacteria (7.86 ± 0.87) than bifidobacteria (7.42 ± 0.59) (\( P < 0.02 \)) or lactobacilli (6.49 ± 1.14) (\( P < 0.001 \)) (Figure 2). During the intervention, there was no significant change in numbers of total bacteria at 0 and 6 mo: (control: 11.29 ± 0.67 and 11.28 ± 0.81, respectively; iron: 11.62 ± 0.94 and 11.78 ± 0.59, respectively), \( Bacteroides \) (control, 10.01 ± 0.67 and 10.23 ± 0.70, respectively; iron, 10.13 ± 0.70 and 10.31 ± 0.85, respectively) or bifidobacteria (Figure 3). However, there was an increase in enterobacteria in the iron group (\( P < 0.005 \)) (Figure 3) and a reduction in lactobacilli (\( P < 0.0001 \)). The enterobacteria population includes many of the enteric pathogens, and 26.6% of the children had positive samples for...
Shigella spp. and enteroinvasive E. coli (these could not be distinguished with IpaH1 and IpaH2 primers) and/or Salmonella spp. at baseline but at low amounts (generally ≤10^3 bacteria/g feces). Of these, Salmonella was the predominant bacterial pathogen and was observed in 78.6% of positive samples, whereas Shigellalenteroinvasive E. coli were detected in 21.4% of positive samples. After 6 mo, more children were positive for Salmonella in the iron group than in the control group (23.3% compared with 16.6% of children, respectively), but this difference was not significant. There were no significant correlations between baseline SF, TfR, or ZPP concentrations and baseline numbers of enterobacteria, bifidobacteria, or lactobacilli, nor were there significant correlations between changes during the study in SF, TfR, or ZPP concentrations and changes in the number of enterobacteria, bifidobacteria, or lactobacilli.

Systemic and gut inflammation

During the intervention, there was no difference between groups in systemic inflammation determined by AGP or CRP concentrations (Table 3). There was also no significant correlation between the change (the difference between baseline and 6-mo values) in systemic inflammation measures (AGP or CRP) and change in the numbers of enterobacteria, lactobacilli or bifidobacteria during the intervention. In contrast, there was a significant increase in the mean calprotectin concentration in fecal samples from the iron group at 6 mo than in the control group (P < 0.01) (Figure 3). There was no significant association between changes in fecal calprotectin concentrations and changes in numbers of bifidobacteria, lactobacilli, or hookworm during the intervention, but there was a positive correlation between change in fecal calprotectin concentrations and changes in numbers of enterobacteria (r = 0.32, P < 0.05).

DISCUSSION

Although each child in the iron group consumed ~1.5 g Fe fortificant over the course of the study, there was no improvement in iron status or anemia. Iron status deteriorated over the course of the study in both groups, likely because of limited food availability caused by the prolonged dry season in Côte d’Ivoire in 2007. Iron absorption was poor for the following reasons: 1) elemental iron powders, such as electrolytic iron, have low bioavailability because of their low solubility (1); 2)
fractional absorption of dietary iron is inversely related to body iron stores, but only 15% of children were iron deficient at baseline; 3) nearly 1 in 4 children had signs of systemic inflammation that may have reduced iron absorption and/or use (39). Our findings are consistent with previous trials of electrolytic iron in African populations that were not effective (40, 41). Thus, it is likely that nearly all of the iron fortificant passed into the colon and was potentially available for the gut microbiota.

In this study, we combined TGGE and PCR to assess and compare the changes in the dominant gut microbiota and other specific populations during the intervention. This approach has proven useful to show nutritional or pathologic modulation of the gut microbiota (18, 19, 42). The baseline composition of the gut microbiota in these African children was markedly different from that reported in European populations (43–45). In humans, the total enterobacteria population represents a subdominant population of $\sim 10^6$ to $10^7$ bacteria/g feces (43–45). In this study, we observed higher numbers of enterobacteria in African children with estimated population amounts close to $10^8$ bacteria/g feces at baseline. Thus, this population was already high and dominant at baseline and was further increased by $\approx 0.5$ log with iron fortification. After iron fortification, the children harbored, on average, one hundred million more enterobacteria per gram of feces, which was a substantial increase. At baseline, 1 in 4 children had fecal samples that contained potential pathogens (mainly Salmonella), and there were greater numbers of enterobacteria than lactobacilli and bifidobacteria (Figure 2). This is a striking reversal of the usual high ratios of lactobacilli and bifidobacteria to enterobacteria observed in healthy white children and adults (43–45). The microbiota profile in our children was likely the result of chronic contamination of the local food and/or water supply, and although it was not associated with clear clinical disease, the high numbers of enterobacteria at baseline may have made these children more susceptible to colonization by enteropathogens (46) when colonic iron was abundant. In healthy humans, high species diversity provides ecologic stability (47, 48) so that after infancy, the composition of the intestinal microbiota at the species level is remarkably stable (49). This characteristic stability was perturbed by iron fortification; TGGE analyses showed profound differences in the dominant gut bacterial species at baseline compared with the endpoint for children in the iron group. However, although the iron group showed a microbiota profile at 6 mo that differed from that at baseline, a specific band pattern related to iron fortification was not detected, which suggests that the overall effects of iron fortification on the gut microbiota balance were host specific. Alternatively, our methods may have been insufficient resolution to identify consistent effects on the less dominant members of the bacterial community.

Iron fortification resulted in an increase in the numbers of enterobacteria and a decrease in the lactobacilli population. The expansion of the enterobacteria was likely mainly due to the increased growth of commensal and nonpathogenic E. coli, and this may be important because abundances of closely related species can predict the susceptibility to intestinal colonization by pathogenic bacteria. In a recent study by Stecher et al (46), the presence of high commensal E. coli densities in animals correlated with higher amounts of Salmonella colonization.

Iron fortification favored the growth of enterobacteria over lactobacilli, and this was likely due to their differing iron requirements. Most enteric gram-negative pathogens, including Salmonella spp., E. coli, Shigella spp. (8), take up iron-side-rophore complexes via specific outer-membrane receptors. In vitro, enteric bacteria display increased virulence in situations of increased iron availability (50), and iron transporter [ferrous iron transporter, protein B (FeoB)]–mediated ferrous iron acquisition is required for bacterial virulence (51) and gastrointestinal tract colonization (52). Thus, it is possible that more soluble forms of iron, such as ferrous sulfate, could have a greater effect on enteropathogen growth than the very poorly soluble electrolytic iron used in this study. Only a few bacteria do not require iron, and Lactobacillus is the major enteric bacterial genera that do not (53). Lactobacilli do not produce siderophores, and their growth is similar in media with and without iron (54). Abundant
lactobacilli and other commensal bacteria in the colon provide an important barrier effect against colonization and invasion by pathogens (55–57). Iron fortification reduced the lactobacilli number and may have weakened this protective effect. The higher ratio of enterobacteria to lactobacilli (10) may have encouraged colonization by Salmonella in the iron group.

The increase in fecal calprotectin concentrations (Figure 3) with iron fortification suggested that changes in the gut microbiota may have increased gut inflammation. Calprotectin is a 36.5-kDa calcium-binding polypeptide observed in the cytosol of neutrophils, monocytes, and activated macrophages (58). Fecal calprotectin concentrations reflect translocation and migration of primarily neutrophils into the intestinal mucosa. It has higher specificity than systemic inflammatory markers for gut inflammation and is more sensitive; this was evident in our data where CRP and AGP concentrations did not increase in the iron group, whereas calprotectin concentrations did increase. Other potential factors that can increase fecal calprotectin concentrations are significant fecal blood (>100 mL) and chronic nonsteroidal antiinflammatory drug use, but these were unlikely in our population with a low intensity of helminth infection. Gastroenteritis in children can elevate fecal calprotectin concentrations; with the use of the same assay as in our study, the median (range) fecal calprotectin concentration in preschool European children with acute gastroenteritis was 110 (0.3–244) μg/g (59). A daily dose of 120 mg supplemental iron for 14 d did not increase already elevated fecal calprotectin concentrations in anemic adults with inflammatory bowel disease (60). The direct correlation between increased fecal calprotectin concentrations and increased enterobacteria numbers in our data suggest the iron-associated increases in enterobacteria may have contributed to the increase in gut inflammation.

Increasing iron intakes may increase diarrheal disease in children; a systematic review (12) concluded that the provision of iron is associated with a significant 11% higher risk of developing diarrhea. Since that review, 2 large trials of iron supplementation in children (12.5 mg Fe/d with 50 μg folic acid) in Nepal (53) and Tanzania (13) reported cause-specific mortality and diarrhea incidence as secondary outcomes. In Nepal, there was a nonsignificant increased risk of death from diarrhea in the iron group [relative risk (RR) (95% CI): 1.21 (0.66, 2.11)] but no significant difference in diarrhea incidence [RR (95% CI): 0.94 (0.84, 1.05)] (61). In Pemba, there was also no significant difference in diarrhea incidence [RR (95% CI): 0.92 (0.68, 1.25)] (13). However, recent controlled iron-supplementation trials (12.5–15 mg Fe/d) in Peru (62) and Bangladesh (63) reported a significant increase in diarrhea.

In contrast, there are no convincing data that iron fortification increased risk of diarrhea, but to our knowledge, there have been no large studies where this effect was rigorously tested. Four iron-fortification-studies in the review of Gera and Sachdev (12) reported diarrheal outcomes; one study (64) showed a significantly higher incidence of diarrhea. In the current study, there was no effect on self-reported diarrhea, but the study was not powered to detect this. Overall, the available data suggest that untargeted oral iron supplementation is associated with a small increase in risk of diarrhea in children. Our findings provide a potential mechanism for this effect.

To our knowledge, this is the first study to examine the effect of an iron intervention on the human gut microbiota in Africa, and our findings need confirmation in other settings and populations. This is important because diarrhea remains a major cause of morbidity and mortality in African children, and iron supplementation and fortification are the most common strategies used in Africa to combat anemia. It is possible that higher doses of iron given in supplements [in children, 2 mg Fe · kg⁻¹ · d⁻¹; up to 30 mg Fe/d (21)] have an even greater effect on the gut microbiota than observed in the current study, in which the children received ~8 mg Fe/d. If iron disturbs gut homeostasis and encourages growth of enteropathogens, particularly in vulnerable periods such as infancy, it may be important to J consider the use of a probiotic to maintain lactobacilli and bifidobacteria

FIGURE 3. Enumeration (means ± SEs) of bifidobacteria (A), lactobacilli (B), and enterobacteria (C) by real-time polymerase chain reaction performed with fecal DNA from Ivorian children in the control and iron-fortification groups as well as fecal calprotectin concentrations (D) at baseline (0) and after 6 mo (6). Relative cell numbers were calculated by normalization of the total rrs gene copy number to the mean copy number per cell for each bacterial group (n = 4.63 for Bacteroides, 7.0 for enterobacteria, and 5.54 for lactobacilli). To compare groups, 2-factor ANOVA or, for lactobacilli, ANCOVA with zero time values as covariates were used with post hoc t tests. Values without a common letter differed significantly, P < 0.01.
populations during the provision of iron and/or 2) minimize the amount of added iron by maximizing the bioavailability of both the fortificant and the native iron in the diet. Strategies to do this are available, including the use of chelated iron and/or absorption enhancers (40, 65).

We thank Andreas B Tschannen and Daniel E Sess for assistance in the study, Mahamadou Bakayoko, Kouadio J Brou, Sostèn S Léon, and Naoussis N Lingue for technical assistance in parasitology, and Samuela Rossi and Doreen Gille for assistance with the gut microbiota analyses.

The authors’ responsibilities were as follows—MBZ, FR, and HG: conducted field work; CC, AD, and CL: performed gut microbiota analyses; MBZ, FR, AD, and CC: analyzed data; MBZ and CC: wrote the first draft of the manuscript; and all authors: helped design the research and edited and approved the final manuscript. FR is affiliated with the Global Alliance for Improved Nutrition, a not-for-profit organization that supports food-fortification programs. MBZ, CC, EKN, CN, AD, JU, HG, CL, and RFH declared no conflicts of interest.

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