

Effects of iron supplementation on dominant bacterial groups in the gut, faecal SCFA and gut inflammation: a randomised, placebo-controlled intervention trial in South African children

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Abstract

Fe supplementation is a common strategy to correct Fe-deficiency anaemia in children; however, it may modify the gut microbiota and increase the risk for enteropathogenic infection. In the present study, we studied the impact of Fe supplementation on the abundance of dominant bacterial groups in the gut, faecal SCFA concentration and gut inflammation in children living in rural South Africa. In a randomised, placebo-controlled intervention trial of 38 weeks, 6- to 11-year-old children with Fe deficiency received orally either tablets containing 50 mg Fe as FeSO₄ (*n* 22) for 4 d/week or identical placebo (*n* 27). In addition, Fe-sufficient children (*n* 24) were included as a non-treated reference group. Faecal samples were analysed at baseline and at 2, 12 and 38 weeks to determine the effects of Fe supplementation on ten bacterial groups in the gut (quantitative PCR), faecal SCFA concentration (HPLC) and gut inflammation (faecal calprotectin concentration). At baseline, concentrations of bacterial groups in the gut, faecal SCFA and faecal calprotectin did not differ between Fe-deficient and Fe-sufficient children. Fe supplementation significantly improved Fe status in Fe-deficient children and did not significantly increase faecal calprotectin concentration. Moreover, no significant effect of Fe treatment or time×treatment interaction on the concentrations of bacterial groups in the gut or faecal SCFA was observed compared with the placebo treatment. Also, there were no significant differences observed in the concentrations of any of the bacterial target groups or faecal SCFA at 2, 12 or 38 weeks between the three groups of children when correcting for baseline values. The present study suggests that in African children with a low enteropathogen burden, Fe status and dietary Fe supplementation did not significantly affect the dominant bacterial groups in the gut, faecal SCFA concentration or gut inflammation.

Key words: Iron supplementation: Iron deficiency: Gut microbiota: Children

Fe deficiency affects more than 2 billion people worldwide, and children, because they require high amounts of Fe for growth and development, are among the most vulnerable⁽¹⁾. Fe deficiency can lead to Fe-deficiency anaemia and impair school performance and cognitive development in children^(2,3). Thus, adequate dietary Fe availability for school-aged children is critical. In the past, the WHO recommended that children living in the regions with a high prevalence of Fe deficiency receive oral supplementation of Fe as FeSO₄^(1,4,5). This recommendation has been modified in recent years because of concerns that Fe supplementation may increase the risk for hospitalisations and mortality from infections^(6–9).

Supplemental Fe is poorly absorbed in the human gastrointestinal tract and most of the dose passes into the colon where it becomes available for the gut microbiota. The symbiotic bacteria in the gut provide the host with many beneficial functions, such as colonisation resistance from pathogens, immunomodulatory properties and degradation of indigestible compounds, while producing bacterial metabolites, such as SCFA, influencing host health and providing additional energy to host cells^(10,11). There are alterations in the composition of the gut microbiota in malnourished children in developing countries, and it has been proposed that the microbiota may contribute to the effects of nutritional deficiencies in these settings^(12–16). Several animal studies

Abbreviations: CRP, C-reactive protein; qPCR, quantitative PCR; SF, serum ferritin; TfR, serum transferrin receptor; ZnPP, zinc protoporphyrin.

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have suggested that host Fe status and dietary Fe availability can influence the microbial ecosystem of the gut^(17–22), and that most bacteria in the gut have a requirement for Fe⁽²³⁾.

However, the impact of Fe status and dietary Fe availability on the gut microbiota in humans is uncertain. A recent study in India has reported decreased numbers of lactobacilli in women with Fe-deficiency anaemia⁽²⁴⁾. Furthermore, two studies in infants^(25,26) and one randomised placebo-controlled trial in Ivorian school children living in a rural area with a high prevalence of environmental pathogens⁽⁹⁾ have reported changes in the composition of the gut microbiota after Fe fortification. It has also been shown that the growth and infectivity of several enteropathogens can be promoted by Fe supplementation *in vitro*⁽²⁷⁾ and also in hosts with Fe overload⁽²⁸⁾. Both a dysbiosis of the gut microbiota, and hence a reduced barrier effect and colonisation resistance against pathogens⁽²⁹⁾, and enhanced pathogen growth due to high-dose Fe supplementation could increase the risk for the development of diarrhoea. Indeed, a systematic review and a recent study in young children in Pakistan have found that Fe supplementation may increase the incidence of diarrhoea in children, especially in areas with a high prevalence of enteric pathogens^(6,30). Moreover, an increase in infection with other pathogens due to Fe supplementation could have an impact on the inflammatory preset of the host, which in turn might alter the composition of the gut microbiota and facilitate colonisation with enteropathogenic bacteria^(8,29,31).

Based on these previous studies and observations, our hypothesis was that high-dose Fe supplementation and also host Fe status can affect the dominant commensal bacterial groups in the gut, their main metabolites and gut inflammation. This may lead to a potential dysbiosis of the gut microbiota with less protection against the establishment of environmental bacteria, such as enteropathogens, and a change in the degradation of dietary compounds. To test this hypothesis, we investigated the impact of oral

supplementation of Fe as FeSO₄ over a time period of 38 weeks on the concentrations of dominant bacterial groups in the gut, faecal SCFA and faecal calprotectin, a gut inflammation marker, in Fe-deficient school children living in rural South Africa compared with a placebo treatment without FeSO₄ over the same time period. We also included non-treated Fe-sufficient children to investigate whether the abundance of dominant bacterial groups, faecal SCFA concentration and faecal calprotectin concentration differs between Fe-sufficient and Fe-deficient children.

Subjects and methods

Study design

Participants included in the present study were 6- to 11-year-old children (*n* 73) from two primary schools that serve low-income rural villages in the Province of KwaZulu-Natal in eastern South Africa (Fig. 1). A total of two groups of children were randomly selected from two arms of a 2×2 study design assessing the effects of Fe and *n*-3 fatty acid supplementation, alone and in combination, on cognition in school children, as reported previously⁽³⁾. One group received Fe supplements (Fe group, *n* 22) and another group received placebo (placebo group, *n* 27). As described previously, these children lived in a malaria-free region and fulfilled the following inclusion criteria: (1) 6 to 11 years of age; (2) Hb concentration >80 g/l; (3) Fe deficient (serum ferritin (SF) concentration <20 µg/l or zinc protoporphyrin (ZnPP) concentration >70 µmol/mol haem or serum transferrin receptor (TfR) concentration >8.3 mg/l); (4) no chronic disease; (5) not using Fe supplements⁽³⁾.

Children in the Fe group were given orally one tablet containing 50 mg Fe as FeSO₄ (Lomapharm; Paul Lohmann GmbH) together with a fruit-flavoured and vitamin C-enriched (approximately 10 mg/serving) beverage (200 ml) for 4 d/week,

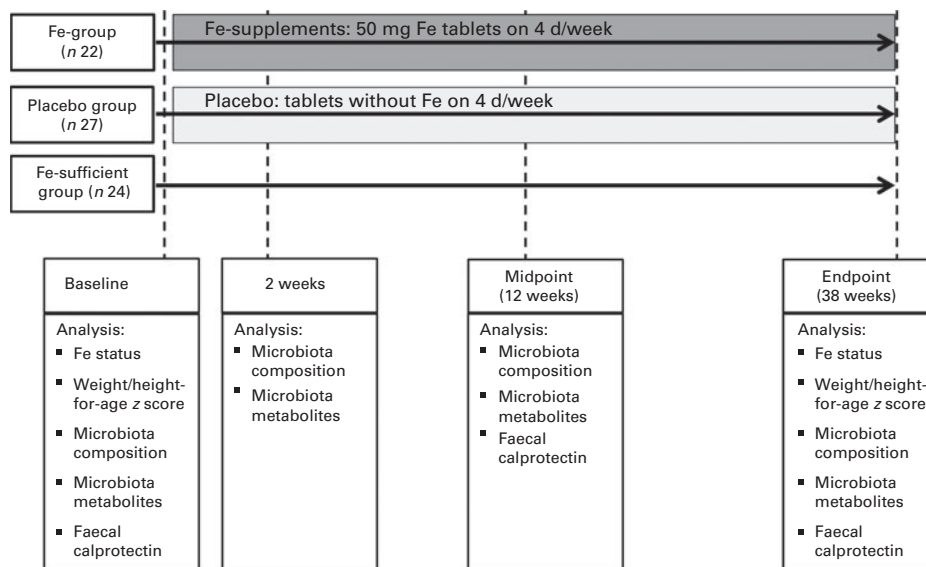


Fig. 1. Summary of the study design with interventions in the placebo and iron groups as well as the different sampling time points and the corresponding analysis.

while those in the placebo group received an identical tablet without Fe and the beverage⁽³⁾. Trained fieldworkers (one fieldworker per group and school) directly supervised the tablet consumption and recorded compliance and self-reported illness symptoms while absent and present. Children in the placebo and Fe groups were dewormed with an oral dose of 400 mg mebendazole (Be-Tabz Pharmaceuticals (Pvt) Limited) 3 and 15 weeks after the start of the intervention trial.

A third group of children (Fe-sufficient group, $n = 24$) with highest SF concentrations (without inflammation, C-reactive protein (CRP) concentration < 5 mg/l) and thereafter lowest TfR and ZnPP concentrations was randomly selected out of 100 children from the same two schools; they participated in the baseline screening, but were not included in the intervention trial due to adequate Fe status. We enrolled this third group as a reference group to compare the composition of the gut microbiota between Fe-deficient and Fe-sufficient children at baseline and to follow the changes in the gut microbiota over time without the intervention.

In a previous study in Ivorian school children, a sample size of thirty children per group was adequate to detect significant differences in the major bacterial groups in the gut microbiota after Fe fortification of 10 mg Fe/d⁽⁹⁾. Since in the present study, a much higher Fe dose (50 mg Fe, 4 d/week) was provided via oral supplementation, we estimated that a sample size between twenty and thirty children per group would be adequate to detect the differences between Fe supplementation and placebo treatment.

Trained local fieldworkers conducted two 24 h dietary recalls 2 weeks apart on different days of the week. The 24 h dietary recalls were administered to the parents/carers of sixty children per participating school in their local language (Zulu). One recall per child was conducted for a week day and one recall was conducted for a weekend day. Dietary data were analysed with the Food Finder computer program (Medical Research Council, 2003). Anthropometric measurements were taken at baseline and endpoint, as described previously⁽³⁾. Age- and sex-specific height-for-age z scores, weight-for-age z scores and BMI-for-age z scores were calculated using the 2007 WHO growth standards for children aged 5 to 19 years with the software WHO Anthro Plus for personal computers (version 1.0.3, WHO, 2010). Weight-for-age z scores were available only for children < 11 years of age. The present study was performed from February to November 2010 and was interrupted by holidays for 2 weeks in March and April, for 5 weeks in June and July, and for 1 week in September. Supplementation was further interrupted for 4.5 weeks by a national strike of teachers in August and September. To catch up on the unexpected loss of intervention days, supplementation was increased from 4 to 5 d/week for 8 weeks, which compensated for 2 weeks of supplementation. Thus, in total, supplements were provided for 105 d over a period of 38 weeks. The present study was conducted according to the guidelines laid down in the Declaration of Helsinki, and all procedures including human subjects were approved by the ethics committees of the North-West University in South Africa and by the Swiss Federal Institute of Technology Zürich in Switzerland. Written

informed consent was obtained from parents, and verbal consent of children was witnessed and formally recorded. The present trial was registered at ClinicalTrials.gov as NCT01092377.

Sample collection and blood sample analysis

Faecal samples were collected from 09.00 to 12.00 hours at four time points throughout the study: at baseline just before the start of the intervention, and at 2 weeks, 12 weeks (midpoint) and 38 weeks (endpoint) of Fe supplementation (Fig. 1). Faecal sample collection was specifically optimised to avoid bacterial cell death by oxygen or bacterial growth due to the lack of a constant low temperature before freezing. The samples were collected immediately after defecation in zip-lock bags containing an Anaerocult A mini bag (Merck Millipore) to maintain anaerobiosis. Samples were kept at 4°C and within 6 h, aliquots were made in several 2 ml Eppendorf tubes and frozen at -80°C until further analysis.

Blood samples were collected at baseline and endpoint, as described previously⁽³⁾. Briefly, venous blood samples (10 ml) were drawn into EDTA-coated and trace-element free tubes (Becton Dickinson) at baseline and endpoint. Hb concentrations were measured on site in the whole blood by the direct cyanmethaemoglobin method (Ames Mini-Pak Hb test pack and Ames Minilab; Bio-Rad Laboratories (PTY) Limited) with Drabkin's solution and a standard miniphotometer. The remaining samples were centrifuged at 500 **g** for 15 min at room temperature, and plasma and serum aliquots were prepared and stored at -20°C for the duration of the fieldwork (4 d) and then at -80°C until analysis. Erythrocytes were washed twice with 0.15 M-NaCl and centrifuged at 500 **g** for 10 min to remove the buffy coat. ZnPP was determined on site on washed erythrocytes, as described previously⁽³⁾. SF, CRP and TfR concentrations were measured in serum, as described previously⁽³⁾. Fe deficiency during the intervention was defined as a SF concentration < 15 $\mu\text{g/l}$ ⁽³²⁾ or a ZnPP concentration > 70 $\mu\text{mol/mol haem}$ ⁽³³⁾ or a TfR concentration > 8.3 mg/l (test kit reference value), and anaemia was defined as a Hb concentration < 115 g/l⁽⁴⁾. For the reporting of prevalence and statistical analyses, a SF concentration < 15 $\mu\text{g/l}$ was used to define Fe deficiency; for inclusion into the study, an SF concentration < 20 $\mu\text{g/l}$ was used. Systemic inflammation was defined as a CRP concentration > 5 mg/l.

Faecal sample DNA extraction and enumeration of bacterial groups

Faecal samples were thawed on ice and total genomic DNA was extracted using the FastDNA SPIN kit for soil (MP Biomedicals), according to the manufacturer's instructions. Bacterial groups prevalent in the gut were enumerated using specific primers for the 16S ribosomal RNA gene or a functional gene (Table 1) by quantitative PCR (qPCR) analysis performed with an ABI PRISM 7500-PCR sequence detection system (Life Technologies), as described previously^(34,35). qPCR consisted of 2 \times SYBR Green Mastermix (Life Technologies) or 2 \times Kapa Sybr Fast qPCR Mastermix (BioLabs

Table 1. Primers used to enumerate selected bacterial target groups by quantitative PCR

Primers	Sequence 5'–3'	Target group	Reference
Eub338F	5'-ACTCCTACGGGAGGCAGCAG-3'	Total bacteria	Guo <i>et al.</i> ⁽⁴⁸⁾
Eub518R	5'-ATTACCGCGGCTGCTGG-3'		
Bac303F	5'-GAAGGTCCCCACATTG-3'	<i>Bacteroides</i> spp.	Ramirez-Farias <i>et al.</i> ⁽⁴⁹⁾
Bfr-Femrev	5'-CGCKACTTGGCTGGTTCAG-3'		
Firm934F	5'-GGAGYATGTGGTTTAATTCGAAGCA-3'	Firmicutes	Guo <i>et al.</i> ⁽⁴⁸⁾
Firm1060R	5'-AGCTGACGACAACCATGCAC-3'		
Clep866mF	5'-TTAACACAATAAGTWATCCACCTGG-3'	<i>Clostridium</i> Cluster IV	Ramirez-Farias <i>et al.</i> ⁽⁴⁹⁾
Clep1240mR	5'-ACCTTCTCCGTTTTGTCAAC-3'		
RrecF	5'-GCGGTRCGGCAAGTCTGA-3'	<i>Roseburia</i> spp./ <i>E. rectale</i>	Furet <i>et al.</i> ⁽⁵⁰⁾
Rrec630mR	5'-CCTCCGACACTCTAGTMCGAC-3'		
Fprau223F	5'-GATGGCCTCGCGTCCGATTAG-3'	<i>Faecalibacterium prausnitzii</i>	Bartosch <i>et al.</i> ⁽⁵¹⁾
Fprau420R	5'-CCGAAGACCTTCTCTCC-3'		
EhalF	5'-AGCAGTAGGGAATCTCCA-3'	<i>Eubacterium hallii</i>	Ramirez-Farias <i>et al.</i> ⁽⁴⁹⁾
EhalR	5'-GCACCGRAGCCTATACGG-3'		
dsrA_F336	5'-CTGCGAATATGCCTGCTACA-3'	SRB, <i>dsrA</i> gene	Pereyra <i>et al.</i> ⁽⁵²⁾
dsrA_R533	5'-TGGTCGARCTTGATGTCGTC-3'		
F_Lacto 05	5'-AGCAGTAGGGAATCTCCA-3'	<i>Lactobacillus</i> / <i>Pediococcus</i> / <i>Leuconostoc</i> spp.	Furet <i>et al.</i> ⁽⁵⁰⁾
R_Lacto 04	5'-CGCCACTGGTGTTCYCCATATA-3'		
xfp-fw	5'-ATCTTCGGACCBGAYGAGAC-3'	Bifidobacteria phosphoketolase	Cleusix <i>et al.</i> ⁽⁵³⁾
xfp-rv	5'-CGATVACGTGVACGAAGGAC-3'		
Eco1457F	5'-CATTGACGTTACCCGCAGAAGAAGC-3'	Enterobacteriaceae	Bartosch <i>et al.</i> ⁽⁵¹⁾
Eco1652R	5'-CTCTACGAGACTCAAGCTTGC-3'		

SRB, sulphate-reducing bacteria; *dsrA*, dissimilatory sulphite-reductase subunit A.

Scientifics Instruments), 0.2 µM of each primer and 1 µl of template genomic DNA in a total volume of 25 µl. Amplification consisted of an initial denaturation step at 95°C for 10 min (20 s for Kapa Sybr Fast qPCR Mastermix) followed by forty cycles of 95°C for 15 s (3 s) and 60°C for 1 min (30 s). A denaturation step was added to check for amplicon specificity. The samples were analysed in duplicate and standard curves with the specific target 16S ribosomal RNA gene or a functional gene were included in each run, as described previously⁽³⁴⁾. Data were analysed with the 7500 Fast System Sequence Detection Software (version 1.4; Life Technologies) and expressed as the log number of 16S ribosomal RNA gene copies or functional gene copies/g faeces.

Faecal SCFA concentration analysis

SCFA concentrations were analysed in the faecal samples of a randomly selected subgroup of children (*n* 10 per group) by HPLC. Briefly, 200–300 mg faeces were homogenised with 1 ml of 0.15 M-H₂SO₄ and subsequently centrifuged at 4°C and 9000 *g* for 20 min. The supernatants were diluted 1:1 with MilliQ water and filtered through a 0.45 µm nylon filter (Infochroma AG) before injection. HPLC (Hitachi LaChrome; Merck) was performed using a Cation-H refill cartridge (30×4.6 mm) connected to an Aminex[®] HPX-87H (300×7.8 mm) column at a flow rate of 0.4 ml/min at 40°C and 10 mM-H₂SO₄ as the eluent solution. The samples were analysed in duplicate and data expressed as µmol/g faeces.

Faecal calprotectin concentration analysis

Calprotectin concentration in the faecal samples of children was measured by immunoassay (Calprest; Eurospital S.p.A), according to the manufacturer's instructions (placebo group, *n* 12; Fe group, *n* 13; Fe-sufficient group, *n* 13), at baseline,

midpoint and endpoint. The samples were analysed in duplicate and data expressed as mg/kg faeces.

Statistical analyses

Statistical analyses were performed using IBM SPSS Statistics (version 19; IBM Company). Data were checked for normal distribution and transformed, if necessary. Outliers (± 3 SD from the mean) were removed from the analysis. At baseline, all variables were compared between the groups using the one-way ANOVA with *post hoc* Bonferroni correction for multiple comparisons. At 2 weeks, midpoint and endpoint of the study, variables were compared between the groups (Fe group, placebo group and Fe-sufficient group) using ANCOVA with corresponding baseline values as covariates. Moreover, potential differences in qPCR data and HPLC data by intervention group only (Fe and placebo groups) over time were analysed using repeated-measures ANOVA, with the sampling time point as the within-subject variable and intervention group (Fe and placebo groups) as the between-subject factor. When significant changes over time were detected, repeated-measures ANOVA was performed for each variable between baseline and the subsequent time points within each group separately to detect deviations from baseline levels. qPCR data, TfR, ZnPP, SF, CRP and calprotectin data were log transformed for statistical analyses. *P* < 0.05 was considered as significant.

Results

Iron status, inflammation and anthropometric measurements of the study subjects

Anthropometric measurements, Hb concentration, Fe status indices and markers of systemic (CRP) and gut (calprotectin) inflammation are shown in Table 2. At baseline, both

Table 2. Baseline and endpoint parameters of iron status, inflammation as well as anthropometric measurements of children included in the study*

(Mean values with their standard errors; medians and maximum and minimum values)

	Placebo group (n 27)		Fe group (n 22)		Fe-sufficient group (n 24)		P†
	Mean	SEM	Mean	SEM	Mean	SEM	
Age (years)	9.1	0.2	9.1	0.3	8.3	0.3	
Sex							
Female							
n	17		11		12		
%	61		50		50		
Hb (g/l)							
Baseline	119.1 ^b	1.7	120.6 ^b	1.6	129.6 ^a	1.1	
Endpoint	125.8	1.9	129.1	1.7			0.266
Serum transferrin receptor (mg/l)‡							
Baseline							
Median	5.9 ^a		6.0 ^a		4.5 ^b		
Minimum–maximum	2.8–11.7		3.3–10.2		3.2–6.7		
Endpoint							
Median	8.4		6.9				< 0.001
Minimum–maximum	6.2–14.4		4.6–8.4				
Zinc protoporphyrin (µmol/mol haem)‡							
Baseline							
Median	76.5 ^a		74.5 ^a		46.0 ^b		
Minimum–maximum	40.0–171.0		43.0–127.0		31.0–73.0		
Endpoint							
Median	77.8		68.5				0.131
Minimum–maximum	35.5–202.5		40.5–126.0				
Serum ferritin§ (µg/l)‡							
Baseline							
Median	18.9 ^b		20.7 ^b		56.9 ^a		
Minimum–maximum	3.8–58.1		11.5–54.7		35.0–105		
Endpoint							
Median	25.4		56.7				< 0.001
Minimum–maximum	4.4–100.0		23.6–178.0				
CRP (mg/l)‡							
Baseline							
Median	0.0		0.4		0.6		
Minimum–maximum	0–4.9		0–12.7		0–19.6		
Endpoint							
Median	0.3		0.3				0.304
Minimum–maximum	0–7.3		0–2.2				
Weight-for-age z score							
Baseline	0.33	0.23	–0.02	0.35			
Endpoint	0.32	0.27	0.44	0.32			0.710
Height-for-age z score							
Baseline	–0.67	0.17	–0.66	0.24			
Endpoint	–0.55	0.19	–0.70	0.25			0.111
BMI-for-age z score							
Baseline	0.61	0.19	0.35	0.24			
Endpoint	0.46	0.18	0.36	0.24			0.293
Faecal calprotectin (mg/kg faeces)‡							
Baseline							
Median	60.3		139.0		78.1		
Minimum–maximum	24.6–501.4		17.3–449.1		16.8–463.8		
Midpoint							
Median	31.2		59.7		115.0		0.501
Minimum–maximum	18.4–306.0		17.8–361.3		16.4–565.6		
Endpoint							
Median	69.8		59.7		60.5		0.865
Minimum–maximum	15.6–490.5		17.1–476.3		19.8–536.7		

CRP, C-reactive protein.

^{a,b} Mean values with unlike superscript letters were significantly different ($P < 0.05$; one-way ANOVA with *post hoc* Bonferroni correction).

* No Fe status indices and anthropometric measurements were available for children in the Fe-sufficient group at the endpoint.

† Midpoint (only for faecal calprotectin) and endpoint variables were compared between the groups using ANCOVA with respective baseline values as covariates.

‡ Data were log transformed for statistical analyses.

§ Only those children were considered whose CRP concentrations were < 5 mg/l.

|| Placebo group, n 12; Fe group, n 13; Fe-sufficient group, n 13.

Fe-deficient groups (placebo and Fe groups) had significantly lower Hb and lower Fe status than the Fe-sufficient group. The prevalence of Fe deficiency based on the concentrations of TfR and SF in the Fe group was 18.2 and 9.1%, respectively, and in the placebo group 18.5 and 29.6%, respectively. Moreover, 25.9% of the children in the placebo group and 13.6% of the children in the Fe group were anaemic, while none of the children in the Fe-sufficient group were anaemic. There was a significant intervention effect (ANCOVA with baseline values as covariates) of Fe supplementation for lower TfR concentrations ($P < 0.001$) and higher SF concentrations ($P < 0.001$) at the endpoint in the Fe group compared with the placebo group. Fe supplementation did not affect the concentration of CRP. Faecal calprotectin concentrations did not differ between the groups at baseline, midpoint and endpoint, and were not affected by Fe supplementation.

Dietary assessment done in the study population found a mean background dietary Fe intake of 9.8 (SEM 0.3) mg/d. The incidence of illness was recorded during the entire trial period, and the mean days absent from school due to illness was 1.3 (SEM 0.3) d and due to gastrointestinal illness (diarrhoea, stomach pain and/or vomiting) was 0.3 (SEM 0.1) d in children included in the present study. Moreover, the mean days absent due to all illness and gastrointestinal illness did not differ between the Fe (all illness: 1.2 (SEM 0.4) d; gastrointestinal illness: 0.2 (SEM 0.1) d) and placebo (all illness: 1.3 (SEM 0.4) d; gastrointestinal illness: 0.3 (SEM 0.2) d) groups.

Concentrations of dominant bacterial groups

Total 16S ribosomal RNA gene copies were stable over the entire trial period, and no differences were observed between the treatment groups across the different time points (Fig. 2).

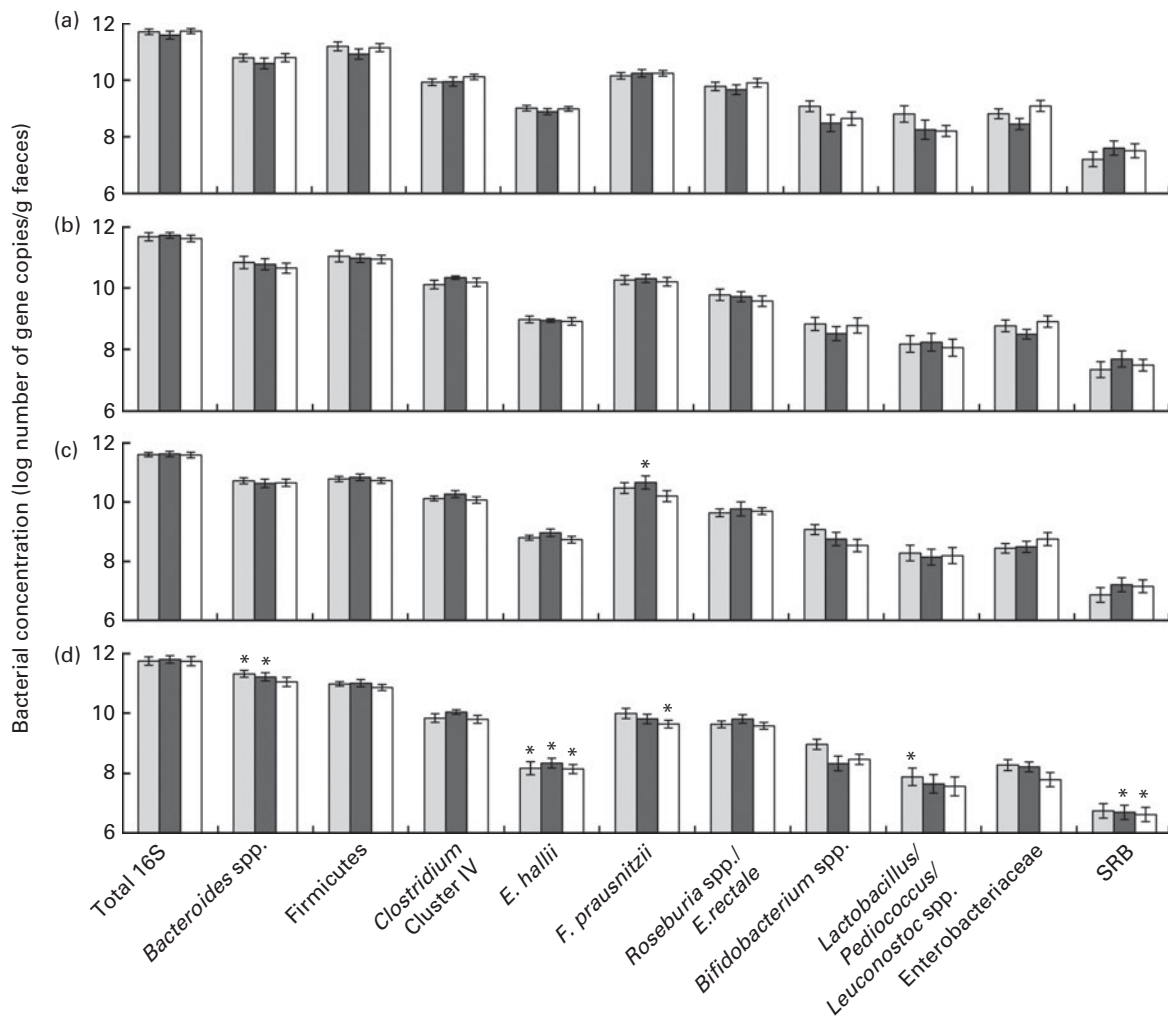


Fig. 2. Log number of 16S ribosomal RNA gene copies or functional gene copies/g faeces of selected bacterial target groups in the gut microbiota of children in the placebo group (□), iron group (■) and iron-sufficient group (□) at (a) baseline, (b) 2 weeks, (c) midpoint and (d) endpoint. No significant treatment×time interaction was detected using repeated-measures ANOVA, with the sampling time point as the within-subject variable and intervention group (iron and placebo groups) as the between-subject factor. Values are means, with their standard errors represented by vertical bars. *Mean value was significantly different from that of baseline concentrations of the same bacterial target group within a treatment group ($P < 0.05$; repeated-measures ANOVA). SRB, sulphate-reducing bacteria.

At baseline, measured gut bacterial populations did not differ between the children who were Fe deficient (placebo and Fe groups) and those with an adequate Fe status (Fe-sufficient group). There was a trend observed towards lower concentrations of Enterobacteriaceae in the faeces of children in the Fe group compared with the Fe-sufficient group ($P=0.064$).

With baseline concentrations as covariates, there were no significant differences observed between the groups in terms of concentrations of any of the measured bacterial target groups at 2 weeks, midpoint or endpoint of the study. Moreover, no significant effects of Fe treatment or time \times treatment interaction were observed when analysing each bacterial target group over time and including only the placebo and Fe groups in the analysis. However, there were significant effects for time within the groups, when comparing bacterial concentrations at 2 weeks, midpoint and endpoint with baseline. As shown in Fig. 2, *Bacteroides* spp. significantly increased in the placebo ($P=0.004$) and Fe ($P=0.004$) groups from baseline to the endpoint, while *Eubacterium hallii* concentrations significantly decreased in the placebo ($P<0.001$), Fe ($P=0.024$) and Fe-sufficient ($P<0.001$) groups, and sulphate-reducing bacteria concentrations significantly decreased only in the Fe-sufficient ($P=0.03$) and Fe ($P=0.008$) groups from baseline to the endpoint. Concentrations of *Lactobacillus/Leuconostoc/Pediococcus* spp. significantly decreased from baseline to the endpoint ($P=0.027$) in the placebo group, and *Faecalibacterium prausnitzii* concentrations decreased from baseline to the endpoint ($P=0.004$) in the Fe-sufficient group. Furthermore, concentrations of *F. prausnitzii* increased from baseline to the midpoint ($P=0.045$) in the Fe group, which was not observed in the placebo group or the Fe-sufficient group (Fig. 3).

Faecal SCFA concentrations

Baseline faecal acetate, propionate and butyrate concentrations did not differ between the placebo, Fe and the Fe-sufficient groups (Fig. 4(a)–(c), respectively). Also, the baseline ratios of acetate:propionate:butyrate did not differ between the placebo (59:28:13), Fe (58:27:15) and Fe-sufficient (56:30:14) groups.

With baseline values as covariates, there were no significant differences observed in faecal acetate, propionate and butyrate concentrations between the three groups at 2 weeks, midpoint or endpoint of the study. Furthermore, no significant effects for Fe treatment and time \times treatment interaction were found for SCFA production by the gut microbiota when including only the Fe and placebo groups in the analysis. However, there were significant effects for time within the Fe and placebo groups (but not within the Fe-sufficient group) when comparing the concentrations of metabolites during the intervention with those at baseline. Acetate concentrations significantly increased from baseline to 2 weeks ($P=0.026$) in the Fe group (Fig. 4(a)). In the placebo group, acetate concentrations significantly increased from baseline to the endpoint ($P=0.009$). In the Fe and placebo groups, butyrate concentrations significantly increased from baseline

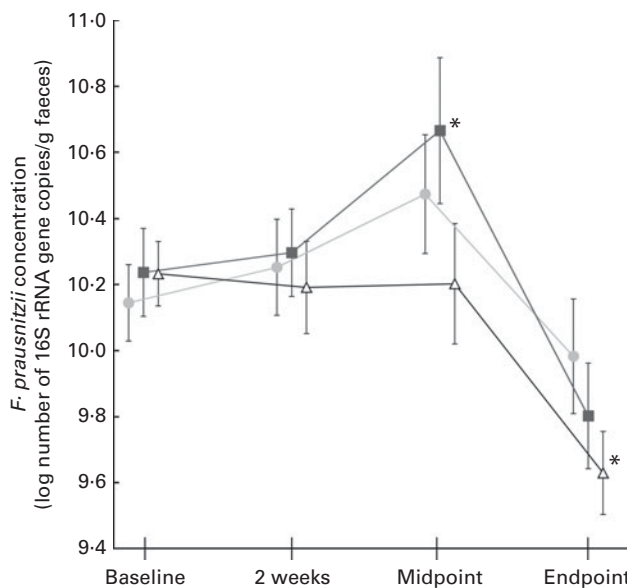


Fig. 3. Log number of 16S ribosomal RNA (rRNA) gene copies/g faeces of *Faecalibacterium prausnitzii* in the faecal samples of children in the placebo group ($n=27$, ○), iron group ($n=22$, ■) and iron-sufficient group ($n=24$, △) measured over time by quantitative PCR. No significant treatment \times time interaction was detected using repeated-measures ANOVA, with the sampling time point as the within-subject variable and intervention group (iron and placebo groups) as the between-subject factor. Values are means, with their standard errors represented by vertical bars. * Mean value was significantly different from that of baseline concentrations within a treatment group ($P<0.05$; repeated-measures ANOVA).

to 2 weeks ($P=0.001$ and $P=0.002$, respectively) and to the endpoint ($P=0.034$ and $P=0.040$, respectively; Fig. 4(c)). At midpoint, butyrate concentrations remained significantly higher than those at baseline in the Fe group ($P=0.040$). There were no significant effects of time on propionate concentrations within the groups (Fig. 4(b)).

Discussion

The present study was the first randomised, placebo-controlled Fe intervention trial to investigate the impact of oral Fe supplementation on gut microbiota and gut inflammation in African children. The major finding of the present study is that high-dose Fe supplementation (50 mg Fe, 4d/week) over a period of 38 weeks did not significantly modify the concentrations of dominant bacterial groups in the gut or faecal SCFA, and did not increase gut inflammation. A second important finding is that the dominant bacterial groups prevalent in the gut and faecal SCFA concentrations of Fe-sufficient children were not different from those of Fe-deficient children.

We found no effect of Fe supplementation on the abundance of dominant bacterial groups in the gut compared with the placebo treatment. These findings are in sharp contrast to the previous study in similarly aged children living in rural Côte d'Ivoire, where daily provision of Fe-fortified biscuits (10 mg Fe as electrolytic Fe) to school children for 6 months significantly increased Enterobacteriaceae and decreased lactobacilli concentrations⁽⁹⁾. This difference may

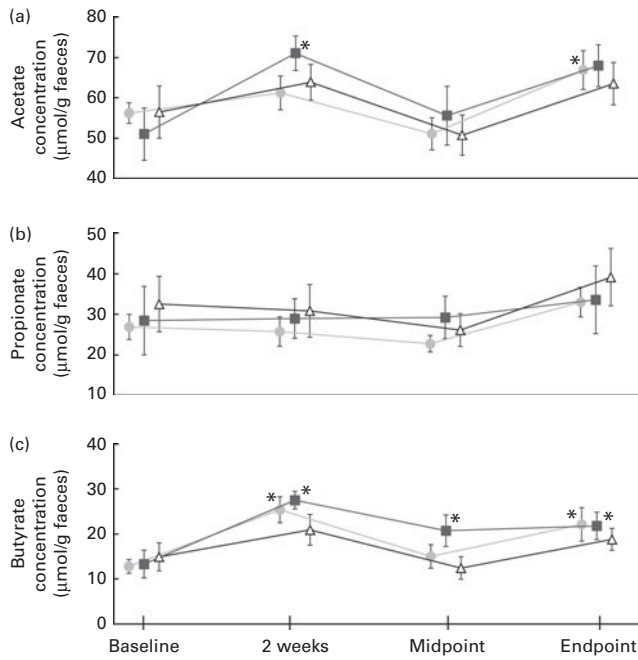


Fig. 4. (a) Acetate, (b) propionate and (c) butyrate concentrations in the faecal samples of children in the placebo group (○), iron group (■) and iron-sufficient group (△) at baseline, 2 weeks, midpoint and endpoint of the study. No significant treatment×time interaction was detected using repeated-measures ANOVA, with the sampling time point as the within-subject variable and intervention group (iron and placebo groups) as the between-subject factor. Values are means (n 9–10 children per group and time point), with their standard errors represented by vertical bars. * Mean value was significantly different from that of baseline concentrations of the same metabolite within a treatment group ($P < 0.05$; repeated-measures ANOVA).

be at least partially explained by the fact that Ivorian children lived in a remote rural area with poorer-quality water and food sanitation, a more monotonous low-quality diet and a higher infectious disease burden (including enteropathogens and malaria). Colonic Fe can promote the growth and virulence of certain pathogens^(36–38). During enteropathogen infection and the resulting inflammation, the composition of the gut microbiota is shifted towards facultative anaerobes such as enterobacteria^(29,39–42), and high colonic Fe concentrations may contribute to these effects by aggravating inflammation^(18,43). The children in the present study were mainly from households with access to relatively clean tap water and lived in a malaria-free environment. In addition, the schools in this area of South Africa participated in the National School Nutrition Program, where children were offered a daily school meal that contributes to nutritional diversity. Nevertheless, studies in infants have shown an impact of additional dietary Fe supplementation on the gut microbiota even in industrialised countries with a low enteropathogen burden^(26,44). However, the gut microbiota in infants is in the process of developing into a fully diversified bacterial ecosystem. This still fragile bacterial consortium may be more vulnerable to alterations in luminal Fe status than a fully diversified and stable gut microbiota such as that found in the 6- to 11-year-old children of the present study.

We also found that the abundance of dominant bacterial groups and faecal SCFA concentrations did not differ between Fe-deficient (Fe and placebo groups) and Fe-sufficient children. This is in contrast with several animal studies that associated Fe deficiency with changes in the composition of the gut microbiota^(17,18,24). Animal and *in vitro* studies have further shown that Fe deficiency modifies the metabolic activity of the gut microbiota, resulting in a decrease in butyrate production^(21,34). These differences from our findings may be due to the differences in the severity of Fe deficiency among the studies. Most of the children included in the present study were only mildly Fe deficient. South Africa fortifies wheat flour and maize meal with Fe, and the mean daily Fe intake in the present study population was found to be 9.8 (SEM 0.3) mg^(3,45). Thus, colonic luminal Fe concentrations in children of the present study were unlikely to have been very low. In previous *in vitro* studies, we have shown that a dietary Fe concentration in this range is sufficient to maintain a stable gut microbiota⁽³⁴⁾.

During the study, there was an overall decrease observed in the concentrations of faecal lactobacilli, *E. ballii*, *F. prausnitzii* and sulphate-reducing bacteria, while concentrations of *Bacteroides* spp. slightly increased from baseline to the endpoint in all the treatment groups. Since these changes were independent of Fe supplementation and also observed in the Fe-sufficient group, we assume that either seasonal changes or other alterations in dietary habits during the intervention (including a long school vacation between the midpoint and the endpoint, with no school lunch provided) may be responsible for these time effects.

SCFA acetate, propionate and butyrate are the major metabolites of the gut microbiota. In the present study, no differences in SCFA were observed between Fe-sufficient and Fe-deficient children at baseline, and Fe supplementation did not affect faecal SCFA concentrations. This is in contrast to previous studies in rats, where Fe supplementation increased the metabolic activity of the microbiota, particularly butyrate^(21,22). However, generally, only about 5% of the SCFA produced by the gut microbiota are excreted in the faeces; the remainder are readily absorbed by the host, which can lead to large variations in faecal SCFA concentrations⁽⁴⁶⁾. Nevertheless, faecal acetate or butyrate concentrations were modified in the Fe and placebo groups over time, which may be explained by seasonal alterations in diet or other factors, as discussed above, for the composition of the gut microbiota.

Because high luminal Fe concentrations can promote inflammation^(18,43), in the present study, we assessed systemic inflammation by serum CRP and local colonic inflammation by faecal calprotectin, a peptide secreted by neutrophils infiltrating the gut mucosa. Serum CRP and faecal calprotectin concentrations did not differ between Fe-sufficient and Fe-deficient children at baseline. Furthermore, in this setting, high-dose Fe supplementation in Fe-deficient children did not measurably increase systemic or gut inflammation. Thus, our findings differ from the study conducted in rural Côte d'Ivoire, where provision of Fe-fortified biscuits increased faecal calprotectin concentrations compared with a control group⁽⁹⁾. However, in that study, Fe fortification increased

enterobacteria numbers (including many potential enteropathogens causing gut inflammation), which correlated with faecal calprotectin. In the present study, the overall incidence of diarrhoea during the intervention was very low with an average of only 0.1 (SEM 0.1) absent due to gastrointestinal illness, and no differences between Fe- and placebo-supplemented children were observed. However, clinical data should be interpreted with caution, as they were based on self-reporting and the sample size of the present study was small. Calprotectin concentrations in our children were generally higher than those previously reported in African children at this age^(9,47).

In summary, in South African school-aged children from a malaria-free rural area with a low gastrointestinal disease burden, we found no significant differences in the abundance of dominant bacterial groups or faecal SCFA concentrations in mildly Fe-deficient and non-Fe-deficient children. This suggests that dietary and luminal Fe levels in both groups were sufficient to maintain the gut microbiota. Furthermore, high-dose Fe supplementation had no measurable impact on the abundance of dominant bacterial groups in the gut, faecal SCFA concentration or gut inflammation. Therefore, it appears that Fe supplementation poses a low risk for negative modulation of the tested bacterial groups and/or adverse intestinal effects at this age and in this setting. Hence, our initial hypothesis that high-dose Fe supplementation would modify the tested bacterial groups and metabolites under all conditions could not be confirmed. The effects of Fe supplementation on the gut microbiota most probably also depend on environmental factors (e.g. presence of enteropathogens) and gut inflammatory preset of the host. Future research should therefore investigate the potential effects of Fe supplementation on the gut microbiota in other age groups, in populations where Fe deficiency is more severe, and in settings where poor-quality water and food supplies increase exposure to potential enteropathogens.

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