SUPPLEMENTARY MATERIALS AND METHODS

Gut microbiome, short-chain fatty acids (SCFAs) and faecal calprotectin

Faecal DNA was extracted with the Fast DNA Spin Kit for Soil, including a bead-beating step (MP Biomedicals, Illkirch, France) and concentration was quantified with a Nanodrop 1000 spectrophotometer (Witec AG, Littau, Switzerland). For the preparation of the amplicon pool for pyrosequencing, the following universal primers were applied for amplification of the V3-V6 region of the 16S rRNA gene: a) forward primer, 5'-*CCATCTCATCCCTGCGTGTCTCCGACTAG*NNNNNN**ACTCCTACGGGAGGCAGCAG**-3' (the italicised sequence is the 454 Life Sciences primer A, and the bold sequence is the broadly conserved bacterial primer 338F; NNNNNN designates the sample-specific six-base barcode used to tag each PCR product); b) reverse primer 5'-*CCTATCCCCTGTGTGCCTTGGCAGTCTCAG***CRRCACGAGCTGACGAC**-3' (the italicised sequence is the 454 Life Sciences primer B, and the bold sequence is the broadly conserved bacterial primer 1061R). PCR amplification mixture contained: 1 µL faecal DNA, 1 µL bar-coded forward primer, 15 µL master mix (1 µL KOD Hot Start DNA Polymerase (1 U/µL; Novagen, Madison, WI, USA), 5 µL KOD-buffer (10×), 3 µL MgSO4 (25 mM), 5 µL dNTP mix (2 mM each), 1 µL (10 µM) of reverse primer) and 33 µL sterile water (total volume 50 µL). PCR conditions were: 95°C for 2 minutes followed by 35 cycles of 95°C for 20 s, 55°C for 10 s, and 70°C for 15 s. The approximately 750 bp PCR amplicon was subsequently purified using the MSB Spin PCRapace kit (Invitek) and the concentration was checked with a Nanodrop 1000 spectrophotometer (Thermo Scientific). A composite sample for pyrosequencing was prepared by pooling 200 ng of these purified PCR products of each sample. The pooled sample was purified using the Purelink PCR Purification kit (Invitrogen), with high-cutoff binding buffer B3, and submitted for pyrosequencing of the V3-V4 region of the 16S rRNA gene on the 454 Life Sciences GS-FLX platform using Titanium sequencing chemistry (GATC-Biotech, Germany). Targeted quantitative real-time polymerase chain reaction (qPCR) was performed using specific primers for bacterial subgroups most prevalent in the human gut and expected low-abundant pathogens (**Supplementary Table 1**). The enumeration of these bacterial groups was performed with a 7500 Fast Real-Time qPCR System (Applied Biosystems Europe BV, Zug, Switzerland) using SYBR Green PCR Master Mix (Applied Biosystems), and taxon-specific primers in a 25 µL volume. Duplicate sample analysis and standard curves were routinely performed in each run. Data were analysed using the 7500 Fast System Sequence Detection Software (Version 1.4, Applied Biosystems).

We measured faecal calprotectin using the Calprest ELISA assay for stools, following the manufacturer's procedures (Eurospital, Trieste, Italy). For the measurement of the SCFAs (acetate, propionate, and butyrate), we homogenised 100-300 mg of stool in 1 mL 0.15 mM sulphuric acid and centrifuged at 9000 rpm and 2°C for 20 minutes.[1] The supernatant was transferred into a microconcentrator and filtered by centrifugation at 4700 rpm and 2°C for 90 minutes.[2] The HPLC analysis was performed using a Phenomenex column (Rezex ROA-Organic Acid H+ (8%), 300*7.8 mm).

Biochemical indicators

Haemoglobin was measured immediately after collection with a HemoCue (HemoCue AB, Ängelholm, Sweden) or a HemoControl device (EKF diagnostics Sales GmbH, Barleben/Magdeburg, Germany). Serum was separated and frozen on collection day. The erythrocytes were washed thrice with normal saline, and zinc protoporphyrin to haem

ratio (ZPP) was measured using a calibrated AVIV hematofluorometer (AVIV Biomedical, Lakewood, USA). Serum ferritin (SF), soluble transferrin receptor (sTfR) and C-reactive protein (CRP) were analysed at Lancet Laboratories in Nairobi using the Cobas Integra (Roche, Basel, Switzerland). We converted the Roche sTfR concentration to the Flowers assay[3] using the regression equation by Pfeiffer (Flowers=1.5*Roche +0.35).[4] Serum hepcidin-25 was measured in Nijmegen (hepcidinanalysis.com) by a combination of weak cation exchange chromatography and time-of-flight mass spectrometry.[5, 6] Body iron stores were calculated according to the equation by Cook et al. (body iron (mg/kg)=-[log10 (sTfR*1000/SF) -2.8229)]/0.1207),[7] and total body iron stores by multiplying with body weight.

Serum levels of IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IFNγ, TNFα, and GM-CSF were determined by using a human cytokine multiplex kit (Cytokine 10-plex panel, Invitrogen, Breda, Netherlands), and IL-12 (p40/p70) and IL-17 were determined by using Singleplex bead kits (Invitrogen) at Radboud University Medical Center, Nijmegen, Netherlands.

Statistical analysis

Data were analysed using IBM SPSS Statistics 20.0.0 (SPSS Inc., Chicago, IL) and Microsoft Office EXCEL 2010 (Microsoft, Redmond, WA). Data were double entered and distribution checked for normality; not normally distributed data were log transformed. Normally distributed data were expressed as means and standard deviations (SD) or standard errors of the mean (SEM). For log transformed data, we obtained geometric means (GM) and corresponding standard deviations (SD) for absolute concentrations by taking the antilog of these values. Correlations of gut microbial subgroups and intestinal inflammation were done using crude values and Kendall's tau. Pyrosequencing data were analysed with a workflow based on QIIME v1.2,[8] and reads were filtered for chimeric sequences using Chimera Slayer.[9] OTU clustering was performed with settings as recommended in the QIIME newsletter of December 17th 2010 (http://qiime.wordpress.com/2010/12/17/new-default-parameters-for-uclust-otu-pickers/) using an identity threshold of 97%. Diversity metrics were calculated as implemented in QIIME 1.2. Hierarchical clustering of samples was performed using UPGMA with weighted UniFrac as a distance measure as implemented in QIIME 1.2. The RDP classifier version 2.2 was performed for taxonomic classification.[10] Visualisation of differences in relative abundance of taxa between different study groups was done in Cytoscape.[11] The baseline gut microbiome composition was illustrated using the approach presented by Sundquist et al..[12] Statistical analysis of the pyrosequencing data was done with SciPy (www.scipy.org). Differences in relative abundance between groups at a single time point (cross-sectional) were compared by Mann-Whitney U (MWU) testing. Comparisons of targets of our primary interest (the phyla Firmicutes and Bacteroidetes, and the taxa lactobacilli, *Roseburia* spp., *Clostridium* spp., bifidobacteria, and enterobacteria) were not corrected for multiple testing.

Longitudinal effects of intervention were statistically assessed by comparing change over time, which were calculated by dividing the relative abundance of a taxon at 4 months or 3 weeks by the relative abundance of a taxon at baseline. These changes over time for two groups were compared by MWU. Changes over time of ratios of enterobacteria to bifidobacteria or lactobacilli (ratio of relative abundances) were compared the same way. The development of specific taxa and phylogenetic diversity over time was assessed by paired testing using the Wilcoxon

matched-pairs signed-rank test. Multivariate redundancy analysis (RDA) was performed in R (http://www.R-project.org) using the vegan package.[13]

For the qPCR analysis, a total of 22 bacterial targets were tested for a treatment effect in univariate general linear models (GLM) for the two MNPs, using baseline variables as covariates (Supplementary Table 1). Moreover, we assessed treatment effects of any iron fortification by pooling data from the two iron groups (+FeMNP) and control groups (-FeMNP) and using univariate GLM, including baseline values as covariates. A summary variable was created for the pathogenic *E. coli* community by summing copy numbers of the *eaeA* (*E. coli* attaching and effacing) gene detecting EPEC and EHEC strains, and of the heat-labile and heat-stable enterotoxin gene detecting ETEC LT and ETEC ST strains.[14]

Treatment effect on weight and height were assessed using GLM with baseline variables as covariates. Treatment effects on the incidence of diarrhoea, malaria, and RTI were assessed using logistic regression. Baseline differences in iron status markers, inflammation markers, and hepcidin-25 were assessed using independent samples *t*-tests. Estimated intervention effects on iron status and hepcidin-25 were assessed with univariate GLM using baseline values as covariates. P values <0.1 were considered as a trend towards significance, and p values <0.05 as statistically significant.

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SUPPLEMENTARY TABLES

Supplementary Table 1 Species, target gene, and primers used for the quantitative real-time polymerase chain reaction.

	Species	Target gene (description)	Primer and sequence (5'-3')		Reference
Commensals	Total Bacteria	16S rRNA gene	Eub 338F	ACTCCTACGGGAGGCAGCAG	(1)
			Eub 518R	ATTACCGCGGCTGCTGG	
	Bacteroides spp.	16S rRNA gene	Bac303F	GAAGGTCCCCCACATTG	(2, 3)
			Bfr-Fmrev	CGCKACTTGGCTGGTTCAG	
	Firmicutes	16S rRNA gene	Firm934F	GGAGYATGTGGTTTAATTCGAAGCA	(1)
			Firm1060R	AGCTGACGACAACCATGCAC	
	Enterobacteriaceae	16S rRNA gene	Eco1457F	CATTGACGTTACCCGCAGAAGAAGC	(2)
			Eco1652R	CTCTACGAGACTCAAGCTTGC	
	Bifidobacteria	xfp gene	xfp-fw	ATCTTCGGACCBGAYGAGAC	(4)
			xfp-rv	CGATVACGTGVACGAAGGAC	
	Lactobacillus/ Leuconostoc/ Pediococcus spp.	16S rRNA gene	F_Lacto 05	AGCAGTAGGGAATCTTCCA	(5)
			R_Lacto 04	CGCCACTGGTGTTCYTCCATATA	
	Roseburia spp./ E. rectale	16S rRNA gene	RrecF	GCGGTRCGGCAAGTCTGA	(6, 7)
			Rrec630mR	CCTCCGACACTCTAGTMCGAC	
	Clostridial Cluster IV	16S rRNA gene	Clep866mF	TTAACACAATAAGTWATCCACCTGG	(7)
			Clep1240mR	ACCTTCCTCCGTTTTGTCAAC	
	Eubacterium hallii	16S rRNA gene	EhalF	GCGTAGGTGGCAGTGCAA	(7, 8)
			EhalR	GCACCGRAGCCTATACGG	
	Faecalibacterium prausnitzii	16S rRNA gene	Fprau223F	GATGGCCTCGCGTCCGATTAG	(2, 9)
			Fprau420R	CCGAAGACCTTCTTCCTCC	
	Sulfate-reducing bacteria	Alpha subunit dissimilatory sulfite reductase	dsrA_290F	CGGCGTTGCGCATTTYCAYACVVT	(10)
			dsrA_660R	GCCGGACGATGCAGHTCRTCCTGRWA	

Pathogens	Salmonella	invA (invasion)	InvA 139	GTGAAATTATCGCCACGTTCGGGCAA	(11, 12)
			InvA 141	TCATCGCACCGTCAAAGGAACC	
	Staphylococcus aureus	Nuclease	SA-1	GCGATTGATGGTGATACGGTT	(12, 13)
			SA-2	CAAGCCTTGACGAACTAAAGC	
	Bacillus cereus	Hemolysin	BC-1	CTGTAGCGAATCGTACGTATC	(9, 12)
			BC-2	TACTGCTCCAGCCACATTAC	
	Clostridium difficile	16S rRNA gene	cdF	TTGAGCGATTTACTTCGGTAAAGA	(14)
			cdR	CCATCCTGTACTGGCTCACCT	
	Clostridium perfringens group	16S rRNA gene	cpF	ATGCAAGTCGAGCGA(G/T)G	(14)
			cpR	TATGCGGTATTAATCT(C/T)CCTTT	
	Vibrio cholera	CT (cholera toxin)	CT-F	ACAGAGTGAGTACTTTGACC	(6, 12)
			CT-R	ATACCATCCATATATTTGGGAG	
	Enteropathogenic Escherichia coli (EPEC)	eaeA (E. coli attaching and effacing)	Eae a	ATGCTTAGTGCTGGTTTAGG	(12)
			Eae b	GCCTTCATCATTTCGCTTTC	
	Enterohemorrhagic Escherichia coli (EHEC) stx1	stx1 (shiga toxin 1)	JMS1F	GTCACAGTAACAAACCGTAACA	(12)
			JMS1R	TCGTTGACTACTTCTTATCTGGA	
	Enterohemorrhagic Escherichia coli (EHEC) stx2	stx2 (shiga toxin 2)	JMS2F	CGACCCCTCTTGAACATA	(12)
			JMS2R	GATAGACATCAAGCCCTCGT	
	Enterotoxigenic Escherichia coli (ETEC) LT	LT (heat-labile enterotoxin)	LT-1	AGCAGGTTTCCCACCGGATCACCA	(12)
			LT-2	GTGCTCAGATTCTGGGTCTC	
	Enterotoxigenic Escherichia coli (ETEC) ST	ST (heat-stable enterotoxin)	ST_f	GCTAAACCAGYAGRGTCTTCAAAA	(15)
			ST_rev	CCCGGTACARGCAGGATTACAACA	

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Supplementary Table 2 Faecal short-chain fatty acid (SCFA) composition over the time of the iron-fortification trial

Intervention	Time point	SCFA (µmol/g)	Acetate (µmol/g)	Propionate (µmol/g)	Butyrate (µmol/g)
+FeMNP	baseline	96.1 ± 1.6	76.9 ± 1.4	12.4 ± 1.8	6.9 ± 1.7
	3 weeks	107.6 ± 1.6	83.3 ± 1.4	15.0 ± 1.7	8.8 ± 1.6
	4 months	106.2 ± 1.5	75.0 ± 1.3	19.4 ± 1.6	11.8 ± 1.7
-FeMNP	baseline	104.2 ± 1.5	83.0 ± 1.3	14.0 ± 1.5	7.2 ± 1.5
	3 weeks	110.3 ± 1.5	85.7 ± 1.3	14.4 ± 1.5	10.1 ± 1.6
	4 months	105.3 ± 1.6	74.5 ± 1.4	17.8 ± 1.8	13.0 ± 1.7
ALL	baseline	99.7 ± 1.6	79.6 ± 1.4	13.1± 1.7	7.0 ± 1.6
	3 weeks	108.8 ± 1.5	84.6 ± 1.4	14.8 ± 1.6	9.4 ± 1.6
	4 months	105.7 ± 1.6	74.8 ± 1.4	18.6 ± 1.7	12.4 ± 1.7

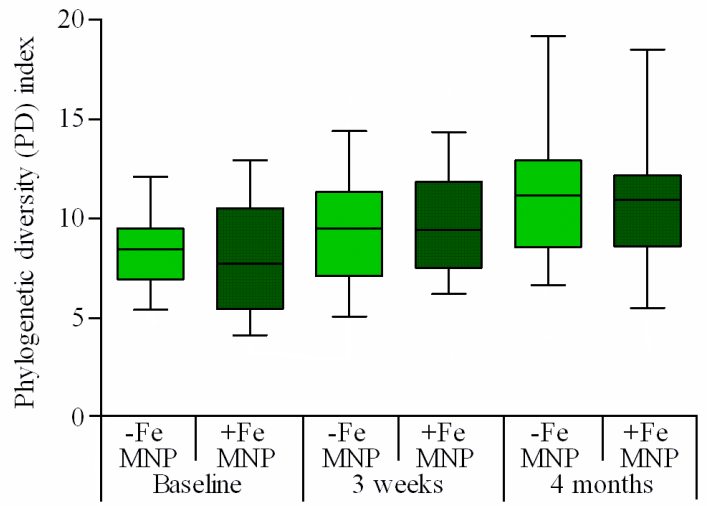
Data are geometric means ±SD. There were no differences between +FeMNP and -FeMNP using GLM with baseline as covariate and $p < 0.05$. In all infants, propionate ($p=0.004$) and butyrate ($p=0.0001$) increased significantly from baseline to endpoint using paired *t*-test; this was also seen in +FeMNP (propionate ($p=0.029$) and butyrate ($p=0.022$) and to some extent in -FeMNP (propionate ($p=0.070$) and butyrate ($p=0.002$)).

Supplementary Table 3 Haematological measurements and anthropometrics performed in study infants.

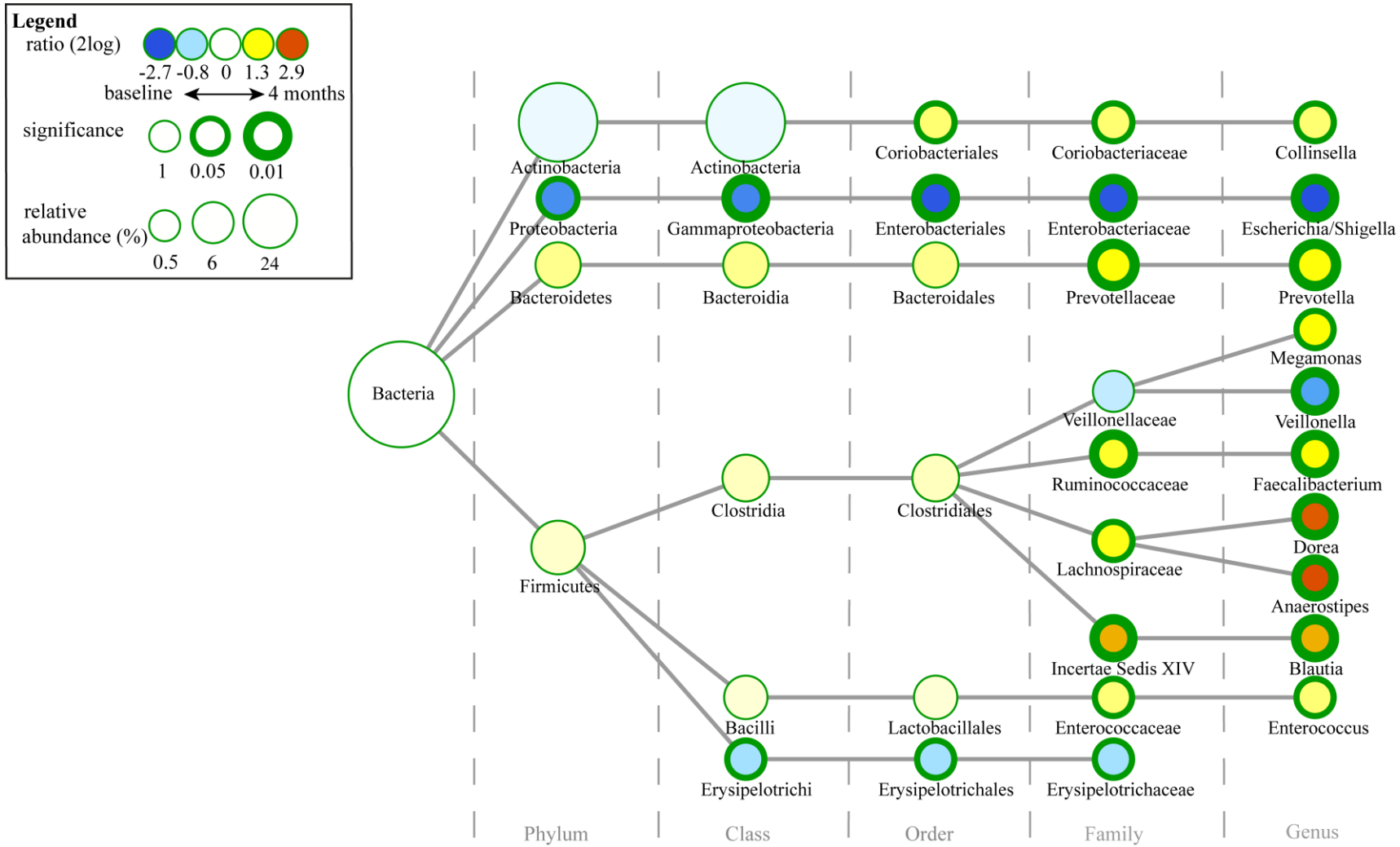
Intervention	Time point (mo)	Hb (g/L)	ZPP (µmol/mol heme)	SF (µg/L)	sTfR (mg/L)	CRP (mg/L)	Hepcidin (nM)	Body iron (mg/kg)	Weight (kg)	Length (cm)
+2.5mgFeMNP	0	104.4 ± 10.6	90.5 ± 1.6	31.3 ± 1.9	8.6 ± 1.2	4.4 ± 1.5	3.3 ± 1.3	2.67 ± 1.0	7.2 ± 1.2	64.7 ± 1.1
	4	100.6 ± 10.6	-	-	-	-	-	-	8.2 ± 1.1	70.1 ± 1.0
	6	102.7 ± 10.7	94.0 ± 1.8	19.8 ± 1.6	9.6 ± 1.2	3.7 ± 2.0	2.5 ± 1.3	0.91 ± 1.0	8.7 ± 1.1	73.8 ± 1.0
-2.5mgFeMNP	0	105.4 ± 10.6	109.0 ± 1.8	25.8 ± 1.7	9.5 ± 1.2	3.4 ± 1.5	2.0 ± 1.2	1.98 ± 1.0	7.4 ± 1.1	65.2 ± 1.0
	4	103.1 (± 10.5)	-	-	-	-	-	-	8.4 ± 1.1	69.5 ± 1.1
	6	103.8 ± 10.7	111.9 ± 1.7	22.4 ± 1.6	11.2 ± 1.4	3.0 ± 1.4	2.1 ± 1.2	1.00 ± 1.0	8.7 ± 1.1	73.7 ± 1.1
+12.5mgFeMNP	0	96.0 ± 10.7	132.9 ± 1.9	29.0 ± 2.0	10.1 ± 1.2	3.2 ± 1.3	2.1 ± 1.2	1.98 ± 1.0	7.1 ± 1.1	63.1 ± 1.1
	4	109.9 ± 10.7	87.6 ± 1.8*	33.7 ± 1.7*	8.75 ± 1.2*	2.8 ± 1.3	3.5 ± 1.3	3.28 ± 1.0*	8.4 ± 1.2	70.2 ± 1.1*
	6	-	-	-	-	-	-	-	-	-
-12.5mgFeMNP	0	102.7 ± 10.8	113.6 ± 1.1	36.0 ± 2.1	10.7 ± 1.2	2.8 ± 1.3	2.5 ± 1.2	2.68 ± 1.0	7.0 ± 1.1	63.7 ± 1.1
	4	106.5 ± 10.7	101.7 ± 2.2*	21.9 ±1.6*	11.5 ± 1.2*	3.9 ± 1.5	2.0 ± 1.3	0.5 ± 1.0*	8.3 ± 1.2	68.1 ± 1.1
	6	-	-	-	-	-	-	-	-	-

Values are geometric means ±SD. There were no differences at baseline using independent samples *t*-tests. *significant treatment effects (between iron and control of the same MNP, ±2.5mgFeMNP and ±12.5mgFeMNP) using GLM with baseline as covariate and $p < 0.05$.

SUPPLEMENTARY FIGURES



Supplementary Figure 1 Alpha diversity of the infant gut microbiome over the time course of the trial in the pooled groups (+FeMNP and -FeMNP). Phylogenetic diversity was not influenced by +FeMNP intervention, but increased significantly over time ($p = 0.004$ for baseline to 3 weeks and $p = 0.005$ for 3 weeks to 4 months). Boxplots are displayed with the 10-90th percentiles.



Supplementary Figure 2 Changes over time from baseline to endpoint in gut microbiome composition of infants in the -FeMNP group. Nodes represent taxa; edges link the different taxonomic levels. The fold increase is calculated as the 2log of the ratio of the relative abundance at the age of ten months old and six months (0=no difference between baseline and endpoint, 1=twice as abundant at endpoint). The significance is expressed as the p value of a Mann-Whitney U test. The node-size corresponds to the relative abundance. In this explorative analysis, the significance is expressed as the p value of a Mann-Whitney U test. The node-size corresponds to the relative abundance. Taxa (that is, nodes) were included in this visualization if they met the following criteria: all samples together have an average relative abundance of > 0.1% for the taxon and the sample groups have a fold-difference of at least 0.5 with a significance of $p < 0.05$ or the taxon has a child (that is, more specific taxonomic classification) meeting the criteria.