ORIGINAL ARTICLE

Oral versus intravenous iron replacement therapy distinctly alters the gut microbiota and metabolome in patients with IBD

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ABSTRACT

Objective Iron deficiency is a common complication in patients with IBD and oral iron therapy is suggested to exacerbate IBD symptoms. We performed an open-labelled clinical trial to compare the effects of per oral (PO) versus intravenous (IV) iron replacement therapy (IRT).

Design The study population included patients with Crohn’s disease (CD; N=31), UC (N=22) and control subjects with iron deficiency (non-inflamed, NI=19). After randomisation, participants received iron sulfate (PO) or iron sucrose (IV) over 3 months. Clinical parameters, faecal bacterial communities and metabolomes were assessed before and after intervention.

Results Both PO and IV treatments ameliorated iron deficiency, but higher ferritin levels were observed with IV. Changes in disease activity were independent of iron treatment types. Faecal samples in IBD were characterised by marked interindividual differences, lower phylotype richness and proportions of Clostridiales. Metabolite analysis also showed separation of both UC and CD from control anaemic participants. Major shifts in bacterial communities and the metabolic phenotypes due to IRT, PO treatment was associated with increased abundances of operational taxonomic units assigned to the species Faecalibacterium prausnitzii, Ruminococcus bromii, Dorea sp. and Collinsella aerofaciens. Clear IV-specific and PO-specific fingerprints were evident at the level of metabolomes, with changes affecting cholesterol-derived host substrates.

Conclusions Shifts in gut bacterial diversity and composition associated with iron treatment are pronounced in IBD participants. Despite similar clinical outcome, oral administration differentially affects bacterial phylotypes and faecal metabolites compared with IV therapy.

Trial registration number clinicaltrial.gov (NCT01067547).

Significance of this study

What is already known on this subject?

► The high prevalence of iron deficiency anaemia in patients with IBD requires therapeutic intervention based on oral or intravenous (IV) iron application.

► Oral iron treatment is standard, but GI side effects and the potency to exacerbate intestinal inflammation support clinical implementation of IV iron therapy.

► High concentrations of luminal iron affect the gut microbiota composition and disease activity in IBD.

What are the new findings?

► Bacterial communities are most sensitive to iron therapy in patients with Crohn’s disease (CD).

► Oral and IV iron therapies differentially affect bacterial communities and the metabolic landscape in patients with IBD.

► Shifts in faecal metabolome and bacterial communities are dissociated from changes in disease activity in patients with IBD.

How might it impact on clinical practice in the foreseeable future?

► IV iron therapy might specifically benefit anaemic patients with CD with an unstable microbiota.

INTRODUCTION

Iron deficiency anaemia is a common clinical issue in patients with IBD, and iron replacement therapy (IRT) improves anaemia and is associated with improved quality of life.1 Conflicting results about clinical outcome regarding the route of IRT have been published.2 3 Total iron replacement is possible with intravenous (IV) iron infusions considered to be safe and efficacious.4 With regard to oral iron, it has been advocated that lower doses of oral iron should be used as it is efficacious with less side effects.

Iron is a critical nutrient for the growth of microorganisms, and the tight regulation of intravascular iron availability forms part of the innate immune defence.5 However, similar iron regulation has not been elucidated in the intraluminal gut environment whereby many gut bacteria are competing for the free iron to maintain their growth. Most of the pathogenic bacteria have enhanced
iron acquisition mechanisms contributing to increased virulence. This was demonstrated in a study involving African children where an increase in enterobacteria and a reduction in lactobacilli accompanied by increased faecal calprotectin levels were noted after 6 months of iron fortified biscuits. Most recently, Jaeggi et al confirmed the effect of iron fortification in Kenyan infants clearly demonstrating changes in faecal bacterial community structure associated with the selection of pathogens.

IBD is a result of disordered immune responses to commensal and pathogenic gut microbes in genetically susceptible individuals. Diversion of faecal stream in the setting of an ileostomy improves colonic inflammation, whereas re-establishment of the faecal stream causes recurrence of disease, clearly demonstrating the critical role of gut microbes in IBD pathogenesis. There is accumulating evidence available implicating the role of gut microbial dysbiosis in the pathogenesis and perpetuation of IBD; however, environmental factors promoting changes in the gut microbial ecosystem are poorly defined.

We previously showed that dietary iron alters dominant gut bacterial communities and exacerbates chronic ileal inflammation in mice. However, as correctly noted in a recent commentary by Guenter Weiss, the relevance of these findings still awaits proof of concept in humans. Hence, we designed and conducted an open-labelled clinical trial with iron-deficient participants with or without IBD and performed pre-iron therapy and post-iron therapy measurements where the individuals served as their own controls. We measured faecal bacterial communities by high-throughput sequencing and metabolite profiles by high-resolution mass spectrometry. Clinical data included changes in serum haemoglobin, ferritin and iron saturation and the effect of iron therapy on the IBD status as captured by clinical disease activity index and C reactive protein (CRP).

METHODS
Cohort and study design
Participants were identified by their treating gastroenterologists and recruited during 2010 from the gastroenterology outpatient clinics in Edmonton (University of Alberta Hospital (UAH), Royal Alexandra Hospital (RAH)). Written informed consent was obtained prior to web-based randomisation. Inclusion criteria were age >18 years and confirmed diagnosis of IBD by standard radiology, endoscopy and histopathology findings. The control group consisted of participants with a recent upper GI bleeding such as peptic ulcer disease, with a deliberate low iron diet (vegetarian), or women with non-specific GI symptoms and menorrhagia. All subjects were iron deficient, that is, iron saturation <16% with or without ferritin <30 μg/L (if normal CRP) or <100 μg/L (if elevated CRP). Exclusion criteria included iron supplementation, blood transfusion or the use of antibiotics within the last 3 months prior to inclusion, untreated concurrent folate and/or vitamin B12 deficiency, history of intolerance to IRT, coeliac disease, known malignancy other than skin cancer and primary haematological disorder. Treatment allocation was 1:1 to either IV or per oral (PO) iron therapy. PO participants took one 300 mg iron sulfate tablet twice a day. IV patients received three or four separate iron sucrose 300 mg infusions if iron deficient only or with anaemia, respectively. Participants were followed up for 12 weeks from the initiation of IRT. The experimental design is summarised in figure 1A. A review of participants’ clinical disease activity status at 12 months from the iron therapy initiation was performed with special emphasis on the need for therapeutic escalation (step-up medical or surgical therapy).

Samples
All participants were reviewed at baseline and 3 months after initiating IRT. At these time points, serum and stool specimens were collected. All participants were given a stool collection kit with a pictorial instruction on how to collect stools specimens and minimise contamination. Stool samples were frozen within 30 min and kept at −80°C until further processing for sequencing and mass spectrometry analysis. We can thus exclude confounding effects of freezing/thawing cycles or storage at −20°C over long periods of time. As the recruitment happened over 18 months, some specimens were kept frozen longer than others. However, this was the case for samples across all groups that were compared, precluding group-specific artefact observations due to the sampling/storage procedure. Quality of life was assessed using Shorten IBD Questionnaire (SIBDQ) and the Euro Quality 5 Dimensions Visual Analogue Scale (EQ5D VAS). At 3 months, subjects were reviewed for medication adherence and possible adverse event. IBD activity was assessed with Modified Harvey Bradshaw Index (HBI) for Crohn’s disease (CD) and Partial Mayo (pMayo) score for UC, in conjunction with serum CRP. Adherence to PO iron therapy was assessed by pill count at week 12 and adherence to IV iron was determined by attendance at the infusion clinic. The disease activity and clinical course of the IBD cohort were also reviewed at 12 months after initiation of treatment.

High-throughput 16S rRNA gene sequencing
Samples were processed and data were analysed as described previously. Detailed information is provided in the online supplementary methods.

High-resolution mass spectrometry and metabolome analysis
Samples were measured via Fourier transform ion cyclotron resonance mass spectrometer (FT-ICR-mass spectrometry (MS)) and acquired data were processed by in-house developed pipelines as described in the online supplementary methods.

Iron measurement
The procedure for quantification of iron content in faeces is detailed in the online supplementary methods.

Statistical analysis
All data sets, including operational taxonomic unit (OTU) counts and abundances of taxonomic groups as well as metabolome data, are provided in online supplementary table S1. Statistical analyses were performed in the R programming environment or using SIMCA-P+12 (Umetrics). Sequencing data were analysed statistically as published previously. Prior to testing for variations in OTU or taxonomic counts, individual counts <0.5% were zeroed, and only prevalent and dominant taxonomic groups or molecular species (ie, detected in at least 30% of the participants (n=124) and with a median sequence abundance >1% in at least one group) were considered. For metabolome analysis, data were autoscaled prior to applying any multivariate tools. Feature selection for a given classification was done according to the absolute values of regression coefficients or variable importance in projection (VIP score) derived from applying orthogonal partial least squares discriminant analysis (OPLS-DA). In order to estimate univariate significance of the selected features, p values were calculated using the Mann–Whitney test. After selecting an adequate set of features (per extension, metabolites), OPLS-DA was reperformed using...
sevenfold cross-validation and the quality of the model was checked by the values of $R^2_Y$ and $Q^2_{CUM}$ (goodness of fit and predictive ability, respectively).

**RESULTS**

IRT restores iron storage, independently of the administration route

A total of 72 participants completed the study (36 in each of the PO and IV group), including 19 non-inflamed (NI) controls, 31 patients with CD and 22 patients with UC. After intervention, the mean iron saturation level in the control group was no different between the PO route (23.3±6.3%) and IV route (23.9±9.9%). Comparable mean iron saturation was achieved at 3 months in the IBD cohort: PO route (24.7±15.8%) and IV route (24.2±13.4%) (figure 1B). A statistically significantly higher serum ferritin level at 3 months was noted in the IV cohort compared with PO in both NI and IBD participants (figure 1B). The route of IRT did not affect haemoglobin levels at 3 months (data not shown).

**Impact of iron treatment on disease activity and quality of life**

The route of IRT did not affect disease activity based on changes in the clinical disease activity indices (MHBI and pMayo) and serum concentrations of CRP (figure 1C). At 12 months follow-up, 37% of the PO (n=27) and 30% of the IV (n=124) cohort required therapeutic escalation requiring biological therapy or surgery ($p=0.63$). Overall, there was a trend towards higher magnitude of improved SIBDQ scores in IBD-IV groups, but results did not reach significance (figure 1D). In contrast, the VAS (EQ5D VAS) indicated improvement in CD participants on the IV treatment (figure 1D), suggesting that IV iron may be associated with a better quality of life in CD. With respect to adverse events, one CD participant in the IV group developed arterial thrombosis of the left leg requiring below knee amputation. Other adverse events included generalised arthralgia and headaches. In the PO cohort, one participant had self-limiting nausea. No adverse event was reported in the control group. Adherence to IV iron therapy was 100% in all

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Gut microbiota

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**Figure 1** Clinical design and outcomes. (A) Anaemic subjects diagnosed with IBD and control anaemic participants without GI disorders were enrolled in the study and randomised into the oral or intravenous iron replacement therapy (IRT) group. Faecal samples were collected at baseline and after the end of intervention (3 months later). Samples were analysed by high-throughput 16S rRNA gene sequencing and Fourier transform ion cyclotron resonance mass spectrometer (FT-ICR-mass spectrometry (MS)) for analysis of bacterial communities and metabolites, respectively. (B) IRT restores iron storage, independently of the route of administration. (C) IBD activity was assessed with Modified Harvey Bradshaw Index (HBI) for CD and Partial Mayo (pMayo) score for UC. Values are % changes. (D) % changes in quality of life per participants category. Quality of life was scored according to Shorten IBD Questionnaire (SIBDQ) and Euro Quality 5 Dimensions Visual Analogue Scale (EQ5D). CD, Crohn’s disease; CRP, C reactive protein; EQ5D, Euro Quality 5 Dimensions; IV, intravenous; NI, non-IBD control anaemic subjects; PO, per oral; SIBDQ, Shorten IBD Questionnaire.
participants. The overall adherence rate in the PO cohort ranged from taking 85–100% of the iron tablets.

**IBD is linked to specific faecal microbiota and metabolome fingerprints**

After sequence processing and filtering, we obtained a total of 6 726 192 chimera-checked 16S rRNA gene sequences (53 382 ±13 153 per sample) spanning a total of 372 OTUs (121±37 per sample). We first looked at the presence of IBD-specific differences in bacterial diversity and composition by analysing all samples independently of the time point of measure. Multidimensional scaling revealed a significant clustering of samples according to the occurrence of IBD (figure 2A). Both IBD groups were characterised by marked interindividual differences in 16S rRNA profiles when compared with NI participants, who represented a more homogenous group of samples. CD participants were most distinguishable from controls, without specific subclustering according to disease state, that is, patients in an active phase of the disease or in remission were scattered across the clusters. Analysis of Shannon effective diversity showed that IBD subjects were characterised by a significant drop in taxa richness, and this was most pronounced in patients with CD, who showed a median effective count <15 species (figure 2B). To see whether differences observed at the level of diversity were associated with changes in the relative abundance of specific bacteria, we looked for significant differences in sequence proportions of taxonomic groups from phyla down to families. *Firmicutes* was the most dominant phylum in the samples analysed, followed by *Actinobacteria* and *Bacteroidetes*. Members of the latter phylum were completely absent in samples from patients with UC in the active state of disease (see online supplementary figure S1A). Among *Firmicutes*, we observed a drop in the relative abundance of *Clostridiales* in patients with CD (figure 2C). At the family level, higher median abundance of Christensenellaceae was specific for NI subjects. Within the phylum *Actinobacteria*, *Bifidobacteriaceae* showed higher abundances in patients with IBD (figure 2C), which might be related to dietary intake of probiotics and prebiotics in the form of commercially available yogurts in 10 participants.

With respect to metabolomics analysis, 7578 m/z values out of the original 204 445 features detected (ca. 4%) passed filtering, of which 2260 m/z values were annotated as putative metabolites without differentiation between human, plant or bacterial origin. An OPLS-DA model based on 1012 features revealed that baseline faecal samples from patients without IBD separated well from patients with CD (figure 2D). Of the 300 m/z values assigned to putative metabolites, 13 were most discriminative for classification of the CD and NI groups, including higher signal intensities for ceramide phosphate and three fatty acids in faeces from patients with CD (figure 2E). The OPLS-DA model built using the samples belonging to NI and UC classes at baseline was less robust than the model discriminating NI and CD classes, with R²Y and Q²CUM values of 0.3 and 0.21, respectively (figure 2F). Of the 331 m/z values assigned to putative metabolites, 13 were most discriminative, including lower intensities of phospholipids in patients with UC (figure 2G).

**Faecal metabolomes and bacterial communities are prone to instability in anaemic IBD versus NI patients following IRT**

We next looked at variations of bacterial communities and metabolomes in faeces when discriminating samples before and after IRT. Multidimensional analysis showed that the effect of treatment overtime did not overrule IBD-specific clustering, that is, we did not observe major effects of oral or IV iron on bacterial diversity that would generate treatment-specific clusters of samples (figure 3A). Changes were individual specific, that is, distances between samples before and after treatment ranged from short to extreme, suggesting that faecal bacterial communities from several subjects are differentially sensitive to changes during IRT. Comparison of phylogenetic distances demonstrated that paired samples (before and after therapy for each individual) were more distantly related in patients with CD and patients with UC than in control anaemic participants, indicating that faecal bacterial communities in IBD subjects are more sensitive to IRT-induced alterations (figure 3B). Dendrogram analysis illustrated that patients with CD (red colour in the outer ring) had the most distinct diversity, but most of all that these patients were characterised by the lowest number of paired samples, defined as two samples from one given individual being most closely related to each other than to any other sample in the data set (black boxes) (figure 3C). When classifying subjects according to changes in the disease status (improved, unchanged, worsen) on the basis of clinical scores and CRP levels, and independently of IBD type (CD or UC), a decrease in sequence proportions of members of the family *Ruminococcaceae* was observed in subjects with improvement of disease (see online supplementary figure S1B).

To assess the impact of IRT on faecal metabolome, we looked at the sample arrangement in a low-dimensional space produced by OPLS-DA for differentiation between NI controls and patients with CD (prediction for NI and UC subjects after IRT was not performed since the corresponding model was weak) (figure 3D). This analysis showed that sample points from the NI and CD groups after IRT (triangles) grouped together compared with sample clouds at baseline (circles), which showed divergence between NI and CD. This indicates that, in comparison with clear CD-specific features at baseline, faecal metabolomes converged after IRT.

**The gut ecosystem is affected by oral iron treatment**

Comparing samples at baseline and after IRT taking into account the route of administration independently of disease phenotype, the iron content in faeces was increased after PO (p = 0.014) but not after IV (p = 0.363) treatment (paired t test) (figure 4A). In contrast, total sulfur concentrations were not significantly different. Because of these changes affecting iron levels in faeces, we assessed whether the route of iron administration influenced changes in bacterial communities and metabolite landscape.

Independently of disease activity phenotype, we searched for differences between PO and IV treatment when considering delta-values calculated as differences in sequence abundances (after—before). We found only five single OTUs that showed differential effects, four of which had lower abundances after oral iron therapy (figure 4B). These OTUs were identified as *Collinsella aerofaciens* (OTU-6), *Faecalibacterium prausnitzii* (OTU-13), *Ruminococcus bromii* (OTU-23) and *Dorea* sp. (OTU-52). In contrast, one OTU belonging to the genus *Bifidobacterium* (OTU-620) was found at higher relative abundances in faeces from subjects treated with iron orally, although four participants in this group consumed prebiotics and probiotics versus six in the IV group.

An OPLS-DA classification model of the routes of administration independently of disease phenotype was built to find treatment-specific differences in faecal metabolomes and showed that IV and PO grouped distinctly (figure 4C). A total of 1040 features were used to build the model and 308 of them
were assigned to putative metabolites via the MassTRIX web server. The top-17 metabolites responsible for separation of IV and PO groups together with the output of univariate statistics are shown in figure 4D. Metabolites displaying higher intensities in the PO group included phosphatidylglycerol, palmitate and derivatives thereof, whereas bile acids, steroids (tetrahydrodeoxycorticosterone) and other cholesterol derivatives were characteristics of the IV group.

DISCUSSION

The high prevalence of iron deficiency anaemia in IBD requires therapeutic intervention based on oral IRT or IV-IRT.20 Oral IRT has long been considered as standard intervention based on the safety profile, low cost and convenient administration. However, the high frequency of GI side effects21 and the potency to exacerbate intestinal inflammation support clinical implementation of IV-IRT.22 23 We previously showed that the
absence of luminal ferrous iron was associated with major changes in the gut microbiota and the prevention of chronic inflammation in an experimental model for CD-like ileitis. To follow up on these findings, the aim of the present study was to investigate the impact of oral (PO) and IV iron treatment on the intestinal milieu via assessment of faecal bacterial diversity and composition as well as metabolome analysis in subjects with iron deficiency suffering from CD or UC.

In accordance with previous reports, distinct bacterial phylogenetic makeups and lower phylotype richness were observed in faeces from patients with IBD compared with NI control anaemic subjects. Most interesting regarding the impact of IRT, faecal bacterial communities in patients with CD were characterised by highest interindividual differences and were most sensitive to changes after 3 months of treatment. Despite limitations of faecal material for bacterial community analysis, the drop observed in the relative abundance of Clostridiales spp. is consistent with the most comprehensive analysis in treatment-naïve paediatric patients with CD. In the latter study, changes in stool samples were actually representative for differences in biopsy material at the terminal ileum and rectum. This implies an important disease-related effect on members of the order Clostridiales independent of medication and sampling procedures. Specifically, reduction of Clostridium cluster IV and XIV members has been proposed to contribute to dysbiosis in patients with CD, and selected mixtures of corresponding clostridia strains were shown to induce T regulatory cells with disease-suppressive phenotypes in IBD-related mouse models. These results support a potential protective role of certain Clostridiales spp. in IBD pathogenesis. In our study, members of the newly described bacterial family

Figure 3  The gut environment in patients with IBD versus control anaemic subjects is more sensitive to overtime changes associated with iron replacement therapy (IRT). (A) NMDS plot of phylogenetic distances showing overall changes in bacterial diversity before and after treatment (connecting lines). (B) Boxplots depict the distribution of generalised UniFrac distances per patient category, indicating that samples before and after IRT are more similar in non-inflamed (NI) control subjects than in patients with IBD. (C) Ward’s clustering dendrogram calculated from phylogenetic distances between all samples. Paired samples (located next to each other before and after IRT for one given individual) are marked with black bars (inner ring). Disease categories were colour-coded in the outer ring as follows: red, Crohn’s disease (CD); green, UC and blue, NI. Percentages of paired samples per patient category are shown in the barplot. The p value was obtained using a χ² test. (D) Influence of iron treatment on classification between control and CD participants. The position of points corresponding to the samples from after IRT (blue and red triangles) was predicted using the orthogonal partial least squares discriminant analysis (OPLS-DA) model built for classification of NI and CD samples at baseline (blue and red circles). ANOVA, analysis of variance; NMDS, non-metric multidimensional scaling.
Christensenellaceae were specific for NI conditions. However, other family members within the order Clostridiales such as Ruminococcaceae as well as the Veillonellaceae within the order Selenomonadales are rather increased in patients with IBD, making general disease-related conclusions at these high taxonomic levels rather difficult.

The transition from a quiescent state to disease exacerbation in patients with IBD was shown to be associated with alterations of faecal bacterial communities, which supports the hypothesis that inflammatory processes in the intestines of both patients with UC and patients with CD affect the microbial milieu. In our study, IRT-induced shifts in bacterial diversity and composition in faeces from patients with IBD were not associated with changes in disease activity, suggesting that patient-specific shifts in faecal microbiota are not necessarily linked to changes in disease severity over short periods of intervention. Regarding the route of iron administration, IV therapy confirmed a slightly more effective management of iron deficiency anemia compared with oral treatment with respect to serum ferritin levels. In addition and consistent with the presence of increased iron in faeces, the luminal milieu in faeces from PO-treated individuals was distinct at the level of both metabolome and the occurrence of specific bacterial phylotypes. In contrast to the increase in total faecal iron after PO administration of iron sulfate tablets, the total sulfur concentration did not increase. Oral administration studies of radioactive sulfate indicate a relatively high efficiency of intestinal sulfate absorption, reaching up to 80%.

Even doses of up to 8 g anhydrous sodium sulfate per day resulted in 50–60% absorption efficiency. Although the colon might contribute to total sulfate absorption, colonic accumulation of this terminal electron acceptor as growth-limiting substrate of sulfate-reducing bacteria would lead to an increased abundance in human faeces, including Desulfovibrio, Desulfomonas or Desulfobacter. However, sequence analysis in our study revealed low relative abundance and no changes in bacterial taxa associated with sulfate metabolism. Changes included higher signals for cholesterol, palmitate and phosphatidylglycerol in PO-IRT, accompanied by a significant decrease in the relative abundance of C. aerofaciens, F. prausnitzii, R. bromii and Dorea sp., and an increase in one OTU within the genus Bifidobacterium. In contrast to the increase in total faecal iron after PO administration of iron sulfate tablets, the total sulfur

Figure 4 PO iron treatment is associated with specific features in faecal samples. (A) Iron and total sulfur content in faeces. (B) Relative abundance of phylotypes that reacted differently to intravenous (IV) or oral iron therapy. IRT groups were compared using Mann–Whitney test. Prior to testing, individual counts <0.5% were zeroed, and only prevalent and dominant molecular species (i.e., detected in at least 30% of the participants (n=124) and with a median sequence abundance >1% in at least one group) were considered. (C) Score plot of orthogonal partial least squares discriminant analysis (OPLS-DA) classification of IV-IRT (beige) versus PO-IRT (brown). Learning the model resulted in one predictive and one orthogonal component (x-axis and y-axis, respectively). (D) Top features responsible for classification in the OPLS-DA model are shown as boxplots. B, baseline; IRT, iron replacement therapy; OTU, operational taxonomic unit; PO, per oral.
concentration did not increase. Oral administration studies of radioactive sulfide indicate a relatively high efficiency of intestinal sulfate absorption, reaching up to 80%. Even doses of up to 8 g anhydrous sodium sulfate per day resulted in 50–60% absorption efficiency. Although the colon might contribute to total sulfate absorption, colonic accumulation of this terminal electron acceptor as growth-limiting substrate of sulfate-reducing bacteria would lead to an increased abundance in human faeces including Desulfovibrio, Desulfomonas or Desulfofaba. However, sequence analysis in our study revealed low relative abundance and no changes in bacterial taxa associated with sulfate metabolism. Opposite to the IBD-related changes that we observed, iron fortification in Kenyan infants showed an increase in clostridia and a decrease in bifidobacteria. Interestingly, *Bifidobacterium* strains isolated from stools of iron-deficient infants efficiently sequestered iron, suggesting an inverse relationship between abundance and functionality. Moreover, we cannot exclude the possibility that the intake of functional foods, including probiotic dairy products, in the present study may have specifically affected results on relative sequence abundances of bifidobacteria. Considering currently available studies, one of the most consistent differences between IBD and NI subjects is the reduced abundance of *E. coli* in stool samples. Lower *E. coli* abundance was also observed in patients with UC compared with controls, whereas signals for C16:2 were higher in the NI group. Of note, C16:2 can be produced from chain-shortening *F. prausnitzii* in stool samples was lower after PO therapy, suggesting that this bacteria is affected not only by disease-intrinsic factors, but also by environmental factors. Different metabolic and inflammatory properties, for example, antioxidant, oestrogenic, anti-inflammatory and anticarcinogenic activities. Altogether, whereas differences in microbiota were observed at the level of a few specific molecular species, the route of iron administration had a clearer discriminative effect at the level of metabolomes, which reflect both host and microbial functions. In conclusion, IRT in patients with iron deficiency or anaemia induced significant shifts in bacterial community structure and metabolite landscape in faeces. Patients with CD seemed to be more prone to IRT-induced shifts and, according to the load of free iron detected in the luminal environment, oral iron therapy affected the presence of specific molecular bacterial species.

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**Competing interests** None declared.

**Patient consent** Obtained.

**Ethics approval** The study was approved by the local ethics committee of the University of Alberta (Canada) and registered with clinicaltrial.gov (NCT01067547).

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**High-throughput sequencing of bacterial communities**

One aliquot (100 mg) of each fecal sample was mixed with 500 µl phenol:chloroform:isoamyl, 900 µl of ASL buffer (Qiagen, QIAamp® stool mini kit) and 200 mg zirconia beads. Cells were lysed using a bead beater (FastPrep-24; MP Biomedicals LLC) at 6.5 M/s, 0.5 min (4 cycles interrupted by 3 minutes on ice) and incubated at 95 °C (5 min, 800 rpm). After centrifugation (1 min, 13,000 g) the aqueous phase was collected into a fresh Eppendorf tube and filled up to 1.2 ml with ASL buffer. DNA was purified using the QIAamp® stool mini kit following the manufacturer’s instructions (Qiagen, QIAamp® stool mini kit), with addition of RNAse treatment (37 °C, 30 min). DNA concentrations and purity (A260/A280 ratio) was determined by spectrophotometric analysis (ND-1000 spectrophotometer, NanoDrop Technologies, Willigton, USA). The V4 region (253 bp) of 16S rRNA genes was amplified (30 cycles) as described previously [1, 2]. Amplicons were purified using the AMPure XP system (Beckmann), pooled in equimolar amount and sequenced in paired-end modus (PE200) using the MiSeq system (Illumina Inc) following the manufacturer’s instructions and a final DNA concentration of 16 pM and 5 % (v/v) PhiX standard library.

**Sequence analysis**

Raw read files were demultiplexed (allowing a maximum of 2 errors in barcodes) and each sample was processed using usearch [3] following the UPARSE approach [4]. First, all reads were trimmed to the position of the first base with quality score <3 and then paired. The resulted sequences were size filtered excluding those with assembled size <220 and >280 nucleotides. Paired reads with expected error >3 were further filtered out and the remaining sequences were trimmed by 10 nucleotides on each side to avoid GC bias and non-random base composition. For each sample, sequences were de-replicated and checked for chimeras with UCHIME [5]. Sequences from all samples were merged, sorted by abundance, and operational taxonomic units (OTUs) were picked at a threshold of 97 % similarity. Finally, all sequences were mapped back to the representative sequences resulting in one
OTU table for all samples. Only those OTUs with a relative abundance above 0.5 % total sequences in at least one sample were kept. RDP classifier [6] was used to assign taxonomic classification to the OTUs representative sequences (80 % confidence). A phylogenetic tree was constructed using fasttree [7]. OTU counts were expressed as proportions of total sequences per sample and normalized to the minimum count of total sequences observed across samples. For estimation of diversity within samples (alpha-diversity), the Shannon index was calculated and transformed to the corresponding effective number of species as described by Jost [8]. Sequences of candidate OTUs were assigned to taxa with valid names in nomenclature using the EzTaxon database [9].

High-resolution mass spectrometry

Sample preparation was performed on dry ice. Fecal samples (5 mg each) were placed into tubes containing ceramic beads (NucleoSpin® Bead Tubes, MACHEREY-NAGEL GmbH & Co. KG) and a stainless metal bead (QIAGEN®, 5 mm). A volume of 1 mL cold methanol (-20 °C) (Fluka® Analytical, LC-MS CHROMASOLV®) was added in each tube followed by homogenization (5 min, 30 1/s) using a TissueLyser II (QIAGEN®). After centrifugation (14,000 rpm, 10 min, 4 °C), supernatants were collected into new Eppendorf tubes and stored at -80 °C until further processed. Prior to MS measurement, a volume of 100 µL of each sample was diluted in 300 µL of methanol and the total volume was placed into maximum recovery vial (Waters®, 12x32 mm). Samples were measured using a Fourier transform ion cyclotron resonance mass spectrometer (FT-ICR-MS) (BRUKER® solariX, Daltonic GmbH, Bremen, Germany) equipped with a 12 Tesla superconducting magnet and an Apollo II ESI source. The instrument was externally calibrated on clusters of arginine (m/z of 173.10440, 347.21607, 521.32775 and 695.43943) dissolved in methanol at a concentration of 5 mg/mL. Samples were placed in an autosampler (GILSON®) at -4 °C in a randomized order and were injected into the mass spectrometer at a flow rate of 120 µL/h. Data were acquired in the negative ion acquisition mode with the following parameters: capillary voltage -3,600 V, ion accumulation time -0.3 s, flight
time to acquisition cell -1 ms, drying gas flow rate -4 L/min, drying gas temperature -180 °C, nebulizer gas pressure -2 bar. A total of 500 scans were accumulated per sample in the mass range of 120-1000 m/z.

**Metabolome analysis**

A sine apodization was performed before Fourier transformation of the time-domain transient. Raw data were processed with DataAnalysis 4.1 (BRUKER®). A FTMS peak picking algorithm was applied to identify peaks in the raw data using the following parameters: relative intensity threshold (relative to the highest peak in a spectrum) -0.001 %, signal-to-noise ratio threshold -4, absolute intensity threshold -100 (default). Identified m/z signals were internally calibrated using a reference list provided by the software. Data were exported as individual .ascii files containing m/z values and corresponding intensities. Peak alignment and data matrix creation were performed by in-house algorithm written in Fortran that searches for mass signals from different spectra that are close to each other (within 1 ppm window) combining them into one group that represents a unique feature. In order to stabilize the variance, m/z values with frequency <10% across all samples were deleted. The data was additionally filtered by an algorithm written in MATLAB that creates a mass-difference network using experimental m/z values as input and, according to the constructed network, annotates the m/z values by unique molecular formulas [10, 11]. The network can be used to reveal clusters of compounds, assign them to possible metabolite classes, and hypothesize their chemical structure. Thereby, after filtering out features that could not be part of the network, every remaining feature corresponds to unique molecular formula. m/z values were also annotated using the MassTRIX webserver [12] that searches for matches between experimental and theoretical m/z values within 1 ppm error window using KEGG, HMDB, and the LipidMAPS databases as input sources. Missing values were substituted by the level of noise from original spectra.
Iron measurement

Fecal samples were freeze-dried (Christ-Heraeus), weighed and introduced into quartz vessels. Subsequently, 1 mL of suprapure HNO₃ (Merck) subjected to sub-boiling distillation (Berghoff distillation apparatus) was added. Vessels were introduced into a pressure digestion system for 10 h at 170 °C. Resulting solutions were filled up to 10 mL with Milli-Q H₂O and iron content was determined using an ICP-AES Spectro Ciros Vision system (SPECTRO Analytical Instruments). Samples were introduced at a flow rate of 0.6 mL/min using a peristaltic pump connected to a Meinhard nebulizer with a cyclone spray chamber. The measured spectral line corresponding to iron was 259.941 nm. Radiofrequency power was set to 1400 W. The flow of plasma and nebulizer gas was set to 13 L Ar/min and to approximately 0.6 L Ar/min, respectively. Three blank samples followed by one control sample of a certified standard were acquired each ten measurements. Iron levels were calculated on a computerized lab-data management system integrating sample measurements with calibration curves, output from blank and control samples, and digested sample weight.

References


Supplementary Figure S1: Boxplots showing sequence abundances of indicated taxonomic groups.