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Research Article

**A decrease in iron availability to human gut microbiome reduces the growth of potentially pathogenic gut bacteria; an *in vitro* colonic fermentation study**

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**Running title:** Iron chelation effects on the human gut microbiota

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**Keywords:** chelation; dysbiosis; gut microbiota; Iron; short chain fatty acids

**Abstract**

Iron-supplements are widely consumed; however most of the iron is not absorbed and enters the colon where potentially pathogenic bacteria can utilise it for growth. This study investigated the effect of iron availability on human gut microbial composition and function using an *in vitro* colonic fermentation model inoculated with faecal microbiota from healthy adult donors, as well as examining the effect of iron on the growth of individual gut bacteria. Batch fermenters were seeded with fresh faecal material and supplemented with the iron chelator, bathophenanthroline disulphonic acid (BPDS). Samples were analysed at regular intervals to assess impact on the gut bacterial communities. The growth of *Escherichia coli* and *Salmonella Typhimurium* was significantly impaired when cultured independently in iron-deficient media. In contrast, depletion of iron did not affect the growth of the beneficial species, *Lactobacillus rhamnosus*, when cultured independently. Analysis of the microbiome composition via 16S-based metataxonomics indicated that under conditions of iron chelation, the relative abundance decreased for several taxa, including a 10% decrease in *Escherichia* and a 15% decrease in *Bifidobacterium*. Metabolomics analysis using <sup>1</sup>H-NMR indicated that the production of SCFAs was reduced under iron-limited conditions. These results support previous studies demonstrating the essentiality of iron for microbial growth and metabolism, but, in addition, they indicate that iron chelation changes the gut microbiota profile and influences human gut microbial homeostasis through both compositional and functional changes.

**Keywords:** chelation; dysbiosis; gut microbiota; Iron; short chain fatty acids

## 1. Introduction

Humans are host to a wide variety of microbes which develop ecosystems that are specific to any one individual but which are subjected to a life-long process of colonisation by foreign microbes [1]. Through evolution, an environment of mutualism has been created whereby many host-bacterial associations have become beneficial relationships; such as is found in the large intestine (colon), which is home to the highest number of microbes (> 100 trillion bacteria) in our body. Commensal bacteria of the mammalian gut have long been recognised for the advantages they confer to the host. The benefits include defence against opportunistic pathogens, metabolism of indigestible compounds, provision of essential nutrients and an involvement in the development of the intestinal structure [2, 3], whilst also producing metabolites, such as SCFAs that contribute towards providing energy to host cells [4-6]. The human gut microbiome also contributes towards the basic developmental features and functions of the immune system [7]. Conversely, perturbations (e.g. antibiotic treatment and/or overgrowth of pathogenic bacteria) in these symbiotic relationships, termed dysbiosis, can lead to a negative impact on the host's health [8]. Dysbiosis has been associated with a range of human disease states, such as autoimmune disorders [9], [10] increased vulnerability to cancers [11], irritable bowel syndrome [12-15], and obesity [16], and research on these associations, including an understanding of the mechanisms involved, is a high priority. The number of studies examining the relationship between diet and the gut microbiome has increased over the past years and has shown that the gut microbiome composition and metabolism are significantly affected by diet [17].

Iron, an essential nutrient for humans, is highly abundant in the environment and involved in numerous biological processes such as hydrogen production, respiration, and DNA biosynthesis. It also functions as a co-factor in numerous metabolic pathways within a host cell and is essential for nearly all prokaryotic and eukaryotic cells. In humans, the absorption of iron by the epithelial cells of the small intestine is a very tightly regulated process due to the absence of excretory pathways [18]. The efficiency of absorption depends on the potential availability of iron in the diet, and is regulated by physiological iron requirements, including body iron stores, with hepcidin having a central role in the control of absorption [19].

Dietary iron has been broadly classified into two types, non-haem and haem iron. Both forms of dietary iron have a separate pathway of uptake by enterocytes, where haem iron is better absorbed (25-30%) compared to the poorly absorbed non-haem iron, (0-15% absorbed) despite being the most common form of iron present in the diet [20]. The unabsorbed iron travels to the large

intestine, and there is accumulating evidence to suggest that this unabsorbed colonic iron can facilitate the growth of intestinal pathogens [21].

The vast majority of bacteria in the gut require iron for growth and development, and they have formulated many strategies to acquire this nutrient, which in its more common state ( $\text{Fe}^{3+}$ ) has low or zero solubility. Potentially pathogenic bacteria make use of a continuous supply of micronutrients, such as iron, for metabolism and replication. Thus, there is constant competition for iron between various bacteria, many of which have developed mechanisms, such as siderophore production, to acquire iron, particularly when availability is limited. Unlike most bacteria, members of the Bifidobacteriaceae and Lactobacillaceae families (two families that are believed to be beneficial to the host [22-25]) have a very limited need for iron [26]. Lactobacilli do not produce siderophores to sequester iron, and their growth is similar in media with and without iron [27]. *Bifidobacterium breve*, an important member of the Bifidobacterial species in breast-fed infants, can acquire luminal iron with the help of a divalent metal permease, however, many of the Bifidobacterium species do not synthesise siderophores or other forms of iron-carriers. The human body has created many ways to promote the growth of these beneficial bacteria, such as the presence of lactoferrin in breast milk which travels intact to the large intestine [28-30], which with its high affinity for iron, renders the iron unavailable to potentially pathogenic bacteria [31]. Along with high amounts of dietary iron in the gut lumen, the oral administration of iron supplements also results in freely available “unbound” iron in the colon [32-34]. This disturbs iron homeostasis consequently modifying the gut microbial composition [35-37].

An emerging link between the gut microbiome and iron availability has been observed, however several studies that have investigated the effects of iron on the human gut microbiota are mainly focussed on the infant microbiome [6, 33-35, 38-40]. Other studies have looked at the role of iron in the gut microbiota in individuals with either metabolic [41] or iron disorders and therefore, this study was designed to use *in vitro* colon models that mimic the microbiological conditions of the human large intestine to assess the impact of iron limitation on structure and function of the complex gut bacterial communities of healthy adults.

## 2. Materials and Methods

### 2.1 Bacterial strains and growth conditions

To evaluate the role of iron on bacterial growth, the following bacterial strains were chosen: *Escherichia coli* 1B04 (isolated from human faeces), *Bifidobacterium longum* B78 F110564 (isolated from human faeces), *Lactobacillus rhamnosus* GG F111027, *Salmonella* Typhimurium (ATCC SL1344), *Clostridium perfringens* (NCTC 3110) and *Bacteroides thetaiotaomicron* VP1-5482 (ATCC 29148). Bacteria were cultured in the following media: Luria (L) Broth (*E. coli* and *S. Typhimurium*); Brain Heart Infusion (BHI; *B. longum*); BHI supplemented with vitamin K (0.5% v/v), hemin (0.5mg/mL), resazurin (0.02% w/v) and L-cysteine (0.5g/L) (*C. perfringens*) and BHI with hemin (*B. thetaiotaomicron*); De Man, Rogosa and Sharpe (MRS) supplemented with glucose (*L. rhamnosus*). All bacteria were grown under anaerobic conditions at 37°C. *E. coli* and *S. Typhimurium* were agitated at medium speed throughout the growth period, whilst the remaining bacteria were grown under static conditions.

### 2.2 The effect of iron chelation on bacterial pure cultures

Bacterial cultures were grown overnight in rich media specified above. Cultures were seeded in 100-well honeycomb plates and cells were then provided with bathophenanthrolinedisulfonic acid disodium salt hydrate (BPDS; B1375, Sigma, United Kingdom) at final concentrations ranging from 0 – 50 µM. Samples were analysed using a Magellan™ Microplate Reader (Tecan Life Sciences, Switzerland), which monitors the growth of microorganisms by measuring the turbidity of the culture (optical density, OD<sub>600</sub>) of the liquid growth medium. The experiments were run for 24 h, with OD<sub>600</sub> taken every 10 minutes. The experiments were performed at 37°C under anaerobic conditions.

### 2.3 *In vitro* colonic fermentations

10 g of fresh fecal samples obtained from healthy volunteers were diluted 1:10 in deoxygenated phosphate buffered saline (pH 7.7), and homogenised using a Stomacher 400 (Seward, United Kingdom) at 230 rpm for 45 s. All samples were processed within 4 h of stool collection.

The working volume of each vessel was set at 150 mL, of which 15 mL was processed faecal inocula and 135 mL was nutritive media (peptone water 2 g/l, yeast extract 2 g/l, NaCl 0.1 g/l, K<sub>2</sub>HPO<sub>4</sub> 0.04 g/l, KH<sub>2</sub>PO<sub>4</sub> 0.04 g/l, MgSO<sub>4</sub>·7H<sub>2</sub>O 0.01 g/l, CaCl<sub>2</sub>·6H<sub>2</sub>O 0.01 g/l, NaHCO<sub>3</sub> 2 g/l, Tween 80 2 ml, glucose 10 g/l, vitamin K1 10 µl, cysteine HCl 0.5 g/l, bile salts 0.5 g/l, pH 7.0). The pH was controlled and

maintained between 6.6 - 7.0 using pH control units Fermac 260 (Electrolab, United Kingdom), connected to 1M NaOH and 1M HCL solutions. Vessels were kept at 37°C by a circulating water jacket and anaerobiosis was maintained by continuous bubbling of the system with oxygen-free nitrogen gas. Conditions tested were either nutritive media with faecal inocula only (Control), or with faecal inocula and BPDS (70 µM). The concentration of BPDS was chosen as a result of preliminary experiments investigating the baseline faecal iron concentrations in multiple healthy donors (data not shown). We used 70 µM BPDS as we calculated that this concentration would be sufficient to chelate most of the iron in the colon. Per donor, 1 vessel was used for each condition. Samples were taken at 0, 4, 8, and 24 h from each vessel, serially diluted in PBS and enumerated on selective agar plates [42]. Faecal samples used in the colon model experiments were obtained from participants recruited onto the QIB Colon Model study. Men and women aged 18 years or older who live or work within 10 miles of the Norwich Research Park were recruited onto the QIB Colon Model study if they satisfied the following criteria. Participants who were assessed to have a normal bowel habit, regular defecation between three times a day and three times a week, with an average stool type of 3 – 5 on the Bristol Stool Chart, and no diagnosed chronic gastrointestinal health problems, such as irritable bowel syndrome, inflammatory bowel disease, or coeliac disease were eligible to enrol onto the study. Demographic information was collected, and a brief health questionnaire was completed during the eligibility screening. Participants were asked additional questions immediately prior to donating a stool sample to confirm that they had not taken antibiotics or probiotics within the last four weeks, had not experienced a gastrointestinal complaint, such as vomiting or diarrhoea, within the last 72 hours, were not currently pregnant or breast-feeding, had not recently had an operation requiring general anaesthetic, and were not taking iron or multivitamin supplements. The study was approved by the Quadram Institute Bioscience (formally Institute of Food Research) Human Research Governance committee (IFR01/2015), and London - Westminster Research Ethics Committee (15/LO/2169). The informed consent of all participating subjects was obtained, and the trial is registered at <http://www.clinicaltrials.gov> (NCT02653001).

#### **2.4 DNA extraction for 16S rDNA based metagenomics**

DNA was extracted from all samples collected from *in vitro* colonic fermentations using a commercially available kit (FastDNA spin kit for soil; MP Biomedicals, USA, Cat No. 6560200). Samples were thawed on ice, homogenized, and approximately 200 mg of each were used to extract DNA following manufacturer's instruction, with an additional bead beating step using FastPrep (MP Biomedicals, USA), as previously described by Kellingray *et al.*, [43].

#### **2.5 16S rRNA gene amplification and sequencing**

The impact of iron on the composition of the human gut microbiome was investigated using high throughput 16S rRNA gene (V4 region) sequencing using the Illumina Miseq platform, followed by data analysis using the Quantitative Insights into Microbial Ecology (QIIME, V1.9) pipeline. Each sample sequence was filtered to read lengths between minimum 200 and maximum 1,000 bp. ChimeraSlayer was used to filter trimmed reads for chimeric sequences after which Shannon index were used to create alpha diversity plots. Weighted UniFrac data were used to produce beta diversity PCoA plots, using the XLSTAT add-on package on Microsoft Excel.

## 2.6 Short chain fatty acid quantification in cultured microbiomes

Faecal water was prepared to quantify short chain fatty acids in stool. Briefly, samples (13 mL) taken from colonic batch fermentations were centrifuged at 3220  $xg$  for 15 minutes at 4°C. 100  $\mu$ L NMR buffer (0.26 g  $\text{NaH}_2\text{PO}_4$  and 1.41 g  $\text{K}_2\text{HPO}_4$  made up in 100 ml  $\text{D}_2\text{O}$ , containing 0.1%  $\text{NaN}_3$  (100 mg), and 1 mM sodium 3-(Trimethylsilyl)-propionate- $d_4$ , (TSP) (17 mg) as a chemical shift reference) was added to 900  $\mu$ L supernatant and analysed using  $^1\text{H}$  NMR spectroscopy (this mixture is defined as 'faecal water'). The  $^1\text{H}$  NMR spectra were recorded at 600 MHz on a Bruker Avance spectrometer (Bruker BioSpin GmbH, Germany) running Topspin 2.0 software and fitted with a cryoprobe and a 60-slot autosampler. Each  $^1\text{H}$  NMR spectrum was acquired with 256 scans, a spectral width of 12,300 Hz, and an acquisition time of 2.67 seconds. The "noesypr1d" pre-saturation sequence was used to suppress the residual water signal with a low-power selective irradiation at the water frequency during the recycle delay and a mixing time of 10 ms. Spectra were transformed with a 0.3 Hz line broadening, and were manually phased, baseline corrected, and referenced by setting the TSP methyl signal to 0 ppm. The metabolites were quantified using the software Chenomx<sup>®</sup> NMR Suite 7.0<sup>™</sup>.

## 2.7 Quantification of iron levels in fermentation samples

A 20  $\mu$ L aliquot of the faecal water prepared in section 2.6 was used to quantify iron concentrations of the fermentation samples at 0, 8 and 24 h using the ferrozine assay as per manual instructions (Iron Assay Kit ab83366, Abcam, UK). Briefly, iron in the sample is reduced using an Fe reducer, provided by the kit, after which iron reacts with Ferene S (an iron chromogen) to produce a stable coloured complex. Absorbance measurements were taken at 593 nm.

## 2.8 Statistical analysis

Data from pure culture experiments and *in vitro* colonic fermentations were expressed as means  $\pm$  standard errors of the means (SEM). Pure cultures were analysed using one-way ANOVA followed by Bonferroni post-tests with GraphPad Prism software (Version 5.04), whilst *in vitro* colonic batch



fermentations were analysed using unpaired t-tests assuming unequal variances on Microsoft Excel.  $P < 0.05$  was considered statistically significant.

### 3. Results

#### 3.1 Iron chelation significantly reduced the growth of selected bacterial species grown in pure cultures

For pure culture experiments, we wanted to test a range of gut bacteria that are known to be affected or unaffected by iron, but the selection, to some extent, depended on what was available in the laboratory. Therefore, various bacterial species were treated with different concentrations (0, 10, 20, and 50  $\mu\text{M}$ ) of BPDS in triplicate for 24 h to determine the effect of iron chelation on their growth. A dose-dependent reduction in the growth of *E. coli*, *S. Typhimurium* and *B. thetaiotaomicron* ( $p < 0.0001$ ), were observed in the presence of BPDS, at 24 h (Fig 1 A-C). As expected, *L. rhamnosus*, a bacterial species known to be able to grow in the absence of iron, exhibited comparable growth to the BPDS-free (0  $\mu\text{M}$ ) culture, confirming previous observations that iron is not an essential nutrient for the survival of this bacterial species (Fig 1 E). A decrease was also observed in the growth of *B. longum* ( $p < 0.01$ ) upon iron chelation (Fig 1 D). These results support the idea that iron is an essential nutrient for bacteria, and in its absence the growth of many bacteria is restricted.

#### 3.2 Reduced iron availability decreased the growth of potentially pathogenic bacteria under *in vitro* colonic fermentation conditions

Batch fermentations were performed for 24 h to elucidate the effects of iron depletion on the human gut microbiome by enumerating specific bacterial groups using selective agar plates. Counts from the control vessel of the three individual experiments were first normalised to 100% and thereafter, viable counts from the iron-chelated vessels were shown as percentages in relation to the Control. Finally, the data from the three individual experiments were combined and average percentages ( $\pm\text{SEM}$ ) presented (Table 1). Results showed a decrease in the viable counts of a number of bacterial groups, including Enterobacteriaceae ( $p < 0.05$  at 8 and 24 h), Bifidobacteria ( $p < 0.001$  at 8 h) and Lactobacilli ( $p < 0.01$  at 8 and 24 h), upon iron chelation at 8 h and 24 h (Table 1). It should be noted that the high viable counts of Bifidobacteria at 24 h is attributed to a single donor.

The remaining two donors displayed a substantial reduction in the viable counts of Bifidobacteria under iron-chelated conditions.

### 3.3 16S rRNA gene sequencing revealed a decrease in the relative abundance of potentially pathogenic bacterial genera in response to iron limitation

High-throughput paired-end sequencing of the 16S rRNA gene (V4 region) was performed on fermentation samples using the Illumina Miseq platform, and microbial communities present in relative abundances  $\geq 0.5\%$  are illustrated in Fig 2. After combining and averaging the abundance values from the three individual experiments, the data revealed that the most abundant genera at 0 h were *Escherichia* (16.1%), followed by *Bacteroides* (8.7%), (Fig 2, SEM values can be found in Table 2). It is worth noting that the relative abundance of *Escherichia* is entirely attributed to one donor only, as the remaining two donors had no more than 0.1% *Escherichia* present at T0.

Briefly, the relative abundance of *Escherichia* was reduced substantially in the iron chelated fermentation vessel in comparison to the control vessel at 8 h (0.8% vs 10.7%) and 24 h (2.3% vs 5.3%) (Fig 2). This correlates well with the reduction in the viable counts for Enterobacteriaceae (Table 1). Again, it is worth noting that the high relative abundance of Enterobacteriaceae at T8 and T24 for both control and chelator conditions, is entirely attributed to the levels observed in one donor only, since in the other two donors, the levels were below the detectable limit. A similar trend was observed for *Bifidobacterium*, where the relative abundance was much lower at T8 and T24 under iron-chelated conditions (4.1% vs 15% and 14% vs 29%, respectively), which is also reflected in the viable counts results for Bifidobacteria at 8 h. Interestingly, 16S rDNA analysis indicated that at 24 h, the relative abundance of *Streptococcus* increased to 36% in the iron chelated condition compared to the starting proportion of 4.7%. Iron-depleted conditions have been shown to proportionally decrease many bacterial groups, and this may provide other bacteria, such as *Streptococcus*, with a competitive advantage resulting in their increased growth. This could also be true for *Bacteroides*, as the relative abundances of this genus increased at both 8 h (10.6% to 18.1%) and 24 h (2.7% to 4.4%) under iron chelated conditions. Finally, 16S rDNA analysis indicated that *Clostridium* abundance was largely unaffected by iron removal (Fig 2), and this was reflected in the viable counts for Clostridia (Table 1).

### 3.4 Iron chelation resulted in a shift in bacterial diversity profiles

Alpha ( $\alpha$ ) – and beta ( $\beta$ ) – diversity was measured for all *in vitro* colonic fermentation samples tested. The Shannon index, which is a measure of  $\alpha$ - diversity, showed a minimal difference in diversity within the population in the absence of the chelator (Fig 3a). However, an increase in population diversity was observed at 8 h in the presence of BPDS. The principal coordinates analysis

(PCoA) plot (Fig 3b), using the weighted UniFrac metric, which depicts  $\beta$ - diversity, indicates that the microbiotas of the subjects before and after iron chelation did not have a large range of taxa in common. Interestingly, at both 8 h and 24 h, a shift in  $\beta$ -diversity was observed upon iron chelation when compared to the control at the respective time points, depicted by the dashed line.

### 3.5 Levels of short chain fatty acids decrease upon iron chelation during *in vitro* colonic fermentation

$^1\text{H-NMR}$  spectroscopy was used to determine the levels of over 70 metabolites from samples taken from the fermenters at 0, 8, and 24 hr. The three metabolites which showed the most substantial changes between treatments, were the short chain fatty acids (SCFAs), acetate, propionate and butyrate (Fig 4).

Acetate concentrations was  $\sim 25.3$  mM under control conditions (i.e. 0  $\mu\text{M}$  BPDS) at 8 h with lower levels ( $\sim 6.3$  mM) found when iron was chelated (with 70  $\mu\text{M}$  BPDS) at the same time-point. The same trend was observed at 24 h where acetate concentrations were  $\sim 33.3$  mM in the control vessel whereas under iron-limitation, its levels were reduced to  $\sim 25.6$  mM. Levels of propionate and butyrate exhibited the same pattern under control and iron-chelated conditions. In the control condition,  $\sim 1.1$  mM (8 h) and  $\sim 6.8$  mM (24 h) of butyrate was measured whereas under iron-chelation, butyrate levels were reduced ( $\sim 0.4$  mM at 8h and  $\sim 3.6$  mM 24 h) representing 47% reduction at 24 h. This is reflected in the reduction observed for the relative abundances of the members of the *Ruminococcus* (3.9% to 1.5%) genera, which are common butyrate producers.

Although similar propionate levels were observed between the different conditions at 8 h, propionate concentrations were lower in the iron-chelated vessel compared to the control at 24 h ( $\sim 10.5$  mM vs  $\sim 3.5$  mM). This represents a 67% decrease in production at 24 h under iron-limiting conditions. This is in line with the 70% decrease observed in the viable counts of *Bacteroides* (a genus containing propionate producers) under low iron conditions (Table 1).

## 4. Discussion

Iron supplements are consumed widely, but the majority of the iron passes through to the colon where it can be utilised by iron-requiring bacteria. This study was designed to assess the effects of colonic iron chelation on the human gut microbiota. We found that the addition of BPDS, a chemical iron chelator, significantly reduced the growth of *E. coli*, *S. Typhimurium*, *B. thetaiotaomicron* and *B. longum* (Figure 1). These results confirm well-established knowledge that iron promotes the growth of potentially pathogenic bacteria. However, Cronin et al., [44] reported that the growth of two *B. longum* strains were largely unaffected in the presence of an iron chelator, even at the highest chelator concentration (5  $\mu\text{M}$ ), suggesting that the effect of iron chelation on *Bifidobacteria* could be

largely strain-dependent. In the same study [44], no effect on the growth of *L. rhamnosus* was observed and it is known that *Lactobacillus* does not require iron for growth due to an absence of haem-containing enzymes. However, they can substitute other metals for iron, such as cobalt and manganese [45], which may give these bacteria a competitive advantage in low-iron conditions.

The net effects of iron on the gut microbial composition are still inconclusive and need further research as the results reported are inconsistent [6, 39, 44, 45]. Our study showed a marked effect on the global microbiome composition when iron was chelated in the fermentation media with BPDS (Figure 2). BPDS acts as a chelator of various metals and, in particular, it has been shown to bind iron with very high affinity and has therefore been used as an iron chelator in many studies [46, 47]. Comparison of the relative abundance of bacterial taxa between the two conditions (control and BPDS) illustrated that the most apparent differences were the decreased relative abundance of the potentially pathogenic *Escherichia*. The observed decrease of *Bifidobacterium* is interesting as other studies have reported different outcomes, which could be due to strain specificity, inter-individual variability of the host, as well as the effects of neighbouring taxa and metabolites. However, the relative abundance of *Clostridium* remained relatively stable, which has been observed in other studies. Notably, *Streptococcus*, a member of the lactic acid bacteria group to which *Lactobacillus* also belongs, was seen to increase upon iron removal, and this could potentially lead to restricted growth of other bacteria in the gut environment.

Studies investigating the effects of iron supplementation report comparable results to those found in this study in relation to the relative abundance of various bacterial taxa, including *Escherichia*. One study examined the effects of low and high doses of in-home iron supplementation on the gut microbiome of Kenyan children [39]. In this setting, provision of iron-fortified porridge led to an increase in pathogen abundance, with proportions of *Enterobacteria*, *Clostridium* and *E. coli* increasing, whilst *Bifidobacteria* decreased. In addition, children given iron-fortified porridge had elevated levels of faecal calprotectin, a marker of gut inflammation, which is likely to reflect an increased pathogenic profile.

Much of the published literature focuses on the effects of iron on the infant microbiome which may not be the same as adults. In addition, geographical location is probably important, as studies have shown that individuals from developing countries tend to have a greater pathogenic microbial profile due to the lack of clean food and water, and, in turn, a compromised gut function. This is reflected in two studies, where one investigated the effects of iron addition on the gut microbiota of children from Cote d'Ivoire [48], the second study focused on children from South Africa [6]. No

detrimental effects of iron supplementation were observed in those that had access to clean water and food, yet the children that did not have access to clean water, had adverse side effects.

We also performed bacterial diversity analysis.  $\alpha$ -diversity analysis using the Shannon index, which accounts for the distribution and richness of OTUs within a population, showed an increase in population diversity at 8 h in the presence of the iron chelator (Figure 3a). We speculate that the chelation of iron may lead to certain taxa exploiting other metals as a means of replacing iron, and therefore temporarily facilitating growth in a micronutrient-restricted environment. However, by 24 h, a visible decrease in diversity was observed under iron-chelated conditions, suggesting an exhaustion of these metals and nutrients.  $\beta$ -diversity analysis demonstrated that there was not a large range of taxa in common when comparing the control samples to those that were cultured in iron-limiting conditions (Figure 3b). Interestingly, at both 8 h and 24 h, there was a shift in  $\beta$ -diversity between the two groups. This is in line with a study performed by Dostal *et al.*, [49], which investigated the effect of iron on butyrate production in the child gut microbiota, where altering the iron concentration in the medium affected microbial community structure as well as causing a shift in  $\beta$ -diversity.

Microbial metabolic activity contributes to human health. When iron was chelated, we observed a decrease in the three main short chain fatty acids (SCFAs) that are produced in the gut (Figure 4), which presumably reflects poor growth of fermentative microbes. Based on the 16S rRNA gene sequencing data from this study and those published elsewhere, we can infer the potential mechanisms behind certain metabolic changes. Firstly, iron-dependent enzymes are critical operators of many metabolic pathways, and therefore these processes can be affected by differing iron concentrations. Moreover, any microbial fermentation that takes place requires the redox balance to be sustained. Due to the dual role of iron as an electron donor and acceptor, we speculate that changes in iron levels could have a large effect on redox balance. During the batch fermentation, the most prominent effect observed on metabolite production was that of acetate levels. Many gut bacteria produce acetate by either the reductive acetyl-CoA pathway, which uses  $H_2$  and  $CO_2$  [50], or via the regular glycolytic pathway through pyruvate metabolism [51]. The former pathway consists of numerous iron-dependent enzymes and can account for >25% of acetate produced in the gut [52]. It is therefore plausible to speculate a lack of conversion of  $H_2$  and  $CO_2$  to acetate under iron-limiting conditions, resulting in an overall decrease in acetate levels. This observation also correlated with the viable counts and 16S rRNA gene analysis, where a decrease in the members of *Bifidobacterium*, a prominent bacterial group which produces acetate, was observed under iron-limiting conditions. Although correlations of metabolite levels with the relative

abundance of bacterial taxa do not provide a causal relationship, it may still provide some indications as to which taxa are responsible for any observed differences.

In conclusion, this *in vitro* study shows that the chelation of iron, achieved with BPDS, resulted in a decrease of the main intestinal SCFAs, a lower relative abundance of potentially pathogenic bacteria within the gut microbial community. This observation was also confirmed in pure culture studies with pathogenic bacteria. Taken together, these data suggest that iron could provide an advantageous niche for potentially pathogenic bacteria and highlights the importance of tight control of iron availability to avoid a pathogenic profile within the colon. The relationship between iron availability and the human gut microbiome is yet to be fully elucidated, and more extensive research on this topic will aid in expanding our knowledge and understanding of this relationship, and in extension, help define improved methods for maintaining gut microbial homeostasis.

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**Declarations**

All authors read and approved the final manuscript. The authors declare that there are no conflicts of interest.

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## Figure legends

### **Figure 1– The effect of iron chelation with BPDS on pure bacterial cultures**

A strain of *E. coli* isolated from human gut (1A), *S. Typhimurium* (1B), *B. thetaiotaomicron* (1C) *B. longum* (1D) and *L. rhamnosus* (1E) were cultured anaerobically overnight in selective rich media. Cultures were supplemented with BPDS, at 0 (control), 10, 20 and 50  $\mu\text{M}$ . Optical density measurements were taken every 10 minutes for a period of 24 h and growth curves were plotted for each strain. Data shown = mean and are representative of 3 independent experiments. The data was statistically analysed using, one-way ANOVA, comparing the different concentrations of BPDS to the control, over a period of 24 h; \*\* $p < 0.01$ , \*\*\*\* $p < 0.0001$  versus control (0  $\mu\text{M}$  BPDS).

### **Figure 2 – Microbial community profiles assessed by 16S rRNA gene analysis**

Microbial composition of both vessels (Control and BPDS). Proportions shown are of the most abundant genera ( $\geq 0.5\%$ , identification to genus level where possible) identified by sequencing of the V4 hypervariable regions of the 16S rRNA gene. Data shown is the average relative abundance from the 3 individual experiments from 3 different donors and analysed using the QIIME pipeline. SEM values can be found in Table 2.

### **Figure 3 – Bacterial diversity profiles of colonic fermentation samples**

(a)  $\alpha$ - diversity analysis of batch fermentation samples with (BPDS) and without iron chelator (No BPDS) using the Shannon index and (b)  $\beta$ - diversity analysis of batch fermentation samples portraying weighted analysis of samples with (BPDS) and without (control) iron chelator using the UniFrac metric and presented as a PCoA plot. Data shown is the average Eigenvalues from the 3 individual experiments from 3 different donors. Each colour represents a different donor. Analysis was performed using QIIME (V1.9) and visualised using the XLSTAT add-on package in Microsoft Excel.

### **Figure 4 – Metabolite concentrations of batch fermentation samples**

Short chain fatty acid concentrations in both vessels were measured using  $^1\text{H-NMR}$  spectroscopy, with samples being screened for multiple metabolites against a spiked standard (TSP) and a validated reference library. Data shown is the average  $\pm$ SEM metabolite concentrations from the 3 individual experiments from 3 different donors.



**Table 1 – The effect of iron chelation via BPDS on gut-derived bacterial communities**

Two vessels were set up containing nutritive media and fecal inocula; one vessel was supplemented with BPDS (70  $\mu$ M BPDS) whilst the other was used as a control (0  $\mu$ M BPDS). Samples were taken from each vessel at 0, 8, and 24 h, and then enumerated using selective agar plates. Data shown = mean of 3 individual experiments  $\pm$  SEM. The data was statistically analysed using unpaired t-test, assuming unequal variance; \* $p$ <0.05, \*\* $p$ <0.01, \*\*\* $p$ <0.001 and \*\*\*\* $p$ <0.0001 for Control versus viable counts at the relevant time-point.

Bacterial Group	Viable Counts % ("Control" normalised to 100%, $\pm$ SEM)			
	T8		T24	
	Control	(+)BPDS	Control	(+)BPDS
Total Anaerobes	100	27.83 $\pm$ 9.47 *	100	29.30 $\pm$ 7.46 *
Bacteroides	100	68.71 $\pm$ 31.61	100	29.70 $\pm$ 18.47
Bifidobacteria	100	5.38 $\pm$ 1.05 ***	100	110 $\pm$ 106.30
Clostridia	100	63.58 $\pm$ 25.84	100	432.89 $\pm$ 299.18
Lactobacilli	100	8.21 $\pm$ 5.10 **	100	35.47 $\pm$ 5.92 **
Enterobacteriaceae	100	19.01 $\pm$ 11.19 *	100	17.65 $\pm$ 15.04 *

Table 2 – Proportions of different bacterial taxa (SEM) illustrated in 16S rRNA gene analysis

Taxa	SEM				
	T0	T8 Control	T8 BPDS	T24 Control	T24 BPDS
g_Bifidobacterium	3.54	6.91	2.09	13.43	6.47
g_Collinsella	0.66	3.55	1.32	3.49	0.71
g_Bacteroides	3.17	3.33	10.06	0.80	2.90
g_Prevotella	5.45	3.07	2.33	0.13	0.30
f_Rikenellaceae	0.41	0.06	0.32	0.19	0.12
g_Enterococcus	0.03	0.03	0.03	0.02	1.25
f_Leuconostocaceae	0.60	0.03	1.97	0.03	3.92
g_Streptococcus	4.45	0.35	0.19	0.30	15.70
g_Turicibacter	0.32	0.19	0.15	0.00	0.03
o_Clostridiales	0.52	0.15	1.83	0.12	0.06
g_Clostridium	1.38	0.06	0.22	0.06	0.09
f_Clostridiaceae;g_SMB53	1.56	0.10	0.47	0.17	0.07
f_Lachnospiraceae	0.30	0.04	0.18	0.29	0.08
f_Lachnospiraceae	0.54	0.44	1.96	0.55	0.17
g_Blautia	1.25	2.10	4.19	2.77	0.55
g_Coprococcus	1.39	2.78	2.17	3.42	0.17
g_Dorea	0.15	0.64	0.55	0.42	0.28
g_Lachnospira	0.20	0.00	0.45	0.00	0.00
g_Roseburia	0.82	0.17	0.85	0.13	0.00
g_[Ruminococcus]	1.10	1.84	2.66	1.91	1.39
f_Ruminococcaceae	0.78	0.00	0.70	0.03	0.00
g_Faecalibacterium	1.66	1.37	5.58	0.35	0.12
g_Oscillospira	1.69	0.19	0.67	0.44	0.22
g_Ruminococcus	2.32	0.25	1.75	0.15	0.45
g_Phascolarctobacterium	0.88	1.15	1.31	0.44	0.21
g_Veillonella	0.12	0.00	0.03	0.55	0.10
f_Erysipelotrichaceae	0.34	1.30	0.29	1.23	0.07
g_Sutterella	0.47	0.93	1.14	0.40	0.73
f_Enterobacteriaceae	0.37	28.13	25.50	25.70	23.90
g_Escherichia	16.08	5.86	0.23	2.61	1.72
g_Klebsiella	0.03	1.36	0.90	1.06	1.17
g_Akkermansia	7.95	0.22	0.39	0.15	0.21

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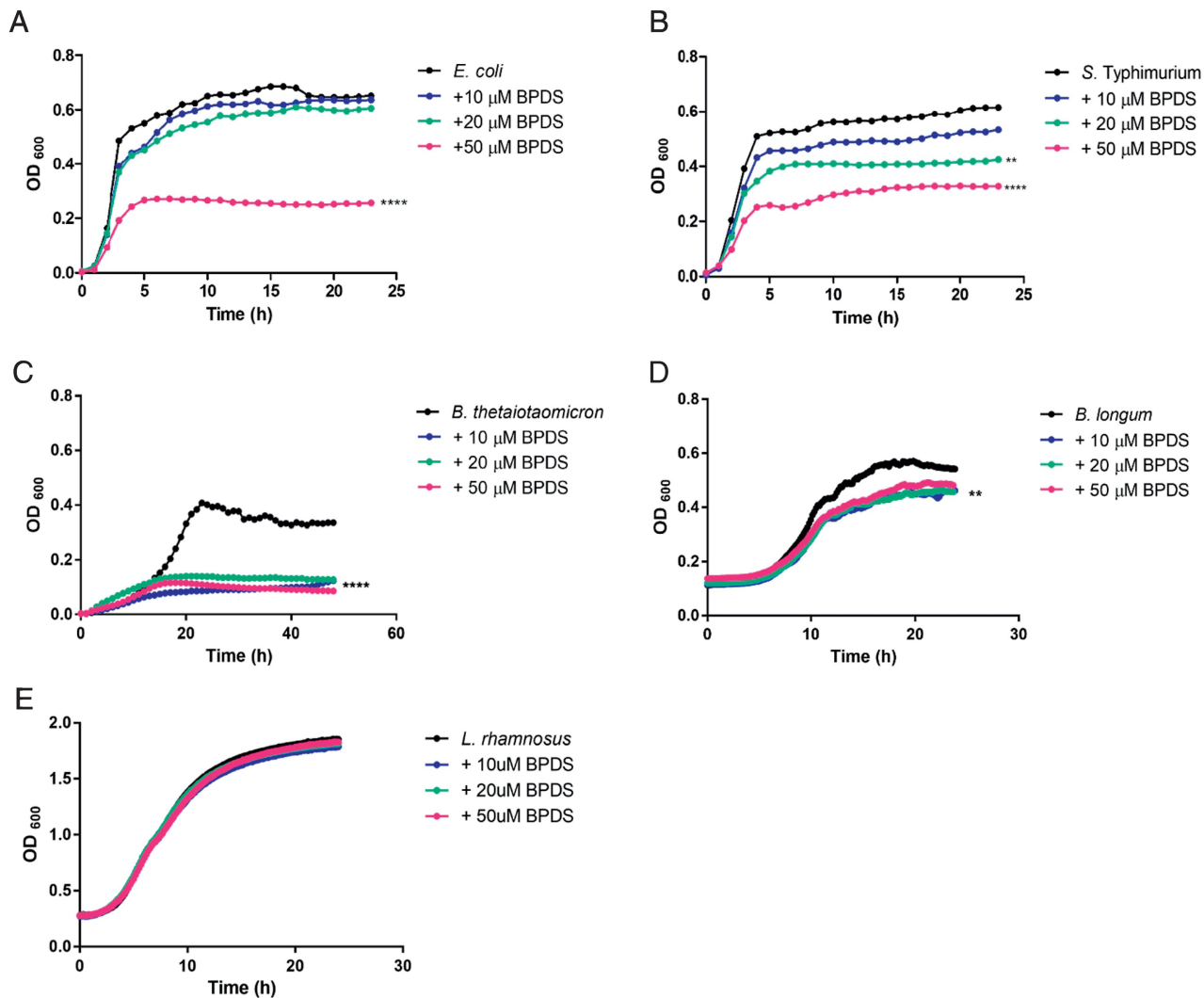


Figure 1

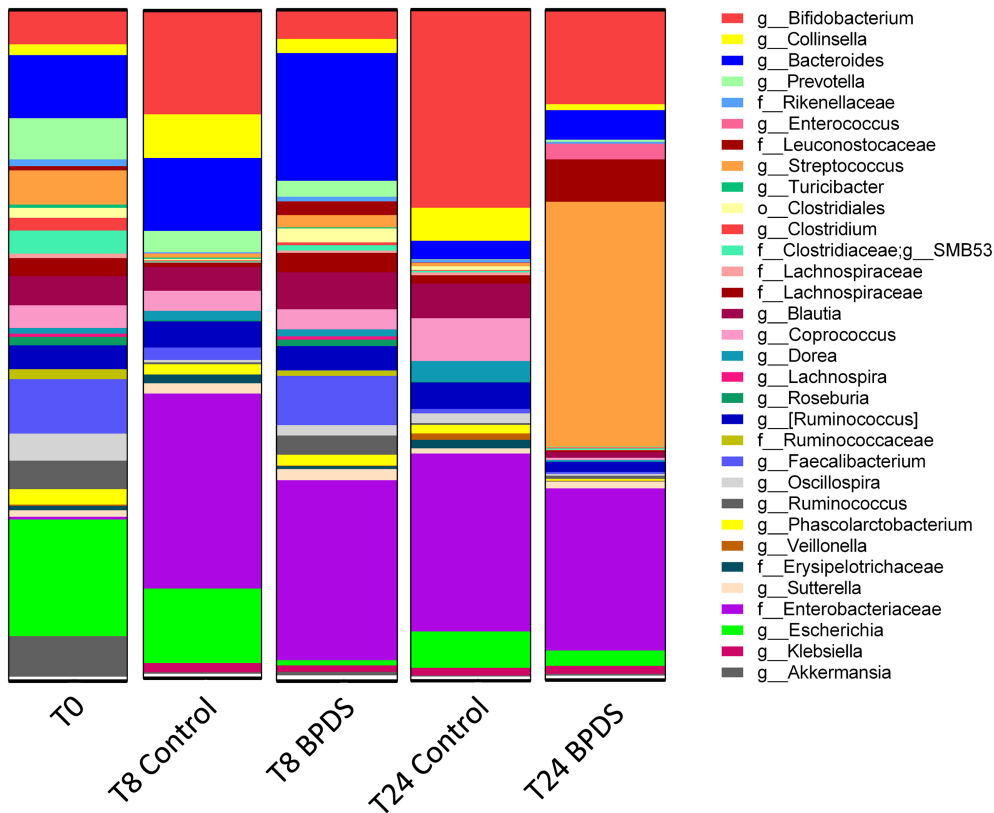


Figure 2

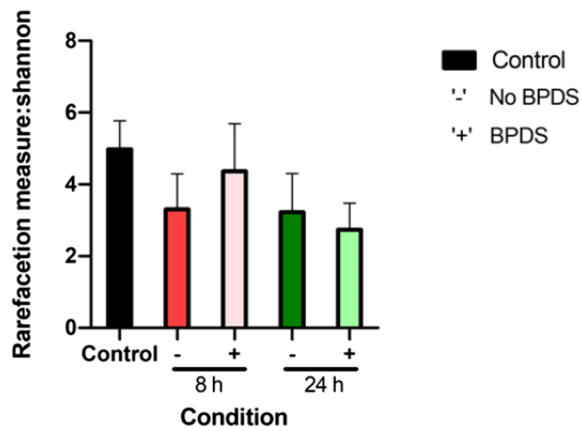
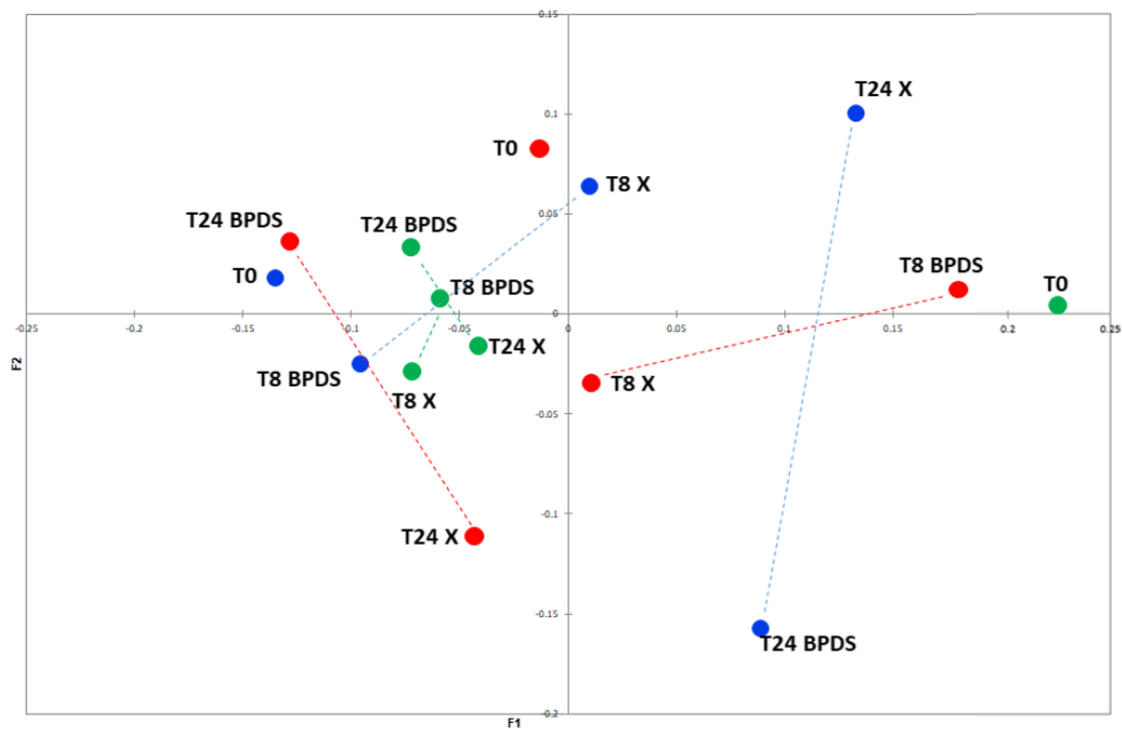
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Figure 3



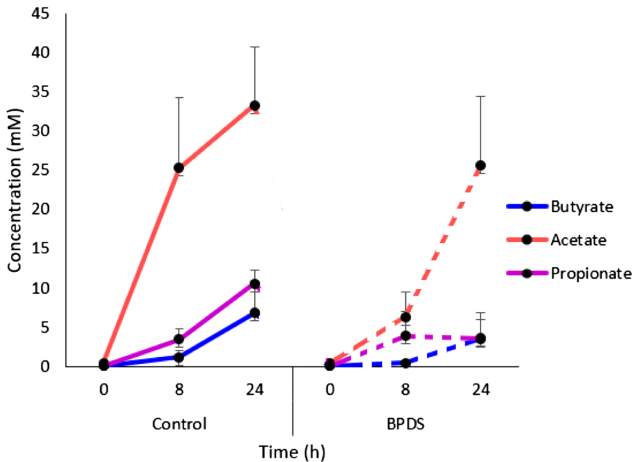


Figure 4