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Low iron availability in continuous *in vitro* colonic fermentations induces strong dysbiosis of the child gut microbial consortium and a decrease of main metabolites

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Abstract

Iron (Fe) deficiency affects an estimated 2 billion people worldwide and Fe supplements are a common corrective strategy. The impact of Fe deficiency and Fe supplementation on the complex microbial community of the child gut was studied using *in vitro* colonic fermentation models inoculated with immobilized fecal microbiota. Chyme media (all Fe chelated by 2,2'-dipyridyl to 26.5 mg Fe L⁻¹) mimicking Fe deficiency and supplementation were continuously fermented. Fermentation effluent samples were analyzed daily on the microbial composition and metabolites by qPCR, 16S rRNA gene 454-pyrosequencing and HPLC. Low Fe conditions (1.56 mg Fe L⁻¹) significantly decreased acetate concentrations and subsequent Fe supplementation (26.5 mg Fe L⁻¹) restored acetate production. High Fe following normal Fe conditions had no impact on the gut microbiota composition and metabolic activity. During very low Fe conditions (0.9 mg Fe L⁻¹ or Fe chelated by 2,2'-dipyridyl), a decrease of *Roseburia* spp./*Eubacterium rectale*, *Clostridium* Cluster IV members and *Bacteroides* spp. was observed while *Lactobacillus* spp. and *Enterobacteriaceae* increased consistent with a decrease of butyrate (-84%) and propionate (-55%). The strong dysbiosis of the gut microbiota together with decrease of main gut microbiota metabolites observed with very low iron conditions could weaken the barrier effect of the microbiota and negatively impact gut health.

Keywords

Iron deficiency; gut microbiota; butyrate; fermentation model

Introduction

Fe deficiency is one of the most common global nutritional deficiencies with more than 2 billion people affected both in industrialized and developing countries (Zimmermann & Hurrell, 2007). Fe deficiency occurs when body Fe requirements are not met by dietary sources and can lead to anemia and other co-morbidities. Fe requirements are higher during growth and pregnancy and it is estimated that 48 % of children (aged 5 – 14 years) and 52 % of pregnant women are anemic in developing countries (WHO, 2002). Fe deficiency anemia

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increases risk for preterm birth and infant mortality (Zimmermann & Hurrell, 2007) and may impair psychomotor and mental development in children (Beard, 2003). Two corrective measures recommended by the World Health Organization are Fe fortification of foods and/or Fe supplementation. FeSO₄ is a highly soluble and bioavailable form of Fe that is widely used in Fe fortification and supplementation (Hilty, *et al.*, 2010). However, despite the high bioavailability of FeSO₄, typical fractional absorption in the duodenum is only 5-20%, resulting in a large fraction passing unabsorbed into the colon and being available for the gut microbiota (Zimmermann, *et al.*, 2010).

The interest in the mammalian gut microbiota and its implications for gut and host health has increased tremendously during the past decade. The complex bacterial ecosystem with a very high bacterial density provides the host with a barrier effect against the colonization with environmental bacteria, such as pathogens (Stecher & Hardt, 2008). Moreover, the anaerobic metabolism of the bacteria in the gut makes indigestible compounds such as fibers available for the host by producing various compounds, like the short chain fatty acids acetate, propionate and butyrate, which have beneficial effects on gut health. Particularly butyrate has been a focus of research because it can act as an energy source for colonocytes and influences a wide array of cellular functions resulting in anti-inflammatory and anti-carcinogenic effects as well as a reduction of oxidative stress (Hamer, *et al.*, 2008).

Dietary composition, such as fibers and micronutrient concentrations, can affect the gut microbiota composition and metabolic activity (Flint, *et al.*, 2007; De Vuyst & Leroy, 2011; Metzler-Zebeli, *et al.*, 2011). The micronutrient Fe is essential for most gut bacteria (Andrews, *et al.*, 2003) except lactobacilli, which are able to grow without Fe in a nucleotide rich medium (Elli, *et al.*, 2000), and thus Fe availability in the gut may impact the dynamics of the gut bacterial ecosystem. However, only very few studies have investigated the effect of Fe deficiency and Fe supplementation on the gut microbiota. Using culture methods, infants given an Fe fortified cow's milk preparation had lower isolation frequencies of bifidobacteria but higher counts of *Bacteroides* spp. and *E. coli* than children receiving an unfortified cow's milk preparation (Mevisen-Verhage, *et al.*, 1985; Mevisen-Verhage, *et al.*, 1985). Zimmermann *et al.* investigated with molecular methods the gut microbiota of school children Fe supplemented for 6 months in Côte d'Ivoire (Zimmermann, *et al.*, 2010). They found lower amounts of lactobacilli and higher concentrations of *Enterobacteriaceae* in fecal samples of children receiving Fe supplemented biscuits compared to a control group receiving non-supplemented biscuits. In contrast, Fe deficiency in young women in India was associated with low levels of lactobacilli belonging to the *Lactobacillus acidophilus*-group (Balamurugan, *et al.*, 2010). In a systematic review, Fe supplementation in children was associated with a slight but significant increase risk for diarrhea (Gera & Sachdev, 2002). Further, it has been reported that total anaerobes, *Enterococcus* spp. as well as lactobacilli were elevated in Fe deprived mice and that Fe supplementation generally perturbed the gut microbiota (Tompkins, *et al.*, 2001; Werner, *et al.*, 2011). We recently reported the impact of Fe deficiency and subsequent Fe supplementation on the gut microbiota composition and metabolic activity in young Sprague-Dawley rats (Dostal, *et al.*, 2012). Fe deficiency increased *Enterobacteriaceae* and *Lactobacillus* / *Leuconostoc* / *Pediococcus* spp., but decreased *Bacteroides* spp. and *Roseburia* spp. / *E. rectale* members. Along with the bacterial composition changes, the gut microbiota metabolites propionate and butyrate were significantly decreased during Fe deficiency. Fe supplementation with FeSO₄ and electrolytic Fe partially reestablished the original gut microbiota composition, and led to a full recovery of metabolic activity in the rats.

In vivo studies have reported controversial results regarding the impact of Fe on specific bacterial groups of the gut microbiota. This may be at least in part due to the complex interactions between the Fe concentration in the gut lumen, the Fe status of the host and the

host-response to differing dietary Fe levels. Moreover, confounding factors such as dietary habits, environmental changes and host physiology can also impact the gut microbiota. The use of *in vitro* gut fermentation models allows investigation of the gut microbiota without effects of the host and other environmental factors via highly controlled parameters (Payne *et al.*, 2012a). The *in vitro* continuous colonic fermentation model developed by Cinquin *et al.* using immobilized child gut microbiota represents a good technological platform to investigate the impact of dietary changes on the gut microbiota (Cinquin, *et al.*, 2004; Cinquin, *et al.*, 2006; Le Blay, *et al.*, 2009; Zihler, *et al.*, 2010; Payne, *et al.*, 2012b). This fermentation model provides a high cell density, biodiversity and long-term stability due to the immobilization of the gut microbiota in gel beads reproducing the free cell and sessile bacterial populations in the colon (Payne, *et al.*, 2012a).

Therefore, the aim of this study was to elucidate the effect of Fe deficiency and dietary Fe supplementation on the child gut microbiota composition and metabolic activity using *in vitro* continuous colonic fermentation models inoculated with immobilized fecal microbiota.

Materials and Methods

Experimental set up

Three different continuous colonic *in vitro* fermentations inoculated with immobilized child gut microbiota using either single-stage reactors or a novel split-single-stage model were carried out to test the impact of different Fe levels, occurring during Fe deficiency and Fe supplementation, on the gut microbiota (**Fig. 1**, A and B). All three fermentations were aimed to mimic the conditions prevalent in the child proximal colon (Cinquin, *et al.*, 2006; Le Blay, *et al.*, 2009; Zihler, *et al.*, 2010; Haug, *et al.*, 2011; Payne, *et al.*, 2012b).

Fermentation 1 was carried out for a total of 70 days, with two single-stage reactors inoculated with immobilized gut microbiota from the same child and run in parallel. Reactors were continuously fed a nutritive medium differing only in Fe concentration to mimic a standard chyme medium, Fe deficiency and Fe supplementation (**Fig. 1A**). Moreover, infection with immobilized *Salmonella enterica* ssp *enterica* serovar Typhimurium N-15 (Le Blay, *et al.*, 2009) was performed during “High Fe” and “Normal Fe” fermentation conditions (period 5, Fermentation 1, reactor 1 and 2) to test the establishment and growth efficiency of the pathogen according to the Fe content of the chyme medium during the last 3 fermentation periods (period 5, 6, 7, Fermentation 1, reactor 1 and 2).

During two other fermentation experiments, Fermentation 2 and 3, a different reactor set up was chosen to mimic the proximal colon of a child (**Fig. 1, B**). A split-single-stage continuous fermentation system with 3 reactors was used: a first reactor inoculated with immobilized gut microbiota was used to continuously inoculate two reactors (control reactor and Fe deficient reactor) operated in parallel and under the conditions of the proximal colon. Fresh “Normal Fe” medium was continuously added to the first reactor with the immobilized gut microbiota and effluent from this reactor containing free bacteria was continuously transferred to the control reactor and Fe deficient reactor, where further medium fermentation by the free bacteria takes place. This fermentation set up allowed the comparison of different fermentation conditions on the exact same gut microbiota. Fermentation 2 and 3 were used to confirm the effects of strong Fe deficiency by continuously adding the high-affinity Fe chelator 2,2'-dipyridyl to the Fe deficient reactor. The control reactor operated with “Normal Fe” medium was used as an indicator for stability and control.

Bacterial immobilization

Fecal samples from three healthy, 6 – 10 year-old children, who had not received antibiotics in the previous 3 months, were collected and maintained in anaerobiosis until bacterial immobilization in gellan-xanthan beads as previously described (Zihler, *et al.*, 2010). Child 1 was used as fecal microbiota donor for Fermentation 1 and child 2 and 3 for Fermentation 2 and 3, respectively. Fecal microbiota was immobilized under anaerobic conditions in 1-2 mm gel beads composed of gellan (2.5%, w/v), xanthan (0.25% w/v) and sodium citrate (0.2%, w/v). Gel beads (60 mL) were immediately transferred to a fermentation reactor (Sixfors, Infors, Bottmingen, Switzerland) containing 140 mL of nutritive medium. This immobilization process was done for each fermentation experiment with a different child donor.

S. Typhimurium N-15 was immobilized as described by Zihler et al in gellan-xanthan beads. After overnight bead cultivation in tryptone soya broth, 2 g of *S. Typhimurium* N-15 beads were added to each reactor of fermentation 1 to mimic infection with a pathogen (Zihler, *et al.*, 2010).

Nutritive medium design

The chyme medium composition was based on the medium design by Macfarlane et al (Macfarlane, *et al.*, 1998) and adapted to mimic the ileal chyme of a child as previously described (Le Blay, *et al.*, 2009). The bile salt concentration was reduced to 0.05 g L⁻¹ and 0.5 mL L⁻¹ vitamin solution (Michel, *et al.*, 1998) was added after autoclaving. The Fe concentration of the medium was controlled to mimic daily Fe reaching the colon of a child during Fe deficiency and Fe supplementation (Fig. 1A). The iron concentration of “Normal Fe” medium containing 5.0 mg L⁻¹ FeSO₄*7H₂O and 50 mg L⁻¹ hemin (Sigma-Aldrich, Buchs, Switzerland) was 8.13 ± 1.8 mg Fe L⁻¹ which approximates the recommended daily Fe intake of 6.3 - 8.9 mg for a 6 – 10 year-old child (WHO, 2002). For Fermentation 1, “Low Fe” medium was formulated with 2.1 mg L⁻¹ FeSO₄*7H₂O and 2.3 mg L⁻¹ hemin and contained 3.91 ± 0.1 mg Fe L⁻¹. The “No Fe” medium contained 1.56 ± 0.1 mg Fe L⁻¹ and no FeSO₄*7H₂O and 0.1 mg L⁻¹ hemin were used to mimic Fe deficiency. Finally, media with very low Fe concentrations were prepared by either treating the “No Fe” medium with the Fe and divalent ion chelator Chelex® 100 (sodium form, Sigma-Aldrich) or by adding the Fe chelator 2,2'-dipyridyl (150 µM or 300 µM for Fermentation 1 or Fermentation 2 and 3, respectively; Sigma-Aldrich). For the Chelex® treated medium, 12.5 g Chelex® 100 were first added to the “No Fe” medium prepared without salts, stirred at 4°C over night, then decanted to remove the Chelex® and finally salts were added (KH₂PO₄, NaHCO₃, NaCl, KCl, MgSO₄, CaCl₂, MnCl₂). This procedure decreased the Fe concentration in the medium to 0.9 ± 0.2 mg Fe L⁻¹. “High Fe” medium contained 26.5 ± 2.2 mg Fe L⁻¹ (100 mg L⁻¹ FeSO₄*7H₂O and 50 mg L⁻¹ hemin) which approximates the daily 30.4 mg Fe reaching the colon (20% absorption in duodenum) of a 19 kg child treated with the recommended daily Fe supplementation of 2 mg Fe kg⁻¹ body weight (CDC, 1998; WHO, 2002). All Fe concentrations of the fermentation medium were measured by atomic absorption spectroscopy (AAS; SpectrAA-240K with GTA-120 Graphite Tube Atomizer Varian Techtron).

Fermentation procedures and sampling

The fermentation was carried out under the conditions of the proximal colon according to previously described procedures (Le Blay, *et al.*, 2009). Fecal beads were first colonized by batch fermentation for 72 hours, during which medium replacement was performed every 12 hours. During the entire fermentation process, pH was controlled and maintained at 5.7 by the addition of 5M NaOH and temperature was kept at 37°C. Anaerobiosis was generated by continuously flushing the headspace of all reactors and medium vessels with CO₂.

In the single-stage Fermentation 1, the working volume of reactor 1 and 2 (Sixfors, Infors, Bottmingen, Switzerland) was set at 200 mL with a continuous inflow of 40 mL h⁻¹ fresh medium resulting in a mean retention time of 5 h and a total medium inflow of 960 mL within 24 h. Different Fe media were fed for 10 days each during 7 experimental periods resulting in 70 days of continuous fermentation (Fig. 1, A). At the beginning of fermentation period 5, 2 g of *S. Typhimurium* N-15 beads (10⁹ cfu g⁻¹) were added aseptically to each reactor to induce *Salmonella* infection (Zihler, *et al.*, 2010).

In split-single-stage Fermentation 2 and 3, beads were first colonized for 72 hours by batch fermentation and then the inoculum reactor was operated in continuous mode for 6 days as described above (Fig. 1, B). The working volume was set at 200 mL but with a high feed flow rate of 80 mL h⁻¹ fresh medium giving a short mean retention time of 2.5 h. Control and test reactors (300 mL total volume) were connected in parallel to the inoculum reactor, whereas each reactor was continuously fed with 40 mL h⁻¹ effluent from the inoculum reactor giving a mean retention time of 7.5 h and an overall mean retention time for the split-single stage system of 10 h. The equipment limitations of the split-single-stage fermentation model lead to a 2 fold longer mean retention time than in Fermentation 1, which is within reported retention times of the child proximal colon of 7.52 ± 5.75 h (Gutierrez, *et al.*, 2002). Fe deficient conditions were generated in the test reactor by continuously adding (1.8 mL h⁻¹) 6.6 mmol L⁻¹ 2,2'-dipyridyl solution using a membrane pump (Stepdos 03S, KNF-flodos, Sursee, Switzerland).

During all three fermentations, daily sampling of all reactors was performed and samples were either frozen at -80°C for quantitative PCR analysis (qPCR) and pyrosequencing or processed immediately for HPLC analysis. Fresh effluents were serially diluted 10-fold with peptone water (0.1 %) and plated on selective CHROMAgar plates (Becton Dickinson, Allschwil, Switzerland) in duplicate for daily *S. Typhimurium* N-15 counts as described previously (Zihler, *et al.*, 2010).

Genomic DNA extraction and gut microbiota composition analysis

Total genomic DNA was extracted from 1.5 mL effluent by using the FastDNA SPIN kit for soil (MP Biomedicals, Illkirch, France). Specific primers (Table 1) were used to enumerate bacterial groups or species prevalent in the gut microbiota by qPCR. qPCR was performed with an ABI PRISM 7500-PCR sequence detection system (Applied Biosystems, Zug, Switzerland) and by using a 2x SYBR Green PCR Master Mix (Applied Biosystems) in a 25 µL volume as previously described (Zihler, *et al.*, 2010). Standard curves and duplicate sample analysis were performed in each run. Standards were generated by amplifying the 16S rRNA gene of a representative bacterial strain of each target group (Table 1). PCR amplicons of the 16S rRNA gene for standards were purified and DNA concentrations were measured on a Nanodrop® ND-1000 Spectrophotometer (Witec AG, Littau, Switzerland) to calculated copy numbers µL⁻¹.

Pyrosequencing analysis

Effluent samples of the last 3 days of each fermentation period were pooled for each reactor (total of 14 samples) and genomic DNA was extracted with the FastDNA SPIN kit for soil (MP Biomedicals, Illkirch, France). The extracted DNA was sent for pyrosequencing analysis and later taxonomic assignment of 16S rRNA gene reads to DNA Vision (Gosselies, Belgium) where the following procedures were performed. V5-V6 hypervariable regions of the 16S rRNA gene were amplified with the primers 784F and 1061R (Andersson, *et al.*, 2008) while the forward primer contained the Titanium A adaptor and the reverse primer contained the Titanium B adaptor and a barcode sequence. PCR reactions were carried out in a total volume of 100 µL using KAPA HiFi Hotstart polymerase (Kapa Biosystems, Woburn,

MA), 300 nmol L⁻¹ of each primer (Eurogentec, Seraing, Belgium) and 60 ng DNA. Amplicons were visualized on a 1% agarose gel cleaned using the Wizard SV Gel and PCR Clean-up System (Promega, Madison, WI).

The amplicons were combined in equimolar ratios into a single tube after the DNA concentration of each amplicon was determined using the Quant-iT PicoGreen dsDNA reagent and kit (Life Technologies, Merelbeke, Belgium). Pyrosequencing was carried out using primer A on a 454 Life Sciences Genome Sequencer FLX instrument (Roche Applied Science, Vilvoorde, Belgium) following Titanium Chemistry.

The obtained sequences were assigned to samples according to sample specific barcodes. The pyrosequencing resulted in an average ($\pm$ SD) of 12712 ± 1894 sequences per sample and their quality was checked for the following criteria: a) match with barcode and primers (only one mismatch/deletion/insertion is allowed); b) length of at least 240 nucleotides (barcodes and primers excluded); c) no more than two undetermined bases (denoted by N). Each sequence passing the quality check was assigned at the family and genus level using the RDP classifier v 2.1 (<http://rdp.cme.msu.edu>) (Cole, *et al.*, 2005) with a confidence estimate cutoff at 80%.

Metabolites analysis

The concentrations of the short chain fatty acids (SCFA) acetate, propionate and butyrate, the branched-chain fatty acids isovalerate and isobutyrate as well as the intermediate products formate and lactate were determined in fermentation effluents by HPLC as described previously (Cleusix, *et al.*, 2008). Mean metabolite concentrations in effluent samples were calculated from duplicate analysis.

Statistical analysis

All statistical analysis were done using JMP 8.0 (SAS Institute Inc., Cary, NC). HPLC and qPCR data are expressed as means $\pm$ SD of the last 3 fermentation days of each fermentation period. qPCR data and cell counts were log₁₀-transformed. In Fermentation 1, comparisons of qPCR data and SCFA concentrations were done between two subsequent fermentation periods using the non-parametric Kruskal-Wallis test. In Fermentation 2 and 3, comparisons of SCFA concentrations were done between control and test reactors also with the non-parametric Kruskal-Wallis test. *P* values < 0.05 were considered significant.

Results

Microbiota analysis by qPCR

The microbial composition in effluents from reactor 1 and 2 of Fermentation 1 was evaluated by qPCR using primers specific for the 16S rRNA gene of bacterial groups (Table 2). For both reactors total 16S rRNA gene copy numbers remained stable over the entire fermentation and were independent of Fe concentrations in the feed medium demonstrating the high stability of the used *in vitro* fermentation system. The predominant bacterial populations during all fermentation periods in both reactors, except during period 7 of reactor 1 with very low Fe concentrations (2,2'-dipyridyl), were *Roseburia* spp / *E. rectale* followed by *Bacteroides* spp. Two different microbiota compositions developed in the two reactors (Table 2, "Normal Fe" reactor 1 and 2, Fermentation 1) probably due to slight changes in initial fermentation conditions, such as pH, inoculation duration and anaerobiosis, which can impact the bead colonization process.

During the first 6 fermentation periods of reactor 1 and 2 corresponding to different Fe concentrations in the feed medium, no major changes were observed in the 16S rRNA gene

copy numbers of *Bacteroides* spp., *Roseburia* spp. / *E. rectale*, *Enterobacteriaceae* and *Lactobacillus* / *Pediococcus* / *Leuconostoc* spp. “Low Fe” and “No Fe” fermentation conditions significantly decreased *F. prausnitzii* 16S rRNA gene copy numbers compared to previous “Normal Fe” and “Low Fe” fermentation periods (Table 2). The “High Fe” fermentation condition applied after “No Fe” fermentation condition significantly increased this species along with *Clostridium* Cluster IV (Table 2, reactor 1).

When the Fe chelator 2,2'-dipyridyl was added to the fermentation medium of reactor 1 to generate very low Fe conditions, a complete reorganization of the gut microbiota was observed. Whereas total 16S rRNA gene copy numbers mL⁻¹ effluent remained stable, *Bacteroides* spp, *Roseburia* spp / *E. rectale* and *Clostridium* Cluster IV 16S rRNA gene copy numbers decreased sharply. In contrast, 16S rRNA gene copy numbers of *Enterobacteriaceae*, *Lactobacillus* / *Pediococcus* / *Leuconostoc* spp. and *Bifidobacterium* spp. increased significantly under very low Fe conditions (2,2'-dipyridyl). The treatment of the fermentation medium with Chelex® to generate very low Fe conditions had similar effects on the gut microbiota composition (Table 2, reactor 2).

Microbiota analysis by pyrosequencing

The V5-V6 sequencing of the entire 16S rRNA gene pool, sampled during the last 3 days of each fermentation period in Fermentation 1, was performed by 454 FLX pyrosequencing (Figs 2 and 3; Supporting Information, Table S1 – S4). After quality check, the number of sequences per sample was reduced from 12712 ± 1894 to 9201 ± 2016 reads (Table S1-S4). The most abundant families in both reactors of Fermentation 1 during the first 6 fermentation periods were *Lachnospiraceae* (55.4% – 84.4%) followed by *Ruminococcaceae* (2.7% - 16.2%) and *Bacteroidaceae* (0.2% - 4.5%). Correlating with the sequence annotation on family level, *Roseburia* spp. and *Dorea* spp. (*Lachnospiraceae*), *Ruminococcus* spp. (*Ruminococcaceae*) and *Bacteroides* spp. (*Bacteroidaceae*) were the most annotated genera (Figs 2 and 3).

As already observed with qPCR analysis, during the first 6 fermentation periods in both reactors Fe availability did not impact *Bacteroidaceae* or *Lachnospiraceae* on family level. However, *Ruminococcaceae* were decreased from 5.47 % (“No Fe” period) to 2.22% during “High Fe” fermentation period. *Blautia* spp (*Lachnospiraceae*) were reduced approximately 50% during “No Fe” period compared to “Normal Fe” or “High Fe” periods.

Pyrosequencing analysis indicated a complete reorganization of the gut microbiota composition during fermentation periods in which Fe was chelated by 2,2'-dipyridyl, (reactor 1) or Chelex® (reactor 2). The addition of 2,2'-dipyridyl in reactor 1 lead to a strong decrease of the most abundant families *Lachnospiraceae* (*Roseburia* spp., *Dorea* spp., *Blautia* spp.) from 79.4 % (“No Fe”, period 6, reactor 1) to 4.5 % , *Bacteroidaceae* from 3.4% to 0.2% and *Ruminococcaceae* from 4.8% to 0.2% (Fig. 2A, SI Table S1). Simultaneously, a strong increase of previously subdominant families like *Bifidobacteriaceae*, *Lactobacillaceae*, *Enterobacteriaceae* and *Enterococcaceae* was observed. Moreover, the addition of 2,2-dipyridyl decreased the number of unclassified reads on family level (Fig. 2A) as well as on genus level (Fig. 2B, SI Table S3).

The treatment of the fermentation medium with Chelex® (reactor 2) also decreased the families *Bacteroidaceae* from 3.87% to 1.39% and *Ruminococcaceae* from 2.73% to 0.26% but had no impact on total *Lachnospiraceae* (Fig. 3A, SI Table S2). On the genus level however, a moderate decrease of the *Lachnospiraceae* members *Roseburia* spp. (6.10% to 3.98%) and *Dorea* spp. (2.98% to 1.35%) was observed compared to the previous “No Fe” period (Fig. 3B, SI Table S4). In addition, an increase of *Bifidobacteriaceae*, *Lactobacillaceae* and *Enterobacteriaceae* was observed.

Metabolite analysis

Short chain fatty acids, isoacids as well as lactate and formate were determined daily in fermentation effluents by HPLC and were used as markers of system stability (**Fig. 4, Table 3**). During all three fermentations inoculated with different microbiota, acetate was the main metabolite followed by either butyrate (Fermentation 1) or propionate (Fermentation 2 and 3).

Metabolites concentrations of the SCFA acetate, butyrate and propionate in reactor 1 are depicted in Fig. 4 for each day during Fermentation 1. Stability was usually reached 6 days after the switch to a medium with a different Fe concentration following a transition period. The “No Fe” periods in Fermentation 1 showed reproducible effects on the metabolic activity of the gut microbiota. Acetate concentrations decreased significantly in fermentation effluents under “No Fe” conditions (reactor 1: period 3, -12%; period 6, -30%; reactor 2: period 6, -18%) compared to previous “Normal Fe” or “High Fe” periods (Table 3, Fig. 4). However, butyrate concentrations remained stable and were unaffected by the switch to “No Fe” medium. A 1:1 ratio of acetate:butyrate was measured during “No Fe” periods while in “Normal Fe” periods this ratio was 2:1. Moreover, isobutyrate and isovalerate concentrations were decreased while formate accumulated in the fermentation effluents during “No Fe” periods.

“High Fe” fermentation conditions applied after “No Fe” period (reactor 1, Fermentation 1) restored the acetate concentration to $63.4 \pm 5.3 \text{ mmol L}^{-1}$ and significantly increased isobutyrate and isovalerate concentrations to concentrations measured during “Normal Fe” period (Table 3, Fig. 4). Butyrate production remained stable also during very high Fe concentrations.

The metabolic activity of the gut microbiota was strongly impacted during very low Fe conditions with 2,2'-dipyridyl. In reactor 1 of Fermentation 1 (period 7), butyrate (-84%) and propionate (-55%) production were significantly decreased while acetate concentrations strongly increased compared to the previous fermentation period (Table 3, Fig. 4). Moreover, intermediate products lactate and formate, which were not detected in the preceding period, were present at high concentrations during very low Fe availability, reaching $14.7 \pm 2.9 \text{ mmol L}^{-1}$ and $22.1 \pm 1.0 \text{ mmol L}^{-1}$, respectively.

During very low Fe fermentation conditions obtained by chelating Fe with Chelex® (Fermentation 1, reactor 2, period 7) similar but less pronounced effects on the gut microbiota metabolic activity were observed than with 2,2'-dipyridyl (Table 3). Butyrate concentrations were significantly reduced while lactate and formate accumulated in the effluent.

The effects of 2,2'-dipyridyl were confirmed during Fermentation 2 and 3 with different microbiotas (**Fig. 5, A and B**). Butyrate concentration was reduced significantly by 55% in the Fe deficient reactors (17.1 ± 2.6 and $14.8 \pm 3.0 \text{ mmol L}^{-1}$, for Fermentation 2 and 3, respectively) compared to the control reactors (38.2 ± 2.6 and $33.5 \pm 4.6 \text{ mmol L}^{-1}$). In contrast to Fermentation 1, no increase of acetate concentration was recorded in the test reactors. However, the ratios acetate:propionate:butyrate show a higher acetate portion in the Fe deficient reactors of Fermentation 2 and 3 (67:20:13; 59:29:12; respectively) compared to control reactors (60:19:21; 49:31:20; respectively).

Salmonella infection simulation

Growth of *S. Typhimurium* N-15 during “High Fe” (period 5) in reactor 1, Fermentation 1, was slower compared to “Normal Fe” (period 5) in reactor 2 resulting in a significantly lower *S. Typhimurium* N-15 count during the last 3 days in reactor 1 compared to reactor 2

($5.0 \pm 0.2 \log \text{ cfu mL}^{-1}$ and $6.2 \pm 0.5 \log \text{ cfu mL}^{-1}$, respectively). During the next “No Fe” periods (period 6), *S. Typhimurium* N-15 reached similar counts in reactor 1 and 2 ($7.0 \pm 0.2 \log \text{ cfu mL}^{-1}$ and $7.2 \pm 0.1 \log \text{ cfu mL}^{-1}$, respectively) (Table 2).

Discussion

Our results highlight the importance of iron for the gut microbiota composition and metabolic activity during *in vitro* colonic fermentation. Especially, Fe deficient conditions (“No Fe” fermentation conditions, 2,2'-dipyridyl and Chelex® treated medium) modulated the metabolites concentrations in the fermentation effluent and the gut microbiota composition.

During fermentation periods mimicking Fe deficiency a significant decrease of acetate was observed in all 3 fermentations. Acetate is produced by nearly all gut bacteria either by the regular glycolytic pathway via pyruvate (Macfarlane & Macfarlane, 2003) or by the reductive acetyl-CoA pathway which uses CO_2 and H_2 (Miller & Wolin, 1996; Leclerc, *et al.*, 1997). The latter pathway involves several Fe-dependent enzymes and can account for 35% of the total acetate (Rey, *et al.*, 2010). Therefore, Fe restricted conditions could inhibit the conversion of CO_2 and H_2 to acetate resulting in a decrease of acetate production. Moreover, bacteria possessing the reductive acetyl-CoA pathway often co-metabolize formate (Rey, *et al.*, 2010; Wolin & Miller, 1993). Indeed, during Fe deficient conditions an accumulation of formate was observed. The bacteria using the reductive acetyl-CoA pathway belong to many different genera, which may explain that no decrease in bacterial numbers was detected by qPCR primers targeting large bacterial groups. However, pyrosequencing analysis of the entire 16S rRNA gene pool revealed a decrease of the genus *Blautia* during very low Fe availability (2,2'-dipyridyl). Some species of this genus possess the reductive acetyl-CoA pathway (Liu, *et al.*, 2008; Rey, *et al.*, 2010). Moreover, during “No Fe” fermentation conditions ($1.56 \pm 0.1 \text{ mg Fe L}^{-1}$), isobutyrate and isovalerate concentrations in fermentation effluents were reduced suggesting a decrease in protein fermentation (Hoyle & Wallace, 2010).

On the other hand, bacterial composition was only marginally affected by “No Fe” fermentation conditions indicating that Fe levels of $1.56 \pm 0.1 \text{ mg Fe L}^{-1}$ mainly affect the metabolic activity of the gut microbiota. However, an increase of *Ruminococcus* spp. and a decrease of *F. prausnitzii* were observed in reactor 1 of Fermentation 1 during “No Fe” conditions, which can explain the stable *Clostridium* Cluster IV numbers.

When very low Fe conditions were generated by either adding 2,2'-dipyridyl or treating the fermentation medium with Chelex® ($0.9 \pm 0.2 \text{ mg Fe L}^{-1}$) a large perturbation of the gut microbiota bacterial composition as well as metabolism was observed. Butyrate was the most affected metabolite with a decrease of up to 84% in correlation with a strong decrease of 16S rRNA gene copy numbers of the butyrate producers *Roseburia* spp. / *E. rectale*. This data was confirmed by pyrosequencing indicating a lower abundance of *Roseburia* spp. during very low Fe fermentation conditions (2,2'-dipyridyl, Chelex®). Butyrate producing bacteria and butyrate production were strongly impacted by Fe deficiency most likely due to the need of Fe as a cofactor in hydrogenases and oxidoreductases present in the butyrate production pathway (Falony, *et al.*, 2009). Some butyrate producing bacteria such as *F. prausnitzii* can convert acetate to butyrate (Pryde, *et al.*, 2002) which could explain the accumulation of acetate when butyrate production was impaired. They can also produce lactate from pyruvate especially when the pyruvate – butyrate pathway is blocked due to the lack of Fe needed for the activity of hydrogenases and oxidoreductases (De Vuyst & Leroy, 2011) as observed in this study during very low Fe fermentation conditions (Table 3). Moreover, propionate concentrations were decreased in fermentation effluents along with a

decrease of the propionate producer *Bacteroides* spp. 16S rRNA gene copy numbers during very low Fe concentrations.

The strong decrease of butyrate producers, *Ruminococcus* spp. and *Bacteroides* spp. can open a niche for the growth of bacteria better adapted to low Fe environments. *Enterobacteriaceae* and *Lactobacillus* / *Leuconostoc* / *Pediococcus* spp. significantly increased during the last two fermentation periods (“No Fe” and 2,2'-dipyridyl or Chelex®) in reactor 1 and 2 during Fermentation 1 (Table 2, Fig. 2A and B, Fig. 3A and B). *Enterobacteriaceae* are very good Fe scavengers (Andrews, *et al.*, 2003) and lactobacilli do not require Fe for growth in nucleotide rich environments (Imbert & Blondeau, 1998; Elli, *et al.*, 2000) which gives both bacterial groups a growth advantage during Fe restricted conditions. Bifidobacteria are reported to bind Fe to their cell walls and membranes which may increase their survival during low Fe environmental conditions (Kot & Bezkorovainy, 1999). The clear growth advantage of bifidobacteria in a complex gut microbiota during very low Fe conditions is demonstrated by their high abundance (64.8 %) during the 2,2'-dipyridyl and Chelex® fermentation period.

Fe supplementation after low Fe conditions restored acetate, isobutyrate and isovalerate concentrations indicating again the dependence of the reductive acetyl-CoA pathway and protein fermentation pathways on Fe. Moreover, *Clostridium* Clust IV members, such as *F. prausnitzii*, were promoted due to Fe supplementation after Fe deficiency.

The findings of this *in vitro* fermentation studies are very consistent with the data of a recent rat study using an Fe depletion-repletion assay to investigate the impact of Fe on gut microbiota (Dostal, *et al.*, 2012). Similarly to our *in vitro* results, Fe deficiency in rats induced a strong decrease in butyrate and propionate production along with a decrease in butyrate and propionate producing bacteria. Fe supplementation also restored metabolic activity of the gut microbiota. An increase of lactobacilli during Fe deficiency was observed in this rat study similarly to the present study, which is in agreement with the mice study of Tompkins *et al.* (Tompkins, *et al.*, 2001). In contrast, a human study carried out in India observed a decrease of the *Lactobacillus acidophilus* group in Fe deficient women (Balamurugan, *et al.*, 2010), indicating that also other mechanisms such as bacterial population dynamics impact this bacterial group.

In Fe deficient rats (Dostal, *et al.*, 2012) and in this *in vitro* study, *Enterobacteriaceae* increased under low Fe conditions. In a nutritional trial in Côte d'Ivoire (Zimmermann, *et al.*, 2010), where children were given an Fe fortified diet over 6 months, and in a study with weanling pigs (Lee, *et al.*, 2008), Fe fortification increased *Enterobacteriaceae*. These contradictory results suggest that changes in *Enterobacteriaceae* numbers might not only be due to Fe concentration in the gut lumen but also react to host responses to Fe and other environmental factors. For example, in the Côte d'Ivoire study (Zimmermann, *et al.*, 2010) calprotectin, a marker for intestinal inflammation, was increased in Fe fortified children and mucosal inflammation can give *Enterobacteriaceae* a growth advantage (Winter, *et al.*, 2010). In *in vitro* fermentations, environmental and host factors are excluded. Thus the lack of host inflammation factors might also be the explanation for the slower growth performance of *S. Typhimurium* N-15 in “High Fe” conditions compared to “Normal Fe” conditions in this *in vitro* study. However, it needs to be considered that virulence factors were not investigated in the present *in vitro* fermentation study and although growth of *S. Typhimurium* N-15 was impaired due to high amounts of Fe, virulence might be promoted and further investigations are needed.

Overall, our data suggest that “No Fe” and very low Fe fermentation conditions could lead to negative impacts on gut health. Especially gut microbiota metabolites influence gut health

to a large extent. During Fe restricted fermentation conditions a significant decrease of the beneficial metabolites acetate, butyrate and propionate was observed. Acetate is mainly used as energy source in colonocytes (Hoyles & Wallace, 2010) and a recent study suggested that the protection from enteropathogenic infection by bifidobacteria is partially attributed to the production of acetate (Fukuda, *et al.*, 2011). The impact of butyrate on gut health has been studied extensively and has been attributed anti-inflammatory properties, anti-carcinogenic effects, regulatory functions in cell proliferation and butyrate can act as an energy source for intestinal cells (Hamer, *et al.*, 2008; Hamer, *et al.*, 2009; Luhrs, *et al.*, 2002; Louis & Flint, 2009). Propionate is involved in cholesterol- and lipid-lowering mechanisms (Delzenne & Williams, 2002). However, not all metabolites have beneficial effects on gut health. The accumulation of lactate in feces has been correlated with inflammatory bowel disease and ulcerative colitis (Hove, *et al.*, 1994; Vernia, *et al.*, 1988) and lactate concentrations increased during low Fe availability during Fermentation 1. Moreover, the strong decrease of dominant bacterial groups such as *Roseburia* spp. / *E. rectale*, *Clostridium* Cluster IV and *Bacteroides* spp. due to low Fe could open nutrient and growth niches for environmental bacteria.

In the present study we demonstrated that the gut microbiota composition as well as the metabolic activity is strongly impacted by Fe availability *in vitro*, and especially very low Fe fermentation conditions induced gut microbiota changes that might have negative effects on gut health. However, the underlying mechanisms of the importance of Fe for the gut microbiota need to be further investigated and elucidated.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1.

A: Continuous single stage fermentation reactor with immobilized gut microbiota used for Fermentation 1 and experimental setup of Fermentation 1 indicating the different medium Fe concentrations in order to mimic Fe deficiency and supplementation. *Immobilized *Salmonella* Typhimurium N-15 was added. **B:** Continuous split-single stage fermentation system used for Fermentations 2 and 3 with a control reactor and a Fe deficient reactor generated by adding continuously 2,2'-dipyridyl.

Fig. 2. Microbial composition in effluents of reactor 1 in Fermentation 1. Percentages of the most abundant families (**A**) and genera (**B**) identified by pyrosequencing of the V5-V6 hypervariable regions of the 16S rRNA gene.

Fig. 3. Microbial composition in effluents of reactor 2 in Fermentation 1. Percentages of the most abundant families (**A**) and genera (**B**) identified by pyrosequencing of the V5-V6 hypervariable regions of the 16S rRNA gene.

Fig. 4. Daily short chain fatty acid concentrations in effluents of reactor 1 during Fermentation 1 measured by HPLC; acetate (○), propionate (□) and butyrate (▲). Data points are means of duplicate analysis.

Fig. 5. Metabolites concentrations in effluents of the control reactor and Fe deficient reactor (addition of 2,2'-dipyridyl) during Fermentation 2 (A) and Fermentation 3 (B) measured by HPLC. Data points are means $\pm$ SD of the last 3 fermentation days. Columns with an asterisk (*) are significantly different from the control reactor within the same metabolite, $P < 0.05$.

Table 1

Primers used to enumerate specific bacterial groups by qPCR

Primer	Sequence 5'-3'	Target	Source
F8 1492R	AGAGTTTGATCMTGGCTC GNTACCTTGTACGACTT	16S rRNA gene for qPCR standard	(Mosoni, <i>et al.</i> , 2007)
Eub 338F Eub 518R	ACTCCTACGGGAGGCAGCAG ATTACCGCGGCTGCTGG	total bacteria	(Guo, <i>et al.</i> , 2008)
Bac303F Bfr-Femrev	GAAGGTCCCCCACAATTG CGCKACTGGCTGGTTCAG	<i>Bacteroides</i> spp.	(Ramirez-Farias, <i>et al.</i> , 2009)
F_Lacto 05 R_Lacto 04	AGC AGT AGG GAA TCT TCC A CGC CAC TGG TGT TCT TCC ATA TA	<i>Lactobacillus/Pedio-coccus/Leuconostoc</i> spp.	(Furet, <i>et al.</i> , 2009)
RrecF Rrec630mR	GCGGTRCGGCAAGTCTGA CCTCCGACACTCTAGTMCAG	<i>Roseburia</i> spp./ <i>E. rectale</i>	(Furet, <i>et al.</i> , 2009)
Clep866mF Clep1240mR	TTAACACAATAAGTWATCCACCTGG ACCTTCCTCCGTTTTGTCAAC	<i>Clostridium</i> Cluster IV	(Ramirez-Farias, <i>et al.</i> , 2009)
Fprau223F Fprau420R	GATGGCTCGCGTCCGATTAG CCGAAGACCTTCTTCCTCC	<i>Faecalibacterium prausnitzii</i>	(Bartosch, <i>et al.</i> , 2005)
xfp-fW xfp-rv	ATCTTCGGACCBGAYGAGAC CGATVACGTGVACGAAGGAC	<i>Bifidobacteria</i> phosphoketolase	(Cleusix, <i>et al.</i> , 2010)
Eco1457F Eco1652R	CATTGACGTTACCCGCAGAAGAAGC CTCTACGAGACTCAAGCTTGC	<i>Enterobacteriaceae</i>	(Bartosch, <i>et al.</i> , 2005)

Table 2

16S rDNA copy numbers (log10 copy numbers mL⁻¹ fermentation effluent) of specific bacterial groups determined by qPCR in reactor 1 and 2 of Fermentation 1 during different Fe availabilities in the feed medium.

Period	total 16S	<i>Bacteroides</i> spp	<i>Roseburia</i> spp / <i>E. rectale</i>	<i>Entero-bacteriaceae</i>	<i>Lactobacillus</i> / <i>Pedococcus</i> / <i>Leuconostoc</i> spp	<i>Bifidobacterium</i> spp	<i>F. prausnitzii</i>	<i>Clostridium</i> Cluster IV	<i>S. Typhimurium</i> N-15 ^a
Donor	10.2	9.6	8.8	3.9	5.4	9.1	9.5	8.9	n.d. ^{\$}
Reactor 1									
Normal Fe	10.5±0.1	8.7±0.3	9.9±0.0	6.4±0.6	6.7±0.4	8.6±0.1	5.8±0.1	7.7±0.5	n.d. ^{\$}
Low Fe	10.6±0.1	9.6±0.3 [*]	9.7±0.1 [*]	7.2±0.4	7.1±0.5	7.6±0.3 [*]	3.4±1.0 [*]	7.1±1.6	n.d.
No Fe	10.7±0.04	9.1±0.1 [*]	9.9±0.1 [*]	7.7±0.5	7.0±0.5	7.6±0.2	3.7±0.1	7.6±0.5	n.d.
High Fe	10.7±0.04	8.8±0.1 [*]	10.1±0.01 [*]	8.2±0.1	7.7±0.6	7.5±0.3	5.1±0.1 [*]	8.1±0.3 [*]	n.d.
High Fe	10.8±0.1	9.1±0.2	10.2±0.1	7.7±0.2 [*]	7.4±0.6	6.3±0.1 [*]	5.6±0.1 [*]	8.7±0.2 [*]	5.0±0.1
No Fe	10.5±0.02 [*]	9.7±0.1 [*]	10.4±0.04 [*]	8.8±0.1 [*]	8.5±0.3 [*]	7.4±0.1 [*]	5.5±0.1	8.8±0.1	7.0±0.2
2'2-Dip	10.8±0.1 [*]	8.3±0.1 [*]	8.4±0.1 [*]	9.2±0.2 [*]	9.0±0.1 [*]	9.2±0.1 [*]	5.3±0.1	4.9±0.3 [*]	8.0±0.1
Reactor 2									
Normal Fe	10.7±0.03	9.8±0.2	9.4±0.2	7.9±0.5	5.4±0.5	7.4±0.3	7.0±0.1	9.2±0.2	n.d.
High Fe	10.7±0.1	9.6±0.2	9.9±0.1 [*]	8.2±0.3	4.7±0.4	7.2±0.4	7.0±0.4	9.0±0.2	n.d.
High Fe	10.7±0.1	9.1±0.2 [*]	9.6±0.04 [*]	8.0±0.3	5.6±0.8	7.3±0.4	7.0±0.2	8.4±0.4	n.d.
Normal Fe	10.7±0.1	8.7±0.2	9.6±0.2	8.3±0.3	6.5±0.3 [*]	7.1±0.2	6.8±0.3	8.8±0.4	n.d.
Normal Fe	10.8±0.1	8.9±0.3	10.3±0.1 [*]	8.4±0.2	7.9±0.03 [*]	6.2±0.1 [*]	6.5±0.1	9.1±0.1	6.2±0.5
No Fe	10.7±0.1	9.6±0.3 [*]	10.5±0.2	9.4±0.2 [*]	7.8±0.4	7.6±0.4 [*]	5.9±0.1 [*]	8.6±0.4	7.2±0.1
Chelex	10.8±0.2	9.2±0.1 [*]	10.1±0.2 [*]	9.5±0.5	8.9±0.1 [*]	8.5±0.1 [*]	5.7±0.1 [*]	7.8±0.1	7.6±0.5

Data are means ± SD of the last three days of each fermentation period; samples were analyzed in duplicate. Means with an asterisk

^{*} differ significantly from the previous treatment period within the same bacterial group, $P<0.05$.

^a cfu mL⁻¹ effluent

δ not determined

\$watermark-text

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Table 3

Concentration of metabolites (mmol L⁻¹ effluent) measured by HPLC in effluent samples of treatment periods in reactor 1 and 2 of Fermentation 1.

	Acetate	Butyrate	Propionate	Iso-butyrate	Iso-valerate	Lactate	Formate
Reactor 1							
Normal Fe	69.6±4.4	44.2±3.0	8.2±1.0	7.3±1.0	5.0±0.5	n.d. ²	n.d.
Low Fe	56.4±2.0 *	44.5±1.3	11.1±0.3 *	3.4±2.1 *	3.6±0.3 *	n.d.	n.d.
No Fe	49.6±2.1 *	49.3±2.9 *	9.9±0.1 *	1.8±3.0	2.0±0.8 *	n.d.	9.7±0.8
High Fe	63.4±5.3 *	49.0±0.9	7.5±0.2 *	7.4±0.6 *	5.6±0.2 *	1.2±1.4	n.d.
High Fe	61.7±3.5	50.7±1.9	9.7±0.6 *	9.8±0.3 *	5.6±1.1	n.d.	n.d.
No Fe	43.1±2.4 *	48.1±2.8	8.9±1.1	4.6±0.7 *	1.3±0.1 *	n.d.	n.d.
2'2-Dip	72.4±4.9 *	7.5±2.3 *	4.0±0.7 *	6.1±0.8	n.d.	14.7±2.9	22.1±1.0
Reactor 2							
Normal Fe	96.1±28.2	40.4±11.2	16.6±4.1	10.7±3.2	5.9±1.5	n.d.	n.d.
High Fe	95.6±16.4	42.6±4.1	13.5±2.4	8.4±3.7	7.2±1.2	n.d.	n.d.
High Fe	88.1±2.3	39.3±3.9	11.7±0.5	6.9±5.1	6.4±0.3	n.d.	n.d.
Normal Fe	89.3±3.1	30.3±1.5 *	9.2±0.0 *	10.9±0.7	5.4±0.1 *	3.2±0.6	n.d.
Normal Fe	62.9±0.9 *	44.4±0.8 *	9.6±0.6	9.0±0.8 *	4.6±0.0 *	n.d.	n.d.
No Fe	51.5±2.5 *	42.4±1.4	7.5±0.2 *	6.6±0.4 *	2.9±0.2 *	n.d.	0.5±0.8
Chelex	43.1±1.1 *	25.6±1.6 *	5.6±1.7	2.0±1.8 *	0.5±0.2 *	2.5±1.1	1.0±1.8

Data are means±SD of the last three days of each fermentation period; samples were analyzed in duplicate. Means with an asterisk

* differ significantly from the previous treatment period within the same metabolite, $P<0.05$.

² not detected