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RESEARCH ARTICLE

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

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MICROBIOLOGY ECOLOGY

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 quantitative PCR, 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 in *Roseburia* spp./*Eubacterium rectale*, *Clostridium* Cluster IV members and *Bacteroides* spp. was observed, while *Lactobacillus* spp. and *Enterobacteriaceae* increased consistent with a decrease in butyrate (−84%) and propionate (−55%). The strong dysbiosis of the gut microbiota together with decrease in main gut microbiota metabolites observed with very low iron conditions could weaken the barrier effect of the microbiota and negatively impact gut health.

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 comorbidities. 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, 2001). Fe-deficiency anemia 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. $FeSO_4$ 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 $FeSO_4$, 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 (SCFA) 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-cancerogenic effects as well as a reduction in 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 *Escherichia coli* than children receiving an unfortified cow's milk preparation (Mevissen-Verhage *et al.*, 1985a, b). Zimmermann *et al.* (2010) investigated with molecular methods the gut microbiota of school children supplemented with Fe for 6 months in Côte d'Ivoire. They found lower amounts of lactobacilli and higher concentrations of *Enterobacteriaceae* in fecal samples of children receiving Fe-supplemented biscuits compared with a control group receiving nonsupplemented 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 increased 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./*Eubacterium rectale* members. Along with the bacterial composition changes, the gut microbiota metabolites propionate and butyrate were significantly decreased during Fe deficiency. Fe

supplementation with $FeSO_4$ and electrolytic Fe partially re-established 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 because of 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, 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 because of 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 setup

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. 1a 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
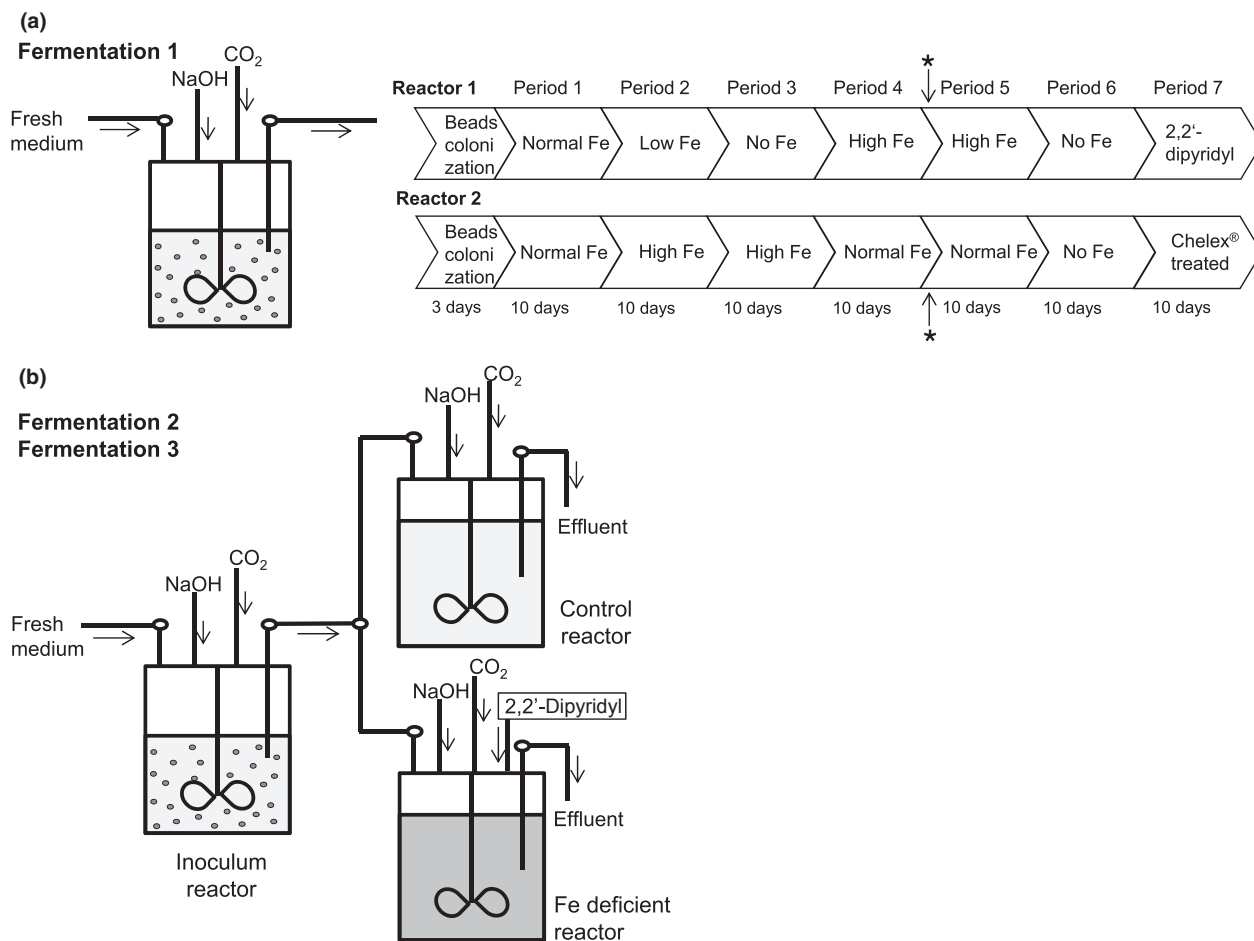
Fig. 1. (a) Continuous single-stage fermentation reactor with immobilized gut microbiota used for fermentation 1 and experimental setup of fermentation 1 indicating the different medium with Fe concentrations to mimic Fe deficiency and supplementation. *Immobilized *Salmonella* Typhimurium N-15 was added. (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.

(Le Blay *et al.*, 2009) was performed during 'High Fe' and 'Normal Fe' fermentation conditions (period 5, fermentation 1, reactors 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 three fermentation periods (period 5, 6, and 7, fermentation 1, reactors 1 and 2).

During two other fermentation experiments, fermentations 2 and 3, a different reactor setup was chosen to mimic the proximal colon of a child (Fig. 1b). 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 setup allowed the comparison of different fermentation conditions on the exact same gut microbiota. Fermentations 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-to-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 fermentations 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 carried out for each fermentation experiment with a different child donor.

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

Nutritive medium design

The chyme medium composition was based on the medium designed 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} $FeSO_4 \cdot 7H_2O$ 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, 2001). For fermentation 1, 'Low Fe' medium was formulated with 2.1 mg L^{-1} $FeSO_4 \cdot 7H_2O$ 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 $FeSO_4 \cdot 7H_2O$ 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 or 300 μM for fermentation 1 or fermentations 2 and 3, respectively; Sigma-Aldrich). For the Chelex®-treated medium, 12.5 g Chelex® 100 was 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 (KH_2PO_4, $NaHCO_3$, NaCl, KCl, $MgSO_4$, $CaCl_2$, $MnCl_2$). 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} $FeSO_4 \cdot 7H_2O$ 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, 2001). All Fe concentrations of the fermentation medium were measured by atomic absorption spectroscopy (SpectrAA-240K with GTA-120 Graphite Tube Atomizer Varion 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 h, during which medium replacement was performed every 12 h. During the entire fermentation process, pH was controlled and maintained at 5.7 by the addition of 5 M NaOH, and temperature was kept at 37 °C. Anaerobiosis was generated by continuously flushing the headspace of all reactors and medium vessels with CO_2.

In the single-stage fermentation 1, the working volume of reactors 1 and 2 (Sixfors; Infors) 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 seven experimental periods resulting in 70 days of continuous fermentation (Fig. 1a). At the beginning of fermentation period 5, 2 g of *S.* Typhimurium N-15 beads (10^9 CFU g^{-1}) was added aseptically to each reactor to induce *Salmonella* infection (Zihler *et al.*, 2010).

In split-single-stage fermentations 2 and 3, beads were first colonized for 72 h by batch fermentation, and then the inoculum reactor was operated in continuous mode for 6 days as described earlier (Fig. 1b). 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) 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 mM 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 CHROM-Agar 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 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 using a 2× 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 calculate copy numbers per μL.

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). The extracted DNA was sent for pyrosequencing analysis and later taxonomic assignment of 16S rRNA gene reads to DNAVision (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. PCRs were carried out in a total volume of 100 μL using KAPA HiFi Hotstart polymerase (Kapabiosystems, Woburn, MA), 300 nM 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 (± SD) of 12712 ± 1894 sequences per sample, and their quality was checked for the following criteria: (1) match with barcode and primers (only one mismatch/deletion/insertion is allowed); (2) length of at least 240 nucleotides (barcodes and primers excluded); and (3) no more than two undetermined bases

Table 1. Primers used to enumerate specific bacterial groups by qPCR

Primer	Sequence 5′–3′	Target	Source
F8	AGAGTTTGATCMTGGCTC	16S rRNA gene for qPCR standard	Mosoni *et al.* (2007)
1492R	GNTACCTTGTTACGACTT		
Eub 338F	ACTCCTACGGGAGGCAGCAG	Total bacteria	Guo *et al.* (2008)
Eub 518R	ATTACCGCGGCTGCTGG		
Bac303F	GAAGGTCCCCCACATTG	*Bacteroides* spp.	Ramirez-Farias *et al.* (2009)
Bfr-Femrev	CGCKACTTGGCTGGTTCAG		
F_Lacto 05	AGC AGT AGG GAA TCT TCC A	*Lactobacillus/Pediococcus/*	Furet *et al.* (2009)
R_Lacto 04	CGC CAC TGG TGT TCY TCC ATA TA	*Leuconostoc* spp.	
RrecF	GCGGTRCGGCAAGTCTGA	*Roseburia* spp./*E. rectale*	Furet *et al.* (2009)
Rrec630mR	CCTCCGACACTCTAGTMCGAC		
Clep866mF	TTAACACAATAAGTWATCCACCTGG	*Clostridium* Cluster IV	Ramirez-Farias *et al.* (2009)
Clep1240mR	ACCTTCCTCCGTTTTGTCAAC		
Fprau223F	GATGGCCTCGCGTCCGATTAG	*Faecalibacterium prausnitzii*	Bartosch *et al.* (2005)
Fprau420R	CCGAAGACCTTCTTCCTCC		
xfp-fw	ATCTTCGGACCBGAYGAGAC	*Bifidobacterium* phosphoketolase	Cleusix *et al.* (2010)
xfp-rv	CGATVACGTGVACGAAGGAC		
Eco1457F	CATTGACGTTACCCGCAGAAGAAGC	*Enterobacteriaceae*	Bartosch *et al.* (2005)
Eco1652R	CTCTACGAGACTCAAGCTTGC		

(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 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 performed using JMP 8.0 (SAS Institute Inc., Cary, NC). HPLC and qPCR data are expressed as means ± SD of the last three 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 made between two subsequent fermentation periods using the nonparametric Kruskal–Wallis test. In fermentations 2 and 3, comparisons of SCFA concentrations were made between control and test reactors also with the nonparametric Kruskal–Wallis test. *P* values < 0.05 were considered significant.

Results

Microbiota analysis by qPCR

The microbial composition in effluents from reactors 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' reactors 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 six fermentation periods of reactors 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 *Faecalibacterium prausnitzii* 16S rRNA gene copy numbers compared with 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 per mL 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, Tables S1–S4). After quality check, the number of sequences per sample was reduced from 12712 ± 1894 to 9201 ± 2016 reads (Tables S1–S4). The most abundant families in both reactors of fermentation 1 during the first six 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 six 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 with '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

Table 2. 16S rRNA gene copy numbers (log_{10} copy numbers mL^{-1} fermentation effluent) of specific bacterial groups determined by qPCR in reactors 1 and 2 of fermentation 1 during different Fe availabilities in the feed medium

Period		Total 16S rRNA gene	Bacteroides spp.	Roseburia spp./ E. rectale	Enterobacteriaceae	Lactobacillus/ Pediococcus/ Leuconostoc spp.	Bifidobacterium spp.	F. prausnitzii	Clostridium Cluster IV	S. Typhimurium N-15[†]
	Donor	10.2	9.6	8.8	3.9	5.4	9.1	9.5	8.9	n.d.
Reactor 1										
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.
2	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.
3	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.
4	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.
5	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
6	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
7	22-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										
1	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.
2	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.
3	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.
4	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.
5	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
6	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
7	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 3 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, $P < 0.05$.
n.d., not determined.
[†]$CFU mL^{-1}$ effluent.
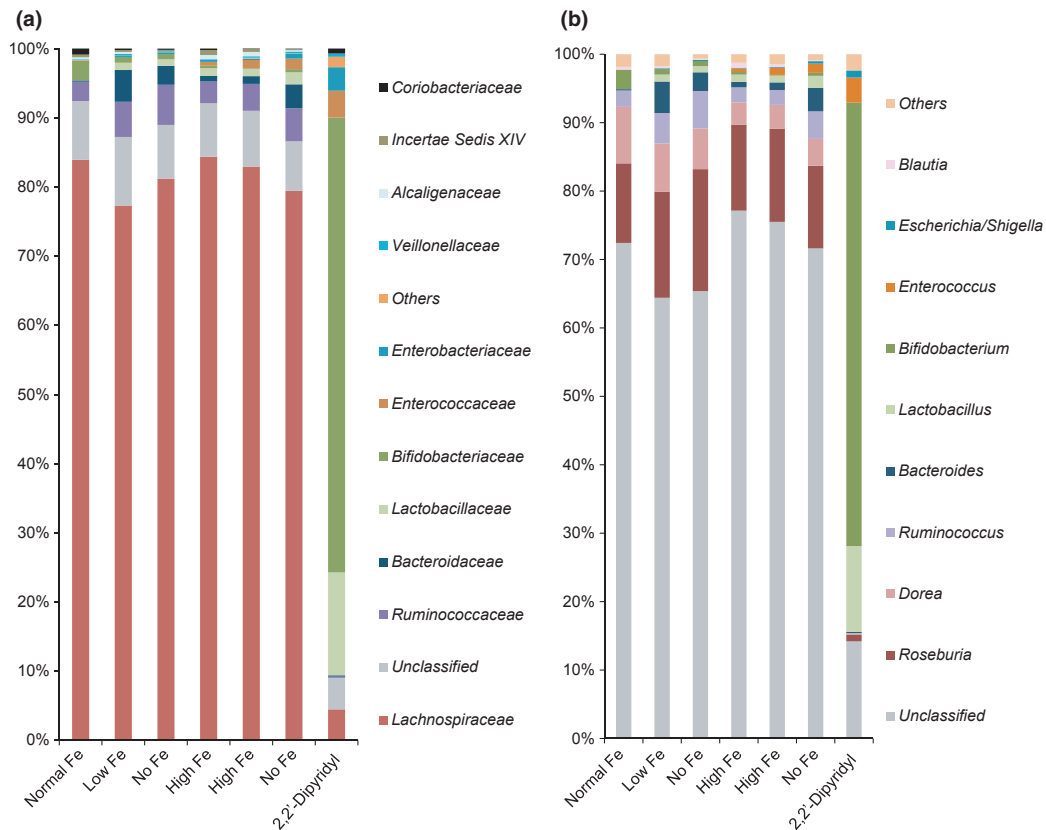
Fig. 2. Microbial composition in effluents of reactor 1 in fermentation 1. Percentages of the most abundant families (a) and genera (b) identified by pyrosequencing of the V5–V6 hypervariable regions of the 16S rRNA gene.

(reactor 1) or Chelex® (reactor 2). The addition of 2,2′-dipyridyl in reactor 1 lead to a strong decrease in the most abundant families *Lachnospiracea* (*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 *Ruminococcacae* from 4.8% to 0.2% (Fig. 2a, Table S1). Simultaneously, a strong increase in previously sub-dominant 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, 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, Table S2). On the genus level, however, a moderate decrease in the *Lachnospiraceae* members *Roseburia* spp. (6.10 to 3.98%) and *Dorea* spp. (2.98 to 1.35%) was observed compared with the previous 'No Fe' period (Fig. 3b, Table S4). In addition, an increase in *Bifidobacteriaceae*, *Lactobacillaceae*, and *Enterobacteriaceae* was observed.

Metabolite analysis

SCFA, 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 (fermentations 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 with 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
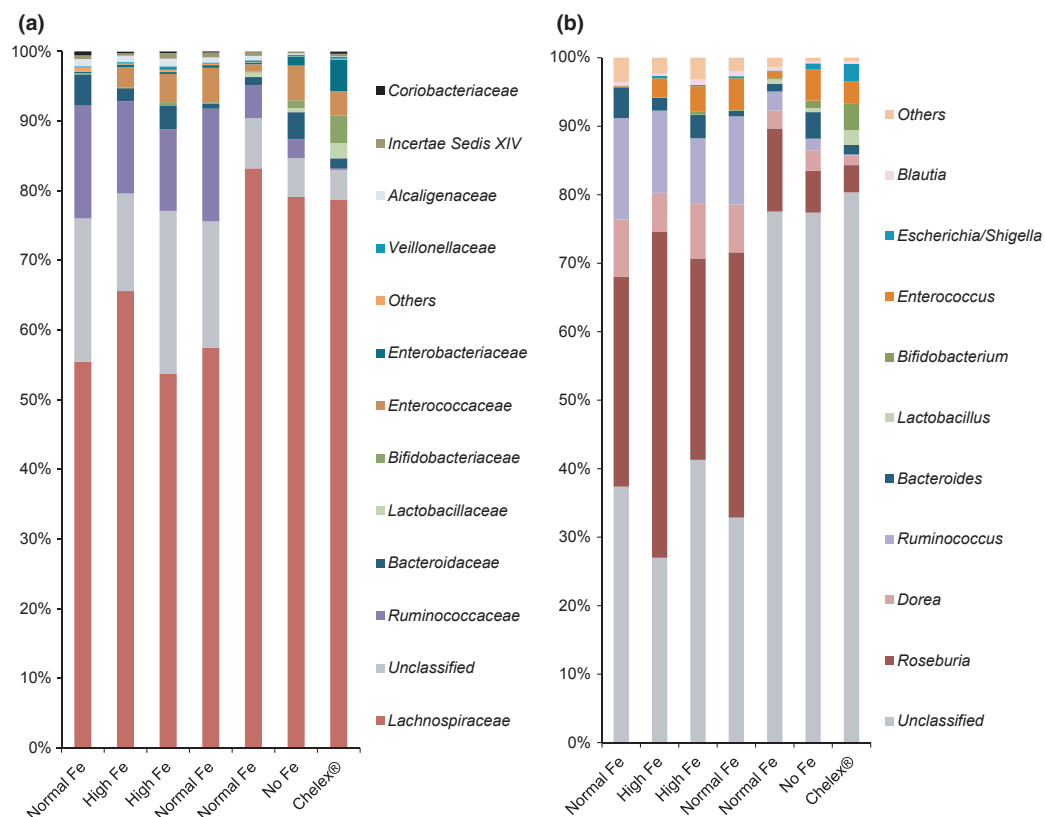
Fig. 3. Microbial composition in effluents of reactor 2 in fermentation 1. Percentages of the most abundant families (a) and genera (b) identified by pyrosequencing of the V5–V6 hypervariable regions of the 16S rRNA gene.
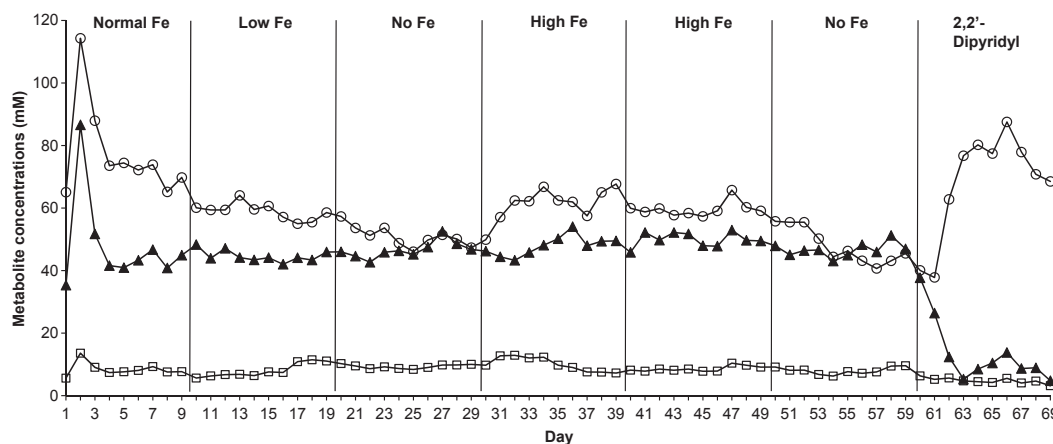
Fig. 4. Daily SCFA concentrations in effluents of reactor 1 during fermentation 1 measured by HPLC: acetate (○), propionate (□), and butyrate (▲). Data points are means of duplicate analysis.

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 ± 5.3 mM and significantly increased isobutyrate and isovalerate concentrations to

Table 3. Concentration of metabolites (mM) measured by HPLC in effluent samples of treatment periods in reactors 1 and 2 of fermentation 1

	Acetate	Butyrate	Propionate	Isobutyrate	Isovalerate	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.	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 3 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, $P < 0.05$.
n.d., not detected.

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 with 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 ± 2.9 and 22.1 ± 1.0 mM, 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 fermentations 2 and 3 with different microbiotas (Fig. 5a and b). Butyrate concentration was reduced significantly by 55% in the Fe-deficient reactors (17.1 ± 2.6 and 14.8 ± 3.0 mM, for fermentations 2 and 3, respectively) compared with the control reactors (38.2 ± 2.6 and 33.5 ± 4.6 mM). In contrast to fermentation 1, no increase in acetate concentration was recorded in the test reactors. However, the ratios of acetate/propionate/butyrate show a higher acetate portion in the Fe-deficient
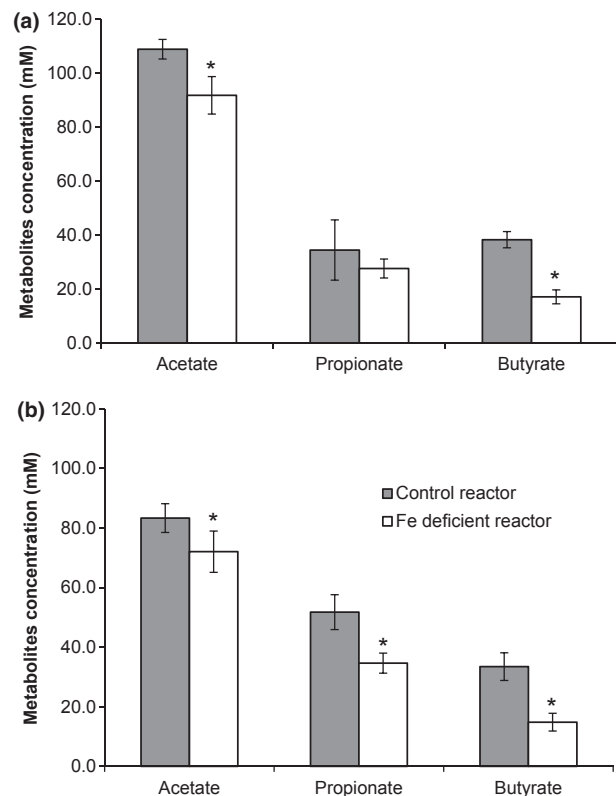
Fig. 5. Metabolite concentrations in effluents of the control reactor and Fe-deficient reactor (addition of 2,2′-dipyridyl) during fermentation 2 (a) and fermentation 3 (b) measured by HPLC. Data points are means ± SD of the last three fermentation days. Columns with an asterisk (*) are significantly different from the control reactor within the same metabolite, $P < 0.05$.

reactors of fermentations 2 and 3 (67 : 20 : 13; 59 : 29 : 12, respectively) compared with 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 with '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 with reactor 2 (5.0 ± 0.2 and 6.2 ± 0.5 log CFU mL^{-1}, respectively). During the next 'No Fe' periods (period 6), *S.* Typhimurium N-15 reached similar counts in reactors 1 and 2 (7.0 ± 0.2 and 7.2 ± 0.1 log CFU mL^{-1}, respectively) (Table 2).

Discussion

Our results highlight the importance of Fe 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 metabolite concentrations in the fermentation effluent and the gut microbiota composition.

During fermentation periods mimicking Fe deficiency, a significant decrease in acetate was observed in all three 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 in acetate production. Moreover, bacteria possessing the reductive acetyl-CoA pathway often co-metabolize formate (Wolin & Miller, 1993; Rey *et al.*, 2010). 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 in 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 ± 0.1 mg Fe L^{-1}), isobutyrate and isovalerate concentrations in fermentation effluents were reduced, suggesting a decrease in protein fermentation (Hoyles & Wallace, 2010).

On the other hand, bacterial composition was only marginally affected by 'No Fe' fermentation conditions, indicating that Fe levels of 1.56 ± 0.1 mg Fe L^{-1} mainly affect the metabolic activity of the gut microbiota. However, an increase in *Ruminococcus* spp. and a decrease in *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 ± 0.2 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 in 16S rRNA gene copy numbers of the butyrate producers *Roseburia* spp./*E. rectale*. These data were 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 because of 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 in the propionate producer *Bacteroides* spp. 16S rRNA gene copy numbers during very low Fe concentrations.

The strong decrease in 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 reactors 1 and 2 during fermentation 1 (Table 2, Figs 2a, b and 3a, 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 because of 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). Similar 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 in lactobacilli during Fe deficiency was observed in this rat study similar to the present study, which is in agreement with the mice study of Tompkins *et al.* (2001). In contrast, a human study carried out in India observed a decrease in the *L. 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 with '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 because of 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 in 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 to anti-inflammatory properties, anticancerogenic effects, and regulatory functions in cell proliferation, and butyrate can act as an energy source for intestinal cells (Luhrs *et al.*, 2002; Hamer *et al.*, 2008, 2009; 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 (Vernia *et al.*, 1988; Hove *et al.*, 1994), and lactate concentrations increased during low Fe availability during fermentation 1. Moreover, the strong decrease in dominant bacterial groups such as *Roseburia* spp./*E. rectale*, *Clostridium* Cluster IV, and *Bacteroides* spp. because of 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.

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Conflict of interest

No conflict of interests were reported by the authors.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. V5-V6 16S rRNA gene regions, obtained from pyrosequencing of fermentation effluents of reactor 1 during Fermentation 1, assigned on family level.

Table S2. V5-V6 16S rRNA gene regions, obtained from pyrosequencing of fermentation effluents of reactor 2 during Fermentation 1, assigned on family level.

Table S3. V5-V6 16S rRNA gene regions, obtained from pyrosequencing of fermentation effluents of reactor 1 during Fermentation 1, assigned on genus level.

Table S4. V5-V6 16S rRNA gene regions, obtained from pyrosequencing of fermentation effluents of reactor 2 during Fermentation 1, assigned on genus level.

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