Responses of the gut microbiota to supplementary iron:

a survey at the host-microbial interface

Guus A.M. Kortman

Responses of the gut microbiota to supplementary iron: a survey at the host-microbial interface
Guus Kortman
Thesis Radboud University Nijmegen – with summary in Dutch
ISBN/EAN: 978-90-9028627-3

Cover design: Guus Kortman, Siegert van der Velde and Proefschrift-aio.nl
design: Proefschrift-aio.nl
Printed by: dpp

The research presented in this thesis was performed at the department of Laboratory Medicine – Laboratory of Genetic, Endocrine and Metabolic Diseases, Radboud University Medical Center, Nijmegen, The Netherlands.

Responses of the gut microbiota to supplementary iron:

a survey at the host-microbial interface

Proefschrift

ter verkrijging van de graad van doctor
aan de Radboud Universiteit Nijmegen
op gezag van de rector magnificus prof. dr. Th.L.M. Engelen,
volgens besluit van het college van decanen
in het openbaar te verdedigen op dinsdag 16 december 2014
om 14.30 uur precies

door

Guus Anton Maria Kortman
geboren op 9 juli 1985
te Oldenzaal

Promotor:
Prof. dr. D.W. Swinkels

Copromotor:
Dr. H. Tjalsma

Manuscriptcommissie:
Prof. dr. M.G. Netea (voorzitter)
Prof. dr. M.A. Huijnen
Prof. dr. M.B. Zimmermann (ETH Zürich, Zwitserland)

Contents

Chapter 1

Nutritional iron turned inside out: intestinal stress from a gut microbial perspective

Guus A.M. Kortman[1], Manuela Raffatellu[2], Dorine W. Swinkels[1] and Harold Tjalsma[1]

[1] Department of Laboratory Medicine – Laboratory of Genetic, Endocrine and Metabolic Diseases, The Radboud Institute for Molecular Life Sciences (RIMLS) of the Radboud University Medical Center, Nijmegen, The Netherlands.

[2] Department of Microbiology and Molecular Genetics; University of California at Irvine; Irvine, CA USA.

This chapter is published in FEMS Microbiology Reviews (September 2014, ahead of print)

Abstract

Iron is abundantly present on earth, essential for most microorganisms and crucial for human health. Human iron deficiency that is nevertheless highly prevalent in developing regions of the world, can be effectively treated by oral iron administration. Accumulating evidence indicates that excess of unabsorbed iron that enters the colonic lumen causes unwanted side effects at the intestinal host–microbiota interface. The chemical properties of iron, the luminal environment and host iron withdrawal mechanisms, especially during inflammation, can turn the intestine in a rather stressful milieu. Certain pathogenic enteric bacteria can however deal with this stress at the expense of other members of the gut microbiota, while their virulence also seems to be stimulated in an iron-rich intestinal environment. This review covers the multifaceted aspects of nutritional iron stress with respect to growth, composition, metabolism and pathogenicity of the gut microbiota in relation to human health. We aim to present an unpreceded view on the dynamic effects and impact of oral iron administration on intestinal host-microbiota interactions to provide leads for future research and other applications.

1 Summary and scope of this review

Iron is a highly abundant metal on earth and is vital for virtually all organisms, including most bacterial species. Nonetheless, iron deficiency is the most prevalent human nutrition disorder worldwide and is generally treated by oral iron administration. Notably, accumulating evidence suggests that unabsorbed iron can stimulate growth and virulence of bacterial pathogens in the intestinal environment. Simultaneously, host iron status influences the local defence against pathogens. *Salmonella enterica* serovar Typhimurium, a highly prevalent intestinal pathogen around the world, is one example of a pathogenic bacterial species that can deal with the stress of iron limitation, iron abundance and host defence factors. It is able to adapt to these environmental triggers and exploit the environment to thrive at the expense of other bacterial species. In this review, we will thoroughly discuss the many different factors that are involved in iron-mediated host-pathogen interactions in the intestinal tract at the molecular level. We will first give a concise introduction in gut microbiology and nutritional stress. The central part of this review will present current knowledge about the effects of luminal iron on gut immunology and on the gut microbiota with respect to bacterial composition, pathogenicity and metabolism. These and other factors will also be related to pathogen stress in the gut lumen and are summarised in **Figure 1**. Furthermore, we will mostly focus on *S.* Typhimurium and its ways to deal with environmental stress and to turn the external situation in favour of its own growth and colonisation. Finally, we will place the topics we discussed in the context of serious threats that persist in tropical Africa, such as infectious diarrhoea and S. Typhimurium infection that is often associated with invasive disease in this region.

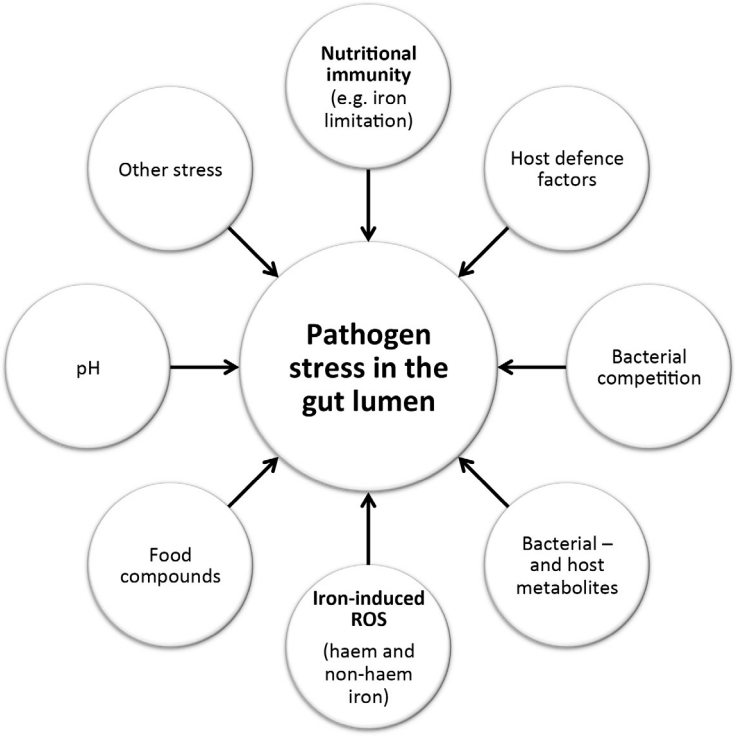


Figure 1. Overview of stress factors towards pathogens in the colon lumen.
Schematic summary of the stress factors to which pathogens in the colon lumen are exposed. This is an incomplete list and only the main factors discussed in this review are depicted.

Fenton reaction:

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + {}^{\bullet}OH + OH^- \quad (1)$$

Iron reduction:

$$Fe^{3+} + O_2^- \rightarrow Fe^{2+} + O_2 \quad (2)$$

Haber-Weiss reaction, in the presence of catalytic amounts of iron (sum of 1 and 2):

$$O_2^- + H_2O_2 \rightarrow O_2 + {}^{\bullet}OH + OH^- \quad (3)$$

Figure 2. The Fenton and Haber-Weiss reaction.
Ferrous iron generates the hydroxyl radical and hydroxyl ion from hydrogen peroxide (1). The superoxide radical (to be formed from dioxygen) can then reduce ferric iron back to the ferrous form (2). The sum of both reactions is therefore described as the formation of molecular oxygen, hydroxyl radicals and hydroxyl anions from the superoxide radical and hydrogen peroxide (3) (Crichton, 2009).

2 General introduction

2.1 Gut microbiology & health

The human gut is the natural habitat for a unique large and dynamic bacterial community that greatly outnumbers human enterocytes. Major functions of the gut microbiota include important trophic effects on intestinal epithelia, on immune structure and function, and protection of the colonized host against invasion by pathogenic microbes via the production of trophic metabolites and immunostimulatory molecules (Abreu 2010, Flint et al 2007, Hooper et al 2012, Macfarlane and Macfarlane 2012, Stecher and Hardt 2008). The healthy gut microbiota is a balanced community that is shaped by host immunity & metabolism and environmental factors (Sekirov et al 2010). Perturbations (e.g. antibiotic treatment and /or overgrowth of enteric pathogens) of the balanced gut microbiota have been associated with gastrointestinal disorders such as diarrhoea, gastroenteritis and chronic inflammatory bowel disease (Sekirov et al 2010). The number of studies investigating the effect of diets on the mammalian gut microbiota composition and metabolism has increased greatly during the past years and revealed that the gut microbiota is clearly influenced by the host diet (Power et al 2014). It is currently not well defined whether the type of diet can significantly contribute to host health or disease via alteration of the gut microbiota composition.

2.2 Factors that shape the gut microbiota

Colonisation of the human gastrointestinal tract starts during birth and is known to be influenced by the place and mode of delivery (Penders et al 2006). Known factors that influence gut colonization after birth are among others hospitalisation, hygiene and the type of feeding (breast feeding or formula feeding) (Del Chierico et al 2012). In later life e.g. diet, hygiene, illness and aging will influence the gut microbial composition (Power et al 2014). Importantly, the gut microbiota composition is not only shaped by environmental factors, but also by genetic factors (e.g. genes related to host immunity (see also section 6) and is thus always a result of a combination of the two. This leads to highly variable inter-individual gut microbiota profiles. The relative contribution of these factors to the shaping of the gut microbiota composition is not well known yet (Spor et al 2011). An interesting study of De Filippo *et al*. in which the gut microbiome of European children was compared with the gut microbiome of children living in African rural area, showed large differences between the groups. In this study ethnicity and many environmental factors were different, but it was suggested that diet played the dominant role over other variables (De Filippo et al 2010).

2.3 Nutritional and metabolic stress in the gut lumen

The gut microbial community thrives on complex carbohydrates and proteins that are not digested and taken up by the upper intestine. The balanced community also depends on the provision of micronutrients for metabolism and replication. There is a continuous competition for these nutrients which is reflected in the different requirements and uptake mechanisms that certain species possess. Metabolic competition and interactions are also ways in which the gut microbiota restrains the growth of unwanted pathogens (Kamada et

al 2013). One of the micronutrients that the vast majority of bacterial species require for their growth and metabolism is iron (Andrews et al 2003). However in the human body, iron availability to microbes is generally extremely limited, due to innate iron withholding mechanisms that aim to prevent growth of pathogenic invaders (Cassat and Skaar 2013). Such a tightly regulated system of nutritional immunity is not known for the lumen of human gut. However, also in the gut lumen with high amounts of dietary iron present, gut microbes have to deal with the stress of iron limitation as the presence of freely available "unbound" iron is probably limited due to the environmental conditions of the colon lumen. The balance of bound and unbound iron can be (re)disturbed by the oral administration of supplementary iron, a common strategy to treat iron deficiency, which is known to cause alterations of the gut microbiota composition and metabolism (see section 7&8). In Figure 1 we summarised the (iron-related) stress factors that will be discussed throughout this review.

Although most knowledge on the effects of iron in the gut originates from studies on the colonic microbiota, we predict that the effects of iron availability on the small intestinal microbiota are likely to be different from the effects on the colonic microbiota. It is important to realize that the small intestinal microbiota has a much lower density of residing microbes, and that its composition and metabolism appears to fluctuate quickly in response to dietary input (Zoetendal et al 2012). Iron may play a large role within this environment where also pathogenic bacteria like *Salmonella* spp, *Vibrio cholera* and pathogenic *E. coli* may colonise. However, relatively little is known about the small intestinal microbiota, as it is much less accessible compared to the colonic (faecal) microbiota, which makes it difficult to study (Zoetendal et al 2012). Therefore, the focus of this review is on the colonic microbiota to which we refer to as 'gut microbiota'.

3 Redox stress and microbes

3.1 The redox chemistry of iron

Under physiological conditions iron is found mostly in the ferrous (Fe^{2+}) or ferric (Fe^{3+}) form and the easy redox cycling properties of this metal make it very well suited as a biocatalyst in proteins, or as an electron carrier (Andrews et al 2003, Crichton 2009). Under aerobic physiological conditions, iron is mostly present in its oxygenic ferric, virtually insoluble, form, with 10^{-9} M being the approximate concentration of ferric ion at pH 7 (Crichton 2009). Fe^{3+} is practically only soluble in water when it is attained in a strong aqueous complex. Thus, despite the high abundance of iron on earth, its bioavailability can be very low. Fe^{3+} prefers oxygen ligands, while Fe^{2+} favours nitrogen and sulphur ligands (Crichton 2009). Ferrous iron is much more soluble than ferric iron, but it is mainly found in anoxic or acidic environments. Importantly, free redox active iron can be very toxic under aerobic conditions via the formation of reactive oxygen species (ROS), generated through the Fenton and Haber-Weiss reaction (**Figure 2**). Of the ROS, one of the most reactive species known is the highly reactive hydroxyl ion OH^-, which can induce severe damage to cells and biological molecules.

3.2 Bacteria and redox stress

The vast majority of life on earth, including most microorganisms in the bacterial kingdom, depends on the presence of the transition metal iron for respiration and various metabolic processes. Only a very limited number of bacteria are known that do not depend on iron for their growth. The most well-known and notable group concerns the beneficial *Lactobacillaceae*, but also the pathogenic *Borrelia burgdorferi* does not use iron for its metabolism, both are dependent on manganese instead (Imbert and Blondeau 1998, Pandey et al 1994, Posey and Gherardini 2000). In addition, certain species of Streptococci that normally use iron can utilise manganese as an alternative transition metal (Jakubovics and Jenkinson 2001). Furthermore, bacteria that utilise iron have to avoid the toxicity of free redox active iron both intracellularly and extracellularly. To this purpose, bacteria possess several mechanisms to detoxify iron or oxidative stress directly, of which a selection is described below.

3.2.1 Detoxification of iron

Iron in its ferric form can be bound to three known types of bacterial high affinity storage proteins; ferritin (similar to what is found eukaryotes), bacterioferritin and DNA-binding protein from starved cells (DPS). DPS is an iron detoxification / iron storage protein that also binds to DNA and plays hereby a role in the protection of DNA from redox stress (Andrews et al 2003). These iron storage proteins lower the concentration of intracellular free iron by storing it in a soluble and non-toxic form. Iron can be released from these bacterial stores in times of iron scarcity, for example when residing inside mammalian host cells or in the blood stream (Andrews et al 2003).

Alternatively, bacteria can export a surplus of iron. *E. coli* can for example export iron via the exporter FieF and an iron-citrate efflux transporter (IceT) has been described for S. Typhimurium; these iron efflux systems have the supposed purpose to prevent stress from high levels of free intracellular iron (Frawley et al 2013, Grass et al 2005). Also a haem export mechanism (HrtAB or orthologous proteins) has been shown to alleviate the iron stress of haem in certain bacterial species (Cassat and Skaar 2013). Finally, *Bifidobacteriaceae* are able to bind iron to their surface, which subsequently reduces radical formation in the surrounding environment and may in the colonic lumen function as a mechanism of iron sequestration to prevent iron uptake by pathogenic species (Kot and Bezkorovainy 1999) (see also **Figure 3**).

3.2.2 Protective enzymes that neutralize oxidants

Several bacterial enzymes are known that directly counteract the formation of oxidative stress. One of these enzymes is Superoxide Dismutase (SOD), which catalyzes the dismutation of superoxide into the less toxic oxygen and hydrogen peroxide. Notably, the various members of the SOD family use different metals as a cofactor for functioning, among which are manganese and zinc. Host sequestration of manganese and zinc by calprotectin (excreted by immune cells) can therefore inhibit this bacterial defence mechanism and this way making bacteria more susceptible for oxidative stress (Damo et al 2012) (See also section 9.1). Another well-known enzyme that neutralizes reactive oxygen

species (ROS) is catalase, which catalyzes the reaction of two hydrogen peroxide molecules to the non-toxic products water and oxygen. Finally, bacteria also possess peroxidases that can contribute to the oxidative stress defence. Peroxidases include glutathione peroxidase and alkylhydroperoxide reductase (Ahp) that can rapidly detoxify hydrogen peroxide, hydroperoxides, or peroxynitrite (Crichton 2009, Mishra and Imlay 2012).

4 Iron, a precious and contested nutrient

4.1 Bacterial iron uptake

Although iron is present in sufficient amounts to sustain their replication in most habitats, bacteria still have to deal with the low solubility of this metal. Therefore, readily available iron is generally scarce and bacteria have evolved multiple iron uptake mechanisms to be able to fulfil their average need of 10^{-7} to 10^{-5} M for optimal growth. Several mechanisms of iron uptake were previously described for various bacterial species, and were most extensively studied in *E. coli*. In the latter bacterium, iron can be taken up in both its ferrous and ferric form. One of the mechanisms of ferrous iron uptake is via the Feo-uptake system (Andrews et al 2003). Moreover, ferric iron can be reduced to ferrous iron via an extracellular reductase, after which it can be taken up in the ferrous form (Cowart 2002). Alternatively, ferric iron can be taken up as ferric citrate, or bound to excreted bacterial siderophores, small iron chelators with a very high affinity for iron (Andrews et al 2003, Miethke and Marahiel 2007). After excretion, bacteria re-internalise the ferric-siderophore complex via binding to specific receptors and transporters. For example the ferric-enterobactin complex binds to the outer membrane receptor FepA of gram-negative bacteria, after which it is transported to the periplasm, a process dependent on the TonB system that provides the energy for this transport. Next, the ferric-enterobactin-complex is further transported into the cytoplasm by ABC-transporters (Chu et al 2010). The role of siderophores in the colonic lumen is further described in section 5.3. Direct uptake of haem-bound iron and scavenging of haem with haemophores are other specialized ways of iron acquisition (Andrews et al 2003). It is important to mention that haem-iron is much more bioavailable for humans compared to non-haem iron (Zimmermann and Hurrell 2007), that haem causes oxidative and cytotoxic stress in the gut lumen and that haem can alter the (mouse) gut microbiota composition (IJssennagger et al 2012, IJssennagger et al 2013). In this review, we will however focus on the effects of non-haem iron on the growth, virulence and metabolism of gut microbes because dietary iron on average consist for 90% of non-haem iron (Hulten et al 1995), which percentage is likely even higher in the in the tropics. Furthermore, oral iron is always given as non-haem iron.

4.2 Iron & bacterial virulence

It has been known for a long time that the scarcity of iron generally limits bacterial growth whereas iron availability often enhances bacterial replication and the expression of virulence factors in pathogenic bacteria, thereby exaggerating infection (Bullen et al 2005, Litwin and Calderwood 1993, Payne and Finkelstein 1978, Weinberg 1978). Furthermore, knockout studies have shown that iron uptake mechanisms such as FeoB are required for

full virulence *in vivo* (Boyer et al 2002, Janakiraman and Slauch 2000, Naikare et al 2006, Tsolis et al 1996). Bacteria sense the availability of iron in the environment and a change in the amount of available iron (for example, from high to low) during invasion of the host is an important signal for the expression of virulence genes. On the one hand, under low iron conditions, the production of siderophores is derepressed, and also the production of toxins may be upregulated (Litwin and Calderwood 1993, Payne 1993). On the other hand, *in vitro* studies have shown that freely available iron can directly induce the expression of virulence genes in S. Typhimurium; especially bacterial adhesion to intestinal epithelial cells seems to be stimulated when iron is highly available (Bjarnason et al 2003, Kortman et al 2012) (**Chapter 5**). Thus both high and low iron conditions seem to be able to stimulate different aspects of bacterial virulence.

4.3 Battle for iron in the human body

Both the host and the bacterial pathogen require iron for their cellular functions. As discussed above, free iron can be very toxic and that is one reason iron is bound to high-affinity host proteins such as ferritin, transferrin, lactoferrin and haemoproteins, resulting in extremely low free iron concentrations of about 10^{-24} M (virtually zero) (Raymond et al 2003). In the human body, tight binding of iron to proteins is also an effective form of innate defence against microorganisms, as they relatively require much higher free concentrations of 10^{-7} to 10^{-5} M for optimal growth, but which is still rather low. These host defence mechanisms are further enhanced upon infection. For instance on the systemic level, the inflammatory response evokes IL-6 production, which induces the production of the iron-regulating peptide hormone hepcidin in the hepatocytes. Hepcidin in turn, binds to the iron exporter ferroportin at the membrane of host cells, e.g. macrophages and duodenal cells, hereby inducing its internalisation and degradation (Kroot et al 2011). The result of these events is a decrease of intestinal iron uptake, an increase of reticulo-endothelial system (RES) iron stores and consequently a decrease in iron availability to extracellular pathogens. Therefore, the ability to overcome iron withdrawal mechanisms of the human body in general is an important virulence trait of bacteria and there is no fight for iron without the armour of the pathogenic opponent. To acquire iron in the iron-limiting conditions created by the host, in particular during an inflammatory response, (invading) pathogens may express receptors for host transferrin and lactoferrin, but more well-known is the secretion of iron scavenging siderophores (Andrews et al 2003, Cairo et al 2006). These molecules mostly have a very high specificity and affinity for ferric iron and can therefore compete with iron bound to host proteins. To illustrate, transferrin has an affinity constant for ferric iron of about 10^{22}, while the association constant of the siderophore enterobactin for ferric iron is approximately 10^{51} (Aisen and Listowsky 1980, Carrano and Raymond 1979). We note that (slightly) different association constants are circulating in literature for transferrin and enterobactin (**Supplementary Table 1**). Fortunately, the fight for iron does not end here as the mammalian host has evolved a mechanism to sequester some bacterial ferric-siderophore complexes with the innate defence peptide lipocalin-2 (a.k.a. neutrophil gelatinase-associated lipocalin (NGAL), siderocalin, 24p3, uterocalin). By binding to the ferric-enterobactin-complex, lipocalin-2 prevents the uptake of ferric

enterobactin by bacteria, thereby enhancing nutritional immunity (Flo et al 2004). However, certain pathogenic bacteria can evade this sophisticated host defence mechanism by the production of 'stealth siderophores' (Allred et al 2013). For instance, S. Typhimurium and pathogenic *E. coli* can produce a C-glucosylated form of enterobactin, termed salmochelin, which escapes from the binding by lipocalin-2 (Hantke et al 2003, Raffatellu and Baumler 2010). Another example is the production of aerobactin, which does not bind to lipocalin-2 either, and is produced by for instance *Klebsiella, Shigella*, some *Salmonella* serovars and pathogenic *E. coli* strains (Flo et al 2004, Wooldridge and Williams 1993). If and to what extent the here discussed host and bacterial mechanisms are involved in a battle for iron at the intestinal host-microbiota interface will be discussed in the next sections.

5 Iron speciation and bacterial iron uptake mechanisms in the colon

5.1 Iron speciation and availability for microbes in the colon

As far as we know, bacteria synthesize siderophores only when the availability of iron in their surround environment is limited. In the colonic lumen, large amounts of iron are regularly present, mostly constituted by excess dietary iron not absorbed in the duodenum (the major site of iron absorption). This is illustrated by the high concentration of iron found in faeces of British adults on a standard western diet and in weaning infants fed with complementary solid foods: approximately 100 µg Fe/g wet weight faeces, which is roughly equal to 1.8 mM, and which is much more than the minimal iron requirement of most bacterial species (Lund et al 1999, Pizarro et al 1987) (see also section 4.3). Moreover, the tight mechanisms of iron withholding for systemic sites as described above are not known to play a role in the gut lumen. Nevertheless, iron speciation and the potential presence of lactoferrin, lipocalin-2 and maybe other yet unidentified defence proteins in the colon mucosa probably contribute to the limitation of iron at this site (**Figure 3**). Notably, lipocalin-2 is only expressed at a low level under healthy conditions (Cowland and Borregaard 1997) and would be more effective when readily available iron in the lumen is limited and thus when siderophores are produced.

Due to the nature of foods and of the oxygenic and acidic environment of the stomach, most of the dietary non-haem iron that enters the intestine is in the ferric form (Jacobs and Miles 1969). When the pH in the duodenum and small intestine rises, the solubility of ferric iron decreases (**Figure 3**). Simultaneously, the increase in pH favours the oxidation of ferrous iron in the presence of oxygen. Further downstream, the reduced environment in the colon may favour the reduction of iron into the ferrous form (Hedrich et al 2011). Also the colonic microbiota may influence the valence state of iron by the action of extracellular reductases (Cowart 2002, Takeuchi et al 2005) (**Figure 3**). The possible effect of colon luminal pH on iron solubility was discussed in a study from Romanowski *et al.*, in which surgically injured mice with the oral provision of a phosphate buffer at pH 7.5 showed an increased colonic pH compared to mice provided with the same buffer at pH 6.0. The expression of bacterial siderophore systems was upregulated within the neutral pH-group, possibly due to a decreased solubility of iron in the intestinal lumen at a higher pH (Romanowski et al

2011). It should however be noted that phosphate also influences siderophore production and that iron is prone to form complexes with phosphate, which could have contributed to their findings. These *in vivo* results are nevertheless consistent with an *in vitro* study, which showed that lowering of the pH increased iron uptake (Salovaara et al 2003).

Iron speciation and solubility in the colon is extremely difficult to predict as many factors can influence this process and the various iron species in the lumen are not easy to measure accurately. Consequently, the amount iron in the colon lumen that is readily available to bacteria is also difficult to estimate. Besides oxygen and pH, which are two main influencers of iron speciation, different food components can affect the valence state and solubility. The most famous food component in this matter is ascorbic acid (vitamin C), which chelates iron and also reduces ferric iron to its ferrous form (**Figure 3**). For this reason ascorbic acid is also known for its positive effect on iron absorption by the host (Miret et al 2003). Other organic acids such as citrate are known to form a soluble chelate with iron and may hereby prevent precipitation of ferric iron when the pH rises after passage through the stomach (van Dokkum 1992) (**Figure 3**). Nonetheless, the neutral to mildly acidic pH of the intestinal lumen favours precipitation with hydroxides and complex formation with mucins, certain amino acids, proteins, and other food components. Therefore, iron species such as iron oxides, iron hydroxides, iron phosphates and iron carbonates may be found in the intestinal lumen, but we do not know how accessible these insoluble forms of iron are for bacteria (Cremonesi et al 2002, Simpson et al 1992) (*Figure 3*). Bacteria may solubilise some of these forms by lowering the external pH, by reducing the iron to the more soluble ferrous form, or by binding to siderophores (Andrews et al 2003, Ratledge and Dover 2000). Ligands that potentially bind to iron and play a role in the colonic lumen are summarised in **Supplementary Table 2**. In **Supplementary information 1** we envisage how orally administered iron will end up in the colon after ingestion. In conclusion, both ferrous and ferric forms of iron are likely to be present in the colon and there are reasons to believe that iron availability to the colonic microbiota is normally limited (see the following paragraphs).

5.2 Quest for food-bound iron sources

Because iron speciation in the colon is difficult to predict and it is likely highly variable, not much is known about the accessibility of colonic luminal iron. Nonetheless, here we discuss a few iron sources that are normally not easy to access, but may become available to certain members of the gut microbiota through specialized mechanisms. It is likely that future research will reveal additional food-bound iron sources that can be utilised by bacteria.

5.2.1 Polyphenol-iron

The human diet often contains polyphenols like tannins and catechols, which are highly present in e.g. tea and coffee. These compounds can bind iron very strongly and hereby prevent uptake of iron by the host, but also prevent the uptake of iron by bacteria. However, in an iron-limited environment, pathogenic bacteria that can produce and/or take up siderophores may benefit from the iron polyphenol withdrawal mechanism, most

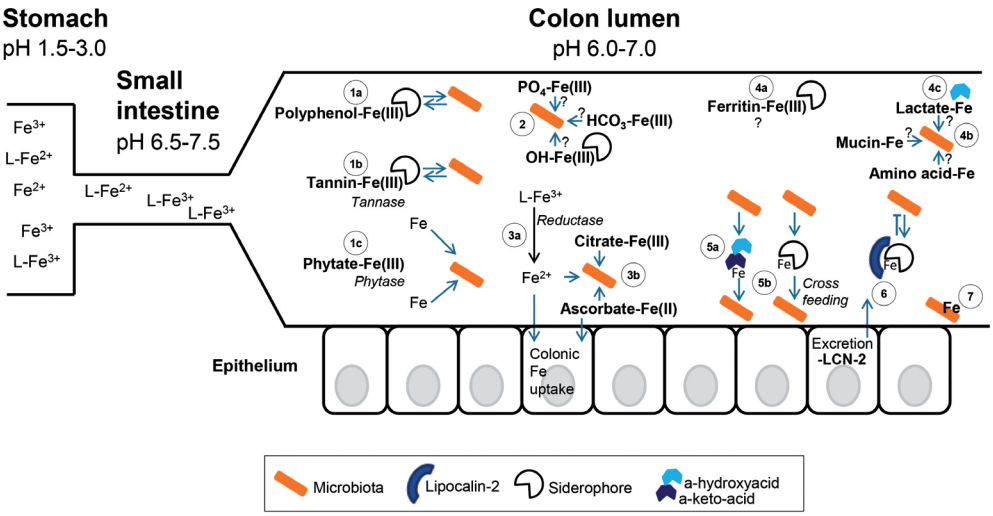


Figure 3. Iron speciation, availability and the battle for iron in the colon lumen.

Iron speciation in the gastrointestinal tract probably plays an important role in the accessibility of iron for the gut microbiota. In the stomach (on the left; very simplified), the low pH favours the solubility of both ferric and ferrous iron, not necessarily requiring a ligand (L) for solubility. When the pH increases in the small intestine, the solubility of mainly ferric iron decreases and more complexes with food components and host excretions will be formed. Within the colon, the pH slightly drops due to the production of e.g. lactate and SCFAs by the microbiota. The colonic part of the figure, where the microbiota depicted in orange represents both (beneficial) resident species and pathogenic species, shows the following aspects:

1) On the left it is shown that iron bound to polyphenols (**1a**), e.g. tannins (**1b**) and phytate (**1c**), may be accessible via enzymatic degradation or via the removal of the iron by siderophores;
2) The rather insoluble forms like iron in a complex with phosphate, carbonate or oxides are likely not readily accessible for the microbiota, but they may be solubilised by bacterial reduction or siderophoric chelation;
3) Soluble forms of ferric iron may be reduced to ferrous iron after which it can be taken up by both the microbiota and the host (**3a**) and for instance, the soluble ferric citrate or ferrous ascorbate may be directly taken up by bacteria (**3b**). Notably, host iron uptake may be a mechanism to withdraw the iron from the colon lumen;
4) On the right, several forms of iron are depicted from which we also do not know very well how accessible they are. We envisage that ferritin-iron (dietary or from sloughed enterocytes) (**4a**) is difficult to access, but iron bound to mucin, amino acids or lactate (**4b**) may be relatively easy to access and that may hypothetically be promoted by the low affinity siderophores α-hydroxyacids and α-keto-acids (**4c**);
5) These low affinity siderophores (**5a**) may also play an important role in the phenomenon of iron cross-feeding by heterologous siderophores within the microbiota (**5b**);
6) The excretion of lipocalin-2 (LCN-2) in the colon lumen may scavenge iron bound to siderophores and hereby prevent uptake by (pathogenic) bacteria;
7) Finally, at the lower right it is depicted that bacteria spp. may bind iron to their cell wall (as has been shown for *Bifidobacterium*), which likely prevents access to this iron by other (pathogenic) species.

probably by scavenging the iron bound to the polyphenols (Freestone et al 2007) (**Figure 3**). It is not known whether this mechanism plays a significant role in the gut lumen, but it might be relevant as both polyphenols and siderophores are likely to be present in the gut environment. In addition, certain bacteria, such as *Streptococcus gallolyticus* or *Staphylococcus lugdunensis*, can degrade the polyphenol tannate and this way provisionally liberate iron from this potent iron binder (Noguchi et al 2007, Smith et al 2005).

5.2.2 Phytate-iron

Another compound with potent iron-binding activity is phytate, whose intestinal availability is high after consumption of a cereal-and-legume-based diet. Similar to tannate, certain gut microbes (e.g. coliforms and *Bifidobacteriaceae*) can degrade phytate, which might be a specialized way of liberating iron, the iron may become available for utilisation by either the degrading organisms or by other bacterial species (Markiewicz et al 2013) (**Figure 3**). Therefore, iron bound to phytate might be a relevant iron source for the colonic gut microbiota. It should however be noted that the iron complexed with phytate that reaches the colon is mostly in an insoluble form, thus being less susceptible to degradation (Schlemmer et al 2009). Nevertheless, degradation products of phytate that could be generated only through microbial action were previously found in the colon of conventional rats, but not in germ-free rats (Schlemmer et al 2009). These findings indicate that phytate degradation by microbes occurs in the colon.

5.3 The evidence and need for siderophores in the colon

Even though some food compounds may be a source of iron, there are strong indications that readily available iron in the gut is generally limited, as studies have reported that the production of siderophores by enteric pathogens in the gut improves their survival and colonisation. The findings described herein suggest that siderophores are commonly produced by enteric pathogens in the colonic lumen, particularly if the intestine is inflamed. In the absence of intestinal inflammation, ferrous iron is likely available in the gut to some extent, due to the reducing environmental conditions of the colon, but it may also be formed through the action of microbial reductases. As such, enteric bacteria may utilise ferrous iron as an iron source via the Feo uptake system. A role for Feo in the gut was shown in both *S.* Typhimurium and *E. coli*, as isogenic mutant strains deficient in FeoB are outcompeted by their wild type strains during colonisation in the mouse intestine (Stojiljkovic et al 1993, Tsolis et al 1996). Similarly, a *Campylobacter jejuni* FeoB mutant is outcompeted during colonisation in rabbit ileal loops, in the chick caecum, and in the intestinal tract of piglets (Naikare et al 2006). Notably, a *S.* Typhimurium strain lacking both FeoB and TonB is further attenuated in the mouse intestine, indicating that both ferrous and ferric iron may be available in the mouse colon (Tsolis et al 1996). However, a single TonB mutant showed similar colonisation levels compared to the wild type strain (Tsolis et al 1996), suggesting that siderophore-mediated iron uptake is only required when the more standard mechanisms of iron uptake are somehow not possible, or when freely available iron is absent. In contrast, other studies reported a disadvantage in intestinal colonisation when siderophore-mediated iron uptake was affected. For instance, a *S.* Typhimurium mutant

in the ferrioxamine receptor FoxA was less able to colonise rabbit ileal loops and showed reduced virulence in a mouse typhoid model compared to the wild type strain, indicating that *S.* Typhimurium can utilise siderophores in favour of their virulence at the intestinal-epithelial interface (Kingsley et al 1999).
Other siderophores like aerobactin have been shown to play a role during colonization of the mouse caecum by a pathogenic *E. coli* strain (Torres et al 2012). In addition, *E. coli* mutants which were affected in the production or uptake of catechol-type siderophores showed impaired colonisation of the mouse intestine compared to the wild type strain (Pi et al 2012). Though all of these studies suggest that siderophores are secreted in the intestinal lumen, as far as we are aware, the study of Pi *et al.* is the only report that has also directly confirmed the presence of enterobactin in the mouse gut lumen (in the mucus) (Pi et al 2012). In future studies, it will be important to provide more information about the types and amounts of siderophores presents in the faeces, but it might be challenging to isolate and identify them from such a complex matrix. Taken together, the results discussed herein suggest that the production and utilisation of siderophores is a special-purpose iron uptake system in the gut lumen and they show that that these molecules are required for full bacterial virulence. The importance of siderophore-mediated iron acquisition appears to be even more important in the inflamed gut, where the iron-laden enterobactin is sequestered by the antimicrobial protein lipocalin-2. Non-pathogenic *Enterobacteriaceae*, such as certain *E. coli* strains, that solely rely on enterobactin for siderophore-mediated iron acquisition are susceptible to lipocalin-2, while pathogens like *S.* Typhimurium circumvent lipocalin-2-based defence via the production of alternative stealth siderophores like salmochelin, which is not recognized by lipocalin-2. Thus, salmochelin-dependent iron acquisition provides those species with a competitive advantage in the inflamed gut (Raffatellu et al 2009)(see also section 9.1). Overall, the aforementioned studies suggest that enteric pathogens constantly have to face the stress of limited iron availability in the normal gut and even more in the inflamed gut, and demonstrate that high affinity iron uptake systems are required for full virulence.

5.4 Iron cross-feeding with heterologous siderophores

An interesting phenomenon is the cross-feeding of heterologous siderophores, i.e. certain bacterial species can compete for and use each other's siderophores, and sometimes they are even able to utilise a type of siderophore that cannot be produced by themselves. This is exemplified by *Salmonella* strains that can take up ferrioxamines (via the receptor FoxA) as mentioned above. They however cannot synthesize ferrioxamines themselves, but may be available from related species and other members of normal gut microbiota (Champomier-Verges et al 1996, Kingsley et al 1999). Examples of other genera that can utilize heterologous siderophores are *Campylobacter* and *Pseudomonas* (Baig et al 1986, Champomier-Verges et al 1996, Llamas et al 2006). A study with microbial isolates from marine sediments indicated that the growth of many bacteria depends on siderophore production by other bacteria within the same community (D'Onofrio et al 2010). Although there is not yet direct evidence, it may be anticipated that cross-feeding with siderophore-iron plays a significant role in the colonic environment with its microbial community of >1000 different species (**Figure 3**).

5.4.1 Low affinity siderophores

A distinct type of iron-binding siderophores, i.e. α-keto-acids and α-hydroxyacids (a reduced form of α-keto-acids), may be important players in the cross-feeding of siderophore-iron. α-Keto-acids and α-hydroxyacids are primary metabolites of both human cells and bacteria, but are also known to bind iron (Drechsel et al 1993). The ferric form of these siderophores can stimulate the growth of bacteria that reside in an iron-limited environment. To our knowledge, the association constant of these siderophores for iron has not been determined yet, but based on a study by Kingsley *et al*. it has been suggested by Reissbrodt *et al.* that these iron-binding acids have a lower affinity for iron compared to conventional siderophores (Kingsley et al 1996, Reissbrodt et al 1997). This is in agreement with the notion that conventional siderophores are synthesized for the purpose of iron scavenging, while α-keto-acids are products of general metabolism (Reissbrodt et al 1997). Notably, it has been shown that the excretion of α-keto-acids by *S.* Typhimurium was increased upon iron limitation (Reissbrodt et al 1997). Although low affinity siderophores may not be very useful for pathogens invading the host with its high affinity iron-withholding proteins, they could significantly contribute to iron sequestration in the gut lumen where iron most probably can be scavenged more easily. Importantly, certain bacteria, among which are *Salmonella* spp. can benefit from the α-keto-acids type of siderophores by using them as iron sources (Kingsley et al 1996) (**Figure 3**). Consistent with these findings, we found two hydroxyacids with (tentative) siderophoric activity in an *in vitro* model of the human proximal colon containing a faecal microbiota (Kortman et al 2014b) (**Chapter 3**). 2-Hydroxyisovaleric acid was only detected in low iron conditions and not in moderate to high iron conditions, and 2-hydroxyisocaproic acid was detected in higher amounts in low iron conditions compared to the moderate to high iron conditions (Kortman et al 2014b) (**Chapter 3**). These results suggest that the reduced iron availability might be a signal to increase the production of hydroxyacids for at least a subset of the gut microbiota.

5.5 Summary of the quest for iron in the colonic lumen

To wrap up the preceding sections on iron speciation and availability in the colon lumen, we here outline the main points. First of all, the total luminal iron content is mainly dependent on dietary iron intake, but is generally considered high. However, the majority of this iron appears not to be readily available for the gut microbiota because much of the iron is bound to food or host derived compounds (ligands) that, together with the environmental conditions in the gastrointestinal tract, make the iron difficult to access. This is supported by the evidence that siderophore production by enteropathogens takes place in the (inflamed) colon, a process that is only required when readily available iron is low. These and other aspects that point at a gut bacterial quest for iron in the colon lumen are visualised in **Figure 3**. Finally, oral iron administration will change the iron speciation balance and it is likely that part of this extra iron becomes readily available for the gut microbiota (see sections 7 & 8).

6 Effect of host iron intake on gut physiology and immunology

To better understand the interplay between nutritional iron, the intestinal microbiota and the host, we review the basic aspect of the effects of luminal iron and host iron status on gut physiology and gut immunology in the following paragraphs.

6.1 Colonic iron absorption

In humans, iron is mainly absorbed in the duodenum, but to a small extent also in the colon. Ferrous iron infused into the colon is much better absorbed than ferric iron, which is in line with findings in the duodenum where iron needs to be in the ferrous state for uptake by enterocytes (Ohkawara et al 1963). It can be envisaged that the effect of iron speciation on iron uptake is larger in the colon compared to the duodenum as the iron within the food matrix has travelled a longer route to the colon with many changes in environmental conditions, allowing the formation of many different iron complexes that possibly affect host iron uptake (see section 5.1 & 5.2). Colonic iron absorption may contribute to the iron needs of the body, but it may also be part of a defence mechanism as iron exclusion from the colonic lumen can contribute to nutritional immunity and restraining the gut microbial community, especially the growth and virulence of enteric pathogens (**Figure 3**).

Interestingly, the microbiota itself also appears to play a role in host iron absorption. It is known that prebiotics can change the microbiota composition and metabolism as shown in humans, which in turn can influence iron absorption, as shown in pigs (Petry et al 2012, Tako et al 2008). A change in bacterial metabolism may for example involve an increase in lactate or SCFAs which can lower the pH and promote solubility and reduction of iron to the ferrous state (Salovaara et al 2003) (**Figure 3**). SCFA can also stimulate the proliferation of epithelial cells, hereby enhancing the absorptive surface, whereas prebiotics or their fermentation products may increase the expression of iron regulatory genes such as Divalent Metal Transporter 1 (DMT1) in both the duodenum and the colon (Tako et al 2008, Yeung et al 2005). It has been shown that iron absorption genes in the colon of mice were higher expressed in iron-deficient mice compared to controls (Takeuchi et al 2005). This study also showed that the reductase Dcytb (highly expressed in the duodenum) was lowly expressed in mouse colon. Dcytb mediates conversion of ferric iron to ferrous iron, which is crucial for the uptake by enterocytes through the iron importer DMT1. Together this may suggest that it is not necessary for colonocytes to express this enzyme because the microbiota possesses reductase activity that already contributes to the conversion of ferric iron to ferrous iron (Takeuchi et al 2005). A role for the gut microbiota in iron absorption is also supported by the finding that germ-free raised rats showed decreased iron uptake compared to conventionally raised rats (Reddy et al 1972). Notably, also oral iron administration can modify the gut microbiota (see section 7) and this implies that the uptake of this administered iron might actually be influenced by the iron-induced changes in microbiota composition and metabolism.

6.2 Effect of gut luminal iron on the host epithelial barrier function

Besides the effects of iron on the gut microbiota, which may cause a shift toward a more

Table 1: most studied bacterial groups in relation to dietary iron content in the past few decades

Bacterial group	Direction	Number of studies	Study 1	Study 2	Study 3	Study 4	Study 5	Study 6	Study 7
Enterobacteriaceae	Up	2	**Zimmerman *et al.* 2010** African schoolchildren	**Jaeggi *et al.* 2014** African infants					
	Down	2	**Dostal *et al.* 2012a** Rats	**Dostal e *et al.* 2012b** *in vitro* fermentation					
	No change	6	**Mevissen-Verhage *et al.* 1985** Dutch infants	**Krebs *et al.* 2013** U.S. infants	**Werner *et al.* 2011** Mice	**Ettreiki *et al.* 2012** Mice and rats	**Dostal *et al.* 2014b** Rats	**Kortman *et al.* 2014** *In vitro* fermentation	
Coliform* bacteria / *E. coli	Up	5	**Mevissen-Verhage *et al.* 1985** Dutch infants	**Balmer *et al.* 1989** UK infants (4d)	**Jaeggi *et al.* 2014** African infants	**Benoni *et al.* 1993** Rats (2wk)	**Lee *et al.* 2008** Piglets		
	Down	0							
	No change	5	**Balmer *et al.* 1989** UK infants (14d)	**Balmer *et al.* 1991** UK infants	**Benoni *et al.* 1993** Rats (4wk)	**Tompkins *et al.* 2001** Mice	**Kortman *et al.* 2014** *In vitro* fermentation		
Lactobacillaceae	Up	1	**Benoni *et al.* 1993** Rats (2 wk)						
	Down	7	**Zimmerman *et al.* 2010** African schoolchildren	**Krebs *et al.* 2013** U.S. infants	**Tompkins *et al.* 2001** Mice	**Werner *et al.* 2011** Mice	**Dostal *et al.* 2012a** Rats	**Dostal *et al.* 2012b** *in vitro* fermentation	**Kortman *et al.* 2014** *In vitro* fermentation
	No change	7	**Mevissen-Verhage *et al.* 1985** Dutch infants	**Balmer *et al.* 1989** UK infants (4 and 14d)	**Balmer e *et al.* 1991** UK infants	**Jaeggi *et al.* 2014** African infants	**Benoni *et al.* 1993** Rats (4wk)	**Lee *et al.* 2008** Piglets	**Dostal *et al.* 2014b** Rats
Bifidobacteriaceae	Up	0							
	Down	6	**Mevissen-Verhage *et al.* 1985** Dutch infants	**Krebs *et al.* 2013** U.S. infants	**Jaeggi *et al.* 2014** African infants	**Werner *et al.* 2011** Mice	**Dostal *et al.* 2012b** *In vitro* fermentation	**Kortman *et al.* 2014** *In vitro* fermentation	
	No change	4	**Balmer *et al.* 1989** UK infants (4 and 14d)	**Balmer *et al.* 1991** UK infants	**Zimmerman *et al.* 2010** African schoolchildren	**Lee *et al.* 2008** Piglets			

Continued, Table 1: most studied bacterial groups in relation to dietary iron content in the past few decades

Bacteroidetes* / *Bacteroides	Up	4	**Mevissen-Verhage *et al.* 1985** Dutch infants	**Krebs *et al.* 2013** U.S. infants	**Werner *et al.* 2011** Mice	**Dostal *et al.* 2012a** Rats	
	Down	3	**Balmer *et al.* 1991** UK infants	**Benoni *et al.* 1993** Rats (4wk)	**Dostal *et al.* 2012b** *in vitro* fermentation		
	No change	5	**Balmer *et al.* 1989** UK infants (4+14d)	**Jaeggi *et al.* 2014** African infants	**Ettreiki *et al.* 2012** Mice and rats	**Dostal *et al.* 2014b** Rats	**Kortman *et al.* 2014** *In vitro* fermentation
Clostridia* / *Clostridium	Up	3	**Balmer *et al.* 1991** UK infants	**Jaeggi *et al.* 2014** African infants	**Benoni *et al.* 1993** Rats (2wk)		
	Down	1	**Werner *et al.* 2011** Mice				
	No change	5	**Mevissen-Verhage *et al.* 1985** Dutch infants	**Balmer *et al.* 1989** UK infants (4 and 14d)	**Krebs *et al.* 2013** US infants	**Lee *et al.* 2008** Piglets	**Kortman *et al.* 2014** *In vitro* fermentation

pathogenic profile and an increase in virulence of enteric pathogens, iron may also directly exert unfavourable effects to the gut epithelium most likely via the promotion of redox stress. Importantly, a decrease in epithelial integrity has been reported *in vitro* with Caco-2 cells exposed to iron (Ferruzza et al 2003, Natoli et al 2009). In contrast, an earlier study did not find a decrease in epithelial integrity when cells were incubated with iron, despite an increase in lipid peroxidation through iron (Courtois et al 2000). Also, in our hands we were not able to reproduce the decrease in epithelial integrity of Caco-2 cells observed by Ferruza *et al*. and Natoli *et al*. (Kortman, unpublished). However, a combined *in vivo-ex vivo* study with calf duodenum showed increased permeability after feeding the calves with a high iron diet, even though duodenal oxidative damage, as measured by malonyldialdehyde (MDA), was not different between diets (Hansen et al 2009). Furthermore, Nchito *et al*. showed that African schoolchildren had increased small intestinal permeability after being orally supplemented with iron, as indicated by an increased lactulose:mannitol ratio (Nchito et al 2006). This increased permeability may allow easier translocation of pathogenic bacteria across the gut wall. It has also been shown that oral iron tablets, due to iron deposition, can cause pathological changes in the mucosa of the upper gastrointestinal tract (Kaye et al 2008). Notably, the studies mentioned above were either focused on the small intestine, or their results were based on *in vitro* data. It is therefore less clear what the effects of iron on the colonic epithelium are *in vivo*. In the colonic lumen, oxygen is hardly available, but close to the epithelium and in the mucous layers there is an oxygen tension, resulting from diffusion from epithelial cells (Van den Abbeele et al 2011). This oxygen may contribute to iron generated radical formation. Indeed, it has been shown that oral iron supplementation can increase lipid peroxidation in the colon of rats, which may have affected epithelial integrity and which may contribute to the development of colorectal carcinoma via the induction of DNA damage (Lund et al 2001). These authors also reported that oral iron supplementation increased the free radical production in the faeces of human healthy volunteers (Lund et al 1999). Also, epidemiological and animal studies may point to a role of luminal iron in colorectal carcinogenesis (Chua et al 2010, Seril et al 2002). As elaborated on in section 8.1 of this review, nutritional iron may be related to the bacterial production of short chain fatty acids, which lower the pH of the intestinal environment. Remarkably, a lower pH is associated with a reduction in the conversion of bile acids to secondary bile acids, which are thought to exert harmful effects to the colonic wall (Boleij and Tjalsma 2012, Macdonald et al 1978). This conversion may also be influenced by iron availability as bacteria use iron-dependent oxidoreductases for this process (Kang et al 2008). To finish this section, also the bacteria may suffer from iron-mediated oxidation stress in the colon lumen. However, oxygen tension is very low in the outer mucous layer and the lumen where the majority of the microbes reside, thus impeding the formation of oxidative stress (Van den Abbeele et al 2011).

6.3 Effect of host iron status on gut immunology and defence

Iron plays an important role in host immunity and host iron status is therefore likely to influence host immunity (**Figure 6**). First of all, as discussed above, iron withholding is an important innate defence mechanism against microbial invaders. Iron is also required by

phagocytic cells to generate the oxidative burst with ROS and reactive nitrogen species to kill the invaders (Cairo et al 2006).
Both iron deficiency and iron overload are associated with impaired immune responses (Weiss 2005). Patients with iron overload (haemochromatosis) can experience oxidative damage to cells and organs, but they also have a higher risk of infection because of the increased availability of iron to pathogenic bacteria (Bullen et al 2005, Cassat and Skaar 2013). Another reason for the increased risk of infection is the direct effect of iron on the immune system as it has been shown that *hfe* knockout mice (haemochromatosis mice) showed an attenuated intestinal inflammatory response, probably due to a reduced production of TNF-α and IL-6 by macrophages when they were stimulated with LPS or *S.* Typhimurium (Cherayil 2010, Wang et al 2008). The production of these pro-inflammatory molecules could be enhanced by pre-incubation of the isolated macrophages with hepcidin, which blocks the iron exporter ferroportin, suggesting that the impaired response is mediated by low hepcidin levels in haemochromatosis mice. It was also reviewed by Wang & Cherayil that alterations in intracellular and extracellular iron concentrations can influence the innate immune response by influencing host gene expression (Wang and Cherayil 2009). Iron-loaded macrophages may fail to kill intracellular pathogens due to a reduced formation of nitric oxide, because iron inhibits Interferon-γ (IFN-γ) mediated pathways (Weiss 2005). In summary, iron levels need to be in good balance to maintain optimal immunity. Too much iron can provide an easy accessible source of iron for pathogenic bacteria and can reduce the immune response to infection, whereas iron deficiency can strengthen nutritional immunity against pathogenic invaders but which can also reduce the effectiveness of immune cells (Weiss 2005).

As host iron status can influence inflammatory pathways, also the intestinal innate mucosal defences are likely to be affected. One interesting aspect is the specific array of antimicrobial defences in the intestine. Enterocytes and Paneth cells secrete antimicrobial peptides (AMPs) such as defensins, cathelicidins, and other AMPs that confront the gut microbiota to another stress factor (Muniz et al 2012). Though little is known about the mechanism of regulation of their expression, AMPs show basal expression levels and they are increasingly expressed in response to inflammatory stimuli. The main site of expression is the small intestine, but to a less extent AMPs are also expressed in the colon, where they play a role in restraining the gut microbiota (Muniz et al 2012). As inflammation upregulates AMP expression, iron could provisionally influence expression levels of AMPs. Future research is needed to further elucidate AMP expression pathways and the putative effects of iron on colonic AMP pathways. One molecule with antimicrobial properties that plays a role in the gastrointestinal tract is indoleamine 2,3-dioxygenase 1 (IDO1), a molecule that starves tryptophan from microbes (Muller et al 2009). IDO1 is expressed in high amounts by macrophages and dendritic cells and its expression can be upregulated by IFNs (Cherayil 2009). Iron overload could therefore reduce IDO1 expression in the gut, as the expression of IFNs is reduced in iron-loaded macrophages (Weiss 2005). Another important molecule with antimicrobial activity concerns lipocalin-2, as discussed in section 9.1.1. We found that both mice on an iron-deficient diet and mice on a high-iron supplemented diet showed a decrease in faecal lipocalin-2 levels

during intestinal inflammation, which may have resulted in an impaired host defence against siderophilic pathogens in these mice (**Chapter 4**). The latter observation suggests that extreme (low and high) iron conditions result in an impaired intestinal host defence, but more research is needed to confirm this hypothesis.

6.4 Effect of host iron status on gut microbiota composition

As we will discuss in section 7, dietary iron can alter the gut microbiota composition. In addition, evidence accumulates that also host iron metabolism or iron status, possibly via effects on host immunity or altered luminal iron uptake, can influence the gut microbiota composition. Vice versa, the gut microbiota may influence host iron uptake, and thereby the host iron status. The former was exemplified by Buhnik-Rosenblau et al. who showed that deletions of genes involved in iron homeostasis altered the gut microbiota of the mutant mice. Interestingly, a *hfe* deletion did not alter faecal iron content or the content of other common minerals, but a *Irp2* (Iron regulatory protein 2) deletion caused an increase in faecal iron levels and other minerals and electrolytes, due to an altered uptake by intestinal epithelial cells. Although they showed that the gut microbiome of the mutant mice was not significantly different from the wildtype, the *hfe* mutant mice showed a significantly different microbiome compared to the *Irp2* mutant mice, possibly due to the greater genetic diversity between the mutant groups than between the single mutants and the wildtype (Buhnik-Rosenblau et al 2012). In a study with pregnant women, increased faecal *Enterobacteriaceae* and *E. coli* numbers, in combination with lower *Bifidobacteriaceae* numbers, correlated with an increase in serum ferritin and in transferrin saturation levels. Although, dietary intake was similar among the women of that study, an effect of dietary iron cannot completely be excluded as its intake was not assessed in this study (Santacruz et al 2010). In another study it has been shown that Indian women with iron deficiency anaemia had lower levels of *Lactobacillaceae* in their faeces, despite a similar dietary iron intake compared to non-anaemic individuals (Balamurugan et al 2010). One limitation of the latter study is that faecal iron levels were not determined and also a limited selection of *Lactobacillaceae* and other bacterial species were assessed. Besides potential differences in faecal iron due to differential duodenal uptake, another possible explanation for the lower abundance of *Lactobacillaceae* in the anaemic women is the potential role of colonic iron absorption (see section 6.1). *Lactobacillaceae* contribute to gut fermentation in a positive way and a decrease in their abundance could therefore be the reason for reduced colonic iron uptake, or it could even exert a more distant effect on duodenal absorption (Balamurugan et al 2010). Of note, modulation of the gut microbiota by prebiotics has been associated with increased iron uptake in rats and pigs and in the latter species also with an increase in colonic *Lactobacillaceae* (Freitas Kde et al 2012, Tako et al 2008). This supports a role of the microbiota in iron absorption, however as far as we are aware, in humans this effect has not been confirmed (yet) (Petry et al 2012).

7 Effect of nutritional iron on the gut microbiota composition

The number of studies investigating the effect of diets on the mammalian gut microbiota has increased greatly during the past years. However, studies investigating the effect of nutritional

iron are relatively limited. So far, *in vivo* (humans and animal models) and *in vitro* studies have reported various outcomes regarding the influence of iron administration or iron deficiency on the composition of the gut microbiota, however some findings can be marked as consistent, as summarised in the following paragraphs and in **Table 1 and Supplementary Table 3**.

7.1 Human studies

It was recently shown that iron fortification resulted in a shift towards a potentially more pathogenic gut microbiota profile (i.e. increased relative abundance of potential pathogenic *Enterobacteriaceae* and a decrease of beneficial *Lactobacillaceae*) among school children in Ivory Coast (Zimmermann et al 2010). Notably, although not statistically significant, numbers of *Salmonella* spp. tended to be higher in the iron-fortification group. The increase in the abundance of faecal *Enterobacteriaceae* correlated with the increase in faecal calprotectin, which is a marker of gut inflammation. Very recently, similar effects were found in a study among Kenyan infants. Iron fortification increased the relative abundance of *Enterobacteriaceae*, among which were pathogenic *E. coli* species, and decreased the relative abundance of *Bifidobacteriaceae*, which also included a higher ratio of *Enterobacteriaceae* to *Lactobacillaceae*. Also in this study faecal calprotectin was elevated in infants receiving iron fortificant compared to the control group (Jaeggi et al 2014) (**Chapter 2**). Earlier studies showed by using culture techniques that an iron fortified cow's milk preparation did not result in differential *Enterobacteriaceae* (other than *E. coli*) and *Lactobacillaceae* counts in neonatal (Dutch infants, first 3 months of life) gut microbiota, but isolation frequencies of *Bacteroides* spp. and *E. coli* were higher and isolation frequencies of *Bifidobacteriaceae* were lower under these conditions (Mevissen-Verhage et al 1985). Other bottle fed UK infants which received milk complemented with lactoferrin or lactoferrin plus iron, showed also increased numbers of *E. coli* in the iron group, but no effect on *Bifidobacteriaceae*, *Lactobacillaceae, Bacteroides* and *Clostridia* were found (Balmer et al 1989).

In a later study with iron fortified formulas (without lactoferrin fortification) the same investigators found an increase in Clostridia and a decrease in *Bacteroides*, with no effect on *E. coli*, coliforms, *Bifidobacteriaceae* and *Lactobacillaceae* counts in faeces of UK infants (Balmer and Wharton 1991). This paragraph is finished with the interesting findings from a study with US infants in which an iron fortified diet was compared to a different meat diet that contained much less iron. Although the different types of diet may have influenced or contributed to differences in the gut microbiota composition, the iron-fortified diet was associated with a decrease in *Bifidobacteriaceae* and *Lactobacillaceae* and an increase in Bacteroidetes. Remarkably, when zinc was added to the iron fortified diet the microbiome responded similar to the meat diet, which contained similar amounts of zinc compared to the iron-zinc-fortified diet (Krebs et al 2013). Notably, zinc has been associated with reduced virulence of enteropathogens in the gut via the induction of bacterial envelope stress (Crane et al 2011, Mellies et al 2012).

7.2 Animal studies

A study with weanling pigs receiving an iron-supplemented diet again reported higher counts of coliform bacteria, and found no effect on the populations of total anaerobic bacteria,

Bifidobacteriaceae, *Lactobacillaceae*, and Clostridia (Lee et al 2008). In two independent mice studies it was found that healthy mice receiving an iron-deficient diet had increased *Lactobacillaceae* numbers compared to the control group (Tompkins et al 2001, Werner et al 2011). Further, Tompkins *et al.* found higher numbers of total anaerobes and *Enterococcus* spp. in mice fed on the iron-deficient diet, while Werner *et al.* found increased numbers of *Bifidobacteriaceae*, *Clostridium* and unclassified *Prevotellaceae*, and decreased numbers of *Bacteroides*. *Enterobacteriaceae* counts were apparently not affected (not reported), but the parent taxon Proteobacteria appeared to be more abundant in the iron group (Werner et al 2011).

In rats, depending on iron dose and time point, several effects were found. There was an increase in Clostridium difficile enterotoxin (only after 24 hours) and at 2 weeks an increase in *E. coli, Lactobacillaceae* and *Clostridium* spp. was found, while *Lactobacillaceae* abundance was not different at 4 weeks and *Clostridium* spp. were again more abundant at 4 weeks (high iron dose only). The latter increase was accompanied by a decrease in enterococci and *Bacteroides* spp. (Benoni et al 1993). A recent study with young rats showed that an iron-deficient diet increased both *Enterobacteriaceae* and *Lactobacillaceae* numbers, while *Bacteroides* spp. and *Roseburia* spp./*Eubacterium* rectale numbers decreased. Subsequent iron repletion did not restore *Roseburia* spp./*E. rectale* numbers, but *Lactobacillaceae* and *Enterobacteriaceae* populations got back to their baseline levels (Dostal et al 2012a). In a very recent study of Dostal *et al.* various iron doses caused rather disperse effects on the abundance of the most dominant bacterial taxa in rats with a humanised gut microbiota (Dostal et al 2014b). In another recent study with rats and mice in which only Bacteroidetes, *Enterobacteriaceae* and Firmicutes were assessed, no effect of ferric iron on these tested gut microbes was found in non-colitis animals. These findings could indicate that ferric iron has less effect on the gut microbiota, but two important limitations of this study were the limited selection of tested taxa and the lack of testing the effects of the ferrous iron control on those taxa (Ettreiki et al 2012).

7.3 *In vitro* studies

The *in vivo* studies described above illustrate that the effects of oral iron supplementation or an iron-deficient diet, leading to high and low amounts of colonic luminal iron respectively, can be various and contradictive. Host factors such as iron status, intestinal immune function, dietary habits and also environmental factors such as hygiene play probably an important role and result in a large variation in the effects of iron on the gut microbiota composition. The effect of such host factors can be better controlled in *in vitro* gut fermentation models, allowing to study the effect of sole iron on the gut microbiota. Obviously the lack of host immunity factors is also a drawback of *in vitro* models. Nevertheless, they are valuable tools for monitoring microbial metabolism during the passage of food. A recent *in vitro* study by Dostal et al. reported an increase in *Lactobacillaceae, Enterobacteriaceae* and *Roseburia* spp./*E. rectale* under low-iron conditions, which was consistent with the outcome of their study in young rats (Dostal et al 2012a, Dostal et al 2012b). Notably, when *S.* Typhimurium was added to this fermentation model, it grew unexpectedly less well under high-iron conditions compared to normal-iron conditions (Dostal et al 2012b). A possible explanation

for this is the absence of host-mediated inflammation on which *S.* Typhimurium can thrive (see section 9.1). In contrast to the rat study, *Bacteroides* spp. increased under low-iron conditions, except for extremely low-iron conditions were a decrease was found. Further, in a study were silage making was simulated it was found that extreme iron depletion by 2,2-dipyridyl had no effect on growth and performance of *Lactobacillaceae*, but repressed the growth of *Enterobacteriaceae* effectively (Bruyneel et al 1990). Recently, we found, using an *in vitro* model that simulates the luminal conditions of the healthy adult proximal colon, that prominent changes were a relative decrease of *Lactobacillaceae* and *Bifidobacteriaceae* and an increase of *Roseburia* and *Prevotellaceae* in iron-rich conditions (Kortman et al 2014b) (**Chapter 3**).

7.4 Summary of the effects of nutritional iron on the gut microbiota composition

From the sections above it becomes clear that oral iron intake can influence the gut microbiota composition, but we note that the reported effects are markedly different from study to study. We also note that infants and young children belong to the population that most often requires oral iron therapy and that altering the gut microbiota composition may have a relative large impact on the developing microbiota of infants that do not yet have developed a stable core microbiota (Yatsunenko et al 2012). However, the potential long term effects of such iron therapy-induced changes are currently not known. The distinct outcomes of the studies described above can originate from many factors. In *in vivo* studies differential results may first of all very well be caused by the host, as the gut microbiota of mice, rats and humans are distinct and are likely to respond differently to iron intervention. The site of sampling of the gut microbiota also varied, as some animals were sampled in their gut (e.g. caecum) after sacrifice, while most studies used samples from excreted stools. Also, the iron source (formulation), dose of iron and length of iron intervention was not the same for all studies (**Supplementary Table 3**). Next, there were studies comparing iron-deficient diets with diets containing normal amounts of iron on the one hand and studies comparing normal iron diets with iron-supplemented/fortified diets on the other hand. Obviously, *in vitro* studies may also result in different outcomes, compared to *in vivo* studies, but it has some advantages like the easy testing of various iron sources and concentrations in a highly controlled setting. It should also be mentioned that analysis techniques have changed tremendously during the last decades. Hence, we here compared older studies that used culture techniques, studies that used qPCR (consequently, the various studies did not assess the effect of iron on exact the same set of taxa) and recent studies that investigated the entire population, i.e. the most abundant genera, by using 16S rRNA pyrosequencing. Importantly, the studies differed in age of the human subjects and animals and they resided in dispersed geographic locations. Hence, the intrinsic gut microbiota might be very different at the start of the study and the environmental influence will be diverse. The geographic location might be of particular importance as the prevalence of iron deficiency and infections is generally higher, and hygiene standards are often lower in developing countries compared to industrialised countries. This is probably the reason why adverse effects of oral iron administration are more prevalent in developing countries. Importantly, two recent studies showed that both African children and infants receiving iron had higher

levels of potentially pathogenic *Enterobacteriaceae* and/or higher levels of pathogenic *E. coli*, which was paralleled lower levels of *Lactobacillaceae* and/or *Bifidobacteriaceae* (Jaeggi et al 2014, Zimmermann et al 2010) (**Chapter 2**). Notwithstanding the difference between studies and their outcomes, this also appears to be the most consistent and robust outcome of the other studies (comparing iron-sufficient or high-iron diets to low or iron-sufficient diets respectively) as mentioned above and as depicted in **Table 1.** *E. coli* was mostly increased and was never reported to decrease, lactobacilli were reported to increase only once and shows basically a net decrease, while bifidobacteria mostly decreased and never increased. In our view the most relevant outcomes are from the two studies among African children/infants (Jaeggi et al 2014, Zimmermann et al 2010) (**Chapter 2**), which are overall supported by studies among Western infants as well as animal or *in vitro* studies. It should also be noted that from a clinical point-of-view, it is most relevant to study the effects of oral iron administration among children in developing countries, but that those studies are not easy to set up and control. In contrast, studies among subjects in industrialised countries and especially studies with animal and **in vitro** models can be controlled much better, but are inferior with respect to clinical relevance. It is therefore a future challenge to design the experimental setup of the latter studies in such a way that they better resemble the clinically relevant environmental setting.

It is tempting to find explanations for the hitherto consensus outcome of iron on the gut microbiota composition as described above. The fact that *Lactobacillaceae* do not require iron (see section 3.2) could be an explanation for their decrease within a microbial community in response to iron, as they might be outcompeted by bacterial species that thrive on the extra iron, e.g. enteropathogens. We note that in older studies *Enterobacteriaceae* as a family were often not assessed, but an increase in coliforms and/or *E. coli* may consequently favour the growth and colonisation of closely related (pathogenic) *Enterobacteriaceae*, a phenomenon known as the like-will-to-like concept postulated by Stecher *et al.* (Stecher et al 2010). The common decrease in *Bifidobacteriaceae* through iron is more difficult to explain as they are known to be dependent on iron as their growth is generally impaired in response to iron limitation, created by the addition of the iron chelator dipyridyl (Bezkorovainy et al 1996, Cronin et al 2012). This growth impairment in response to dipyridyl indicates the absence of a high affinity siderophore mediated iron uptake system in most *Bifidobacterium* species, but it also indicates that they require iron for their growth. The common decrease of *Bifidobacteriaceae* in response to luminal iron could therefore be due to an altered balance in competition with other microbes or an effect in response to a changed environment such as the presence of stressful metabolites or oxidative stress.

8 The effect of nutritional iron on bacterial metabolism in the gut

Next to microbiota composition, nutritional iron will affect metabolic pathways within the gut microbiota. In the following paragraphs we review the current knowledge on the bacterial fatty acid synthesis and the subsequent effect on virulence of pathogenic bacteria.

8.1 Effect of iron on the production of health promoting Short Chain Fatty Acids

Bacterial metabolism is important for our gut health because indigestible food components (mainly complex carbohydrates) are predominantly metabolized to the main Short Chain Fatty Acids (SCFAs) acetate, propionate and butyrate. These SCFAs are the principal metabolites in the colon, very beneficial for gut health and an energy source for enterocytes and more distant tissues (Macfarlane and Macfarlane 2011, Macfarlane and Macfarlane

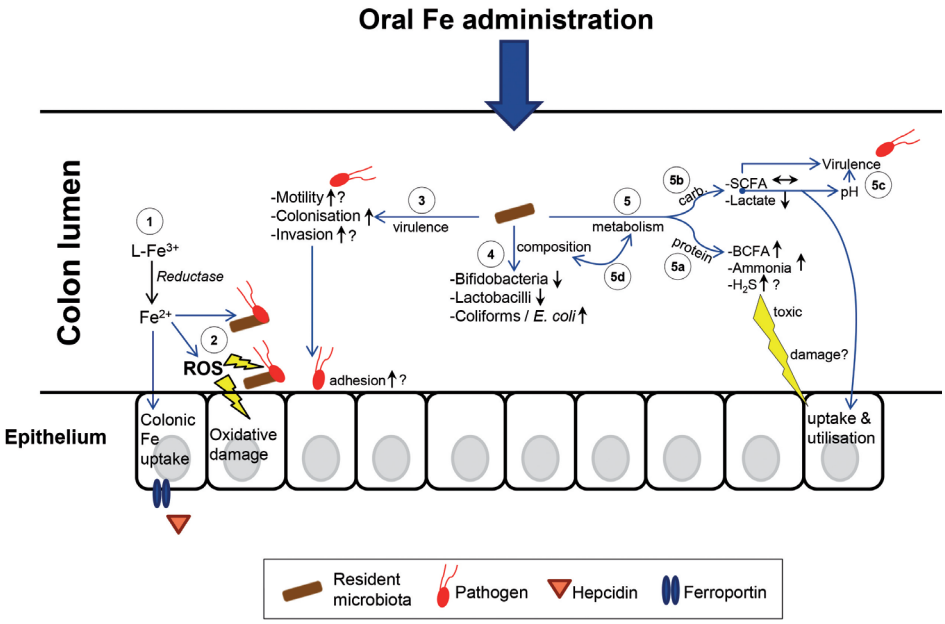


Figure 4. Potential effects of oral iron administration on the microbiota in the non-inflamed colon.

Oral iron administration:

1) Primarily increases the amount of iron in the colon, which may consist of both ferric and ferrous forms, available for uptake by the microbiota and the host (depicted on the left);
2) Can induce the formation of ROS close to the epithelium, which may elicit damage to the epithelium and stress to the local microbiota;
3) May enhance the virulence of enteric pathogens, favouring colonisation and possibly the ability to adhere to and invade the epithelium;
4) Will alter the gut microbiota composition, which mostly involves a decrease in *Bifidobacteriaceae*, *Lactobacillaceae* and an increase in coliforms / *E. coli*;
5) Likely influences gut microbial metabolism (directly and/or via an altered microbial composition), which likely increases protein fermentation, resulting in the formation of toxic products (**5a**) and which may also affect beneficial carbohydrate (carb.) metabolism (**5b**) It is known that iron limitation may lower SCFA production and increase lactate production, which in turn can influence virulence of enteric pathogens either directly or via a change in luminal pH (**5c**). Notably, a changed microbial metabolism may influence the gut microbiota composition (**5d**).

2012). The connection between iron and SCFA production was first described in two studies of Dostal *et al.* (Dostal et al 2012a, Dostal et al 2012b) (see also **Figure 4**). Specifically, the *in vivo* levels of butyrate and propionate were lower during luminal iron deficiency in rats, and were restored by iron repletion. Although it cannot be excluded that intestinal SCFA uptake was altered by the dietary interventions, these results suggested that iron supplementation could have a beneficial effect on gut health by increasing SCFA production. In contrast, however, *in vitro* SCFA production seemed not much stimulated by high iron conditions, and butyrate and propionate production was most clearly impaired during extremely low iron conditions. This decreased production was accompanied by a decrease in the SCFA-producing species *Roseburia* spp./ *E. rectale* and *Clostridium* Cluster IV members (Dostal et al 2012b). Our *in vitro* fermentation studies showed that supplementary iron increased propionate levels slightly, but that total SCFA levels were not significantly changed (Kortman et al 2014b) (**Chapter 3**). A third (and very recent) study of Dostal *et al.* showed reduced caecal levels of acetate, propionate and butyrate in rats on an iron-deficient diet compared to rats on iron-supplemented diets. They additionally showed that propionate and butyrate levels were higher in rats on an iron-supplemented diet compared to rats on a control diet (and which were not first depleted from iron) (Dostal et al 2014b). It can thus be hypothesized that an iron-deficient diet (in combination with iron deficiency) may be unbeneficial for gut health because luminal SCFA levels can decrease under these conditions. Conversely, iron supplementation may increase health promoting luminal SCFA levels. Notably, enteric pathogens might benefit from a decrease in luminal SCFA levels, as SCFAs may influence their virulence (described in section 8.2). A decrease in butyrate may also decrease the intestinal expression of the AMP cathelicidin, and hereby weaken the host defence (Raqib et al 2006, Sekirov and Finlay 2009).

A decrease in SCFA production under (extremely) low iron conditions can, besides changes in microbiota composition, possibly be explained by inhibition of iron-dependent enzymes involved in the pathways of butyrate and acetate synthesis. Approximately one third of acetate production can be assigned to the reductive acetyl-CoA-pathway (a.k.a. Wood-Ljungdahl pathway) (Miller and Wolin 1996). This pathway contains several enzymes that depend on iron as a cofactor and acetate production may therefore decrease when iron availability is limited (Dostal et al 2012b, Ragsdale and Pierce 2008). Similarly, butyrate production also involves iron-dependent enzymes and may thus be lowered during iron limitation, which could also involve a reduction in acetate to butyrate conversion by certain species (De Vuyst and Leroy 2011). More (*in vivo*) research is needed to elucidate the effects of iron on the SCFA production by the gut microbiota, the metabolic pathways involved and the possible effects on gut health. One limitation may however be the rapid absorption of SCFAs and other bacterial metabolites by the host (Montagne et al 2003). Caecal, colonic, or faecal SCFA levels do therefore not necessarily reflect the actual production by the gut microbiota, and which also differs along the intestinal tract (Mortensen and Clausen 1996). This makes complementary *in vitro* studies very useful. Interestingly, African children had much higher faecal SCFA levels compared to European children, thus microbial production appeared to exceed absorption rates in these African children. This difference was likely

due to the plant-based diets of the African children, which may result in an enrichment of beneficial SCFA producing bacteria (De Filippo et al 2010) (see also section 2.2), but it should also be realized that the poorer health status of the African children may have compromised their capacity to absorb SCFA, which also would result in higher faecal SCFA levels.

8.2 Short Chain Fatty Acids and bacterial virulence

One of the health effects described to SCFAs relies in the lowering of the luminal pH, which is known to inhibit the growth of (pathogenic) *E. coli* (Duncan et al 2009), but also direct effects of SCFAs may cause growth inhibition of *E. coli* and *Salmonella* spp. (Ricke 2003, Topping and Clifton 2001). Notably, SCFAs and/or lowering of the pH may contribute to an increased solubility of luminal iron and other minerals (Freitas Kde et al 2012, Salovaara et al 2003). This in turn may enhance host mineral absorption, but also bacterial mineral uptake, from which both the host and bacteria could benefit. Another beneficial effect of SCFAs from the host' perspective can be an increased host resistance against pathogenic *E. coli* infection through acetate production of for example *Bifidobacteriaceae* and decreased virulence of pathogens (Fukuda et al 2011). In addition, enteric pathogens have to deal with the stress of butyrate and propionate as they have been reported to down-regulate invasion genes (*HilA, HilD, invF, SipC*) of *Salmonella* pathogenicity island 1 (SPI-1) (Van Immerseel et al 2006). Notably, *HilA, invF* and *SipC* expression is also regulated by oxygen, pH and the iron metabolism regulator Fur (ferric-uptake regulator protein), resulting in upregulation of *HilA* under iron sufficiency (Teixido et al 2011, Troxell et al 2011). Low oxygen is also a signal for upregulation, but an acidic pH is associated with a down-regulation (Bajaj et al 1996, Teixido et al 2011). In contrast to the downregulation of invasion genes by SCFAs, also upregulation of a virulence factor, i.e. the *iha* gene (involved in adhesion), in pathogenic *E. coli* has been reported. This SCFA-induced regulation appeared to be independent of iron (Herold et al 2009). It has also been shown that SCFAs can stimulate *S.* Typhimurium adhesion and invasion, via the induction of the acid tolerance system in response to the stress of a lower pH, however the lowering of virulence by SCFA appear to prevail (Ricke 2003, Van Immerseel et al 2006)(see also **Figure 4**).

8.3 Effect of iron on the production of potentially toxic Branched Chain Fatty Acids

In contrast to carbohydrate fermentation, protein fermentation by gut bacteria can result in toxic or potentially toxic metabolites such as ammonia, H2S, Branched Chain Fatty Acids (BCFAs) (e.g. isobutyrate and isovalerate), indolic and phenolic compounds (Macfarlane and Macfarlane 2012).

Dostal *et. al*. showed that the production of BCFAs was decreased under low-iron conditions and increased under high-iron condition *in vitro*, indicating decreased and increased unbeneficial protein fermentation respectively (Dostal et al 2012b). This is in line with our *in vitro* findings that iron increased the production of BCFAs, and also toxic ammonia production, by an adult faecal microbiota (Kortman et al 2014b) (**Chapter 3**). Notably, BCFAs and ammonia are considered to be indicators of protein fermentation (Hoyles and

Wallace 2010) and these *in vitro* studies therefore indicate that iron stimulates protein fermentation, which may result in a more putrefactive and potentially toxic or carcinogenic environment (Macfarlane and Macfarlane 2012, Nyangale et al 2012)(**Figure 4**). In contrast, lactate (mostly derived from carbohydrate) levels have been found to decrease in response to iron in *in vitro* fermentation studies (Dostal et al 2012b, Kortman et al 2014b) (**Chapter 3**). Notwithstanding the evidence for the toxicity of products originated from protein fermentation shown in multiple *in vitro* studies, we need to note that evidence for the *in vivo* toxicity is limited and a recent trial did not support a role of protein fermentation in human gut toxicity (Windey et al 2012a, Windey et al 2012b). On the other hand, protein fermentation is often associated with the growth of pathogenic bacteria (Rist et al 2013). Future *in vivo* research on the effect of iron on protein fermentation and toxicity is therefore warranted. Finally, it is important to realize that the effects of microbial metabolites are not limited to the gut as they are taken up and can have effects on distant sites and on systemic host metabolism (Mortensen and Clausen 1996, Nicholson et al 2012, Zheng et al 2011). We do not yet know how this affects human health and disease, but it implicates that iron-induced changes of gut microbial activity may also have systemic effects.

9 How pathogens can thrive on the altered nutritional conditions during intestinal inflammation

9.1 Life in the inflamed intestine

It is currently well known that intestinal inflammation can cause dysbiosis of the gut microbiota and that this often results in an overgrowth of enteric pathogens (Stecher and Hardt 2008, Winter et al 2013a) (**Figure 5**). Pathogens can overcome the intestinal barrier and eventually cause intestinal inflammation when they get the opportunity to breach the colonisation resistance of beneficial resident strains (Kaiser and Hardt 2011, Lawley and Walker 2013). This may for example occur after antibiotic treatment that disrupts this resident microbiota (Stecher and Hardt 2008). Paradoxically, also host defence systems can contribute to dysbiosis at the site of infection. The secretion of AMPs and the production of reactive oxygen and nitrogen intermediates will also affect the non-pathogenic resident microbiota. Their susceptibility to radical species may even be enhanced by host-mediated sequestration of zinc and manganese that are required for members of the SOD family (that can neutralize these radicals); a process part of the nutritional immunity (Diaz-Ochoa et al 2014, Kehl-Fie et al 2011). Once inflammation is established, enteric pathogens can exploit this inflammation and outgrow the resident microbiota (Barman et al 2008, Lawley et al 2008, Lupp et al 2007, Stecher et al 2007, Winter et al 2010). First, some general mechanisms adopted by pathogens to achieve this will be discussed in the next paragraphs followed by paragraphs that describe the potential role of iron in such processes.

9.1.1 Evasion of nutritional immunity

One example of a pathogen that benefits from an inflamed gut is *S.* Typhimurium. A first step of *S.* Typhimurium infection is the invasion of non-phagocytic intestinal cells by injecting effector proteins with the Type-three secretion system encoded by the Salmonella

pathogenicity island (SPI)-1 (T3SS-1) (Moest and Meresse 2013). Secondly, other effector proteins secreted by a second T3SS encoded on SPI-2 contribute to *S.* Typhimurium replication in macrophages (Figueira and Holden 2012). Both T3SS are important for the initiation of the inflammatory diarrhoea (Hapfelmeier et al 2005, Tsolis et al 1999), however, the amplification of the host response requires the activation of the resident T cells (mainly Th17 cells) to produce cytokines like IL-17 and IL-22, mediated by the macrophage cytokine IL-23 (Godinez et al 2008, Godinez et al 2009). In turn, IL-17 and IL-22 stimulate intestinal epithelial cells to secrete neutrophil chemoattractants (e.g. CXCL-1, CXCL-8) and antimicrobial proteins including lipocalin-2 (Behnsen et al 2014, Raffatellu et al 2009). Moreover, the neutrophils recruited to the gut also secrete large amounts of antimicrobial proteins, including lactoferrin, calprotectin and lipocalin-2 (Blaschitz and Raffatellu 2010) (**Figure 5**). The goal of this host response is to limit micronutrient availability to pathogenic bacteria, however, also non-pathogenic resident strains have to face this armour of host defence. Pathogenic strains that express a lactoferrin receptor or can "steal" iron from lactoferrin by the use of siderophores have a growth advantage over strains unable to do this (Andrews et al 2003). Moreover, as lipocalin-2 sequesters the iron-laden siderophore enterobactin, bacterial pathogens that evade this response by secreting stealth siderophores such as salmochelin, have a competitive advantage for colonising the inflamed gut (Bachman et al 2011, Raffatellu et al 2009) (**Figure 5**). Furthermore, *S.* Typhimurium expresses a high affinity zinc transporter (ZnuABC) with which it can evade the sequestering of zinc by host calprotectin (Liu et al 2012). In addition to the evasion of nutritional immunity, other mechanisms that allow *S.* Typhimurium to thrive in the inflamed gut have been discovered and are highlighted below.

9.1.2 Utilisation of alternative respiration molecules by pathogens

In 2010, Winter *et al.* discovered that *S.* Typhimurium can thrive on tetrathionate, a molecule formed in the mouse gut during inflammation (Winter et al 2010). The formation of tetrathionate starts at the site of the gut microbiota, but involves an important host factor: protein fermentation by the gut microbiota produces H_2S, which is converted to thiosulphate by the intestinal mucosa. It was postulated that thiosulphate can be converted to tetrathionate via the reaction of thiosulphate with reactive oxygen species, the latter being formed by the host in the inflammatory process. The newly formed tetrathionate can then be used as an electron acceptor by *S.* Typhimurium via the action of tetrathionate reductase (**Figure 5**). In addition to *S.* Typhimurium, several other pathogenic members of the phylum Proteobacteria possess this rather unique virulence trait, whereas most members of the gut microbiota are not likely to respire on tetrathionate (Winter and Baumler 2011). Therefore, tetrathionate respiration in the inflamed intestine appears to provide a luminal growth advantage for these Proteobacteria.

One year later, work in the same laboratory showed that tetrathionate respiration by *S.* Typhimurium supported this pathogen's utilization of luminal host-derived ethanolamine (Thiennimitr et al 2011). Ethanolamine is a substrate that is probably not ready fermentable by competing obligate anaerobic bacteria, thus providing a growth advantage for *S.* Typhimurium (Winter et al 2013a) (**Figure 5**). Next, it was discovered that *E. coli* can respire

on nitrate, a feature likely to be present in other members of the *Enterobacteriaceae* as well (Winter et al 2013b). Like tetrathionate, luminal nitrate levels also increase during intestinal inflammation (**Figure 5**). In summary, the release of reactive nitrogen and oxygen species leads to formation of nitrate, S-oxides and N-oxides on which certain pathogens can respire by the action of nitrate reductase, DMSO reductase and TMAO reductase respectively; this is in contrast to the many obligate anaerobe members of the gut microbiome that lack these oxidoreductases (Winter et al 2013a, Winter et al 2013b). Taken together, the alternative electron acceptors formed through the oxidative burst of inflammation can stimulate the growth and colonisation of enteric pathogens such as *S.* Typhimurium, which in turn can exaggerate inflammation. Freely available luminal iron may play a significant role in these processes, which is hypothesized below.

9.1.3 Motility of S. Typhimurium during inflammation

Another mechanism contributing to the exploitation of intestinal inflammation can be found in the motility and chemotaxis. Stecher *et al.*, showed that *S.* Typhimurium defective in flagellar assembly and the ability to chemotax were only impaired in the inflamed intestine and not in the healthy intestine of mice. It was proposed that fully motile and chemotactic *S.* Typhimurium has better access to specific energy rich carbohydrates that are released from mucins during inflammation (Stecher et al 2008). We envisage that this may provisionally also apply for the access to certain iron sources in the inflamed colon, but this is subject of further investigations. Furthermore, it has been shown that *S.* Typhimurium uses chemotaxis to seek for niches in which tetrathionate and nitrate are present. The sensing of favourable energy containing sources and motility are thus important for the capability to thrive in the inflamed gut (Rivera-Chavez et al 2013).

9.1.4 The potential effect of iron on exploitation of inflammation by enteric pathogens

Iron administration programmes often take place in infection endemic regions, implicating an increased chance of iron ending up in an already inflamed gut. The mechanisms of pathogens to thrive in an inflamed gut described above inspired us to hypothesize about the possible effects of an increase in luminal iron on these pathogenic mechanisms. To the best of our knowledge this has not yet been investigated experimentally, therefore we here can only speculate on the potential effects of iron on exploitation of inflammation by enteric pathogens. These speculations add to the potential effects of iron on the gut microbiota as already discussed above, and which are summarised in **Figure 4**.

With regarding to nutritional immunity it can be envisaged that an excess of luminal iron decreases the need for the production of siderophores. Consequently, pathogens that can produce a stealth siderophore do not have an advantage over non-producers if iron is available, because siderophore production will be repressed (**Figure 5**). However, these pathogens are likely to benefit from the extra iron directly and enhance the expression of virulence proteins. Nevertheless, the resident microbiota would at least not be outcompeted via the stealth siderophore-mediated system. It should be noted,

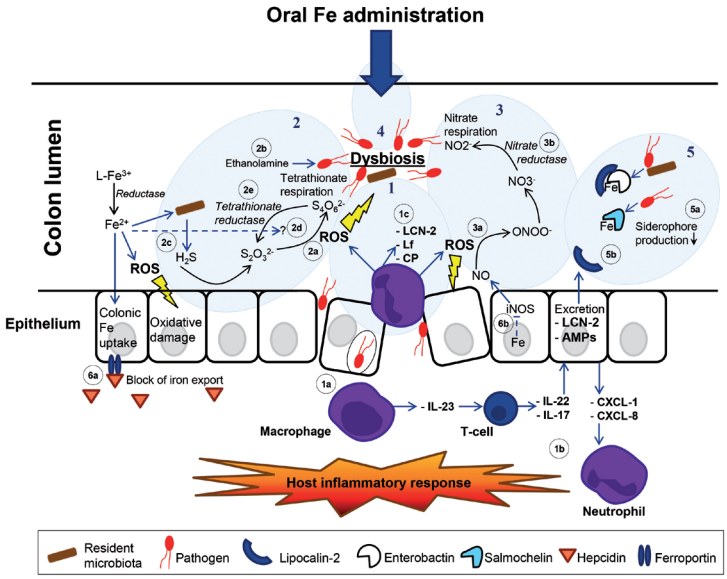


Figure 5. Pathogen exploitation of intestinal inflammation leading to dysbiosis, and the potential effects of oral iron administration.

The figure shows processes 1-5 contributing to dysbiosis, and process 6 that might be connected:

1) The inflammatory response, triggered by intestinal pathogens, activates macrophages (**1a**), which in turn stimulates T-cells to secrete IL-22 and IL-17. This triggers secretion of chemokines by the epithelium, which attract neutrophils to the site of inflammation (**1b**). Importantly, the infiltrated neutrophils release ROS into the intestinal lumen (potentially strengthened by iron) as an innate defence mechanism, together with antimicrobial proteins (AMPs) such as lipocalin-2 (LCN-2), lactoferrin (Lf) and calprotectin (CP) (**1c**).This process, and also the secretion of other AMPs by the epithelium, cause dysbiosis because pathogens like *S.* Typhimurium confer an advantage in this inflamed environment;
2) The ROS generation by the neutrophils may be enhanced by luminal iron and which in turn favours the formation of tetrathionate (**2a**) on which *S.* Typhimurium can respire, and supports the utilisation of host-derived ethanolamine (**2b**). The formation of tetrathionate may potentially also be increased by iron-stimulated microbial H2S production (**2c**). Furthermore, extra iron may directly interfere with alternative respiration by pathogens (**2d**) and favour the ubiquitous expression of tetrathionate reductase, which contains an iron-sulfur cluster (**2e**);
3) Similarly, induction of ROS possibly favours formation of the alternative respiration molecule nitrate (**3a**) and also the nitrate reductase is dependent on an iron-sulfur cluster (**3b**);
4) Extra iron (coming from the upper GI-tract) is likely to also enhance the dysbiosis process directly via its effects on gut microbiota composition;
5) The production of alternative stealth siderophores such as salmochelin normally also promotes dysbiosis, but extra iron is likely to inhibit both siderophore and stealth siderophore production (**5a**). Lipocalin-2 (LCN-2) based defence is likely not effective in the absence of siderophores (**5b**);
6) Inflammation will increase the blood hepcidin levels, which in turns binds to the cellular iron exporter ferroportin at the basolateral side of the epithelial cell. This may cause an increase in intracellular epithelial iron, providing an iron source for invading pathogens (**6a**). This iron potentially influences inducible nitric oxide synthase (iNOS) production by the epithelium, as has been shown for macrophages (Weiss, 2005) (**6b**).

however, that intestinal inflammation would still suppress the growth of Bacteroidetes and Firmicutes, while enhancing the colonization of Proteobacteria. Thus, iron administration is unlikely to increase the colonization with beneficial microbes in this environment. An unfortunate paradox is that both iron withdrawal mechanisms and iron overload may enhance the colonization of pathogens during inflammation; nevertheless, the best way to limit pathogens' growth is most likely by iron limitation because this best prevents access to the essential iron. One should also realize that a decrease or absence of siderophores would make lipocalin-2 based defence useless as it only prevents bacterial iron uptake via siderophores. It would therefore make sense that the host only expresses lipocalin-2 when siderophores are present and/or readily available iron is low. It is not yet known whether siderophores or luminal iron concentration can influence lipocalin-2 expression, but we know that this antimicrobial protein is only weakly expressed under normal conditions (Cowland and Borregaard 1997, Nielsen et al 1996, Raffatellu et al 2009).

Another argument for the benefit of iron limitation in the inflamed gut is that iron excess could possibly enhance the alternative respiration pathway via the following mechanisms because: I) Increased luminal iron is associated with increased intestinal permeability and lipid peroxidation and the respiratory burst during inflammation may be enhanced by the redox properties of iron. In this way iron may increase the production of both tetrathionate ions and nitrate, thus promote tetrathionate and nitrate respiration by enteric pathogens (**Figure 5**); II) As described (see section 8.3), extra iron can increase microbial protein fermentation, which in turns leads to increased levels of H_2S. As H_2S is a precursor of tetrathionate, this may further support growth of enteric pathogens during inflammation (**Figure 5**); III) Crucial for the alternative respiration is the expression of the oxidoreductases, which require iron-sulfur clusters (Winter and Baumler 2011); if iron acquisition by the pathogen becomes easier through iron excess, the expression of the iron-containing oxidoreductases might be unhampered. It should be noted that it is not known whether the expression of these oxidoreductases is normally limited due to difficult accessible iron (**Figure 5**); IV) Finally, the reduction of ferric iron is considered an early form of microbial respiration, by which bacteria can generate energy (Pierre et al 2002). We therefore envisage that iron itself may function as an electron donor (Fe^{2+}) or acceptor (Fe^{3+}) for certain members of the gut microbiota, but also for pathogens that might use it for alternative respiration during inflammation (**Figure 5**). This is in-line with the view that reduction of ferric iron is considered an early form of microbial respiration and by which bacteria can generate energy (Pierre et al 2002). We want to emphasize that these hypothetical mechanisms and their potential contribution to exploitation of inflammation need to be examined experimentally in both *in vitro* and *in vivo* models.

9.2 Iron and inflammatory bowel disease (IBD)

Compelling evidence that the provision of extra iron to an inflamed gut is contraindicated comes from patients with inflammatory bowel disease (IBD). These patients often get anaemia of the chronic disease (anaemia of inflammation), because iron uptake and iron homeostasis is unbalanced due to chronically increased systemic hepcidin levels (Cherayil

2010). These patients therefore require iron supplementation therapy, but caution has to be taken as oral iron has been shown to worsen the symptoms of IBD, while intravenous iron does not (Zhu et al 2010). In rats and mice where colitis was induced, it was shown that oral iron exaggerated the inflammation (Carrier et al 2006, Reifen et al 2000, Seril et al 2002). Moreover, mutant mice ($TNF^{\Delta ARE/WT}$) that develop distal ileitis remained healthy on an iron-deficient diet, also in combination with intravenous iron administration (Werner et al 2011). These studies strongly suggested that oral iron can worsen gut inflammation, which might be (partly) caused by iron-induced radical formation, but also might be a consequence of the increased growth of pathogenic microbes that thrive on iron and inflammation, and hereby might contribute to the pathogenesis of IBD. It is known that the gut microbiota of patients with IBD differs from healthy controls, often showing that enteropathogenic strains are increased in IBD patients (Boleij and Tjalsma 2012). This difference might be enlarged by iron, as Werner *et al.* showed that ferrous sulphate caused marked alterations of the mouse caecal microbiota (Werner et al 2011). Amongst other differences, mice with ileitis on a diet supplemented with ferrous sulphate showed a reduction in beneficial *Bifidobacteriaceae* and *Lactobacillaceae* and an increase in *Bacteroidaceae*, but a similar abundance of Proteobacteria, when compared to mice with ileitis on a low-iron diet without ferrous sulphate (Werner et al 2011). Remarkably, Ettreiki *et al.*, showed that juvenile treatment with ferric iron could prevent 2,4,6-trinitrobenzenesulfonic acid (TNBS)-induced colitis in mice and rats, whereas treatment with ferrous iron did not. Ferric iron could also prevent gut microbial dysbiosis, as it prevented the increase in *Enterobacteriaceae* during colitis (Ettreiki et al 2012). Results on the effects of ferrous iron on the microbiota were unfortunately not reported.

Also in human IBD patients, administration of iron intravenously is preferred over oral iron administration. A recent systematic review concluded that intravenous iron is more efficient in restoring iron status, with less discontinuation of intervention due to adverse effects, compared to oral iron (Avni et al 2013). Another recent systematic review highlighted the gastrointestinal adverse effects due to oral iron administration, among which were nausea, diarrhoea and abdominal pain (Lee et al 2012). It is therefore concluded that intravenous iron therapy is preferred for IBD patients, which is in line with the conclusion of Zhu *et al.*, a few years earlier (Zhu et al 2010). We however note that also intravenous iron therapy may not be without risk as this may induce iron overload (of reticulo-endothelial stores in patients with inflammation) (Rostoker et al 2012). Finally, a recent study concluded that iron fortification is well tolerated in patients with quiescent IBD (absence of inflammation), but is contra-indicated in patients with relapses (Tolkien et al 2013). A similar recent study confirmed the detrimental effect of oral iron administration in patients with forms of mildly active IBD (Powell et al 2013). To our knowledge, no one has investigated the effects of oral iron on the human gut microbiome during IBD yet, but it might very well be that alterations of the gut microbiota during oral iron therapy contribute to the exaggeration of intestinal inflammation. If not directly influenced by iron, certain bacterial species are likely to thrive on the inflammation, which is associated with elevated lipocalin-2 and calprotectin levels, also in IBD (Diaz-Ochoa et al 2014) (see section 9.1). Furthermore, at the site of intestinal

inflammation luminal iron may exaggerate this inflammation by catalysing the formation of toxic oxygen radicals. This scenario could promote alternative microbial respiration, but could also lead to cellular apoptosis, tissue damage and ER stress (Weiss 2011). It goes without saying that the adverse effects of oral iron in IBD patients are likely to apply for patients with acute forms of gastroenteritis as well.

10 Iron challenges of enteric invasive pathogens during the infection route

This section will specifically discuss the aspects of iron in relation to virulence traits of invasive enteric pathogens that can also cause inflammatory diarrhoea, such as *Salmonella, Shigella* and *Campylobacter* (Navaneethan and Giannella 2008), from the colonic lumen to epithelial invasion, mainly using *S.* Typhimurium as a model organism. As mentioned in section 4.2, iron availability is an important signal for the expression of virulence genes by bacterial pathogens. Although readily available iron is not likely to be present in large amounts in the colon lumen, it is probably still high compared to readily available iron after entering the host tissues, with its excellent iron withdrawal mechanisms. Herein lies the iron challenge: for full virulence invasive enteric bacteria need mechanisms to deal with the changes in iron concentrations while they simultaneously have to be able to enter the host. To establish host invasion, epithelial adhesion is a first key step and requires different virulence mechanisms compared to the actual invasion, which is the next step to an infection. Moreover, evasion of the host defences is also indispensable to the success of the pathogen.

10.1 Reaching the site of adhesion

The very first step that leads to infection may be the expression of flagella by the pathogen. Flagella enable microbes to swim in liquids and swarm over surfaces and have also been implicated in the adhesion to epithelial cells (Moens and Vanderleyden 1996). Motility is thus an important virulence factor and may be essential in the colon lumen to reach the epithelium and ultimately to invade the host. The expression of flagellum protein appears to be post-transcriptionally regulated by iron and oxidative stress, and it was proposed that iron inhibits flagellin synthesis of *S.* Typhimurium via the stabilisation of the aconitaseB (AcnB) clusters (Tang et al 2004, Tang et al 2005). In contrast, in *Campylobacter jejuni,* iron replete conditions were highly associated with the upregulation of flagellin biosynthesis genes (Butcher and Stintzi 2013). Results from our laboratory have also suggested that iron can increase the expression of flagellum protein in *S.* Typhimurium *in vitro* (Kortman, unpublished). It is thus difficult to predict how flagella synthesis responds to luminal iron, but the increased iron availability may increase the motility of pathogens in the relative iron-rich environment of the lumen in a non-inflamed environment (**Figure 4**), which would make sense in an evolutionary view from the pathogen's perspective. Furthermore, it also fits with the finding that flagella synthesis is usually downregulated inside host cells, an environment with extremely low amounts of freely available iron (Ibarra and Steele-Mortimer 2009).

10.2 Epithelial adhesion

Once the motile (and invasive) pathogen has reached the epithelium, it should adhere to the cells to be able to invade the epithelium in a second step. We found that adhesion of several enteric pathogens to intestinal epithelial cells was markedly increased by iron *in vitro* (Kortman et al 2012). A recent study confirmed this effect, but also showed that iron-induced adhesion was minimized when *S.* Typhimurium was in an environment with bacterial metabolites, originating from an *in vitro* fermentation model (Dostal et al 2014a). Other factors such as iron availability in the different media (cell culture medium vs. fermentation medium) or the deteriorating effect of fermentation medium on cultured intestinal epithelial cells, may however have contributed to those findings. *In vitro* data thus show that iron can increase bacterial adhesion (see also **Figure 4**), while in vivo studies point at an increased growth and colonisation of potentially pathogenic *Enterobactericeae in vivo* (Jaeggi et al 2014, Zimmermann et al 2010). There is however no direct evidence from *in vivo* studies that adhesion of enteric pathogens to the colon epithelium is increased by iron, thus further investigations on this issue, and on the effect of iron on molecular bacterial adhesion mechanisms, is necessary. Below we describe a few molecular bacterial adhesion mechanisms that are possibly involved.

The most commonly known adhesive molecules that many bacteria, among which is *S.* Typhimurium, express on their surface are the fimbriae, which can reversibly bind to the surface of eukaryotic cell surfaces (Wagner and Hensel 2011). Little is known about the expression of fimbriae in response to iron. A study with enterotoxigenic *E. coli* indicated that iron represses fimbriae, but it has also been shown that iron can upregulate the expression of the Fimbrial protein Z (FimZ) in *S.* Typhimurium (Bjarnason et al 2003, Karjalainen et al 1991). The large non-fimbrial protein SiiE, also expressed by *S.* Typhimurium, is another factor known to be involved in adhesion to the epithelial surface (Wagner and Hensel 2011). Not much is known about the expression of SiiE in response to iron, however its expression coincides with the expression of invasion genes and it is regulated by the same global regulator (sirA) as SiiE (Gerlach et al 2007). Remarkably, invasion genes are known to be upregulated in response to iron (discussed in section 10.3) and it is therefore likely that SiiE expression will also be increased by iron availability. Our *in vitro* experiments have indeed shown that iron can increase the expression of the SiiE-protein (Kortman GAM, unpublished). Finally, there is a potential link between iron availability and LPS-mediated adhesion. LPS is a known modulator of cellular adhesion and it has been described that iron limitation alters the LPS structure and it reduces the LPS expression in *Helicobacter pylori* (Jacques 1996, Keenan et al 2008). Furthermore, iron can up-regulate the expression of the *rfaJ* gene, which encodes UDP-D-glucose: (galactosyl) lipopolysaccharide glucosyltransferase, an enzyme involved in the LPS synthesis (Bjarnason et al 2003). These studies indicate that iron may stimulate both LPS biosynthesis and LPS-mediated adhesion.

10.3 Epithelial invasion

For invasive pathogens the next step for the establishment of an infection is the invasion of the intestinal epithelium. In *Salmonella* and *Shigella*, the largely studied type 3 secretion system (T3SS-1 and T3SS-2) is important in this step, as well as in the initial adhesion

to the epithelium. Misselwitz *et al.* showed that T3SS-1 mediates irreversible docking of *S.* Typhimurium to HeLa cells (Misselwitz et al 2011). Notably, T3SS-1 is considered an essential virulence factor in causing host infections (Que et al 2013). The expression of T3SS-1, which includes invasion genes, is regulated by the action of multiple genes and regulators. This topic will not be discussed in great detail here, but can be found elsewhere in literature (Moest and Meresse 2013, Teixido et al 2011). Importantly, one of the regulators of T3SS is Fur (described in section 8b) and activation of Fur under iron sufficiency, together with a low oxygen tension, ultimately leads to increased T3SS-1 expression (Ellermeier and Slauch 2008, Teixido et al 2011, Troxell et al 2011). Vice versa, iron limitation will lead to decreased T3SS-1 expression. Notably, iron has shown to upregulate the expression of the *invH* gene in *S.* Typhimurium, which encodes an invasion protein (Bjarnason et al 2003).

When we translate these findings to the gut, it makes sense that the T3SS-1 will be switched on when pathogens transit in the lumen of the non-inflamed gut, where iron availability is probably limited, but relatively high compared to the environment inside the host. This scenario supports the pathogen's expression of this important virulence mechanism for invading the host. After invasion, the availability of intracellular iron to pathogens is very limited, which, together with increased oxygen tension, may directly form a signal for the pathogen to switch off the T3SS-1 (Teixido et al 2011). Instead, the invaded pathogen may increase the expression of different virulence factors, such as the expression of high affinity iron uptake systems to acquire iron in the iron-limited environment of the host cell, and to switch on the T3SS-2 system to promote intracellular survival (Ibarra and Steele-Mortimer 2009, Zaharik et al 2002). Interestingly, increased iron status of enterocytes promoted the invasion of *Salmonella enteritidis* into differentiated Caco-2 cells. Thus, the increased iron status of enterocytes may also further support the invasion process (Foster et al 2001). The iron content of enterocytes might increase during inflammation because of an increase in systemic hepcidin levels, which in turn prevents iron export from cells, presumably causing the trap of iron inside enterocytes (**Figure 5**). It can be envisaged that also oral supplementation can increase enterocyte iron status, although the relevance for the colon has to be investigated further. Notably, *in vitro* experiments show that moderate (extracellular) iron levels can increase invasion of *S.* Typhimurium into cultured intestinal epithelial cells, while high iron levels can conversely decrease invasion (Dostal et al 2014a, Kortman et al 2012). Finally, once the pathogen has breached the colonic epithelium it will highly trigger the host immune response. Survival of the pathogen may partly depend on the host iron status as this can affect the local immune response and the ability to quickly clear the infection (see section 7.2).
To conclude this section, it is clear that iron availability can have a large impact on the infection cycle (involving the flagellar, fimbrial and T3SS-1 system (Saini et al 2010)) of a pathogen and that increased luminal iron, but also increased intracellular iron levels of enterocytes may exaggerate the virulence of enteric pathogens. More research is however needed to investigate these aspects into more detail.

11 Clinical relevance of nutritional iron stress

11.1 Iron deficiency in the tropics and oral iron administration programmes

As outlined above, iron is a highly abundant metal on earth and is vital for virtually all organisms (Cairo et al 2006). Despite its abundance, iron deficiency is the most prevalent nutrition disorder worldwide and especially infants and young children in the tropics are in a vulnerable age for developing iron deficiency anaemia. These children require oral iron therapy to prevent serious health consequences such as developmental impairment (De-Regil et al 2011, WHO and UNICEF 2004). It has been shown that iron deficiency can be effectively controlled by both iron supplementation and fortification programmes (Zimmermann and Hurrell 2007). Also iron-containing micronutrient powders (MNPs), which are added to food after cooking (in-home fortification), can be effective in reducing iron deficiency rates (Adu-Afarwuah et al 2008). Recent trials have, however, questioned the safety of untargeted iron supplementation and fortification in developing countries (Oppenheimer 2001, Sazawal et al 2006, Soofi et al 2013, WHO and UNICEF 2006, Zlotkin et al 2013). In 2007 the WHO advised against universal iron supplementation and fortification in malaria endemic regions with low standards of health care, considering the dramatic results of the Pemba trial of Sazawal *et al.* (Sazawal et al 2006, WHO 2007). Reported increases in infections during oral iron administration might be at least partly ascribed to bacteria that could originate from the gut and that thrive on extra iron.

11.2 Oral iron administration and morbidity

Importantly, as discussed in section 7.1 it has recently been shown in two studies that oral iron administration can shift the gut microbiota towards a more pathogenic profile (Jaeggi et al 2014, Zimmermann et al 2010) (**Chapter 2**). Although morbidity rates were not significantly higher, the increase in enteric pathogens was associated with increased gut inflammation (Zimmermann et al 2010). Notably, a systematic review of oral iron supplementation trials reported a slight increase in the risk of developing diarrhoea upon oral iron administration (Gera and Sachdev 2002). Two recent trials with the administration of iron-containing MNPs to young children have also raised concerns with regarding to diarrhoea. In Pakistan a significant increase in severe and bloody diarrhoea and respiratory disease was reported and in Ghana there was an increased number of hospitalisations that was possibly partly due to diarrhoea (Soofi et al 2013, Zlotkin et al 2013). It should be noted that these MNPs contained multiple micronutrients and vitamins next to iron.

It thus appears that oral iron increases the risk of getting diarrhoea in children. This is an important adverse effect, as diarrhoea contributed for 11% to the mortality of children < 5 years old in the WHO African region in 2010 (WHO 2013). Although iron-induced diarrhoea so far has not directly been linked to the stimulation of enteric pathogens, it is important to realize that diarrhoea is often a sign of gastrointestinal infection and that luminal iron might play a role in its aetiology (WHO and UNICEF 2009). Therefore, a prior recommendation of both the WHO and the U.S. National Institutes of Health Working group (NIH TWG) is to investigate the impact of iron preparations on the gut microbiota (WHO and UNICEF 2006).

11.3 Effects of nutritional iron on *Salmonella* infection and other intestinal pathogens

S. Typhimurium is a common cause of diarrhoea and is a major cause of gastroenteritis and invasive disease in humans worldwide. Remarkably, in tropical Africa this pathogen is often associated with severe invasive disease rather than with gastroenteritis and diarrhoea (Feasey et al 2012, Graham 2010). Furthermore, invasive disease caused by *Salmonella* often co-occurs with malaria infection as malaria appears to predispose children for systemic *Salmonella* infection, while both infections clearly have a link with host iron homeostasis (van Santen et al 2013). As pointed out above, there is a concurrent high prevalence of iron deficiency among children in those countries. These children require oral iron therapy to prevent developmental impairment (WHO and UNICEF 2004). Precariously, *S.* Typhimurium appears to be stimulated by increased iron availability in several but not all published studies, as extensively discussed above. Moreover, *S.* Typhimurium has shown to possess mechanisms to exploit inflammation and outcompete the residing microbiota as discussed in section 9.1 (Winter et al 2013a). The effect of luminal iron content and host iron status in these exploitation mechanisms are largely unknown and needs therefore more attention. So far, relatively little is known about a potential link of nutritional iron with intestinal infection caused by other enteric pathogens, but which may be important to investigate for its clinical implications. For example *Campylobacter* requires iron for successful colonisation of the host intestine and it is known that iron can induce flagella biosynthesis (an important virulence trait) in *Campylobacter jejuni*, (Feldmann et al 2007, Miller et al 2009). Moreover, certain pathogenic *E. coli* strains are known for their production and utilization of stealth siderophores, suggesting an important role of iron in their virulence (Feldmann et al 2007, Hantke et al 2003). Importantly, a recent study showed that Kenyan infants receiving iron-containing micronutrient powder had increased faecal pathogenic *E. coli* numbers compared to infants receiving micronutrient powder without iron (Jaeggi et al 2014) (**Chapter 2**).

11.4 Challenges in oral iron administration

A big challenge of oral iron administration in infection endemic regions is to increase the bioavailability of iron to the host, without enhancing the risk of (gut borne) bacterial infections. As known for years, the bioavailability (i.e. uptake) of non-haem iron including iron supplements, is low compared to haem iron in meat (Zimmermann and Hurrell 2007). Although much research has focused on finding a preparation and dose schedule with optimal absorption, the predicted uptake of (isotope-labeled) iron preparations from a standard diet in industrialized countries is only 2-18%, depending on host iron status as was recently reported by a systematic review (Collings et al 2013). Especially in geographic areas where infection pressure is high and diets are monotonously cereal- and legume-based, oral iron administration is very challenging. First of all, the cereal- and legume-based diets are commonly rich in polyphenols and phytate which bind iron (see sections 5.2.1 & 5.2.2) and therefore potent inhibitors of iron absorption and such diets can result in an absorption of iron from the diet of often less than 10%. This food matrix will also lower iron absorption from supplements of fortificants (Zimmermann and Hurrell 2007). Interestingly, although

phytate reduces host iron uptake, it may also lower iron-induced colonic lipid peroxidation during moderate to high iron intake as has been shown for pigs, which is by itself a beneficial effect (Schlemmer et al 2009).

11.5 The iron administration paradox during infection

Chronic infection can cause anaemia. Ironically, uptake of dietary and supplemented iron will in these cases be impaired during inflammation due to malaria and other infections, which are highly prevalent in tropical Africa. As pointed out above, inflammation will generally increase circulating hepcidin, which is likely to lead to reduced intestinal iron absorption. Consequently, increased amounts of unabsorbed iron will enter the colon, where it may promote the growth and virulence of enteric pathogens and also alter gut

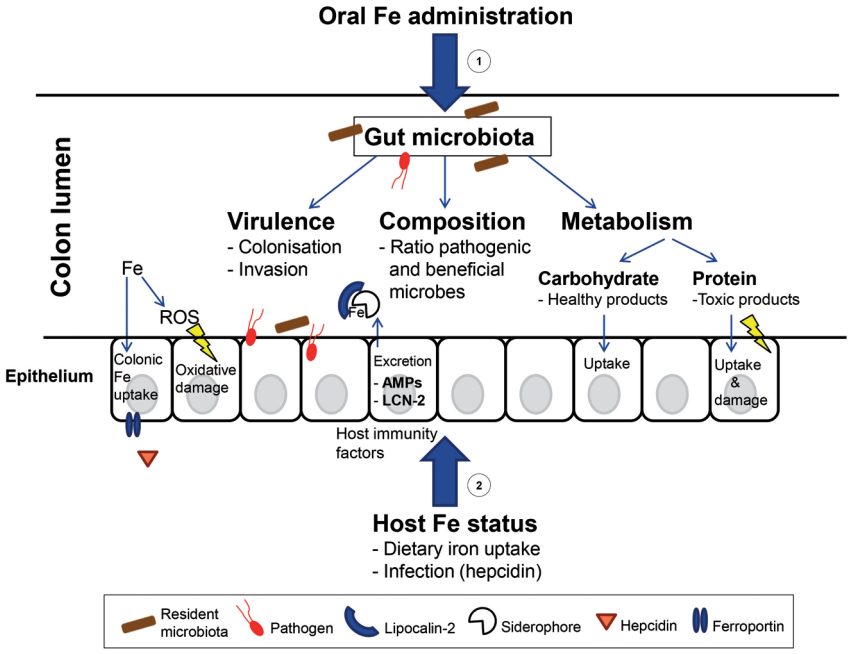


Figure 6. Schematic overview of the main gut microbial factors that are influenced by oral iron administration and host iron status.

This figure summarises the main two iron-related components that influence the gut microbiota composition, virulence and metabolism.

1) Orally administered iron can have direct impact on the gut microbiota composition (and the ratio of pathogenic bacteria to beneficial bacteria) and metabolism, and potentially on the virulence of enteric pathogens.
2) Host iron status is influenced by dietary iron uptake and infection, this in turn influences host immunity factors which can affect the gut microbiota. Intestinal inflammation (not depicted in this figure) will have a large impact on the gut microbiota (Figure 5).

microbial metabolism, especially during oral iron administration involving relative high amounts of iron (**Figure 4**).

11.6 Targeted iron to those in need

It would probably be best to screen infants and children in the tropics for iron deficiency before giving them extra iron, as it appeared that especially iron replete children suffered from adverse effects compared to iron deficient children (Sazawal et al 2006, WHO 2007). However, screening in rural areas of developing countries is complicated due to logistics, inflammation (making iron parameters less proper measures of body iron status) and its expensiveness. With regarding to the generally high rate of iron deficiency, untargeted oral iron administration is preferred above targeted treatment. Nonetheless, hepcidin has been proposed as a valuable biomarker in a point-of-care test to identify anaemic children as it can in theory discriminate between iron deficiency anaemia (IDA) (low hepcidin) and anaemia due to infection (ACD) (high hepcidin) (Jaeggi et al 2013, Prentice et al 2012). In the latter case it would be better to first treat the infection before starting with iron supplementation. However, recent data from a study in Kenya indicate that circulating hepcidin levels do not correlate with gut inflammation (during infancy), meaning that potential local injurious effects of iron supplements on infectious or inflammatory intestinal disease may not be properly predicted (Jaeggi et al 2013). In addition, a study in Malawian children showed that hepcidin was a poor predictor of bone marrow iron deficiency. Upregulation of hepcidin by inflammation and iron status was blunted by erythropoietin in this population, and therefore would have limitations as a biomarker to differentiate between IDA and ACD in this category of children (Jonker et al 2013). Thus, although promising, these recent studies underscore that the potential value of serum hepcidin to guide safe iron supplementation in infancy needs further clarification.

11.7 Approaches for safe oral iron administration without affecting the microbiota composition and metabolism

In the absence of robust and affordable biomarkers for targeted supplementation of iron, several strategies to increase bioavailability and safety of iron formulas for universal use have been investigated. Moreover, there are several additional approaches that can be thought of based on theoretical considerations. All these tested and hypothetical approaches will be discussed below.

11.7.1 Improving absorption of dietary iron

Usually, most diets contain sufficient amounts of endogenous iron, even plant-based diets, but the uptake is hampered by food components that bind and precipitate the iron. Improving the absorption of endogenous dietary iron is one strategy to prevent extra amounts of iron entering the colon. Nevertheless, when making dietary iron more bioavailable, also the availability for gut microbiota most likely increases. One approach, also mentioned above, is the addition of ascorbic acid as this compound supports the solubility of iron during passage in the stomach and intestine, even when polyphenols and phytate are present. A drawback is that ascorbic acid is susceptible to losses during storage and cooking (Hurrell 2002).

Another approach which has already been successfully tested, is the reduction of dietary phytate by enzymatic degradation via the activation of endogenous phytase or the addition of a commercial phytase. It is important to greatly reduce the phytate content to achieve a significant increase in iron absorption (Hurrell 2002). Interestingly, it has been shown that the addition of a phytase, active at the pH within the stomach and intestine can significantly increase the iron absorption of a meal fortified with $FeSO_4$ or NaFeEDTA (Nielsen et al 2013, Troesch et al 2009). The effects of dietary phytate digestion on the gut microbiota is however not known and needs further investigation. Above all, it is preferred to increase the diversity of monotonous diets, which also can enhance the absorption of iron and other essential micronutrients. Unfortunately, this can be very challenging in poorer countries (WHO and FAO 2006).

11.7.2 Low dose, highly bioavailable iron

As already mentioned in section 11.4, most oral iron administration studies have focused on the bioavailability of iron preparations. Other important classical aspects taken into account are the stability (e.g. as fortificant to those in flour), sensory experience, and cost. Hence, the majority of these studies did not evaluate the causes of the adverse gastrointestinal side effects that are frequently reported. Nevertheless, increasing the bioavailability of an iron preparation in the host is also a strategy to prevent large amounts of unabsorbed iron entering the colon. If iron bioavailability is high, then the dose might be lowered, while maintaining sufficient host iron absorption. The bioavailability might be improved by adding ascorbic acid as a well known enhancer of iron absorption; alternatively, the iron might be provided as NaFeEDTA to improve iron absorption from diets high in phytate, or soluble ionic iron can be encapsulated to increase stability and to prevent unwanted sensory effects (Hurrell 2002). A slightly different strategy could be the slow release of iron from a preparation, this way maximizing duodenal absorption. Providing a low dose, but highly bioavailable iron is a promising strategy for preventing large amounts of iron entering the colonic lumen, although it remains challenging to find the optimal dose and preparation. This is underscored by a very recent study which showed that micronutrient powder containing a low dose of 2.5 mg highly bioavailable iron as FeNaEDTA did not improve the body iron status of Kenyan infants, while their gut microbiota was still shifted towards a potentially more pathogenic profile (Jaeggi et al 2014).

11.7.3 Iron containing micronutrient powders (MNPs)

A promising approach for the low dose strategy was the application of MNPs with highly bioavailable iron for untargeted in-home fortification plus the provision of other important minerals and vitamins. These MNPs are recommended by the WHO to treat iron deficiency, with the important remark that measures to prevent, diagnose and treat malaria in malaria-endemic areas have to be established (WHO 2011). MNPs could avoid the need to screen infants for iron deficiency anaemia (IDA) and are expected to have less impact on the gut microbiota compared to iron supplementation doses, as the MNP iron dosage is generally lower and is always accompanied by a food matrix. However, MNPs with iron have recently been associated with increased hospital admission and bloody diarrhoea, but the effects of

MNPs on the gut microbiota have not been extensively studied yet (Soofi et al 2013, Zlotkin et al 2013).

11.7.4 Provision of probiotics and/or prebiotics together with iron

As oral iron administration tend to decrease numbers of the beneficial gut microbe families *Lactobacillaceae* and *Bifidobacteriaceae* (discussed in section 7.5), the simultaneous administration of probiotic bacteria may counteract this effect and contribute to the maintenance of these beneficial strains in the colonic lumen. To our knowledge, the combination of oral iron therapy and probiotic administration has not been studied yet and thus needs further investigation. Here we propose a few probiotic candidates to be provided during oral administration of iron based on the current knowledge. First of all, *Bifidobacterium* strains have a great potential as probiotics, because they can utilise and possibly bind iron to their surfaces, thereby making iron less available to pathogenic strains in the colon (discussed in section 3.2.1). Furthermore, some strains of *Bifidobacterium* have been reported to decrease the duration or incidence of diarrhoea in infants (Di Gioia et al 2014). Bailey *et al*. discovered an iron-responsive probiotic strain of the species *Streptococcus thermophilus*. This strain showed enhanced growth in response to iron and might therefore be better able to compete with enteric pathogens during increased iron availability (Bailey et al 2011). Another very interesting probiotic strain is *E. coli* Nissle 1917, which can also thrive on iron (Deriu et al.) and has been shown to reduce *S.* Typhimurium adhesion to, and invasion into intestinal epithelial cells (Schierack et al 2011). Moreover, *E. coli* Nissle 1917 is capable of reducing *S.* Typhimurium colonisation of the mouse inflamed gut because it can compete for iron with *S*. Typhimurium via several high affinity iron uptake mechanisms (Deriu et al 2013). It should however be noted that, the differential response to probiotics among individuals may limit the overall health benefit when a single strain is universally administrated to large populations (van Baarlen et al 2013). Future research will be needed to assess the potential benefit, and also to assess the safety of probiotic administration in infection endemic regions.

Finally, a slightly different approach may increase iron uptake by the host and improve gut health. Prebiotics such as inulin and fructo-oligosaccharides have been shown to increase the number of beneficial *Bifidobacteriaceae* and to decrease colonic pH (Vieira et al 2013). This study suggest that the simultaneous provision of prebiotics with iron might be a promising approach to both stimulate iron uptake and the colonisation of beneficial *Bifidobacteriaceae* (as discussed in section 6.1). It has indeed been shown that, whereas inulin can increase the *Bifidobacteriaceae* population and decrease colonic pH, it did not significantly increase iron uptake in women with low iron status (Petry et al 2012). Nevertheless, a trend towards increased iron absorption was reported, and future studies should reveal the benefit and safety of this approach.

11.7.5 Provision of antimicrobial agents

As iron appears to increase the number of enteric pathogens, one strategy is to suppress these “ironphilic” pathogens by antimicrobial agents). This way, the risk of getting infectious

diarrhoea may be lowered and the prevention of gut inflammation in general would lower the risk of pathogenic overgrowth. The provision of antibiotics is not advisable, as this treatment will have a (temporary) detrimental effect on the gut microbiota. One approach could be the provision of the natural antimicrobial compound carvacrol. We recently showed that carvacrol can reduce iron-induced virulence (i.e. adhesion) of *S.* Typhimurium and earlier it was shown that carvacrol improved gut health in piglets, thus carvacrol is a potential candidate for specifically reducing the iron-induced virulence of enteric pathogens (Kortman et al 2014a, Michiels et al 2010) (**Chapter 6**). Future studies should focus on the targeted delivery of carvacrol in the colon as well as on its effects on the gut microbiota. As mentioned in section 7.1, the provision of zinc may counteract the effects of iron, as zinc can reduce the virulence of enteropathogens (Crane et al 2011, Mellies et al 2012) but contradictory results have been obtained thus far. On the one hand, zinc appeared to reduce iron-induced gut microbial changes In US infants (Krebs et al 2013). On the other hand, MNP including iron and zinc (but also other micronutrients) did not reduce the incidence of bloody diarrhoea in Pakistani children compared to MNP including iron, but without zinc (Soofi et al 2013). Notably, zinc is also an pivotal metal for many bacteria and may therefore also support bacterial growth in general (Hood and Skaar 2012).

11.7.6 Limitation of accessibility of orally administered iron for enteric pathogens

The ideal oral iron preparation can be given in a low dose and is well absorbed by the host, resulting in only slightly increased amounts of iron entering the colon. However, host iron uptake will likely always comprise a rather low percentage of the dose given. An iron source highly bioavailable for the host, but hardly accessible for enteric pathogens, would therefore be most perfect. This may be very challenging with regarding to the effects of the environmental conditions of the stomach and intestine on iron speciation, but is may not be impossible. As already mentioned, it has been shown, with the limitations of the study, that ferric iron did not alter the gut microbiota of rodents and may therefore indicate that ferric iron ends up in less accessible form (e.g. insoluble) in the colon (Ettreiki et al 2012). Ferric iron is however generally not as bioavailable for the host as ferrous iron, although NaFe(III)EDTA has shown good bioavailability because of its solubility (Zimmermann and Hurrell 2007).

A rather new form of iron preparations is nanocompound iron. This nanostructured iron is poorly water-soluble but is surprisingly well absorbed by intestinal epithelial cells and rodents. It has been shown that nanocompound iron can be taken up via endocytosis by intestinal epithelial cells and it also showed good bioavailability in a mouse and rat study (Hilty et al 2010, Pereira et al 2013, Powell et al 2014). The effect of nanocompound iron on the gut microbiota remains to be investigated and will depend on the integrity of these nanocompounds in the intestinal tract and/or the capability of bacteria to utilise iron from these particles.

11.7.7 Natural iron complexes

Other more natural forms of iron such as lactoferrin and ferritin can also be good candidates, although the effect on the gut microbiota needs to be investigated for these iron sources as

well. Plant ferritin as a whole can be absorbed by the human intestine via an endocytosis mediated pathway and has the potential to be used as a readily bioavailable iron supplement as reviewed by Theil *et al.* (San Martin et al 2008, Theil 2004). For example ferritin rich legume seeds such as soy beans can potentially be used as an iron source (Lönnerdal 2009). The stability of ferritin and availability of ferritin iron for gut bacteria in the colonic lumen is not yet known but it can be envisaged that this form is relatively difficult to access. Next, it has been shown that supplementation of lactoferrin can increase the iron status of infants and pregnant women (Lonnerdal 2009). This form of iron is generally not well available for bacteria, but certain pathogenic species have developed ways to sequester iron from lactoferrin via a lactoferrin receptor or siderophore-mediated uptake (as discussed in section 9.1.1) and may therefore provide these pathogenic species with a competitive advantage over other (beneficial) microbes.

To wrap up, many of the approaches mentioned in this section are already tested with regard to bioavailability to the host, but to assess their safety it would be required to study their effects on the gut microbiota. It remains difficult to predict in which form the originally administered iron will end up in the colon and to what extent it can be utilised by the microbes, which is especially important with regard to enteric pathogens.
The perfect iron preparation shows good bioavailability for the host but not for (pathogenic) gut microbes, shows no adverse effects and is low cost for application in developing countries. When new iron preparations are tested in regions with high infection pressure it is advisable to also assess the effects on the gut microbiota. It goes without saying that preparations can first be tested *in vitro* gut models and animal models that closely resemble the relevant *in vivo* conditions.

12 Conclusion and future perspectives

Iron deficiency anaemia is widespread among the world and should generally be corrected to avoid serious health consequences. Although in industrialised countries oral iron therapy is commonly associated with gastrointestinal side effects, it is not known to increase the burden of infection in this setting. In contrast, in an African region with high infection pressure (e.g. malaria) and low hygiene standards oral iron supplementation has been associated with increased morbidity and mortality of children. Furthermore, oral administration can increase the incidence of diarrhoea, which may partly be caused by the outgrowth of enteric pathogens. In this review we extensively discussed the multifaceted effects of nutritional iron, oral iron administration, host iron status, intestinal inflammation and nutritional stress on the gut microbiota (summarised in **Figure 6**). From a gut microbial perspective it becomes clear that life in the colon lumen is not always easy and that many stress factors play a role, especially for enteric pathogens that are usually very well restrained in the healthy colon (**Figure 1**). Above all, it becomes clear that oral iron administration changes the gut microbiota profile on several fronts. Although the effects appear to be variable among studies, together they point at a shift towards a potentially pathogenic profile. The clinical relevance of these effects are not fully clear yet but it is highly recommended

that potentially pathogenic effects on the gut microbiota need to be avoided as much as possible. We therefore provide leads for the development of alternative iron preparations that provisionally have less impact on the gut microbiome. It goes without saying that more research on this matter will help us to increase the understanding of how the gut microbiome deals with iron and which way the iron can be administered best.

13 Aim and outline of this thesis

Oral iron administration in infection endemic regions is associated with an increased susceptibility of children to infections. The aim of this thesis was to investigate the impact of oral iron administration at the intestinal epithelial interface on i) the pathogenicity of intestinal pathogens, ii) the gut microbiota composition, iii) gut microbial metabolism, and iv) host responses. Knowledge on the response of gut microbes to iron is pivotal for the design of oral iron supplementation strategies or alternative iron preparations with less adverse effects on the intestinal microbiota and the gastrointestinal tract.

Chapter 1 comprehensively reviews the current knowledge on many (clinical) aspects of oral iron administration with regard to effects on the gut microbiota and the host. **Chapter 2** shows the effects of oral iron fortification on the gut microbiota of African infants in a rural environment, a group of individuals highly vulnerable to iron deficiency and infections. Iron fortification caused a shift towards a more pathogenic gut microbiota profile and was associated with increased gut inflammation. Importantly, this outcome implies that oral administration programmes in similar areas need to be set up with adequate health measures, and stresses the importance of further research on the effects of iron on the gut microbiota as described in the following chapters of this thesis. In **Chapter 3** we describe the effect of multiple iron preparations and doses on gut microbial composition and metabolism in a model for the human large intestine. The explorative and multi-omics approach gave valuable insights and showed that gut microbial metabolism shifted towards a more toxic profile. Next, in **Chapter 4** the effects of oral iron administration on the host immune response in a mouse model for human intestinal bacterial infection are described. It shows that both the host immune response and innate defense can be attenuated by both iron deprivation and iron supplementation. This illustrates the complex interplay between bacterial pathogenicity, host iron status, host immunity and the gut microbiota, which can be further elucidated in future research. **Chapter 5** describes that increased iron availability increases the virulence of intestinal pathogens, which was in particular evident by increased adhesion of pathogens to a monolayer of intestinal epithelial cells *in vitro*. In **Chapter 6**, the potential of the natural antimicrobial carvacrol to prevent iron-induced virulence *in vitro* was investigated. The results indicate that supplementation of carvacrol can potentially prevent the stimulation of enteric pathogens during oral iron administration, but further research is required to assess this approach. Finally, **Chapter 7** summarizes the research in this thesis, highlights the gain in insights and provides directions for future research.

Supplementary Tables

Supplementary Table 1. A selection of papers which illustrate that (slightly) different association constants (Ka) for transferrin and enterobactin circulate in literature. Also for estimated free iron concentration in normal human plasma different values can be found.

Study	Transferrin (Ka)1	Enterobactin (Ka)1	Free [Fe] in plasma
(Bullen et al 2000)	10^{36}	10^{52}	10^{-18} M
(Miethke and Skerra 2010)	10^{22}	10^{49}	10^{-24} M
(Aisen and Listowsky 1980)	10^{22}		
(Aisen et al 1978)	10^{20}		
(Raymond et al 2003)			10^{-24} M
(Carrano and Raymond 1979)		10^{51}	

[1] Note that association constants may have been determined at different conditions in the different studies

Supplementary Table 2. Dietary ligands or host secreted ligands for iron that may be found within the colonic lumen.[1]

Ligand/iron species	Reference
Organic acids	
Ascorbate	(Cremonesi et al 2002, Hurrell and Egli 2010, Jacobs and Miles 1969, Miret et al 2003, van Dokkum 1992)
Citrate	(Cremonesi et al 2002, Jacobs and Miles 1969, Salovaara et al 2003, van Dokkum 1992)
Lactate	(Cremonesi et al 2002, Miret et al 2003)
Oxalate	(Conrad and Umbreit 2002, Salovaara et al 2003, van Dokkum 1992)
Succinate	(Cremonesi et al 2002, Salovaara et al 2003)
Tartrate	(Salovaara et al 2003)
Polyphenols	
Tannate	(Conrad and Umbreit 2002, van Dokkum 1992)
Catechols	(Freestone et al 2007)
Phytate	(Conrad and Umbreit 2002, Hurrell and Egli 2010)
Oxides / hydroxide	(Simpson et al 1992)
Carbonate	(Conrad and Umbreit 2002, Simpson et al 1992)
Phosphate	(Conrad and Umbreit 2002, Simpson et al 1992)
Lactose[2]	(van Dokkum 1992)
Ferritin	(Lönnerdal 2009, Vereecke et al 2011)
Lactoferrin	(Lonnerdal 2009)
Mucins	(Conrad and Umbreit 2002, Cremonesi et al 2002, Jacobs and Miles 1969, Miret et al 2003)
Amino acids	(Conrad and Umbreit 2002, Cremonesi et al 2002, Hurrell and Egli 2010, Miret et al 2003, van Dokkum 1992)
Protein (animal)	(Hurrell and Egli 2010, van Dokkum 1992)
Bile constituents	(Cremonesi et al 2002, Jacobs and Miles 1970)

[1]Many of these ligands allow iron to bind in either the ferrous or the ferric state. Most likely, this list represents only a subset of the food or host derived ligands that can precipitate or solubilise luminal iron.
[2]In lactose tolerant people lactose is digested in the small intestine, so it is likely that lactose normally does not enter the colon in significant amounts.

Supplementary Table 3: setting and set up of studies included in Table 1.

	In vivo human (n=6)	In vivo animal (n=7)	In vitro (n=2)
Study	**Mevissen-Verhage *et al.* 1985**	**Benoni *et al.* 1993**	**Dostal *et al*. 2012b**
Subjects	Infants, newborns	Rats (Sprague-Dawley)	Children faecal samples [5]
Setting	European, The Netherlands	Laboratory	In vitro fermentation
Iron source & dose	5mg/L in cow-milk	$FeSO_4$, 10 or 30 mg Fe/kg water [3]	$FeSO_4$, various
Duration	First 3 months of life [2]	2 and 4 weeks	Long (70 days, with intervals)
Technique	Culture techniques (faecal)	Culture techniques (faecal)	qPCR & pyrosequencing
Study	**Balmer *et al.* 1989**	**Tompkins *et al.* 2001**	**Kortman *et al.* 2014**
Subjects	Infants, newborns	Mice Swiss-Webster, 5 weeks old	Dutch adult faecal samples
Setting	European, United Kingdom	Laboratory	In vitro fermentation
Iron source & dose	9 mg Fe/L (+ lactoferrin)	$FeCl_3$, 121 or 1590 mg/kg diet [3]	$FeSO_4$ / Ferric citrate, medium-high
Duration	4 and 14 days	12 weeks	72 hours
Technique	Culture techniques (faecal)	Culture techniques (colonic)	Pyrosequencing & microarray
Study	**Balmer *et al.* 1991**	**Lee *et al.* 2008**	
Subjects	Infants, newborns	Weaning piglets, 3-d-old	
Setting	European, United Kingdom	Controlled, concrete floors	
Iron source & dose	6 mg Fe/L	$FeSO_4$; 50, 100 or 250 mg Fe/kg diet [3]	
Duration	2-15 weeks [2]	2 and 4 weeks [2]	
Technique	Culture techniques (faecal)	Culture techniques (faecal)	
Study	**Zimmerman *et al.* 2010**	**Werner *et al.* 2011**	
Subjects	School Children, 6-14-y-old	Mice C57BL/6, 7 weeks old	
Setting	African, Ivory Coast, rural	Laboratory	
Iron source & dose	Electrolytic iron, 20 mg/day	$FeSO_4$, 180 mg Fe/kg diet	
Duration	6 months	11 weeks	
Technique	TGGE & qPCR (faecal)	Pyrosequencing (caecal)	
Study	**Krebs *et al.* 2013**	**Dostal *et al.* 2012a**	
Subjects	Infants, 5-mo-old	Rats (Sprague-Dawley), 21-d-old	
Setting	US	Laboratory	
Iron source & dose	~6 mg/serving [1]	$FeSO_4$; 10 or 20 mg/kg diet [3]	
Duration	4 months	24 d depletion, then 13 d repletion	
Technique	Pyrosequencing (faecal)	TGGE & qPCR (faecal)	
Study	**Jaeggi *et al.* 2014**	**Ettreiki *et al.* 2012**	
Subjects	Infants, 6-mo-old	Mice (BalbC) and Rats (Wistar) [4]	
Setting	African, Kenya, rural	Laboratory	
Iron source & dose	2.5 or 12.5 mg/day [3,7]	Ferric, 75 or 150 mg/kg water [3]	
Duration	4 months	6 weeks	
Technique	Pyrosequencing & qPCR (faecal)	qPCR, small selection of taxa (caecal)	
Study		**Dostal *et al.* 2014b**	
Subjects		Rats (Fischer 344), 5-wk-old	
Setting		Laboratory, humanised microbiota	
Iron source & dose		$FeSO_4$, 35 or 70 mg Fe/kg diet [3,6]	
Duration		12 + 4 weeks	
Technique		Pyrosequencing & qPCR (caecal)	

[1] compared to a different low iron meat diet

[2] consensus result over the whole period was deduced

[3] consensus result for iron doses was deduced

[4] consensus result for both species was deduced

[5] faecal samples were used as starting microbiota for the in vitro fermentation

[6] for the 70 mg/kg diets a mixture of ferrous sulphate and ferric citrate was used

[7] the 2.5 mg dose was given as NaFeEDTA, the 12.5 mg dose was given as ferrous fumarate

Supplementary information 1

Iron speciation of oral iron preparations

Iron speciation of dietary iron from the source to the colon is extensively discussed in the main article, but it is also intriguing to envisage in which form an oral supplement or fortificant will end up in the colon. Oral iron preparations are most commonly provided in a soluble and ferrous form (e.g. ferrous sulphate), but can also be given in a soluble and ferric form (e.g. FeNaEDTA). Although difficult to predict, we here envisage what happens with the iron preparation upon ingestion in the gastrointestinal tract.

Ferrous iron

Despite the presence of oxygen in stomach, the gastric acid will promote to keep the iron in the ferrous state. Probably not for 100% as it has been shown in mice that ferrous iron ends up mainly in the ferric form in the duodenum, resulting in a decreased solubility (Cremonesi et al 2002). When pH rises, oxidation of ferrous iron to the ferric form likely occurs, but oxygen levels in the small intestine lumen are low and very low in the colon. The environment of the colon lumen may therefore favour the ferrous state. Notably, host iron uptake mainly takes place in the duodenum, quickly after the rise in pH.

Ferric iron

The presence of oxygen and the low pH of the gastric acid will promote to keep the iron in its ferric form. When pH rises, its solubility rapidly decreases which in turn favours complexation with e.g. hydroxides. However, e.g. organic acids can keep the ferric iron in a soluble form (e.g. citrate), or reduce it to the ferrous form (e.g. ascorbate). If the ferric iron is provided in e.g. an EDTA complex, this also favours solubility (if the complex is stable in the stomach). Notably, for uptake via DMT1, the iron needs to be in the ferrous form, which requires action of the host reductase DcytB if iron is in the ferric (and soluble) state.

Conclusion

Bioavailability of ferrous iron to the host is generally better than that of ferric iron, which supports the above theories. Ferrous iron will largely be converted to the ferric form when pH rises after passage through the stomach, but is likely to remain partly in the ferrous form, which can explain the better host bioavailability compared to ferric iron. This is in line with the finding that orally administered ferrous iron can promote ROS formation in

faecal water (Lund et al 1999) and it also fits with the idea that the less reactive ferric iron has less adverse effects on the gut wall as it probably will not be converted into ferrous iron to a large extent. Finally, we note that large amounts of food appear to decrease iron uptake, because of an increase in gastric acid pH which decreases solubility of iron (Simpson and Peters 1990). High food intake together with oral iron administration is therefore likely to promote early iron speciation, affecting host iron uptake and possibly also the accessibility for the gut microbiota. It goes without saying that specific food components not mentioned here will also affect iron speciation and solubility of the administered iron. This in turn affects accessibility of the iron for the gut microbiota. For more information on this matter, we refer back to section 5 (Iron speciation and bacterial iron uptake mechanisms in the colon) of the main article and **Supplementary Table 2**. After all, concomitant food can thus decrease intestinal absorption of iron, but the simultaneous intake of food can also reduce gastrointestinal side effects and it is therefore advised to take iron preparations together with small amounts of food (Macdougall and Geisser 2013).

14 References

- Abreu MT (2010). Toll-like receptor signalling in the intestinal epithelium: how bacterial recognition shapes intestinal function. Nature reviews Immunology 10: 131-144.
- Adu-Afarwuah S, Lartey A, Brown KH, Zlotkin S, Briend A, Dewey KG (2008). Home fortification of complementary foods with micronutrient supplements is well accepted and has positive effects on infant iron status in Ghana. The American journal of clinical nutrition 87: 929-938.
- Aisen P, Leibman A, Zweier J (1978). Stoichiometric and site characteristics of the binding of iron to human transferrin. J Biol Chem 253: 1930-1937.
- Aisen P, Listowsky I (1980). Iron transport and storage proteins. Annual review of biochemistry 49: 357-393.
- Allred BE, Correnti C, Clifton MC, Strong RK, Raymond KN (2013). Siderocalin outwits the coordination chemistry of vibriobactin, a siderophore of Vibrio cholerae. ACS chemical biology 8: 1882-1887.
- Andrews SC, Robinson AK, Rodriguez-Quinones F (2003). Bacterial iron homeostasis. FEMS microbiology reviews 27: 215-237.
- Avni T, Bieber A, Steinmetz T, Leibovici L, Gafter-Gvili A (2013). Treatment of anemia in inflammatory bowel disease- systematic review and meta-analysis. PloS one 8: e75540.
- Bachman MA, Oyler JE, Burns SH, Caza M, Lepine F, Dozois CM et al (2011). Klebsiella pneumoniae yersiniabactin promotes respiratory tract infection through evasion of lipocalin 2. Infection and immunity 79: 3309-3316.
- Baig BH, Wachsmuth IK, Morris GK (1986). Utilization of exogenous siderophores by Campylobacter species. Journal of clinical microbiology 23: 431-433.
- Bailey JR, Probert CS, Cogan TA (2011). Identification and characterisation of an iron-responsive candidate probiotic. PloS one 6: e26507.
- Bajaj V, Lucas RL, Hwang C, Lee CA (1996). Co-ordinate regulation of Salmonella typhimurium invasion genes by environmental and regulatory factors is mediated by control of hilA expression. Mol Microbiol 22: 703-714.
- Balamurugan R, Mary RR, Chittaranjan S, Jancy H, Shobana Devi R, Ramakrishna BS (2010). Low levels of faecal lactobacilli in women with iron-deficiency anaemia in south India. The British journal of nutrition 104: 931-934.
- Balmer SE, Scott PH, Wharton BA (1989). Diet and faecal flora in the newborn: lactoferrin. Archives of disease in childhood 64: 1685-1690.
- Balmer SE, Wharton BA (1991). Diet and faecal flora in the newborn: iron. Archives of disease in childhood 66: 1390-1394.
- Barman M, Unold D, Shifley K, Amir E, Hung K, Bos N et al (2008). Enteric salmonellosis disrupts the microbial ecology of the murine gastrointestinal tract. Infection and immunity 76: 907-915.
- Behnsen J, Jellbauer S, Wong CP, Edwards RA, George MD, Ouyang W et al (2014). The Cytokine IL-22 Promotes Pathogen Colonization by Suppressing Related Commensal Bacteria. Immunity 40: 262-273.
- Benoni G, Cuzzolin L, Zambreri D, Donini M, Del Soldato P, Caramazza I (1993). Gastrointestinal effects of single and repeated doses of ferrous sulphate in rats. Pharmacological research : the official journal of the Italian Pharmacological Society 27: 73-80.
- Bezkorovainy A, Kot E, Miller-Catchpole R, Haloftis G, Furmanov S (1996). Iron metabolism in bifidobacteria. International Dairy Journal 6: 905-919.

- Bjarnason J, Southward CM, Surette MG (2003). Genomic profiling of iron-responsive genes in Salmonella enterica serovar typhimurium by high-throughput screening of a random promoter library. Journal of bacteriology 185: 4973-4982.
- Blaschitz C, Raffatellu M (2010). Th17 cytokines and the gut mucosal barrier. Journal of clinical immunology 30: 196-203.
- Boleij A, Tjalsma H (2012). Gut bacteria in health and disease: a survey on the interface between intestinal microbiology and colorectal cancer. Biological reviews of the Cambridge Philosophical Society 87: 701-730.
- Boyer E, Bergevin I, Malo D, Gros P, Cellier MF (2002). Acquisition of Mn(II) in addition to Fe(II) is required for full virulence of Salmonella enterica serovar Typhimurium. Infection and immunity 70: 6032-6042.
- Bruyneel BJ, Woestyne MMV, Verstraete WH (1990). Effect of 2,2′-dipyridyl on silage fermentation. Journal of the Science of Food and Agriculture 51: 179-183.
- Buhnik-Rosenblau K, Moshe-Belizowski S, Danin-Poleg Y, Meyron-Holtz EG (2012). Genetic modification of iron metabolism in mice affects the gut microbiota. Biometals 25: 883-892.
- Bullen J, Griffiths E, Rogers H, Ward G (2000). Sepsis: the critical role of iron. Microbes and infection / Institut Pasteur 2: 409-415.
- Bullen JJ, Rogers HJ, Spalding PB, Ward CG (2005). Iron and infection: the heart of the matter. FEMS immunology and medical microbiology 43: 325-330.
- Butcher J, Stintzi A (2013). The Transcriptional Landscape of Campylobacter jejuni under Iron Replete and Iron Limited Growth Conditions. PloS one 8: e79475.
- Cairo G, Bernuzzi F, Recalcati S (2006). A precious metal: Iron, an essential nutrient for all cells. Genes & nutrition 1: 25-39.
- Carrano CJ, Raymond KN (1979). Ferric ion sequestering agents. 2. Kinetics and mechanism of iron removal from transferrin by enterobactin and synthetic tricatechols. Journal of the American Chemical Society 101: 5401-5404.
- Carrier JC, Aghdassi E, Jeejeebhoy K, Allard JP (2006). Exacerbation of dextran sulfate sodium-induced colitis by dietary iron supplementation: role of NF-kappaB. International journal of colorectal disease 21: 381-387.
- Cassat JE, Skaar EP (2013). Iron in infection and immunity. Cell host & microbe 13: 509-519.
- Champomier-Verges MC, Stintzi A, Meyer JM (1996). Acquisition of iron by the non-siderophore-producing Pseudomonas fragi. Microbiology (Reading, England) 142 (Pt 5): 1191-1199.
- Cherayil BJ (2009). Indoleamine 2,3-dioxygenase in intestinal immunity and inflammation. Inflammatory bowel diseases 15: 1391-1396.
- Cherayil BJ (2010). Cross-talk between iron homeostasis and intestinal inflammation. Gut microbes 1: 65-69.
- Chu BC, Garcia-Herrero A, Johanson TH, Krewulak KD, Lau CK, Peacock RS et al (2010). Siderophore uptake in bacteria and the battle for iron with the host; a bird's eye view. Biometals 23: 601-611.
- Chua AC, Klopcic B, Lawrance IC, Olynyk JK, Trinder D (2010). Iron: an emerging factor in colorectal carcinogenesis. World J Gastroenterol 16: 663-672.

- Collings R, Harvey LJ, Hooper L, Hurst R, Brown TJ, Ansett J et al (2013). The absorption of iron from whole diets: a systematic review. The American journal of clinical nutrition 98: 65-81.
- Conrad ME, Umbreit JN (2002). Pathways of iron absorption. Blood cells, molecules & diseases 29: 336-355.
- Courtois F, Suc I, Garofalo C, Ledoux M, Seidman E, Levy E (2000). Iron-ascorbate alters the efficiency of Caco-2 cells to assemble and secrete lipoproteins. American journal of physiology 279: G12-19.
- Cowart RE (2002). Reduction of iron by extracellular iron reductases: implications for microbial iron acquisition. Archives of biochemistry and biophysics 400: 273-281.
- Cowland JB, Borregaard N (1997). Molecular characterization and pattern of tissue expression of the gene for neutrophil gelatinase-associated lipocalin from humans. Genomics 45: 17-23.
- Crane JK, Byrd IW, Boedeker EC (2011). Virulence inhibition by zinc in shiga-toxigenic Escherichia coli. Infection and immunity 79: 1696-1705.
- Cremonesi P, Acebron A, Raja KB, Simpson RJ (2002). Iron absorption: biochemical and molecular insights into the importance of iron species for intestinal uptake. Pharmacology & toxicology 91: 97-102.
- Crichton RR (2009). Iron Metabolism - From Molecular Mechanisms to Clinical Consequences 3rd ed. edn. John Wiley & Sons Ltd: Chichester, UK.
- Cronin M, Zomer A, Fitzgerald GF, van Sinderen D (2012). Identification of iron-regulated genes of Bifidobacterium breve UCC2003 as a basis for controlled gene expression. Bioengineered bugs 3: 157-167.
- D'Onofrio A, Crawford JM, Stewart EJ, Witt K, Gavrish E, Epstein S et al (2010). Siderophores from neighboring organisms promote the growth of uncultured bacteria. Chemistry & biology 17: 254-264.
- Damo S, Chazin WJ, Skaar EP, Kehl-Fie TE (2012). Inhibition of bacterial superoxide defense: a new front in the struggle between host and pathogen. Virulence 3: 325-328.
- De-Regil LM, Jefferds ME, Sylvetsky AC, Dowswell T (2011). Intermittent iron supplementation for improving nutrition and development in children under 12 years of age. The Cochrane database of systematic reviews: CD009085.
- De Filippo C, Cavalieri D, Di Paola M, Ramazzotti M, Poullet JB, Massart S et al (2010). Impact of diet in shaping gut microbiota revealed by a comparative study in children from Europe and rural Africa. Proc Natl Acad Sci U S A 107: 14691-14696.
- De Vuyst L, Leroy F (2011). Cross-feeding between bifidobacteria and butyrate-producing colon bacteria explains bifdobacterial competitiveness, butyrate production, and gas production. International journal of food microbiology 149: 73-80.
- Del Chierico F, Vernocchi P, Bonizzi L, Carsetti R, Castellazzi AM, Dallapiccola B et al (2012). Early-life gut microbiota under physiological and pathological conditions: the central role of combined meta-omics-based approaches. Journal of proteomics 75: 4580-4587.
- Deriu E, Liu JZ, Pezeshki M, Edwards RA, Ochoa RJ, Contreras H et al (2013). Probiotic bacteria reduce salmonella typhimurium intestinal colonization by competing for iron. Cell host & microbe 14: 26-37.
- Di Gioia D, Aloisio I, Mazzola G, Biavati B (2014). Bifidobacteria: their impact on gut microbiota composition and their applications as probiotics in infants. Appl Microbiol Biotechnol 98: 563-577.
- Diaz-Ochoa VE, Jellbauer S, Klaus S, Raffatellu M (2014). Transition metal ions at the crossroads of mucosal immunity and microbial pathogenesis. Front Cell Infect Microbiol 4: 2.
- Dostal A, Chassard C, Hilty FM, Zimmermann MB, Jaeggi T, Rossi S et al (2012a). Iron depletion and repletion with ferrous sulfate or electrolytic iron modifies the composition and metabolic activity of the gut microbiota in rats. The Journal of nutrition 142: 271-277.

- Dostal A, Fehlbaum S, Chassard C, Zimmermann MB, Lacroix C (2012b). Low iron availability in continuous in vitro colonic fermentations induces strong dysbiosis of the child gut microbial consortium and a decrease in main metabolites. FEMS microbiology ecology 83: 161-175.
- Dostal A, Gagnon M, Chassard C, Zimmermann MB, O'Mahony L, Lacroix C (2014a). Salmonella adhesion, invasion and cellular immune responses are differentially affected by iron concentrations in a combined in vitro gut fermentation-cell model. PloS one 9: e93549.
- Dostal A, Lacroix C, Pham VT, Zimmermann MB, Del'homme C, Bernalier-Donadille A et al (2014b). Iron supplementation promotes gut microbiota metabolic activity but not colitis markers in human gut microbiota-associated rats. The British journal of nutrition 111: 2135-2145.
- Drechsel H, Thieken A, Reissbrodt R, Jung G, Winkelmann G (1993). Alpha-keto acids are novel siderophores in the genera Proteus, Providencia, and Morganella and are produced by amino acid deaminases. Journal of bacteriology 175: 2727-2733.
- Duncan SH, Louis P, Thomson JM, Flint HJ (2009). The role of pH in determining the species composition of the human colonic microbiota. Environmental microbiology 11: 2112-2122.
- Ellermeier JR, Slauch JM (2008). Fur regulates expression of the Salmonella pathogenicity island 1 type III secretion system through HilD. Journal of bacteriology 190: 476-486.
- Ettreiki C, Gadonna-Widehem P, Mangin I, Coeffier M, Delayre-Orthez C, Anton PM (2012). Juvenile ferric iron prevents microbiota dysbiosis and colitis in adult rodents. World J Gastroenterol 18: 2619-2629.
- Feasey NA, Dougan G, Kingsley RA, Heyderman RS, Gordon MA (2012). Invasive non-typhoidal salmonella disease: an emerging and neglected tropical disease in Africa. Lancet 379: 2489-2499.
- Feldmann F, Sorsa LJ, Hildinger K, Schubert S (2007). The salmochelin siderophore receptor IroN contributes to invasion of urothelial cells by extraintestinal pathogenic Escherichia coli in vitro. Infection and immunity 75: 3183-3187.
- Ferruzza S, Scarino ML, Gambling L, Natella F, Sambuy Y (2003). Biphasic effect of iron on human intestinal Caco-2 cells: early effect on tight junction permeability with delayed onset of oxidative cytotoxic damage. Cellular and molecular biology (Noisy-le-Grand, France) 49: 89-99.
- Figueira R, Holden DW (2012). Functions of the Salmonella pathogenicity island 2 (SPI-2) type III secretion system effectors. Microbiology (Reading, England) 158: 1147-1161.
- Flint HJ, Duncan SH, Scott KP, Louis P (2007). Interactions and competition within the microbial community of the human colon: links between diet and health. Environmental microbiology 9: 1101-1111.
- Flo TH, Smith KD, Sato S, Rodriguez DJ, Holmes MA, Strong RK et al (2004). Lipocalin 2 mediates an innate immune response to bacterial infection by sequestrating iron. Nature 432: 917-921.
- Foster SL, Richardson SH, Failla ML (2001). Elevated iron status increases bacterial invasion and survival and alters cytokine/chemokine mRNA expression in Caco-2 human intestinal cells. The Journal of nutrition 131: 1452-1458.
- Frawley ER, Crouch ML, Bingham-Ramos LK, Robbins HF, Wang W, Wright GD et al (2013). Iron and citrate export by a major facilitator superfamily pump regulates metabolism and stress resistance in Salmonella Typhimurium. Proc Natl Acad Sci U S A 110: 12054-12059.
- Freestone PP, Walton NJ, Haigh RD, Lyte M (2007). Influence of dietary catechols on the growth of enteropathogenic bacteria. International journal of food microbiology 119: 159-169.
- Freitas Kde C, Amancio OM, de Morais MB (2012). High-performance inulin and oligofructose prebiotics increase the intestinal absorption of iron in rats with iron deficiency anaemia during the growth phase. The British journal of nutrition 108: 1008-1016.

- Fukuda S, Toh H, Hase K, Oshima K, Nakanishi Y, Yoshimura K et al (2011). Bifidobacteria can protect from enteropathogenic infection through production of acetate. Nature 469: 543-547.
- Gera T, Sachdev HP (2002). Effect of iron supplementation on incidence of infectious illness in children: systematic review. BMJ (Clinical research ed 325: 1142.
- Gerlach RG, Jackel D, Stecher B, Wagner C, Lupas A, Hardt WD et al (2007). Salmonella Pathogenicity Island 4 encodes a giant non-fimbrial adhesin and the cognate type 1 secretion system. Cellular microbiology 9: 1834-1850.
- Godinez I, Haneda T, Raffatellu M, George MD, Paixao TA, Rolan HG et al (2008). T cells help to amplify inflammatory responses induced by Salmonella enterica serotype Typhimurium in the intestinal mucosa. Infection and immunity 76: 2008-2017.
- Godinez I, Raffatellu M, Chu H, Paixao TA, Haneda T, Santos RL et al (2009). Interleukin-23 orchestrates mucosal responses to Salmonella enterica serotype Typhimurium in the intestine. Infection and immunity 77: 387-398.
- Graham SM (2010). Nontyphoidal salmonellosis in Africa. Current opinion in infectious diseases 23: 409-414.
- Grass G, Otto M, Fricke B, Haney CJ, Rensing C, Nies DH et al (2005). FieF (YiiP) from Escherichia coli mediates decreased cellular accumulation of iron and relieves iron stress. Archives of microbiology 183: 9-18.
- Hansen SL, Ashwell MS, Moeser AJ, Fry RS, Knutson MD, Spears JW (2009). High dietary iron reduces transporters involved in iron and manganese metabolism and increases intestinal permeability in calves. Journal of dairy science 93: 656-665.
- Hantke K, Nicholson G, Rabsch W, Winkelmann G (2003). Salmochelins, siderophores of Salmonella enterica and uropathogenic Escherichia coli strains, are recognized by the outer membrane receptor IroN. Proc Natl Acad Sci U S A 100: 3677-3682.
- Hapfelmeier S, Stecher B, Barthel M, Kremer M, Muller AJ, Heikenwalder M et al (2005). The Salmonella pathogenicity island (SPI)-2 and SPI-1 type III secretion systems allow Salmonella serovar typhimurium to trigger colitis via MyD88-dependent and MyD88-independent mechanisms. Journal of immunology 174: 1675-1685.
- Hedrich S, Schlomann M, Johnson DB (2011). The iron-oxidizing proteobacteria. Microbiology (Reading, England) 157: 1551-1564.
- Herold S, Paton JC, Srimanote P, Paton AW (2009). Differential effects of short-chain fatty acids and iron on expression of iha in Shiga-toxigenic Escherichia coli. Microbiology (Reading, England) 155: 3554-3563.
- Hilty FM, Arnold M, Hilbe M, Teleki A, Knijnenburg JT, Ehrensperger F et al (2010). Iron from nanocompounds containing iron and zinc is highly bioavailable in rats without tissue accumulation. Nat Nanotechnol 5: 374-380.
- Hood MI, Skaar EP (2012). Nutritional immunity: transition metals at the pathogen-host interface. Nature reviews 10: 525-537.
- Hooper LV, Littman DR, Macpherson AJ (2012). Interactions between the microbiota and the immune system. Science (New York, NY 336: 1268-1273.
- Hoyles L, Wallace RJ (2010). Gastrointestinal tract: intestinal fatty acid metabolism and implications for health. In: Timmis K (ed). Handbook of hydrocarbon and lipid microbiology. Springer: Berlin. pp 3119-3132.
- Hulten L, Gramatkovski E, Gleerup A, Hallberg L (1995). Iron absorption from the whole diet. Relation to meal composition, iron requirements and iron stores. European journal of clinical nutrition 49: 794-808.

- Hurrell R, Egli I (2010). Iron bioavailability and dietary reference values. The American journal of clinical nutrition 91: 1461S-1467S.
- Hurrell RF (2002). Fortification: overcoming technical and practical barriers. The Journal of nutrition 132: 806S-812S.
- Ibarra JA, Steele-Mortimer O (2009). Salmonella--the ultimate insider. Salmonella virulence factors that modulate intracellular survival. Cellular microbiology 11: 1579-1586.
- IJssennagger N, Derrien M, van Doorn GM, Rijnierse A, van den Bogert B, Muller M et al (2012). Dietary heme alters microbiota and mucosa of mouse colon without functional changes in host-microbe cross-talk. PloS one 7: e49868.
- IJssennagger N, Rijnierse A, de Wit NJ, Boekschoten MV, Dekker J, Schonewille A et al (2013). Dietary heme induces acute oxidative stress, but delayed cytotoxicity and compensatory hyperproliferation in mouse colon. Carcinogenesis 34: 1628-1635.
- Imbert M, Blondeau R (1998). On the iron requirement of lactobacilli grown in chemically defined medium. Current microbiology 37: 64-66.
- Jacobs A, Miles PM (1969). Intraluminal transport of iron from stomach to small-intestinal mucosa. British medical journal 4: 778-781.
- Jacobs A, Miles PM (1970). The formation of iron complexes with bile and bile constituents. Gut 11: 732-734.
- Jacques M (1996). Role of lipo-oligosaccharides and lipopolysaccharides in bacterial adherence. Trends in microbiology 4: 408-409.
- Jaeggi T, Moretti D, Kvalsvig J, Holding PA, Tjalsma H, Kortman GAM et al (2013). Iron Status and Systemic Inflammation, but Not Gut Inflammation, Strongly Predict Gender-Specific Concentrations of Serum Hepcidin in Infants in Rural Kenya. PloS one 8: e57513.
- Jaeggi T, Kortman GA, Moretti D, Chassard C, Holding P, Dostal A et al (2014). Iron fortification adversely affects the gut microbiome, increases pathogen abundance and induces intestinal inflammation in Kenyan infants. Gut.
- Jakubovics NS, Jenkinson HF (2001). Out of the iron age: new insights into the critical role of manganese homeostasis in bacteria. Microbiology (Reading, England) 147: 1709-1718.
- Janakiraman A, Slauch JM (2000). The putative iron transport system SitABCD encoded on SPI1 is required for full virulence of Salmonella typhimurium. Mol Microbiol 35: 1146-1155.
- Jonker FAM, Calis JCJ, Phiri K, Kraaijenhagen RJ, Brabin BJ, Faragher B et al (2013). Low Hepcidin Levels in Severely Anemic Malawian Children with High Incidence of Infectious Diseases and Bone Marrow Iron Deficiency. PloS one 8: e78964.
- Kaiser P, Hardt WD (2011). Salmonella typhimurium diarrhea: switching the mucosal epithelium from homeostasis to defense. Current opinion in immunology 23: 456-463.
- Kamada N, Chen GY, Inohara N, Nunez G (2013). Control of pathogens and pathobionts by the gut microbiota. Nature immunology 14: 685-690.
- Kang DJ, Ridlon JM, Moore DR, 2nd, Barnes S, Hylemon PB (2008). Clostridium scindens baiCD and baiH genes encode stereo-specific 7alpha/7beta-hydroxy-3-oxo-delta4-cholenoic acid oxidoreductases. Biochimica et biophysica acta 1781: 16-25.
- Karjalainen TK, Evans DG, Evans DJ, Jr., Graham DY, Lee CH (1991). Iron represses the expression of CFA/I fimbriae of enterotoxigenic E. coli. Microbial pathogenesis 11: 317-323.

- Kaye P, Abdulla K, Wood J, James P, Foley S, Ragunath K et al (2008). Iron-induced mucosal pathology of the upper gastrointestinal tract: a common finding in patients on oral iron therapy. Histopathology 53: 311-317.
- Keenan JI, Davis KA, Beaugie CR, McGovern JJ, Moran AP (2008). Alterations in Helicobacter pylori outer membrane and outer membrane vesicle-associated lipopolysaccharides under iron-limiting growth conditions. Innate immunity 14: 279-290.
- Kehl-Fie TE, Chitayat S, Hood MI, Damo S, Restrepo N, Garcia C et al (2011). Nutrient metal sequestration by calprotectin inhibits bacterial superoxide defense, enhancing neutrophil killing of Staphylococcus aureus. Cell host & microbe 10: 158-164.
- Kingsley R, Rabsch W, Roberts M, Reissbrodt R, Williams PH (1996). TonB-dependent iron supply in Salmonella by alpha-ketoacids and alpha-hydroxyacids. FEMS microbiology letters 140: 65-70.
- Kingsley RA, Reissbrodt R, Rabsch W, Ketley JM, Tsolis RM, Everest P et al (1999). Ferrioxamine-mediated Iron(III) utilization by Salmonella enterica. Appl Environ Microbiol 65: 1610-1618.
- Kortman GA, Boleij A, Swinkels DW, Tjalsma H (2012). Iron availability increases the pathogenic potential of Salmonella typhimurium and other enteric pathogens at the intestinal epithelial interface. PloS one 7: e29968.
- Kortman GA, Roelofs RW, Swinkels DW, de Jonge MI, Burt SA, Tjalsma H (2014a). Iron-Induced Virulence of Salmonella enterica Serovar Typhimurium at the Intestinal Epithelial Interface Can Be Suppressed by Carvacrol. Antimicrobial agents and chemotherapy 58: 1664-1670.
- Kortman GAM, Dutilh BE, Maathuis AJH, Engelke UF, Boekhorst J, Keegan KP et al (2014b). Gut microbial metabolism shifts towards a more toxic profile with supplementary iron in a kinetic model of the human large intestine. Abstract: Meeting of the European Iron Club
- Kot E, Bezkorovainy A (1999). Binding of ferric iron to the cell walls and membranes of Bifidobacterium thermophilum: effect of free radicals. Journal of agricultural and food chemistry 47: 4606-4610.
- Krebs NF, Sherlock LG, Westcott J, Culbertson D, Hambidge KM, Feazel LM et al (2013). Effects of different complementary feeding regimens on iron status and enteric microbiota in breastfed infants. The Journal of pediatrics 163: 416-423.
- Kroot JJ, Tjalsma H, Fleming RE, Swinkels DW (2011). Hepcidin in human iron disorders: diagnostic implications. Clinical chemistry 57: 1650-1669.
- Lawley TD, Bouley DM, Hoy YE, Gerke C, Relman DA, Monack DM (2008). Host transmission of Salmonella enterica serovar Typhimurium is controlled by virulence factors and indigenous intestinal microbiota. Infection and immunity 76: 403-416.
- Lawley TD, Walker AW (2013). Intestinal colonization resistance. Immunology 138: 1-11.
- Lee SH, Shinde P, Choi J, Park M, Ohh S, Kwon IK et al (2008). Effects of dietary iron levels on growth performance, hematological status, liver mineral concentration, fecal microflora, and diarrhea incidence in weanling pigs. Biological trace element research 126 Suppl 1: S57-68.
- Lee TW, Kolber MR, Fedorak RN, van Zanten SV (2012). Iron replacement therapy in inflammatory bowel disease patients with iron deficiency anemia: a systematic review and meta-analysis. Journal of Crohn's & colitis 6: 267-275.
- Litwin CM, Calderwood SB (1993). Role of iron in regulation of virulence genes. Clinical microbiology reviews 6: 137-149.
- Liu JZ, Jellbauer S, Poe AJ, Ton V, Pesciaroli M, Kehl-Fie TE et al (2012). Zinc sequestration by the neutrophil protein calprotectin enhances Salmonella growth in the inflamed gut. Cell host & microbe 11: 227-239.

- Llamas MA, Sparrius M, Kloet R, Jimenez CR, Vandenbroucke-Grauls C, Bitter W (2006). The heterologous siderophores ferrioxamine B and ferrichrome activate signaling pathways in Pseudomonas aeruginosa. Journal of bacteriology 188: 1882-1891.
- Lonnerdal B (2009). Nutritional roles of lactoferrin. Current opinion in clinical nutrition and metabolic care 12: 293-297.
- Lönnerdal B (2009). Soybean ferritin: implications for iron status of vegetarians. The American journal of clinical nutrition 89: 1680S-1685S.
- Lund EK, Wharf SG, Fairweather-Tait SJ, Johnson IT (1999). Oral ferrous sulfate supplements increase the free radical-generating capacity of feces from healthy volunteers. The American journal of clinical nutrition 69: 250-255.
- Lund EK, Fairweather-Tait SJ, Wharf SG, Johnson IT (2001). Chronic exposure to high levels of dietary iron fortification increases lipid peroxidation in the mucosa of the rat large intestine. The Journal of nutrition 131: 2928-2931.
- Lupp C, Robertson ML, Wickham ME, Sekirov I, Champion OL, Gaynor EC et al (2007). Host-mediated inflammation disrupts the intestinal microbiota and promotes the overgrowth of Enterobacteriaceae. Cell host & microbe 2: 204.
- Macdonald IA, Singh G, Mahony DE, Meier CE (1978). Effect of pH on bile salt degradation by mixed fecal cultures. Steroids 32: 245-256.
- Macdougall IC, Geisser P (2013). Use of intravenous iron supplementation in chronic kidney disease: an update. Iranian journal of kidney diseases 7: 9-22.
- Macfarlane GT, Macfarlane S (2011). Fermentation in the human large intestine: its physiologic consequences and the potential contribution of prebiotics. Journal of clinical gastroenterology 45 Suppl: S120-127.
- Macfarlane GT, Macfarlane S (2012). Bacteria, colonic fermentation, and gastrointestinal health. Journal of AOAC International 95: 50-60.
- Markiewicz LH, Honke J, Haros M, Swiatecka D, Wroblewska B (2013). Diet shapes the ability of human intestinal microbiota to degrade phytate--in vitro studies. Journal of applied microbiology 115: 247-259.
- Mellies JL, Thomas K, Turvey M, Evans NR, Crane J, Boedeker E et al (2012). Zinc-induced envelope stress diminishes type III secretion in enteropathogenic Escherichia coli. BMC microbiology 12: 123.
- Mevissen-Verhage EA, Marcelis JH, Harmsen-Van Amerongen WC, de Vos NM, Verhoef J (1985). Effect of iron on neonatal gut flora during the first three months of life. European journal of clinical microbiology 4: 273-278.
- Michiels J, Missotten J, Van Hoorick A, Ovyn A, Fremaut D, De Smet S et al (2010). Effects of dose and formulation of carvacrol and thymol on bacteria and some functional traits of the gut in piglets after weaning. Archives of animal nutrition 64: 136-154.
- Miethke M, Marahiel MA (2007). Siderophore-based iron acquisition and pathogen control. Microbiol Mol Biol Rev 71: 413-451.
- Miethke M, Skerra A (2010). Neutrophil gelatinase-associated lipocalin expresses antimicrobial activity by interfering with L-norepinephrine-mediated bacterial iron acquisition. Antimicrobial agents and chemotherapy 54: 1580-1589.
- Miller CE, Williams PH, Ketley JM (2009). Pumping iron: mechanisms for iron uptake by Campylobacter. Microbiology (Reading, England) 155: 3157-3165.

- Miller TL, Wolin MJ (1996). Pathways of acetate, propionate, and butyrate formation by the human fecal microbial flora. Appl Environ Microbiol 62: 1589-1592.
- Miret S, Simpson RJ, McKie AT (2003). Physiology and molecular biology of dietary iron absorption. Annual review of nutrition 23: 283-301.
- Mishra S, Imlay J (2012). Why do bacteria use so many enzymes to scavenge hydrogen peroxide? Archives of biochemistry and biophysics 525: 145-160.
- Misselwitz B, Kreibich SK, Rout S, Stecher B, Periaswamy B, Hardt WD (2011). Salmonella enterica serovar Typhimurium binds to HeLa cells via Fim-mediated reversible adhesion and irreversible type three secretion system 1-mediated docking. Infection and immunity 79: 330-341.
- Moens S, Vanderleyden J (1996). Functions of bacterial flagella. Critical reviews in microbiology 22: 67-100.
- Moest TP, Meresse S (2013). Salmonella T3SSs: successful mission of the secret(ion) agents. Current opinion in microbiology 16: 38-44.
- Montagne L, Pluske JR, Hampson DJ (2003). A review of interactions between dietary fibre and the intestinal mucosa, and their consequences on digestive health in young non-ruminant animals. Animal Feed Science and Technology 108: 95-117.
- Mortensen PB, Clausen MR (1996). Short-chain fatty acids in the human colon: relation to gastrointestinal health and disease. Scandinavian journal of gastroenterology Supplement 216: 132-148.
- Muller A, Heseler K, Schmidt SK, Spekker K, Mackenzie CR, Daubener W (2009). The missing link between indoleamine 2,3-dioxygenase mediated antibacterial and immunoregulatory effects. Journal of cellular and molecular medicine 13: 1125-1135.
- Muniz LR, Knosp C, Yeretssian G (2012). Intestinal antimicrobial peptides during homeostasis, infection, and disease. Frontiers in immunology 3: 310.
- Naikare H, Palyada K, Panciera R, Marlow D, Stintzi A (2006). Major role for FeoB in Campylobacter jejuni ferrous iron acquisition, gut colonization, and intracellular survival. Infection and immunity 74: 5433-5444.
- Natoli M, Felsani A, Ferruzza S, Sambuy Y, Canali R, Scarino ML (2009). Mechanisms of defence from Fe(II) toxicity in human intestinal Caco-2 cells. Toxicol In Vitro 23: 1510-1515.
- Navaneethan U, Giannella RA (2008). Mechanisms of infectious diarrhea. Nature clinical practice Gastroenterology & hepatology 5: 637-647.
- Nchito M, Friis H, Michaelsen KF, Mubila L, Olsen A (2006). Iron supplementation increases small intestine permeability in primary schoolchildren in Lusaka, Zambia. Transactions of the Royal Society of Tropical Medicine and Hygiene 100: 791-794.
- Nicholson JK, Holmes E, Kinross J, Burcelin R, Gibson G, Jia W et al (2012). Host-gut microbiota metabolic interactions. Science (New York, NY 336: 1262-1267.
- Nielsen AV, Tetens I, Meyer AS (2013). Potential of phytase-mediated iron release from cereal-based foods: a quantitative view. Nutrients 5: 3074-3098.
- Nielsen BS, Borregaard N, Bundgaard JR, Timshel S, Sehested M, Kjeldsen L (1996). Induction of NGAL synthesis in epithelial cells of human colorectal neoplasia and inflammatory bowel diseases. Gut 38: 414-420.
- Noguchi N, Ohashi T, Shiratori T, Narui K, Hagiwara T, Ko M et al (2007). Association of tannase-producing Staphylococcus lugdunensis with colon cancer and characterization of a novel tannase gene. Journal of gastroenterology 42: 346-351.
- Nyangale EP, Mottram DS, Gibson GR (2012). Gut microbial activity, implications for health and disease: the potential role of metabolite analysis. Journal of proteome research 11: 5573-5585.

- Ohkawara Y, Bamba M, Nakai I, Kinka S, Masuda M (1963). The absorption of iron from the human large intestine. Gastroenterology 44: 611-614.
- Oppenheimer SJ (2001). Iron and its relation to immunity and infectious disease. The Journal of nutrition 131: 616S-633S; discussion 633S-635S.
- Pandey A, Bringel F, Meyer J-M (1994). Iron requirement and search for siderophores in lactic acid bacteria. Applied Microbiology and Biotechnology 40: 735-739.
- Payne SM, Finkelstein RA (1978). The critical role of iron in host-bacterial interactions. The Journal of clinical investigation 61: 1428-1440.
- Payne SM (1993). Iron acquisition in microbial pathogenesis. Trends in microbiology 1: 66-69.
- Penders J, Thijs C, Vink C, Stelma FF, Snijders B, Kummeling I et al (2006). Factors influencing the composition of the intestinal microbiota in early infancy. Pediatrics 118: 511-521.
- Pereira DI, Mergler BI, Faria N, Bruggraber SF, Aslam MF, Poots LK et al (2013). Caco-2 cell acquisition of dietary iron(III) invokes a nanoparticulate endocytic pathway. PloS one 8: e81250.
- Petry N, Egli I, Chassard C, Lacroix C, Hurrell R (2012). Inulin modifies the bifidobacteria population, fecal lactate concentration, and fecal pH but does not influence iron absorption in women with low iron status. The American journal of clinical nutrition 96: 325-331.
- Pi H, Jones SA, Mercer LE, Meador JP, Caughron JE, Jordan L et al (2012). Role of catecholate siderophores in gram-negative bacterial colonization of the mouse gut. PloS one 7: e50020.
- Pierre JL, Fontecave M, Crichton RR (2002). Chemistry for an essential biological process: the reduction of ferric iron. Biometals 15: 341-346.
- Pizarro F, Amar M, Stekel A (1987). Determination of iron in stools as a method to monitor consumption of iron-fortified products in infants. The American journal of clinical nutrition 45: 484-487.
- Posey JE, Gherardini FC (2000). Lack of a role for iron in the Lyme disease pathogen. Science (New York, NY 288: 1651-1653.
- Powell JJ, Cook WB, Hutchinson C, Tolkien Z, Chatfield M, Pereira D et al (2013). Dietary fortificant iron intake is negatively associated with quality of life in patients with mildly active inflammatory bowel disease. Nutrition & metabolism 10: 9.
- Powell JJ, Bruggraber SF, Faria N, Poots LK, Hondow N, Pennycook TJ et al (2014). A nano-disperse ferritin-core mimetic that efficiently corrects anemia without luminal iron redox activity. Nanomedicine : nanotechnology, biology, and medicine.
- Power SE, O'Toole PW, Stanton C, Ross RP, Fitzgerald GF (2014). Intestinal microbiota, diet and health. The British journal of nutrition 111: 387-402.
- Prentice AM, Doherty CP, Abrams SA, Cox SE, Atkinson SH, Verhoef H et al (2012). Hepcidin is the major predictor of erythrocyte iron incorporation in anemic African children. Blood 119: 1922-1928.
- Que F, Wu S, Huang R (2013). Salmonella pathogenicity island 1(SPI-1) at work. Current microbiology 66: 582-587.
- Raffatellu M, George MD, Akiyama Y, Hornsby MJ, Nuccio SP, Paixao TA et al (2009). Lipocalin-2 resistance confers an advantage to Salmonella enterica serotype Typhimurium for growth and survival in the inflamed intestine. Cell host & microbe 5: 476-486.
- Raffatellu M, Baumler AJ (2010). Salmonella's iron armor for battling the host and its microbiota. Gut microbes 1: 70-72.
- Ragsdale SW, Pierce E (2008). Acetogenesis and the Wood-Ljungdahl pathway of CO(2) fixation. Biochimica et biophysica acta 1784: 1873-1898.

- Raqib R, Sarker P, Bergman P, Ara G, Lindh M, Sack DA et al (2006). Improved outcome in shigellosis associated with butyrate induction of an endogenous peptide antibiotic. Proc Natl Acad Sci U S A 103: 9178-9183.
- Ratledge C, Dover LG (2000). Iron metabolism in pathogenic bacteria. Annual review of microbiology 54: 881-941.
- Raymond KN, Dertz EA, Kim SS (2003). Enterobactin: an archetype for microbial iron transport. Proc Natl Acad Sci U S A 100: 3584-3588.
- Reddy BS, Pleasants JR, Wostmann BS (1972). Effect of intestinal microflora on iron and zinc metabolism, and on activities of metalloenzymes in rats. The Journal of nutrition 102: 101-107.
- Reifen R, Matas Z, Zeidel L, Berkovitch Z, Bujanover Y (2000). Iron supplementation may aggravate inflammatory status of colitis in a rat model. Digestive diseases and sciences 45: 394-397.
- Reissbrodt R, Kingsley R, Rabsch W, Beer W, Roberts M, Williams PH (1997). Iron-regulated excretion of alpha-keto acids by Salmonella typhimurium. Journal of bacteriology 179: 4538-4544.
- Ricke SC (2003). Perspectives on the use of organic acids and short chain fatty acids as antimicrobials. Poultry science 82: 632-639.
- Rist VT, Weiss E, Eklund M, Mosenthin R (2013). Impact of dietary protein on microbiota composition and activity in the gastrointestinal tract of piglets in relation to gut health: a review. Animal : an international journal of animal bioscience 7: 1067-1078.
- Rivera-Chavez F, Winter SE, Lopez CA, Xavier MN, Winter MG, Nuccio SP et al (2013). Salmonella uses energy taxis to benefit from intestinal inflammation. PLoS pathogens 9: e1003267.
- Romanowski K, Zaborin A, Fernandez H, Poroyko V, Valuckaite V, Gerdes S et al (2011). Prevention of siderophore- mediated gut-derived sepsis due to P. aeruginosa can be achieved without iron provision by maintaining local phosphate abundance: role of pH. BMC microbiology 11: 212.
- Rostoker G, Griuncelli M, Loridon C, Couprie R, Benmaadi A, Bounhiol C et al (2012). Hemodialysis-associated hemosiderosis in the era of erythropoiesis-stimulating agents: a MRI study. The American journal of medicine 125: 991-999 e991.
- Saini S, Slauch JM, Aldridge PD, Rao CV (2010). Role of cross talk in regulating the dynamic expression of the flagellar Salmonella pathogenicity island 1 and type 1 fimbrial genes. Journal of bacteriology 192: 5767-5777.
- Salovaara S, Sandberg AS, Andlid T (2003). Combined impact of pH and organic acids on iron uptake by Caco-2 cells. Journal of agricultural and food chemistry 51: 7820-7824.
- San Martin CD, Garri C, Pizarro F, Walter T, Theil EC, Núñez MT (2008). Caco-2 Intestinal Epithelial Cells Absorb Soybean Ferritin by μ2 (AP2)-Dependent Endocytosis. The Journal of nutrition 138: 659-666.
- Santacruz A, Collado MC, Garcia-Valdes L, Segura MT, Martin-Lagos JA, Anjos T et al (2010). Gut microbiota composition is associated with body weight, weight gain and biochemical parameters in pregnant women. The British journal of nutrition 104: 83-92.
- Sazawal S, Black RE, Ramsan M, Chwaya HM, Stoltzfus RJ, Dutta A et al (2006). Effects of routine prophylactic supplementation with iron and folic acid on admission to hospital and mortality in preschool children in a high malaria transmission setting: community-based, randomised, placebo-controlled trial. Lancet 367: 133-143.
- Schierack P, Kleta S, Tedin K, Babila JT, Oswald S, Oelschlaeger TA et al (2011). E. coli Nissle 1917 Affects Salmonella Adhesion to Porcine Intestinal Epithelial Cells. PloS one 6: e14712.

- Schlemmer U, Frolich W, Prieto RM, Grases F (2009). Phytate in foods and significance for humans: food sources, intake, processing, bioavailability, protective role and analysis. Molecular nutrition & food research 53 Suppl 2: S330-375.
- Sekirov I, Finlay BB (2009). The role of the intestinal microbiota in enteric infection. The Journal of physiology 587: 4159-4167.
- Sekirov I, Russell SL, Antunes LC, Finlay BB (2010). Gut microbiota in health and disease. Physiological reviews 90: 859-904.
- Seril DN, Liao J, Ho KL, Warsi A, Yang CS, Yang GY (2002). Dietary iron supplementation enhances DSS-induced colitis and associated colorectal carcinoma development in mice. Digestive diseases and sciences 47: 1266-1278.
- Simpson RJ, Peters TJ (1990). Forms of soluble iron in mouse stomach and duodenal lumen: significance for mucosal uptake. The British journal of nutrition 63: 79-89.
- Simpson RJ, Sidhar S, Peters TJ (1992). Application of selective extraction to the study of iron species present in diet and rat gastrointestinal tract contents. The British journal of nutrition 67: 437-444.
- Smith AH, Zoetendal E, Mackie RI (2005). Bacterial mechanisms to overcome inhibitory effects of dietary tannins. Microbial ecology 50: 197-205.
- Soofi S, Cousens S, Iqbal SP, Akhund T, Khan J, Ahmed I et al (2013). Effect of provision of daily zinc and iron with several micronutrients on growth and morbidity among young children in Pakistan: a cluster-randomised trial. Lancet 382: 29-40.
- Spor A, Koren O, Ley R (2011). Unravelling the effects of the environment and host genotype on the gut microbiome. Nature reviews 9: 279-290.
- Stecher B, Robbiani R, Walker AW, Westendorf AM, Barthel M, Kremer M et al (2007). Salmonella enterica serovar typhimurium exploits inflammation to compete with the intestinal microbiota. PLoS biology 5: 2177-2189.
- Stecher B, Barthel M, Schlumberger MC, Haberli L, Rabsch W, Kremer M et al (2008). Motility allows S. Typhimurium to benefit from the mucosal defence. Cellular microbiology 10: 1166-1180.
- Stecher B, Hardt WD (2008). The role of microbiota in infectious disease. Trends in microbiology 16: 107-114.
- Stecher B, Chaffron S, Kappeli R, Hapfelmeier S, Freedrich S, Weber TC et al (2010). Like will to like: abundances of closely related species can predict susceptibility to intestinal colonization by pathogenic and commensal bacteria. PLoS pathogens 6: e1000711.
- Stojiljkovic I, Cobeljic M, Hantke K (1993). Escherichia coli K-12 ferrous iron uptake mutants are impaired in their ability to colonize the mouse intestine. FEMS microbiology letters 108: 111-115.
- Takeuchi K, Bjarnason I, Laftah AH, Latunde-Dada GO, Simpson RJ, McKie AT (2005). Expression of iron absorption genes in mouse large intestine. Scandinavian journal of gastroenterology 40: 169-177.
- Tako E, Glahn RP, Welch RM, Lei X, Yasuda K, Miller DD (2008). Dietary inulin affects the expression of intestinal enterocyte iron transporters, receptors and storage protein and alters the microbiota in the pig intestine. The British journal of nutrition 99: 472-480.
- Tang Y, Guest JR, Artymiuk PJ, Read RC, Green J (2004). Post-transcriptional regulation of bacterial motility by aconitase proteins. Mol Microbiol 51: 1817-1826.
- Tang Y, Guest JR, Artymiuk PJ, Green J (2005). Switching aconitase B between catalytic and regulatory modes involves iron-dependent dimer formation. Mol Microbiol 56: 1149-1158.

- Teixido L, Carrasco B, Alonso JC, Barbe J, Campoy S (2011). Fur activates the expression of Salmonella enterica pathogenicity island 1 by directly interacting with the hilD operator in vivo and in vitro. PloS one 6: e19711.
- Theil EC (2004). Iron, ferritin, and nutrition. Annual review of nutrition 24: 327-343.
- Thiennimitr P, Winter SE, Winter MG, Xavier MN, Tolstikov V, Huseby DL et al (2011). Intestinal inflammation allows Salmonella to use ethanolamine to compete with the microbiota. Proc Natl Acad Sci U S A 108: 17480-17485.
- Tolkien Z, Pereira DI, Prassmayer L, Fitt E, Pot G, Greenfield SM et al (2013). Dietary iron does not impact the quality of life of patients with quiescent ulcerative colitis: an observational study. Nutrition journal 12: 152.
- Tompkins GR, O'Dell NL, Bryson IT, Pennington CB (2001). The effects of dietary ferric iron and iron deprivation on the bacterial composition of the mouse intestine. Current microbiology 43: 38-42.
- Topping DL, Clifton PM (2001). Short-chain fatty acids and human colonic function: roles of resistant starch and nonstarch polysaccharides. Physiological reviews 81: 1031-1064.
- Torres AG, Cieza RJ, Rojas-Lopez M, Blumentritt CA, Souza CS, Johnston RK et al (2012). In vivo bioluminescence imaging of Escherichia coli O104:H4 and role of aerobactin during colonization of a mouse model of infection. BMC microbiology 12: 112.
- Troesch B, Egli I, Zeder C, Hurrell RF, de Pee S, Zimmermann MB (2009). Optimization of a phytase-containing micronutrient powder with low amounts of highly bioavailable iron for in-home fortification of complementary foods. The American journal of clinical nutrition 89: 539-544.
- Troxell B, Sikes ML, Fink RC, Vazquez-Torres A, Jones-Carson J, Hassan HM (2011). Fur negatively regulates hns and is required for the expression of HilA and virulence in Salmonella enterica serovar Typhimurium. Journal of bacteriology 193: 497-505.
- Tsolis RM, Baumler AJ, Heffron F, Stojiljkovic I (1996). Contribution of TonB- and Feo-mediated iron uptake to growth of Salmonella typhimurium in the mouse. Infection and immunity 64: 4549-4556.
- Tsolis RM, Adams LG, Ficht TA, Baumler AJ (1999). Contribution of Salmonella typhimurium virulence factors to diarrheal disease in calves. Infection and immunity 67: 4879-4885.
- van Baarlen P, Wells JM, Kleerebezem M (2013). Regulation of intestinal homeostasis and immunity with probiotic lactobacilli. Trends in immunology 34: 208-215.
- Van den Abbeele P, Van de Wiele T, Verstraete W, Possemiers S (2011). The host selects mucosal and luminal associations of coevolved gut microorganisms: a novel concept. FEMS microbiology reviews 35: 681-704.
- van Dokkum W (1992). Significance of iron bioavailability for iron recommendations. Biological trace element research 35: 1-11.
- Van Immerseel F, Russell JB, Flythe MD, Gantois I, Timbermont L, Pasmans F et al (2006). The use of organic acids to combat Salmonella in poultry: a mechanistic explanation of the efficacy. Avian pathology : journal of the WVPA 35: 182-188.
- van Santen S, de Mast Q, Swinkels DW, van der Ven AJ (2013). The iron link between malaria and invasive non-typhoid Salmonella infections. Trends in parasitology 29: 220-227.
- Vereecke L, Beyaert R, van Loo G (2011). Enterocyte death and intestinal barrier maintenance in homeostasis and disease. Trends in molecular medicine 17: 584-593.
- Vieira AT, Teixeira MM, Martins FS (2013). The Role of Probiotics and Prebiotics in Inducing Gut Immunity. Frontiers in immunology 4: 445.

- Wagner C, Hensel M (2011). Adhesive mechanisms of Salmonella enterica. Advances in experimental medicine and biology 715: 17-34.
- Wang L, Johnson EE, Shi HN, Walker WA, Wessling-Resnick M, Cherayil BJ (2008). Attenuated inflammatory responses in hemochromatosis reveal a role for iron in the regulation of macrophage cytokine translation. Journal of immunology 181: 2723-2731.
- Wang L, Cherayil BJ (2009). Ironing out the wrinkles in host defense: interactions between iron homeostasis and innate immunity. Journal of innate immunity 1: 455-464.
- Weinberg ED (1978). Iron and infection. Microbiological reviews 42: 45-66.
- Weiss G (2005). Modification of iron regulation by the inflammatory response. Best practice & research Clinical haematology 18: 183-201.
- Weiss G (2011). Iron in the inflammed gut: another pro-inflammatory hit? Gut 60: 287-288.
- Werner T, Wagner SJ, Martinez I, Walter J, Chang JS, Clavel T et al (2011). Depletion of luminal iron alters the gut microbiota and prevents Crohn's disease-like ileitis. Gut 60: 325-333.
- WHO, UNICEF (2004). Focusing on anaemia: Towards an integrated approach for effective anaemia control.
- WHO, FAO (2006). Guidelines on food fortification with micronutrients.
- WHO, UNICEF (2006). Joint Statement: Iron supplementation of young children in regions where malaria transmission is intense and infectious disease highly prevalent.
- WHO (2007). Conclusions and recommendations of the WHO Consultation on prevention and control of iron deficiency in infants and young children in malaria-endemic areas. Food Nutr Bull 28: S621-627.
- WHO, UNICEF (2009). Diarrhoea: why children are still dying and what can be done.
- WHO (2011). Guideline: Use of multiple micronutrient powders for home fortification of foods consumed by infants and children 6–23 months of age. Geneva, World Health Organization.
- WHO (2013). World Health Statistics 2013.
- Windey K, De Preter V, Louat T, Schuit F, Herman J, Vansant G et al (2012a). Modulation of protein fermentation does not affect fecal water toxicity: a randomized cross-over study in healthy subjects. PloS one 7: e52387.
- Windey K, De Preter V, Verbeke K (2012b). Relevance of protein fermentation to gut health. Molecular nutrition & food research 56: 184-196.
- Winter SE, Thiennimitr P, Winter MG, Butler BP, Huseby DL, Crawford RW et al (2010). Gut inflammation provides a respiratory electron acceptor for Salmonella. Nature 467: 426-429.
- Winter SE, Baumler AJ (2011). A breathtaking feat: to compete with the gut microbiota, Salmonella drives its host to provide a respiratory electron acceptor. Gut microbes 2: 58-60.
- Winter SE, Lopez CA, Baumler AJ (2013a). The dynamics of gut-associated microbial communities during inflammation. EMBO reports 14: 319-327.
- Winter SE, Winter MG, Xavier MN, Thiennimitr P, Poon V, Keestra AM et al (2013b). Host-derived nitrate boosts growth of E. coli in the inflamed gut. Science (New York, NY 339: 708-711.
- Wooldridge KG, Williams PH (1993). Iron uptake mechanisms of pathogenic bacteria. FEMS microbiology reviews 12: 325-348.
- Yatsunenko T, Rey FE, Manary MJ, Trehan I, Dominguez-Bello MG, Contreras M et al (2012). Human gut microbiome viewed across age and geography. Nature 486: 222-227.
- Yeung CK, Glahn RE, Welch RM, Miller DD (2005). Prebiotics and Iron Bioavailability—Is There a Connection? Journal of Food Science 70: R88-R92.

- Zaharik ML, Vallance BA, Puente JL, Gros P, Finlay BB (2002). Host-pathogen interactions: Host resistance factor Nramp1 up-regulates the expression of Salmonella pathogenicity island-2 virulence genes. Proc Natl Acad Sci U S A 99: 15705-15710.
- Zheng X, Xie G, Zhao A, Zhao L, Yao C, Chiu NH et al (2011). The footprints of gut microbial-mammalian co-metabolism. Journal of proteome research 10: 5512-5522.
- Zhu A, Kaneshiro M, Kaunitz JD (2010). Evaluation and treatment of iron deficiency anemia: a gastroenterological perspective. Digestive diseases and sciences 55: 548-559.
- Zimmermann MB, Hurrell RF (2007). Nutritional iron deficiency. Lancet 370: 511-520.
- Zimmermann MB, Chassard C, Rohner F, N'Goran E K, Nindjin C, Dostal A et al (2010). The effects of iron fortification on the gut microbiota in African children: a randomized controlled trial in Cote d'Ivoire. The American journal of clinical nutrition 92: 1406-1415.
- Zlotkin S, Newton S, Aimone AM, Azindow I, Amenga-Etego S, Tchum K et al (2013). Effect of iron fortification on malaria incidence in infants and young children in Ghana: a randomized trial. JAMA : the journal of the American Medical Association 310: 938-947.
- Zoetendal EG, Raes J, van den Bogert B, Arumugam M, Booijink CC, Troost FJ et al (2012). The human small intestinal microbiota is driven by rapid uptake and conversion of simple carbohydrates. The ISME journal 6: 1415-1426.

Chapter 2

Iron fortification adversely affects the gut microbiome, increases pathogen abundance and induces intestinal inflammation in Kenyan infants

Tanja Jaeggi[1], Guus AM Kortman[2], Diego Moretti[1], Christophe Chassard[1], Penny Holding[3], Alexandra Dostal[1], Jos Boekhorst[4], Harro M Timmerman[4], Dorine W Swinkels[2], Harold Tjalsma[2], Jane Njenga[5], Alice Mwangi[5], Jane Kvalsvig[6], Christophe Lacroix[1] and Michael B Zimmermann[1]

[1] Department of Health Sciences and Technology, ETH Zurich, Zurich, Switzerland
[2] Department of Laboratory Medicine – Laboratory of Genetic, Endocrine and Metabolic Diseases, Radboud University Medical Center, Nijmegen, The Netherlands
[3] International Centre for Behavioural Studies, Mombasa, Kenya
[4] NIZO Food Research B.V., Ede, The Netherlands
[5] Department of Food, Technology and Nutrition, University of Nairobi, Nairobi, Kenya
[6] University of KwaZulu-Natal, Durban, South Africa

This chapter is published in Gut (August 2014, ahead of print)

Abstract

In-home iron fortification for infants in developing countries is recommended to control anaemia but low absorption typically results in >80% of the iron passing into the colon. Iron is essential for growth and virulence of many pathogenic enterobacteria. We determined the effect of high and low dose in-home iron fortification on the infant gut microbiome and intestinal inflammation.

We performed two double-blind randomised controlled trials in 6 month old Kenyan infants (n=115) consuming home-fortified maize porridge daily for four months. In the first, infants received a micronutrient powder (MNP) containing 2.5 mg iron as NaFeEDTA or the MNP without iron. In the second, they received a different MNP containing 12.5 mg iron as ferrous fumarate or control. The primary outcome was gut microbiome composition analysed by 16S pyrosequencing and targeted real-time PCR (qPCR). Secondary outcomes included faecal calprotectin (marker of intestinal inflammation) and incidence of diarrhoea. We analysed the trials separately and combined. The study is registered with ClinicalTrials.gov, number NCT01111864.

At baseline, 63% of the total microbial 16S rRNA could be assigned to Bifidobacteriaceae but there were high prevalences of pathogens, including *Salmonella, C. difficile, C. perfringens*, and pathogenic *E. coli.* Using pyrosequencing, +FeMNPs increased enterobacteria, particularly *Escherichia/Shigella* ($p = 0.048$), the enterobacteria/bifidobacteria ratio ($p = 0.020$), and *Clostridium* ($p = 0.030$). Most of these effects were confirmed using qPCR; e.g., +FeMNPs increased pathogenic E. coli strains ($p = 0.029$). +FeMNPs also increased faecal calprotectin ($p = 0.002$). During the trial, 27.3% of infants in +12.5mgFeMNP required treatment for diarrhoea versus 8.3% in -12.5mgFeMNP ($p = 0.092$). There were no study-related serious adverse events in either group.

In this setting, provision of iron-containing MNPs to weaning infants adversely affects the gut microbiome, increasing pathogen abundance and causing intestinal inflammation.

Introduction

While infants have the highest rates of iron deficiency anaemia (IDA), they are also the group less well covered by universal fortification programmes. Micronutrient powders (MNPs), added directly to complementary foods after cooking (in-home fortification), can be an effective approach for providing additional dietary iron and reducing anaemia rates (Adu-Afarwuah et al 2008). Currently, in-home fortification programmes are in place or planned in 36 countries including 10 in Sub-Saharan Africa (UNICEF-CDC 2013).

However, the safety of iron-containing MNPs (+FeMNPs) is uncertain. A 2007 WHO Consultation, on the results of the Pemba trial where iron and folic acid supplementation increased child mortality (Sazawal et al 2006), did not recommend the use of +FeMNPs in malaria-endemic areas because of concerns about potential increases in infection (Fontaine 2007). Two recent fortification trials in infants with an MNP containing 12.5 mg ferrous fumarate have raised safety concerns: in Ghana, there was an increased rate of hospitalisations possibly due to diarrhoea (Zlotkin et al 2013), and in Pakistan a small but significant increase in overall diarrhoea prevalence, bloody diarrhoea, and respiratory illness (Soofi et al 2013). An earlier systematic review concluded iron supplementation, but not fortification, may increase risk for diarrhoea (Gera and Sachdev 2002). If +FeMNPs increase risk for infection and diarrhoeal disease, this would be an important adverse effect, as diarrhoea contributes to the death of ≈1 in 9 under 5-year-old children in Sub-Saharan Africa (WHO 2013).

The food matrix of most cereal- and legume-based complementary foods is rich in phytic acid, a potent inhibitor of iron absorption; therefore usually less than 20% of iron added to these foods is absorbed (Tondeur et al 2004, Zimmermann and Hurrell 2007). In rural African populations with high levels of inflammation and infection, absorption is likely to be even lower, as inflammation increases circulating hepcidin the major iron regulator, which reduces dietary iron absorption through binding and degradation of the iron efflux protein ferroportin at the basolateral membrane of the enterocytes (Nemeth et al 2004). Thus, providing +FeMNPs to individuals with concurrent inflammation results in most of the iron passing unabsorbed into the colon. Once absorbed, iron in the body is bound to proteins limiting iron access to potential pathogens, and during infection iron supply is further reduced in the extracellular compartment and remains in macrophages and enterocytes (Cassat and Skaar 2013). There is no comparable system for sequestration of dietary iron known in the gut lumen, although neutral pH and the presence of defence molecules such as lipocalin-2 (Raffatellu et al 2009) may reduce iron solubility and availability to gut microbes.

Iron is an essential, growth-limiting nutrient for many gut bacteria, competing for unabsorbed dietary iron (Andrews et al 2003). For most enteric gram-negative bacteria (e.g. *Salmonella*, *Shigella* or pathogenic *Escherichia coli*), iron acquisition plays an essential role in virulence and colonization (Naikare et al 2006). In contrast, lactobacilli, a major group of beneficial 'barrier' bacteria improving gut integrity and reducing colonization by enteric pathogens (Anderson et al 2010), do not require iron, but instead rely on manganese (Weinberg 1997). Therefore, an increase in unabsorbed dietary iron through fortification or supplementation could modify the colonic microbiota equilibrium and favour growth of pathogenic strains over 'barrier' strains.

Colonization of the human gastrointestinal tract begins at birth and depends on the mode of delivery, hygiene and prematurity (Penders et al 2006). The iron-binding protein lactoferrin in breast milk limits iron availability to the gut microbiota (Yen et al 2011), and may have a protective effect in breastfed infants. The relatively simple gut microbiota of breast-fed infants further diversifies with the introduction of complementary feeding (Vael and Desager 2009). In a study comparing the gut microbiome among infants from the US, Venezuela and Malawi, there were significant differences between sites but a common pattern was the dominance of bifidobacteria throughout the first year of life, thereafter bifidobacteria diminish steadily leading to the establishment of an adult-like gut microbiome at about 3 years of age (Yatsunenko 2012).
In a controlled trial of iron fortification in schoolchildren in Côte d'Ivoire, iron increased enterobacteria and intestinal inflammation, and decreased lactobacilli (Zimmermann et al 2010). In older studies in European infants using culture methods, iron induced an increase in *E. coli* and a decrease in bifidobacteria (Balmer et al 1989), along with higher counts of *Bacteroides* (Mevissen-Verhage et al 1985). A recent small study in American infants (n=14) receiving complementary feeding regimens providing iron from fortified cereals or meat reported an increase in Bacteroidales and decreases in bifidobacteria, Lactobacillales and *Rothia* (Krebs et al 2013). In infants in Pakistan, +FeMNPs caused an increase in *Aeromonas* spp. compared to the non-supplemented control group (Soofi et al 2013).
Our study aim was to determine the effects of two widely used high and low dose +FeMNPs on the gut microbiome, using both barcoded 16S rRNA pyrosequencing and targeted real-time PCR (quantitative polymerase chain reaction, qPCR), and intestinal inflammation in Kenyan infants prone to diarrhoeal disease. Our hypotheses were that iron fortification would: a) increase faecal enterobacteria and its ratio to bifidobacteria and/or lactobacilli; b) favour colonization by potential pathogens; and c) increase intestinal inflammation.

Materials and methods

Study site and participants

The study was conducted in Msambweni County, in southern coastal Kenya. This is a malaria-endemic area, where 40% of the paediatric hospital admissions in 2006 were due to plasmodial infections (Okiro et al 2007). The region experiences a long rainy season from April to July and short rains from October to November. Farming is the main economic activity and maize the staple food crop, in this sparsely populated area. The typical local weaning food is the liquid maize porridge *uji*; a regular portion consists of about 8-10 g maize flour boiled in 100-150 mL water and sweetened with sugar.

In the catchment area of the Kikoneni health centre, we continuously recruited infants aged 5.5 months. We recruited 80 infants from March 2010 until September 2011 and randomly assigned them to receive either an MNP with or without 2.5 mg iron as sodium iron ethylenediaminetetraacetic acid (NaFeEDTA, ±2.5mgFeMNP, MixMe®, DSM Nutritional Products Europe Ltd, Basel, Switzerland). We recruited 80 more infants from September 2011 to May 2012 and randomly assigned them to receive either an MNP with or without

Table 1: Composition of the two micronutrient powders, with and without iron*

	+2.5mgFeMNP	-2.5mgFeMNP	+12.5mgFeMNP	-12.5mgFeMNP
Iron, mg	2.5	-	12.5	-
Zinc, mg	2.5	2.5	5	5
Vitamin A, µg	100	100	300	300
Vitamin D, µg	5	5	5	5
Vitamin C, mg	60	60	30	30
Copper, mg	0.34	0.34	-	-
Tocopherol Equivalent, mg	5	5	-	-
Iodine, µg	30	30	-	-
Vitamin K1, µg	30	30	-	-
Selenium, µg	17	17	-	-
Thiamine, mg	0.5	0.5	-	-
Riboflavin, mg	0.5	0.5	-	-
Pyridoxine, mg	0.5	0.5	-	-
Folic Acid Anhydrous, µg	90	90	-	-
Niacinamide, mg	6	6	-	-
Vitamin B12, µg	0.9	0.9	-	-

*amounts per 1g powder

12.5 mg iron as ferrous fumarate (±12.5mgFeMNP, Sprinkles®, Hexagon Nutrition, Mumbai). The composition of the MNPs is shown in **Table 1**. Inclusion criteria were an infant age of 5.5 months (± 3 weeks), mother at least ≥15 years of age, infant haemoglobin (Hb) ≥70 g/L, and no maternal or infant chronic diseases. The MNPs were packed in group-coded sachets (containing one daily dose).

Study design

Prior intervention, we conducted triangle sensory tests (Meilgaard et al 2007) in local adults (n=25 per MNP). In these tests, the +FeMNPs were indistinguishable from the corresponding -FeMNPs and their acceptability was high (data not shown).
Trained field workers instructed the mothers in cooking the *uji* and its fortification with the MNPs. Further, the participating mothers were trained on the home collection of the infant stool samples. Before starting the intervention with MNPs, we conducted a two week run-in period to familiarise the families with the stool collection method and the introduction of the maize porridge.

Then, weekly for 4 months, we dispensed 7 MNP sachets and 2 kg of maize flour (Dola, Kitui Flour Mills Ltd, Mombasa, Kenya) directly to the participating mothers from six distribution points. We analysed triplicate samples of the maize flour for iron concentration by using atomic absorption spectroscopy, and for phytic acid (Makower 1970, Vanveldhoven and Mannaerts 1987) at the ETH Zurich. During the weekly distribution, field workers collected the previous week's used and unused MNP sachets to assess compliance, recorded the infant feeding history and the health status by using a multiple choice questionnaire. If

a mother reported any illness the dedicated study nurse examined the child and recorded treated episodes of malaria, diarrhoea and respiratory tract infections (RTI). The study nurse used a forced choice questionnaire to record treated cases of malaria, diarrhoea and respiratory tract infections at baseline (covering the last 3 months) and after 4 months (covering the entire intervention period).

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

Stool samples were collected at baseline, 3 weeks and 4. The mothers were provided with plastic diapers, containers, spatulas, and Anaerocult sachets (Merck KGaA, Darmstadt, Germany) to generate an anaerobic environment, a falcon tube for the measurement of 8 mL water to wet the Anaerocult, along with an illustrated pamphlet to reinforce the sampling instructions. The stool samples were collected in the morning, kept anaerobic, and aliquots were frozen at -20°C the same day.

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

analysed using the 7500 Fast System Sequence Detection Software (Version 1.4, Applied Biosystems).

We measured faecal calprotectin using the Calprest ELISA assay for stools, following the manufacturer's procedures (Eurospital, Trieste, Italy). For the measurement of the SCFAs (acetate, propionate, and butyrate), we homogenised 100-300 mg of stool in 1 mL 0.15 mM sulphuric acid and centrifuged at 9000 rpm and 2°C for 20 minutes (Underwood et al 2009). The supernatant was transferred into a microconcentrator and filtered by centrifugation at 4700 rpm and 2°C for 90 minutes (Chen and Lifschitz 1989). The HPLC analysis was performed using a Phenomenex column (Rezex ROA-Organic Acid H+ (8%), 300*7.8 mm).

Biochemical indicators

At baseline, after 4 (±12.5mgFeMNP arms) and 6 months (±2.5mgFeMNP arms), venous blood samples (3 mL) were drawn using heparin vacutainers and butterfly needles. Haemoglobin was measured immediately after collection on site with a HemoCue (HemoCue AB, Ängelholm, Sweden) or a HemoControl device (EKF diagnostics Sales GmbH, Barleben/Magdeburg, Germany). Serum was separated by centrifugation and frozen on collection day. The remaining erythrocytes were washed thrice with normal saline, and zinc protoporphyrin to haem ratio (ZPP) was measured using a calibrated AVIV hematofluorometer (AVIV Biomedical, Lakewood, USA). Serum ferritin (SF), soluble transferrin receptor (sTfR) and C-reactive protein (CRP) were analysed at Lancet Laboratories in Nairobi using the Cobas Integra (Roche, Basel, Switzerland). We converted the Roche sTfR concentration to the Flowers assay (Flowers et al 1989) using the regression equation by Pfeiffer (Flowers=1.5*Roche +0.35) (Pfeiffer et al 2007). Serum hepcidin-25 was measured in Nijmegen (hepcidinanalysis.com, the Netherlands) by a combination of weak cation exchange chromatography and time-of-flight mass spectrometry (WCX-TOF MS) (Jaeggi et al 2013, Kroot et al 2010). Body iron stores (mg/kg body weight) were calculated from the ratio of sTfR to SF according to the equation by Cook et al. (body iron (mg/kg)=-[log10 (sTfR*1000/SF) -2.8229)]/0.1207) (Cook et al 2003), and total body iron stores (mg) by multiplying with body weight. The following cut-offs were used: a) anaemia: Hb <110 g/L (WHO 2001), b) ID: body iron stores <0 mg/kg (Cook et al 2003); and c) inflammation: CRP ≥4.1 mg/L (manufacturer's reference range).

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

Anthropometric indicators

At baseline and after 4 months, infant weight was recorded using a hanging scale (Salter 235-6S, 25 kg x 100 g; Salter Brecknell, UK) and length using a measurement board (Shorr Production, LLC., Olney, MD). We calculated weight-for-age (WAZ), height-for-age (HAZ), weight-for-height (WHZ) and BMI-for-age (BAZ) Z-scores using the WHO Anthro software (Habil et al 2011).

Statistical analysis

Data were analysed using IBM SPSS Statistics 20.0.0 (SPSS Inc., Chicago, IL) and Microsoft Office EXCEL 2010 (Microsoft, Redmond, WA). Data were double entered and distribution checked for normality; not normally distributed data were log transformed. Normally distributed data were expressed as means and standard deviations (SD) or standard errors of the mean (SEM). For log transformed data, we obtained geometric means (GM) and corresponding standard deviations (SD) for absolute concentrations by taking the antilog of these values. Correlations of gut microbial subgroups and intestinal inflammation were done using crude values and Kendall's tau. Pyrosequencing data were analysed with a workflow based on QIIME v1.2,(Caporaso et al 2010) and reads were filtered for chimeric sequences using Chimera Slayer.(Haas et al 2011) OTU clustering was performed with settings as recommended in the QIIME newsletter of December 17th 2010 (http://qiime.wordpress.com/2010/12/17/new-default-parameters-for-uclust-otu-pickers/) using an identity threshold of 97%. Diversity metrics were calculated as implemented in QIIME 1.2. Hierarchical clustering of samples was performed using UPGMA with weighted UniFrac as a distance measure as implemented in QIIME 1.2. The RDP classifier version 2.2 was performed for taxonomic classification (Cole et al 2009). Visualisation of differences in relative abundance of taxa between different study groups was done in Cytoscape (Shannon et al 2003). The baseline gut microbiome composition was illustrated using the approach presented by Sundquist et al. (Sundquist et al 2007). Statistical analysis of the pyrosequencing data was done with SciPy (www.scipy.org). Differences in relative abundance between groups at a single time point (cross-sectional) were compared by Mann-Whitney U (MWU) testing. Comparisons of targets of our primary interest (the phyla Firmicutes and Bacteroidetes, and the taxa lactobacilli, *Roseburia* spp., *Clostridium* spp., bifidobacteria, and enterobacteria) were not corrected for multiple testing.

Longitudinal effects of intervention were statistically assessed by comparing change over time, which were calculated by dividing the relative abundance of a taxon at 4 months or 3 weeks by the relative abundance of a taxon at baseline. These changes over time for two groups were compared by MWU. Changes over time of ratios of enterobacteria to bifidobacteria or lactobacilli (ratio of relative abundances) were compared the same way. The development of specific taxa and phylogenetic diversity over time was assessed by paired testing using the Wilcoxon matched-pairs signed-rank test. Multivariate redundancy analysis (RDA) was performed in R (http://www.R-project.org) using the vegan package (Oksanen et al 2012).

For the qPCR analysis, a total of 22 bacterial targets were tested for a treatment effect in univariate general linear models (GLM) for the two MNPs, using baseline variables as covariates (**Supplementary Table 1**). Moreover, we assessed treatment effects of any iron fortification by pooling data from the two iron groups (+FeMNP) and control groups (-FeMNP) and using univariate GLM, including baseline values as covariates. A summary variable was created for the pathogenic *E. coli* community by summing copy numbers of the *eaeA* (*E. coli* attaching and effacing) gene detecting EPEC and EHEC strains, and of the heat-labile and heat-stable enterotoxin gene detecting ETEC LT and ETEC ST strains (Fukushima et al 2003).

Treatment effect on weight and height were assessed using GLM with baseline variables as covariates. Treatment effects on the incidence of diarrhoea, malaria, and RTI were assessed

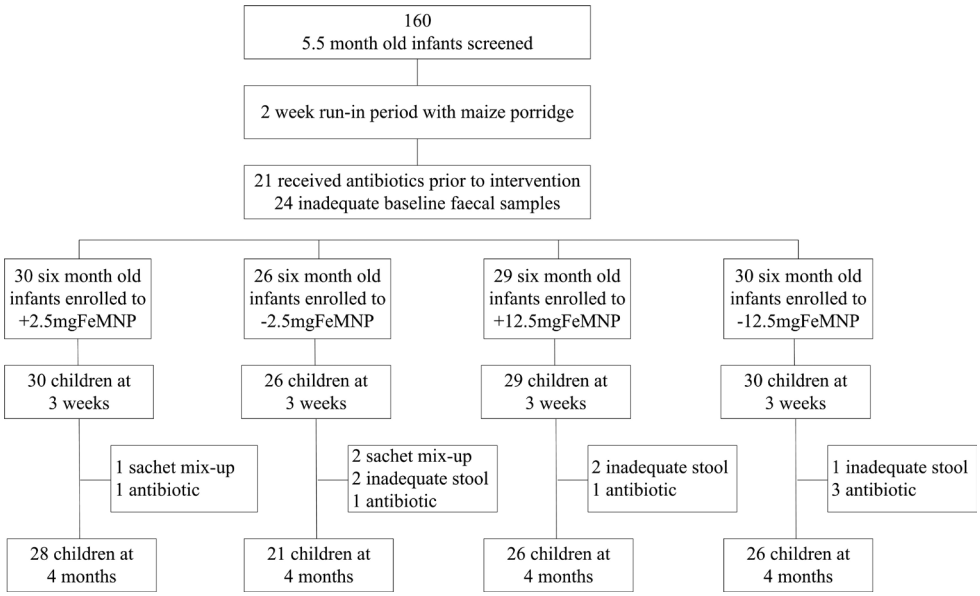


Figure 1. Trial profile

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

Ethical aspects

This study was approved by the ethics and research committees of the Kenyatta National Hospital/ University of Nairobi (P167/6/2009 and P168/05/2011) and the Swiss Federal Institute of Technology Zurich (EK 2009-N-53). Caregivers of the infants gave written informed consent. A data safety monitoring board, including a paediatrician and a nutritionist, performed safety monitoring for the study.

Results

Our estimated sample size was 100-120 infants, and we enrolled 115 infants into the study (**Figure 1, trial profile**). We originally recruited 160 infants, but during the two week run-in period, 21 infants were excluded because they had received antibiotics in the previous two months and 24 infants did not provide an adequate baseline stool sample. During the study a further 11 infants were excluded because they received antibiotics or were unable to provide adequate stool samples. Use of antibiotics was unexpectedly high in this setting; this was the local standard of care. Due to a labelling error at the factory in a second batch of the ±2.5mgFeMNP sachets, 3 infants received mixed treatments and were excluded.

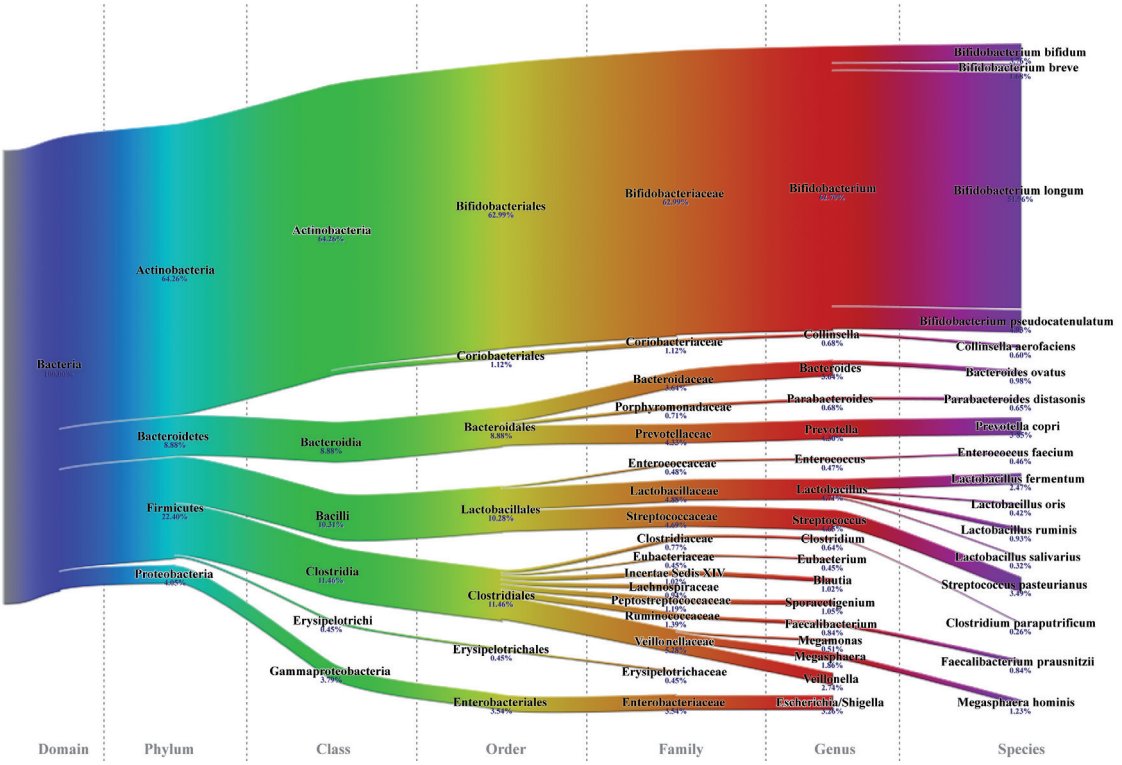


Figure 2. Baseline gut microbiome of the 6 month-old Kenyan infants enumerated by 16S rRNA pyrosequencing (n=101). The fraction of 16S rRNA reads (in %) attributed to specific taxonomic level is given below the taxon name.

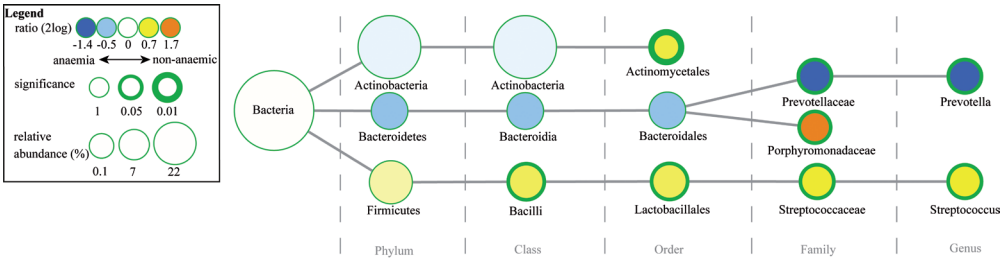


Figure 3 Differences in baseline gut microbiome composition in anaemic versus non-anaemic 6 month-old Kenyan infants. Nodes represent taxa; edges link the different taxonomic levels. The fold increase is calculated as the 2log of the ratio of the relative abundance in anaemic and non-anaemic (0= no difference between anaemia status, 1= twice as abundant in non-anaemic). The significance is expressed as the p value of a Mann-Whitney U test of the anaemic and non-anaemic infant samples. The node-size corresponds to the relative abundance. Taxa (that is, nodes) were included in this visualization if they met the following criteria: all samples together have an average relative abundance of > 0.1% for the taxon and the study groups have a fold-difference of at least 0.1 with a significance of $p < 0.05$ or the taxon has a child (that is, more specific taxonomic classification) meeting the criteria.

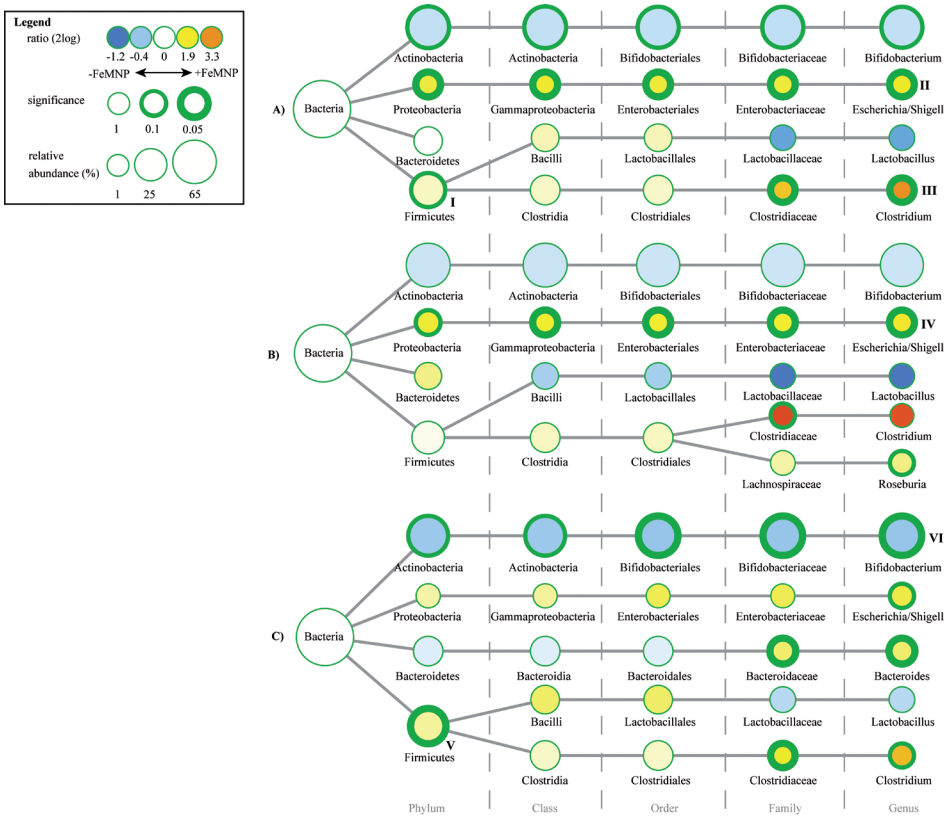


Figure 4. Differences in gut microbial composition after 4 months in Kenyan infants receiving iron containing micronutrient powders (+FeMNP) versus no-iron micronutrient powders (-FeMNP). A) +FeMNP vs. -FeMNP; B) +2.5mgFeMNP vs. -2.5mgFeMNP; C) +12.5mgFeMNP vs. -12.5mgFeMNP. Nodes represent taxa; edges link the different taxonomic levels. The fold increase is calculated as the 2log of the ratio of the relative abundance in +FeMNP and -FeMNP (0= no difference between group, 1= twice as abundant in +FeMNP, etc.). The significance is expressed as the p value of a Mann-Whitney U test. The node-size corresponds to the relative abundance (in %). Taxa displayed were selected based on the list of targets of our primary interest.

Therefore the final analysis was completed on 101 infants: 28 from +2.5mgFeMNP, 21 from -2.5mgFeMNP, 26 from +12.5mgFeMNP, and 26 from -12.5mgFeMNP.

Baseline prevalence of anaemia, iron deficiency (negative body iron stores) and systemic inflammation were 67.3%, 25.5% and 29.7%, respectively. Nearly all infants were still being breastfed (99.0%), but 80.2% had already been introduced to complementary foods, predominantly *uji* starting on average at four months of age. Compliance with the sachets was 99.4% for ±2.5mgFeMNP and 96.2% for ±12.5mgFeMNP. The native iron and phytic acid concentrations in the maize flour were 1.15 ±0.06 mg and 310 ±20 mg per 100 g, respectively.

Baseline infant gut microbiome and its development over time

In total, 934,853 bacterial 16S rRNA sequences were analysed by pyrosequencing. At baseline, the infant gut microbiome consisted of the phyla Actinobacteria (64.3% of reads), Firmicutes (22.4%; including 4.7% *Lactobacillus*), Bacteroidetes (8.9%; including 3.6% *Bacteroides* and 4.3% *Prevotella*), and Proteobacteria (4.1%; including 3.3% *Escherichia/ Shigella*); and was highly dominated by the family of *Bifidobacteriaceae*, contributing 63.0% of the total 16S rRNA (**Figure 2**). There was no significant effect of baseline anaemia status on phylogenetic diversity among the infants (data not shown). However, an exploratory analysis revealed significant differences in taxa between anaemic and non-anaemic infants at baseline: non-anaemic infants harboured lower abundances of *Prevotella* (2.0% vs. 4.5%, p = 0.014), whereas they showed higher abundances of *Actinomycetales* (0.14 % vs. 0.09%, p = 0.004) and *Streptococcus* (6.3% vs. 3.9%, p = 0.023) (**Figure 3**).

The phylogenetic diversity in all stool samples increased after 3 weeks (p = 0.004) and further increased to 4 months (p = 0.005, **Supplementary Figure 1**). Redundancy analysis throughout the study found: a) a strong inter-individual variation of the gut microbial composition (p = 0.001); b) a significant difference in the gut microbiome of anaemic and non-anaemic infants (p = 0.031); and c) a significant difference in the gut microbiome of infants in the two MNP trials (±2.5mgFeMNP and ±12.5mgFeMNP, p = 0.015). Furthermore, strong time specific signatures were found (p = 0.001). Gender, season in which the intervention started, and the starting date of complementary feeding did not significantly affect the gut microbiome. Significant changes over time in the infants gut microbiome composition of the -FeMNP group from baseline (six months old) to endpoint (10 months old) are illustrated in **Supplementary Figure 2**. These changes involved an increase in *Faecalibacterium* and *Prevotella*, and a decrease in *Enterobacteriaceae* (p = 0.010, p = 0.006, p = 0.002 respectively).

Composition of the infant gut microbiome during iron fortification

The phylogenetic diversity of the gut microbiome was not significantly modified by +FeMNP versus -FeMNP (**Supplementary Figure 1**). Differences in the gut microbiome at endpoint for the combined ±FeMNP groups and the separate analysis of the ±2.5mgFeMNP and ±12.5mgFeMNP are shown in **Figure 4A-C**. The changes over time in the relative abundance of a taxon between the ±FeMNP groups and the ±2.5mgFeMNP and ±12.5mgFeMNP are shown in **Figure 5I-VI**. Comparing +FeMNP versus -FeMNP, there were significantly higher abundances of the genera *Clostridium*, and *Escherichia/ Shigella* (p = 0.033, p = 0.010, respectively), and a trend towards lower abundances of the genus *Bifidobacterium* (p = 0.085) in the +FeMNP group at endpoint (Figure 4A). In addition, the change over time in relative abundances (4 months vs. baseline) was significantly different in +FeMNP versus -FeMNP for *Firmicutes, Escherichia/ Shigella*, and *Clostridium* (p = 0.034, p = 0.030, p = 0.048, respectively; Figure 5I-III). Comparing +2.5mgFeMNP to -2.5mgFeMNP, there were significantly higher abundances of the genera *Escherichia/ Shigella* (p = 0.044) and a trend towards higher in *Roseburia* (p = 0.083) in +2.5mgFeMNP versus -2.5mgFeMNP at endpoint (Figure 4B). In addition, the change over time in relative abundances (4 months vs. baseline) was significantly different in +2.5mgFeMNP compared to -2.5mgFeMNP for *Escherichia/*

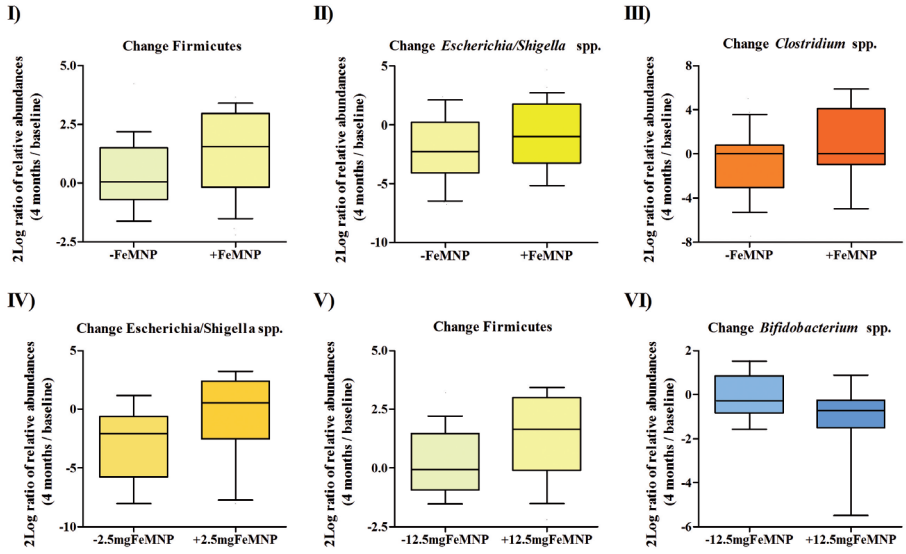


Figure 5. Change from baseline to 4 months in taxa that differed significantly between +FeMNP and -FeMNP infants at 4 months. The boxplots (I-VI) report on significant changes over time from baseline to endpoint (I: p = 0.0.034, II: p = 0.030, III: p = 0.048, IV: p = 0.034, V: p = 0.046, VI: p = 0.049) of taxa being different in groups at endpoint (corresponding roman numerals and colours in Figure 4). Boxplots of 2log ratios (0= no difference for the two time points, 1= twice as abundant at 4 months, etc.) are displayed with the 10-90th percentiles.

Shigella (p = 0.034; Figure 5IV). Comparing +12.5mgFeMNP to -12.5mgFeMNP, there were significantly higher abundances of *Firmicutes* (p = 0.018), *Bacteroides* (p = 0.045), a trend towards higher abundances of *Clostridium* spp. (p = 0.052) and *Escherichia/ Shigella* (p = 0.067), and significantly lower abundance of *Bifidobacterium* (p = 0.047) in +12.5mgFeMNP versus -12.5mgFeMNP at endpoint (Figure 4C). In addition, the change over time in relative abundances (4 months vs. baseline) was significantly higher in +12.5mgFeMNP compared to -12.5mgFeMNP for *Firmicutes* (p = 0.046; Figure 5V) and showed a significantly larger decrease for *Bifidobacterium* (p = 0.049, Figure 5VI).

Comparing +FeMNP versus -FeMNP by qPCR analysis, revealed a borderline significant effect of iron on enterobacteria (p = 0.061), with higher concentrations in +FeMNP (8.9 ±0.3 log number of gene copies/g faeces) versus -FeMNP (8.0 ±0.4) at endpoint. Furthermore, there was a significant treatment effect on *Roseburia* spp./ *Eubacterium rectale* (p = 0.020), with lower concentrations in +FeMNP (4.4 ±0.4) versus -FeMNP (5.8 ±0.4). In the separate analyses of the two MNPs, a significant treatment effect was found on *Roseburia* spp./ *E. rectal*e in ±12.5mgFeMNP (p<0.0001), with lower concentrations in +12.5mgFeMNP (3.2 ±0.4) versus -12.5mgFeMNP (6.2 ±0.5) at endpoint; while a significant treatment effect was seen for *Eubacterium hallii* in ±2.5mgFeMNP (p = 0.009), with higher concentrations in +2.5mgFeMNP (8.4 ± 0.6) versus -2.5mgFeMNP (7.1 ±0.5) at endpoint.

Examining the effect of iron on pathogenic gut microbiota, in all analysed stool samples, we detected *Bacillus cereus* in 39.5% (mean in detected samples: 4.6 log copies/g faeces (range: 3.3-7.2)), *Staphylococcus aureus* in 65.4% (6.6 (3.0-10.0)), *Clostridium difficile* in 56.5% (7.2 (3.4-10.4)), members of the *Clostridium perfringens* group in 89.7% (7.7 (3.1-10.4)), *Salmonella* in 22.4% (5.7 (4.4-7.9)) and *Vibrio cholera* in 0%. Further, we detected enteropathogenic *E. coli* (EPEC) in 65.0% (mean in detected samples: 6.0 log copies/g faeces (range: 3.2-9.3)), enterotoxigenic *E. coli* producing heat-labile toxin (ETEC LT) in 49.2% (6.0 (3.0-10.5)), ETEC producing heat-stable toxin (ETEC ST) in 7.0% (5.7 (3.5-8.7)), enterohaemorrhagic *E. coli* producing shiga-like toxin 1 (EHEC stx1) in 9.6% (4.7 (3.4-7.4)), and EHEC stx2 in 8.5% (4.6 (3.6-6.3)). There was a significant treatment effect on the sum of the pathogenic *E. coli* at endpoint ($p = 0.029$), with higher concentration in the +FeMNP (6.0±0.5 log numbers of copies/g faeces) versus -FeMNP (4.5±0.5). In the separate analyses of the two MNPs, there was a significant treatment effect on the sum of the pathogenic *E. coli* in ±2.5mgFeMNP at midpoint ($p = 0.012$), and at endpoint ($p = 0.043$), with transient lower concentrations in the +2.5mgFeMNP (4.9±0.7) versus -2.5mgFeMNP (7.0±0.5) at midpoint and higher concentrations in +2.5mgFeMNP (6.5±0.6) versus -2.5mgFeMNP (4.1±0.8) at endpoint. No effect of iron on pathogenic *E. coli* could be detected in the ±12.5mgFeMNP. The increase in the sum of pathogenic *E. coli* through iron fortification was significant in infants with baseline iron deficiency ($p = 0.012$) but not in infants who were iron sufficient ($p = 0.327$).

Figure 6A-D shows the ratio of abundances of enterobacteria to bifidobacteria and lactobacilli analysed by pyrosequencing and qPCR. Using pyrosequencing, the ratio of the relative abundances of enterobacteria to bifidobacteria changed significantly over time from baseline to endpoint in the +FeMNP versus -FeMNP ($p = 0.020$; Figure 6A). At endpoint, the enterobacteria to bifidobacteria ratio was significantly higher in the +FeMNP versus -FeMNP ($p = 0.004$); similarly in the separate analysis of the MNPs, the enterobacteria to bifidobacteria ratio was significantly higher in +2.5mgFeMNP ($p = 0.030$) and in +12.5mgFeMNP ($p = 0.049$), compared to the respective control groups at endpoint. The qPCR analysis confirmed this, showing a significant effect on the ratio of enterobacteria to bifidobacteria in +FeMNP compared to -FeMNP group at endpoint ($p = 0.008$, Figure 6B); with a trend towards a significant effect in the separate analysis of ±2.5mgFeMNP ($p = 0.053$) and ±12.5mgFeMNP ($p = 0.098$). Using pyrosequencing, the ratio of the relative abundance of enterobacteria to lactobacilli in +FeMNP did not change over time from baseline to endpoint, after a temporary decrease at 3 weeks ($p = 0.018$) in +FeMNP versus -FeMNP; however, at endpoint, the ratio enterobacteria to lactobacilli was significantly higher in +FeMNP ($p = 0.023$; Figure 6C). Using qPCR, a similar trend towards a higher ratio of enterobacteria to lactobacilli was detected at endpoint in +FeMNP compared to -FeMNP ($p = 0.062$, Figure 6D). Furthermore, pyrosequencing analysis of ±2.5mgFeMNP and ±12.5mgFeMNP separately showed a borderline significantly higher ratio of enterobacteria to lactobacilli in +12.5mgFeMNP versus -12.5mgFeMNP ($p = 0.055$) at endpoint, with a temporary decrease after 3 weeks ($p = 0.021$); but did not differ at any time point in +2.5mgFeMNP versus -2.5mgFeMNP.

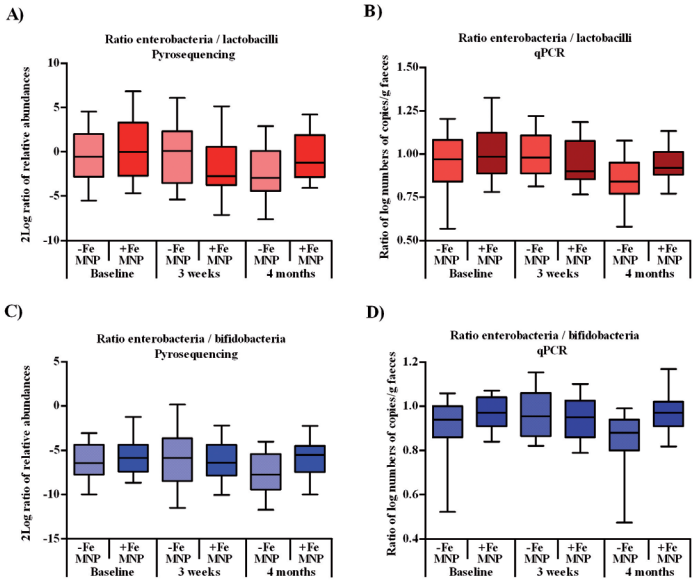


Figure 6. Ratio of infant enterobacteria to bifidobacteria at baseline, 3 weeks and 4 months comparing +FeMNP and -FeMNP assessed by: A) pyrosequencing (log2 ratio of the relative abundance); and B) qPCR (ratio of log numbers of copies/g faeces). Ratio of infant enterobacteria to lactobacilli assessed by: **C**) pyrosequencing; and **D**) qPCR. Boxplots are displayed with the 10-90th percentiles. Values differed significantly between groups (+FeMNP and -FeMNP): **A**) the change over time from baseline to endpoint in ratios was significant different between groups (p = 0.020); at endpoint the ratios were significantly higher in +FeMNP versus -FeMNP (p = 0.004). **B**) the ratios were significantly different between groups at 4 months using GLM and adjusted for baseline differences (p = 0.008). **C**) the change over time from baseline to 3 weeks in ratios was significantly different (p = 0.018), but the change over time from baseline to endpoint was not different between +FeMNP versus -FeMNP; at endpoint the ratios were significantly higher in +FeMNP versus -FeMNP at endpoint (p = 0.023). **D**) the ratios were borderline significantly different between groups at 4 months using GLM and adjusted for baseline differences (p = 0.062).

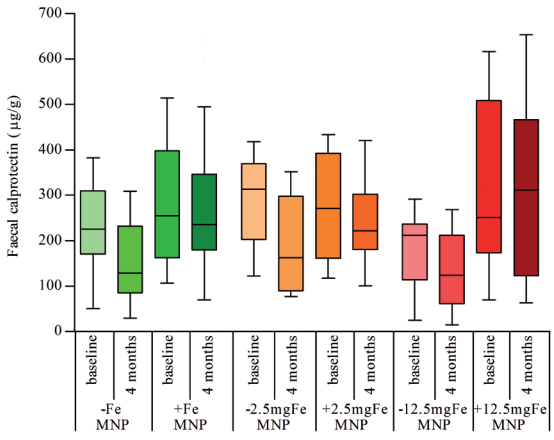


Figure 7. Infant faecal calprotectin concentrations at baseline and 4 months in the pooled data from the –FeMNP versus +FeMNP groups, in the -2.5mgFeMNP versus +2.5mgFeMNP, and in the -12.5mgFeMNP versus +12.5mgFeMNP. Values differed significantly over the trial between the ±12.5mgFeMNP groups (p = 0.008) and the pooled MNP groups (±FeMNP, p = 0.002), using GLM and baseline variables as covariates. Boxplots are displayed with the 10-90th percentiles.

Faecal calprotectin, SCFAs, iron status, and systemic inflammation

Intestinal inflammation, assessed by faecal calprotectin, was significantly higher in infants receiving +FeMNP (229.2 ±1.9 μg/g) versus -FeMNP (123.3 ±2.1 μg/g, p = 0.002). In the separate analyses of the MNPs, faecal calprotectin values were significantly elevated in +12.5mgFeMNP (248.9 ±2.2 μg/g) versus -12.5mgFeMNP (102.5 ±2.2 μg/g, p = 0.008), but were not significantly higher in +2.5mgFeMNP (215.6±1.6 μg/g) versus -2.5mgFeMNP (165.6±1.8 μg/g, p = 0.164, **Figure 7**). The increase in faecal calprotectin through iron fortification was significant in infants who were iron sufficient at baseline (p = 0.0002) but not in infants with iron deficiency (p = 0.912). There were no significant correlations of faecal calprotectin with the overall gut microbiome composition assessed by pyrosequencing. The qPCR data revealed intra-sample correlations of faecal calprotectin with none of the commensal bacteria but with the sum of pathogenic *E. coli* (p = 0.011, t=.177), and enterobacteria (p = 0.008, t=.201), and members of the *C. perfringens* group (p = 0.007, t=-.206) at 3 weeks, and EPEC (p = 0.024, t=.190) at 4 months. There were no significant differences in faecal acetate, propionate, or butyrate concentrations between +FeMNPs and -FeMNPs during the intervention (**Supplementary Table 2**).

There was a significant treatment effect of +12.5mgFeMNP versus -12.5mgFeMNP on body iron (p = 0.001), SF (p = 0.004), sTfR (p = 0.008), ZPP (p = 0.039) and a trend towards an effect on hepcidin-25 (p = 0.052, Supplementary Table 3). In contrast, there was no significant treatment effect of +2.5mgFeMNP versus -2.5mgFeMNP on any iron status indicator or hepcidin-25. There was no treatment effect of either +FeMNP on serum CRP (**Supplementary Table 3**) or serum IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-17, IFNγ, TNFα or GM-CSF (data not shown). There was a significant treatment effect of +2.5mgFeMNP versus -2.5mgFeMNP on IL-12, which was higher in +2.5mgFeMNP (523.6 ±1.5 μg/g vs. 431.9 ±1.4 μg/g, p = 0.028).

Growth

There was no significant treatment effect of iron on weight gain. However, we identified a significant increase of linear growth in +FeMNPs (70.2 ±1.1 cm vs. 68.5 ±1.1 cm at endpoint, p = 0.017, Supplementary Table 3). This effect was significant in the infants of ±12.5mgFeMNP (70.2 ±1.1 cm vs. 68.1 ±1.1 cm at endpoint, p = 0.011), but not in ±2.5mgFeMNP. Linear growth was not correlated to gut microbiota or faecal calprotectin.

Morbidity

On enrolment, 19.2% of mothers reported a treated episode of diarrhoea in their infant during the previous 3 months, 29.7% a treated RTI, and 3.8% a treated malaria episode. During the intervention, incidence of treated RTI and malaria did not significantly differ between +FeMNP versus -FeMNP. However, there was a trend towards a greater incidence of treated episodes of diarrhoea in +12.5mgFeMNP versus -12.5mgFeMNP: 27.3% (n=6/22) versus 8.3% (n=2/24, p = 0.092).

Discussion

This study shows that in breastfed, 6 months old infants from rural Africa, the gut microbiome is dominated by *Bifidobacteriaceae*, but harbours many gram negative and gram positive pathogens. Our findings indicate that the iron in MNPs favours growth of several of these potentially pathogenic subgroups. At the same time, iron decreases abundances of bifidobacteria, shifting gut microbial balance away from beneficial 'barrier' strains towards a potentially more pathogenic profile. This is accompanied by an increase in intestinal inflammation. Our findings in Kenyan infants are comparable to those of a recent controlled trial in school-aged children in Côte d'Ivoire receiving iron-fortified biscuits containing 20 mg iron/day as electrolytic iron for 6 months (Zimmermann et al 2010). The expected absorption of electrolytic iron in that setting was <5%, and there was no decrease in anaemia or iron deficiency in the iron fortified group. Although the study was a secondary analysis, only qPCR methods were used, and the authors did not report increases in specific pathogens; it did demonstrate that iron fortification can extensively modify the gut microbiota, increasing enterobacteria and decreasing lactobacilli, along with increased intestinal inflammation measured by faecal calprotectin. The differences between these two studies are likely due to age-related differences in the gut microbiota between infants and older children, but may also reflect differences in methods used to characterise the gut microbiome, differences in geographic setting, and possibly, differences in the iron compound and/or dose given. A recent high dose iron supplementation trial in low-to-middle income South African schoolchildren residing in an area with an improved water supply and a lower risk of contaminated food (Dostal et al 2014), supports the suggestion that environmental variables modulate the effects of iron on intestinal inflammation and gut microbiota. Despite the high iron dose, there were no measurable effects on intestinal inflammation measured by faecal calprotectin, nor on gut microbiota evaluated by qPCR. Overall, these data suggest that the effects of supplemental or fortification iron on the gut microbiota and intestinal inflammation in children are more pronounced in settings where hygiene standards are low and the microbiome is likely to be populated by opportunistic enteropathogens.

Iron fortification in both the present study and in the Ivorian study (Zimmermann et al 2010) favoured growth of enterobacteria over bifidobacteria and/or lactobacilli, and this could be due to their different iron requirements and metabolism. Many pathogenic enterobacteria require iron acquisition for bacterial virulence (Boyer et al 2002, Bullen et al 2000) and/or gastrointestinal tract colonization (Tsolis et al 1996). Only few bacteria do not require iron, of which lactobacilli is the major group (Weinberg 1997). Lactobacilli do not produce siderophores and their growth is similar in media with and without iron (Pandey et al 1994). *Bifidobacterium breve*, an important bifidobacteria species in breast-fed infants, can sequester luminal iron using a divalent metal permease (Bezkorovainy and Solberg 1989, Bezkorovainy et al 1996), but the majority of bifidobacteria do not produce siderophores or other active iron carriers. Abundant bifidobacteria, lactobacilli and other beneficial bacteria in the colon provide an important 'barrier effect' against colonization and

invasion by pathogens (Coconnier et al 1997, Lievin et al 2000, Weinberg 1997). Our findings suggest that +FeMNPs weaken this protective effect. If +FeMNPs promote expansion of enterobacteria, this may be important because abundances of closely related species can predict susceptibility to intestinal colonization by pathogenic bacteria (Nakamura et al 2009). In our study, this effect may have encouraged colonization by potentially pathogenic members of the genus *Escherichia/ Shigella* spp., evidenced by the higher abundances of this genus in the +FeMNP groups at endpoint, and in particular of the five pathogenic E. coli subgroups (EPEC, ETEC LT, ETEC ST, EHEC stx1, and stx2).

Calprotectin is a calcium- and zinc-binding protein found in the cytosol of neutrophils, monocytes and activated macrophages (Gisbert and McNicholl 2009). Faecal calprotectin levels mainly reflect migration of neutrophils into the gut mucosa and is a non-specific marker of intestinal inflammation elevated in children with gastroenteritis (Berni Canani et al 2004). Faecal calprotectin is markedly higher during infancy than in later childhood (Konikoff and Denson 2006). In healthy Ugandan children, median faecal calprotectin were 278 μg/g at 3-6 months, 183 μg/g at 6-12 months of age and fell to 28 μg/g at school-age (Hestvik et al 2011). We used a different assay than the Ugandan study, but found comparable levels of faecal calprotectin at baseline. In -FeMNP groups, faecal calprotectin decreased as expected over the 4 month intervention while in +FeMNP groups, and particularly in +12.5mgFeMNP, levels remained significantly elevated. In the Ivorian fortification study, where school-age children received ≈9 mg of iron per day, faecal calprotectin increased sharply and was correlated with the increase in gut enterobacteria (Zimmermann et al 2010). Although we found no significant difference in systemic concentrations of most cytokines, circulating IL-12 concentrations were significantly higher in +2.5mgFeMNP versus -2.5mgFeMNP at endpoint. Gut microbial antigens can stimulate secretion of IL-12 by gut macrophages and induce development of Th1 cells (Maynard et al 2012). Thus, in our study, IL-12 secretion may have been triggered by invasive pathogens or dysbiosis resulting from increased luminal iron; notably, in +2.5mgFeMNP versus -2.5mgFeMNP, the *Escherichia/ Shigella* taxon was significantly higher at endpoint, an effect not seen in +12.5mgFeMNP. Furthermore, dietary iron has shown to increase intestinal inflammation in this study and others (Carrier et al 2006, Zimmermann et al 2010); through different potential pathways such as neutrophil infiltration, lipid peroxidation, NF-κB activation and proinflammatory cytokines (Carrier et al 2006). Electron acceptors generated as by-products of host inflammatory response have been proposed to favour facultative anaerobes, in particular enterobacteria (Winter et al 2013).

The +FeMNPs we used in this study contained iron as two different forms, ferrous and ferric, and the ferric iron was bound to a chelator (EDTA). Our data does not clarify whether the form of dietary iron is a potential determinant of the changes induced in the gut microbiota, as the relative concentration of ferrous versus ferric iron in the human colon resulting from dietary iron ingestion is unknown. However, even if different forms of iron are ingested, it is likely that most of the unabsorbed dietary iron that enters the colon is in the oxidised, ferric (Fe^{3+}) form, mainly as ferric oxides or hydroxides (Simpson

et al 1992). In the colon, low-oxygen tension would favour the reduction of ferric to ferrous (Fe^{2+}) iron. Systems for acquisition of both ferrous and ferric iron have been identified in many gastrointestinal pathogens. Although ferric ion has a solubility of only 10^{-17} M at pH 7 and bacteria generally require iron at around 10^{-7} to 10^{-5} M to achieve optimal growth (Andrews et al 2003), bacteria can reduce ferric iron to the more soluble ferrous form and/ or use extracellular ferric iron chelators, such as siderophores, as solubilizing agents prior to uptake (Koster 2001). Many enteric pathogens, including *Salmonella* spp. and *Escherichia/ Shigella* spp. (Naikare et al 2006), take up iron-siderophore complexes via specific outer membrane receptors. Most enteric gram-negative bacteria also have active transport systems for ferrous iron (Andrews et al 2003), and iron transporter FeoB-mediated ferrous iron acquisition may increase virulence (Boyer et al 2002).

In our study, the ferrous fumarate-containing +12.5mgFeMNP tended to cause greater modifications of the gut microbiome and more intestinal inflammation; this was likely due to the fact that its iron dose was 5-fold that of the chelated ferric iron in the +2.5mgFeMNP. However, it is not clear from our findings if the higher iron dose was more detrimental: although +12.5mgFeMNP resulted in lower abundances of *Bifidobacterium*, and higher levels of faecal calprotectin, as well as a trend towards higher rates of treated diarrhoea, the effect on *Escherichia/ Shigella*, the ratio of enterobacteria to bifidobacteria, and on the increase of pathogenic *E. coli* was stronger with +2.5mgFeMNP. Therefore, our data do not clarify whether the lower iron dose MNP would have a better safety profile.

In accordance with previous studies on human gut microbiota (Backhed et al 2005, De Filippo et al 2010, Fallani et al 2011, Qin et al 2010), the four dominant phyla in our infants at baseline were Actinobacteria (63%, mainly *Bifidobacteriaceae*), Firmicutes (22%), Bacteroidetes (9%), and Proteobacteria (4%). We found more distinct treatment effects after 4 months than after 3 weeks, likely due to the rapid fluctuations in the gut microbiome at 3 weeks caused by the introduction of the complementary feeding (Vael and Desager 2009). This finding underscores the importance of allowing the gut microbiome time to adapt to changes in diet when evaluating an intervention and comparing different trials. Our data from the gut microbiome of African infants are comparable to those reported in Dutch infants using qPCR methods that found similar abundances of bifidobacteria, *E. coli*, and lactobacilli (Penders et al 2006). However, in contrast, many of our infants carried enteropathogens, with over half of the faecal samples containing *C. difficile, S. aureus*, members of the *C. perfringens* group, enteropathogenic and/or enterotoxigenic *E. coli*, and nearly one in four harbouring *B. cereus* and/or *Salmonella*. Our data also suggest the baseline gut microbiome of anaemic versus non-anaemic infants may differ, as may their response to +FeMNPs. Anaemic infants showed higher *Prevotella* (Bacteroidetes) and lower *Actinomycetales* (Actinobacteria) and *Streptococcus* (Firmicutes) abundances than non-anaemic infants. A previous study comparing the gut microbiome of older children from Burkina Faso and Italy found higher concentrations of Bacteroidetes (especially *Prevotella* and *Xylanibacter*) and lower concentrations of Firmicutes and *Escherichia/ Shigella* in the Burkinabe than Italian children (De Filippo et al 2010). Thus, our findings suggest

higher rates of anaemia in African children may contribute to differences observed in gut microbiota in African versus European children.

In rats, the availability of colonic iron may modulate gut microbiota metabolites, and in particular, production of caecal SCFAs (Dostal et al 2012). This could have important gut modulatory effects, as SCFAs have been reported to inhibit growth or reduce concentrations of *Salmonella enteritidis, Salmonella enterica* serovar Typhimurium*, E. coli, Shigella flexneri* and *Campylobacter jejuni* (Ricke 2003, Topping and Clifton 2001), and butyrate can down-regulate expression of genes involved in *Salmonella* invasion at low doses (Van Immerseel et al 2006). In *in vitro* fermentations, low iron concentrations decreased numbers of SCFA-producers, including *Roseburia* spp./ *E. rectale, Clostridium* Cluster IV members and *Bacteroides* spp., and decreased butyrate and propionate concentrations in the effluent (Dostal et al 2013). In the present study, we found no effect of iron-containing MNPs on faecal SCFA concentrations. This difference may be due to the fact that 95-99% of SCFAs produced by bacterial fermentation are absorbed in the colon, so concentrations measured in faeces likely do not reflect levels in the colonic lumen (Scheppach 1994). In Swedish infants, faecal butyrate and propionate concentrations steadily increased over the first two years of life, while faecal acetate increased rapidly in the first few weeks after birth and then plateaued (Midtvedt and Midtvedt 1992). A similar pattern from 6 to 10 months of age was visible in the Kenyan infants in this study.

Data on the effect of iron interventions on diarrhoeal incidence in children disagree. In a systematic review of controlled trials of oral iron supplementation or fortification, provision of iron was associated with a 11% higher risk of developing diarrhoea ($p = 0.04$) (Gera and Sachdev 2002). Four food fortification studies reported diarrhoeal outcomes, three provided iron-fortified infant formula (Brunser et al 1993, Power et al 1991, Singhal et al 2000), and one provided an iron-fortified infant food (Javaid et al 1991). Since that review, two small iron fortification trials done in school-age children have not reported an increased risk for diarrhoea (Manger et al 2008, Moretti et al 2006). However, a controlled trial in Swedish and Honduran infants providing iron supplements from 6 to 9 months, among infants with Hb ≥110 g/L, iron treatment increased risk for diarrhoea (Dewey et al 2002). Recent controlled iron supplementation trials (12.5-15 mg Fe/day) in Peru (Richard et al 2006) and Bangladesh (Chang et al 2010) reported a significant increase in diarrhoea. Two large trials of iron and folic acid supplementation in infants and children (subjects aged 12-35 months received 12.5 mg Fe/day, younger children received half the dose) in Nepal (Tielsch et al 2006) and Tanzania (Sazawal et al 2006) reported no difference in diarrhoea incidence. However, in a controlled study in Ghanaian children that used the same +12.5mgFeMNP as in our study, there were significantly more hospital admissions in the iron group [RR (95% CI) 1.23 (1.02-1.49)], and based on data from the outpatient register, 83% of the additional cases in the iron group were due to diarrhoea, but this was not significant [RR (95% CI) 1.12 (0.86–1.46)] (Zlotkin et al 2013). A recent study in Pakistan found an increase in diarrhoea in infants receiving +12.5mgFeMNP, with or without zinc, compared to untreated controls [without zinc: OR (95%CI) 1.15 (1.00-1.3); with zinc: 1.31 (1.13-1.51)] (Soofi et al 2013). In

the present study, there was a trend towards a higher rate of diarrhoea requiring treatment in the infants receiving the +12.5mgFeMNP. Therefore, the available data suggest oral iron supplements and +FeMNPs may modestly increase in risk for diarrhoea in infants. Our findings of a shift in the gut microbiome towards a potentially more pathogenic profile along with the increase in intestinal inflammation could provide a potential mechanism for this adverse effect.

This is the first controlled intervention trial to examine the effect of +FeMNPs on the African infant gut microbiome and our findings need confirmation in other settings and populations. Both IDA and diarrhoea are major causes of morbidity and mortality in infants in developing countries. In-home fortification with +FeMNPs has repeatedly been shown to reduce IDA rates in infants and children, but whether they increase risk for diarrhoeal disease remains uncertain. The limited available clinical evidence and our findings of their effects on the gut microbiome and inflammation, suggest +FeMNPs may not be entirely safe in settings with high infectious disease burdens. Currently, WHO recommends fortification using MNPs containing iron, vitamin A, and zinc for children aged 6 to 23 months irrespective of their iron status in settings with an anaemia prevalence of >20% and in conjunction with measures to treat malaria, promote improved sanitation, and improve overall management of diarrhoea (WHO 2011). At the same time, WHO recommends iron supplementation only be targeted to infants with IDA, while providing adequate protection from malaria.[4] Our findings, together with others (Soofi et al 2013, Zlotkin et al 2013), suggest that, until safer formulations are available, +FeMNPs should be targeted to infants with IDA, while providing adequate protection from malaria and diarrhoea.

Supplementary Tables

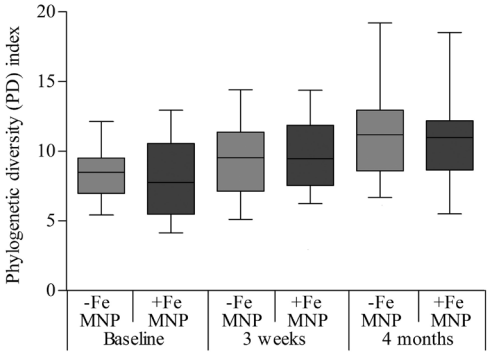
Supplementary Table 1. Species, target gene, and primers used for the quantitative real-time polymerase chain reaction.

Species	Target gene (description)	Primer and sequence (5'-3')		Reference
Total Bacteria	16S rRNA gene	Eub 338F Eub 518R	ACTCCTACGGGAGGCAGCAG ATTACCGCGGCTGCTGG	(Fierer et al 2005)
Bacteroides spp.	16S rRNA gene	Bac303F Bfr-Fmrev	GAAGGTCCCCCACATTG CGCKACTTGGCTGGTTCAG	(Bartosch et al 2004, Liu et al 2003)
Firmicutes	16S rRNA gene	Firm934F Firm1060R	GGAGYATGTGGTTTAATTCGAAGCA AGCTGACGACAACCATGCAC	(Fierer et al 2005)
Enterobacteriaceae	16S rRNA gene	Eco1457F Eco1652R	CATTGACGTTACCCGCAGAAGAAGC CTCTACGAGACTCAAGCTTGC	(Bartosch et al 2004)
Bifidobacteria	xfp gene	xfp-fw xfp-rv	ATCTTCGGACCBGAYGAGAC CGATVACGTGVACGAAGGAC	(Cleusix et al 2009)
Lactobacillus/ Leuconostoc/ Pediococcus spp.	16S rRNA gene	F_Lacto 05 R_Lacto 04	AGCAGTAGGGAATCTTCCA CGCCACTGGTGTTCYTCCATATA	(Furet et al 2009)
Roseburia spp./ E. rectale	16S rRNA gene	RrecF Rrec630mR	GCGGTRCGGCAAGTCTGA CCTCCGACACTCTAGTMCGAC	(Ramirez-Farias et al 2008, Walker et al 2005)
Clostridial Cluster IV	16S rRNA gene	Clep866mF Clep1240mR	TTAACACAATAAGTWATCCACCTGG ACCTTCCTCCGTTTTGTCAAC	(Ramirez-Farias et al 2008)
Eubacterium hallii	16S rRNA gene	EhalF EhalR	GCGTAGGTGGCAGTGCAA GCACCGRAGCCTATACGG	(Hold et al 2003, Ramirez-Farias et al 2008)
Faecalibacterium prausnitzii	16S rRNA gene	Fprau223F Fprau420R	GATGGCCTCGCGTCCGATTAG CCGAAGACCTTCTTCCTCC	(Bartosch et al 2004, Wang et al 1996)
Sulfate-reducing bacteria	Alpha subunit dissimilatory sulfite reductase	dsrA_290F dsrA_660R	CGGCGTTGCGCATTTYCAYACVVT GCCGGACGATGCAGHTCRTCCTGRWA	(Pereyra et al 2010)
Salmonella	invA (invasion)	InvA 139 InvA 141	GTGAAATTATCGCCACGTTCGGGCAA TCATCGCACCGTCAAAGGAACC	(Fukushima et al 2003, Rahn et al 1992)
Staphylococcus aureus	Nuclease	SA-1 SA-2	GCGATTGATGGTGATACGGTT CAAGCCTTGACGAACTAAAGC	(Brakstad et al 1992, Fukushima et al 2003)
Bacillus cereus	Hemolysin	BC-1 BC-2	CTGTAGCGAATCGTACGTATC TACTGCTCCAGCCACATTAC	(Fukushima et al 2003, Wang et al 1996)
Clostridium difficile	16S rRNA gene	cdF cdR	TTGAGCGATTTACTTCGGTAAAGA CCATCCTGTACTGGCTCACCT	(Rinttila et al 2004)
Clostridium perfringens group	16S rRNA gene	pF cpR	ATGCAAGTCGAGCGA(G/T)G TATGCGGTATTAATCT(C/T)CCTTT	(Rinttila et al 2004)
Vibrio cholera	CT (cholera toxin)	CT-F CT-R	ACAGAGTGAGTACTTTGACC ATACCATCCATATATTTGGGAG	(Fukushima et al 2003, Walker et al 2005
Enteropathogenic Escherichia coli (EPEC)	eaeA (E. coli attaching and effacing)	Eae a Eae b	ATGCTTAGTGCTGGTTTAGG GCCTTCATCATTTCGCTTTC	(Fukushima et al 2003)
Enterohemorrhagic Escherichia coli (EHEC) stx1	stx1 (shiga toxin 1)	JMS1F JMS1R	GTCACAGTAACAAACCGTAACA TCGTTGACTACTTCTTATCTGGA	(Fukushima et al 2003)
Enterohemorrhagic Escherichia coli (EHEC) stx2	stx2 (shiga toxin 2)	JMS2F JMS2R	CGACCCCTCTTGAACATA GATAGACATCAAGCCCTCGT	(Fukushima et al 2003)
Enterotoxigenic Escherichia coli (ETEC) LT	LT (heat-labile enterotoxin)	LT-1 LT-2	AGCAGGTTTCCCACCGGATCACCA GTGCTCAGATTCTGGGTCTC	(Fukushima et al 2003)
Enterotoxigenic Escherichia coli (ETEC) ST	ST (heat-stable enterotoxin)	ST_f ST_rev	GCTAAACCAGYAGRGTCTTCAAAA CCCGGTACARGCAGGATTACAACA	Liu et al 2013)

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

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

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

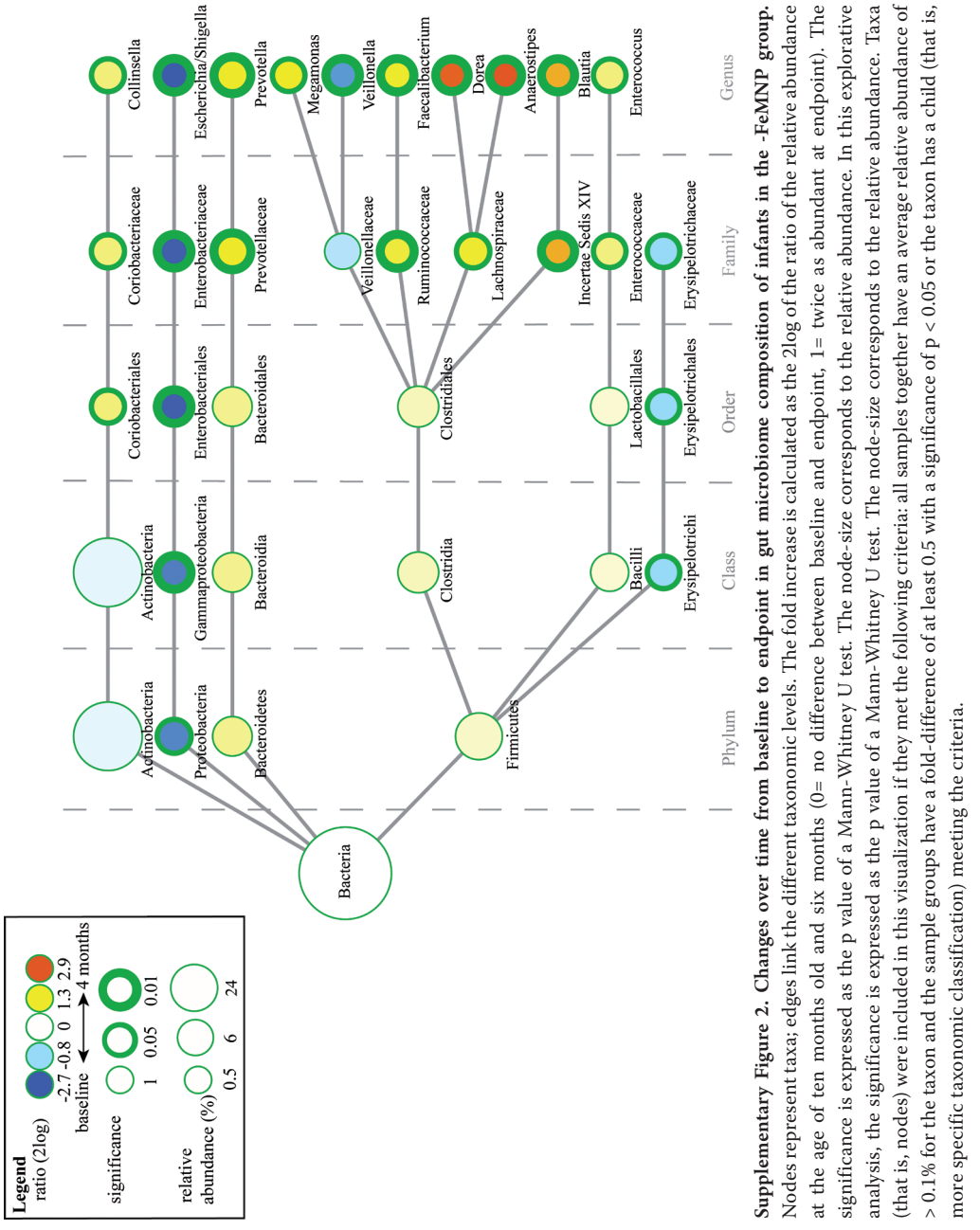


Supplementary Figure 1. Alpha diversity of the infant gut microbiome over the time course of the trial in the pooled groups (+FeMNP and -FeMNP). Phylogenetic diversity was not influenced by +FeMNP intervention, but increased significantly over time (p = 0.004 for baseline to 3 weeks and p = 0.005 for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

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

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

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



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

References

- Adu-Afarwuah S, Lartey A, Brown KH, Zlotkin S, Briend A, Dewey KG (2008). Home fortification of complementary foods with micronutrient supplements is well accepted and has positive effects on infant iron status in Ghana. The American journal of clinical nutrition 87: 929-938.
- Anderson RC, Cookson AL, McNabb WC, Kelly WJ, Roy NC (2010). Lactobacillus plantarum DSM 2648 is a potential probiotic that enhances intestinal barrier function. FEMS Microbiol Lett 309: 184-192.
- Andrews SC, Robinson AK, Rodriguez-Quinones F (2003). Bacterial iron homeostasis. FEMS microbiology reviews 27: 215-237.
- Backhed F, Ley RE, Sonnenburg JL, Peterson DA, Gordon JI (2005). Host-bacterial mutualism in the human intestine. Science 307: 1915-1920.
- Balmer SE, Scott PH, Wharton BA (1989). Diet and faecal flora in the newborn: lactoferrin. Archives of disease in childhood 64: 1685-1690.
- Bartosch S, Fite A, Macfarlane GT, McMurdo ME (2004). Characterization of bacterial communities in feces from healthy elderly volunteers and hospitalized elderly patients by using real-time PCR and effects of antibiotic treatment on the fecal microbiota. Appl Environ Microbiol 70: 3575-3581.
- Berni Canani R, Rapacciuolo L, Romano MT, Tanturri de Horatio L, Terrin G, Manguso F et al (2004). Diagnostic value of faecal calprotectin in paediatric gastroenterology clinical practice. Dig Liver Dis 36: 467-470.
- Bezkorovainy A, Solberg L (1989). Ferrous iron uptake by Bifidobacterium breve. Biol Trace Elem Res 20: 251-267.
- Bezkorovainy A, Kot E, Miller-Catchpole R, Haloftis G, Furmanov S (1996). Iron Metabolism in Bifidobacteria. Int Dairy Journal 6: 905-919.
- Boyer E, Bergevin I, Malo D, Gros P, Cellier MF (2002). Acquisition of Mn(II) in addition to Fe(II) is required for full virulence of Salmonella enterica serovar Typhimurium. Infection and immunity 70: 6032-6042.
- Brakstad OG, Aasbakk K, Maeland JA (1992). Detection of Staphylococcus aureus by polymerase chain reaction amplification of the nuc gene. J Clin Microbiol 30: 1654-1660.
- Brunser O, Espinoza J, Araya M, Pacheco I, Cruchet S (1993). Chronic iron intake and diarrhoeal disease in infants. A field study in a less-developed country. Eur J Clin Nutr 47: 317-326.
- Bullen J, Griffiths E, Rogers H, Ward G (2000). Sepsis: the critical role of iron. Microbes and infection / Institut Pasteur 2: 409-415.
- Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK et al (2010). QIIME allows analysis of high-throughput community sequencing data. Nature methods 7: 335-336.
- Carrier JC, Aghdassi E, Jeejeebhoy K, Allard JP (2006). Exacerbation of dextran sulfate sodium-induced colitis by dietary iron supplementation: role of NF-kappaB. International journal of colorectal disease 21: 381-387.
- Cassat JE, Skaar EP (2013). Iron in infection and immunity. Cell host & microbe 13: 509-519.
- Chang S, El Arifeen S, Bari S, Wahed MA, Rahman KM, Rahman MT et al (2010). Supplementing iron and zinc: double blind, randomized evaluation of separate or combined delivery. Eur J Clin Nutr 64: 153-160.
- Chen HM, Lifschitz CH (1989). Preparation of fecal samples for assay of volatile fatty acids by gas-liquid chromatography and high-performance liquid chromatography. Clin Chem 35: 74-76.

- Cleusix V, Lacroix C, Dasen G, Leo M, Le Blay G (2009). Comparative study of a new quantitative real-time PCR targeting the xylulose-5-phosphate/fructose-6-phosphate phosphoketolase bifidobacterial gene (xfp) in faecal samples with two fluorescence in situ hybridization methods. J Appl Microbiol 108: 181-193.
- Coconnier MH, Lievin V, Bernet-Camard MF, Hudault S, Servin AL (1997). Antibacterial effect of the adhering human Lactobacillus acidophilus strain LB. Antimicrob Agents Chemother 41: 1046-1052.
- Cole JR, Wang Q, Cardenas E, Fish J, Chai B, Farris RJ et al (2009). The Ribosomal Database Project: improved alignments and new tools for rRNA analysis. Nucleic acids research 37: D141-145.
- Cook JD, Flowers CH, Skikne BS (2003). The quantitative assessment of body iron. Blood 101: 3359-3364.
- De Filippo C, Cavalieri D, Di Paola M, Ramazzotti M, Poullet JB, Massart S et al (2010). Impact of diet in shaping gut microbiota revealed by a comparative study in children from Europe and rural Africa. Proc Natl Acad Sci U S A.
- Dewey KG, Domellof M, Cohen RJ, Landa Rivera L, Hernell O, Lonnerdal B (2002). Iron supplementation affects growth and morbidity of breast-fed infants: results of a randomized trial in Sweden and Honduras. J Nutr 132: 3249-3255.
- Dostal A, Chassard C, Hilty FM, Zimmermann MB, Jaeggi T, Rossi S et al (2012). Iron depletion and repletion with ferrous sulfate or electrolytic iron modifies the composition and metabolic activity of the gut microbiota in rats. The Journal of nutrition 142: 271-277.
- Dostal A, Fehlbaum S, Chassard C, Zimmermann MB, Lacroix C (2013). Low iron availability in continuous in vitro colonic fermentations induces strong dysbiosis of the child gut microbial consortium and a decrease in main metabolites. FEMS Microbiol Ecol 83: 161-175.
- Dostal A, Baumgartner J, Riesen N, Chassard C, Smuts CM, Zimmermann MB et al (2014). Effects of iron supplementation on dominant bacterial groups in the gut, faecal SCFA and gut inflammation: a randomised, placebo-controlled intervention trial in South African children. Br J Nutr: 1-10.
- Fallani M, Amarri S, Uusijarvi A, Adam R, Khanna S, Aguilera M et al (2011). Determinants of the human infant intestinal microbiota after the introduction of first complementary foods in infant samples from five European centres. Microbiology 157: 1385-1392.
- Fierer N, Jackson JA, Vilgalys R, Jackson RB (2005). Assessment of soil microbial community structure by use of taxon-specific quantitative PCR assays. Appl Environ Microbiol 71: 4117-4120.
- Flowers CH, Skikne BS, Covell AM, Cook JD (1989). The clinical measurement of serum transferrin receptor. The Journal of laboratory and clinical medicine 114: 368-377.
- Fontaine O (2007). Conclusions and recommendations of the WHO Consultation on prevention and control of iron deficiency in infants and young children in malaria-endemic areas. Food Nutr Bull 28: S621-627.
- Fukushima H, Tsunomori Y, Seki R (2003). Duplex real-time SYBR green PCR assays for detection of 17 species of food- or waterborne pathogens in stools. J Clin Microbiol 41: 5134-5146.
- Furet JP, Firmesse O, Gourmelon M, Bridonneau C, Tap J, Mondot S et al (2009). Comparative assessment of human and farm animal faecal microbiota using real-time quantitative PCR. FEMS Microbiol Ecol 68: 351-362.
- Gera T, Sachdev HP (2002). Effect of iron supplementation on incidence of infectious illness in children: systematic review. BMJ (Clinical research ed 325: 1142.

- Gisbert JP, McNicholl AG (2009). Questions and answers on the role of faecal calprotectin as a biological marker in inflammatory bowel disease. Dig Liver Dis 41: 56-66.
- Haas BJ, Gevers D, Earl AM, Feldgarden M, Ward DV, Giannoukos G et al (2011). Chimeric 16S rRNA sequence formation and detection in Sanger and 454-pyrosequenced PCR amplicons. Genome Res 21: 494-504.
- Habil N, Al-Murrani W, Beal J, Foey AD (2011). Probiotic bacterial strains differentially modulate macrophage cytokine production in a strain-dependent and cell subset-specific manner. Beneficial microbes 2: 283-293.
- Hestvik E, Tumwine JK, Tylleskar T, Grahnquist L, Ndeezi G, Kaddu-Mulindwa DH et al (2011). Faecal calprotectin concentrations in apparently healthy children aged 0-12 years in urban Kampala, Uganda: a community-based survey. BMC Pediatr 11: 9.
- Hold GL, Schwiertz A, Aminov RI, Blaut M, Flint HJ (2003). Oligonucleotide probes that detect quantitatively significant groups of butyrate-producing bacteria in human feces. Appl Environ Microbiol 69: 4320-4324.
- Jaeggi T, Moretti D, Kvalsvig J, Holding PA, Tjalsma H, Kortman GA et al (2013). Iron status and systemic inflammation, but not gut inflammation, strongly predict gender-specific concentrations of serum hepcidin in infants in rural Kenya. PLoS One 8: e57513.
- Javaid N, Haschke F, Pietschnig B, Schuster E, Huemer C, Shebaz A et al (1991). Interactions between infections, malnutrition and iron nutritional status in Pakistani infants. A longitudinal study. Acta paediatrica Scandinavica Supplement 374: 141-150.
- Konikoff MR, Denson LA (2006). Role of fecal calprotectin as a biomarker of intestinal inflammation in inflammatory bowel disease. Inflamm Bowel Dis 12: 524-534.
- Koster W (2001). ABC transporter-mediated uptake of iron, siderophores, heme and vitamin B12. Res Microbiol 152: 291-301.
- Krebs NF, Sherlock LG, Westcott J, Culbertson D, Hambidge KM, Feazel LM et al (2013). Effects of Different Complementary Feeding Regimens on Iron Status and Enteric Microbiota in Breastfed Infants. J Pediatr.
- Kroot JJ, Laarakkers CM, Geurts-Moespot AJ, Grebenchtchikov N, Pickkers P, van Ede AE et al (2010). Immunochemical and mass-spectrometry-based serum hepcidin assays for iron metabolism disorders. Clin Chem 56: 1570-1579.
- Lievin V, Peiffer I, Hudault S, Rochat F, Brassart D, Neeser JR et al (2000). Bifidobacterium strains from resident infant human gastrointestinal microflora exert antimicrobial activity. Gut 47: 646-652.
- Liu C, Song Y, McTeague M, Vu AW, Wexler H, Finegold SM (2003). Rapid identification of the species of the Bacteroides fragilis group by multiplex PCR assays using group- and species-specific primers. FEMS Microbiol Lett 222: 9-16.
- Liu J, Gratz J, Amour C, Kibiki G, Becker S, Janaki L et al (2013). A laboratory-developed TaqMan Array Card for simultaneous detection of 19 enteropathogens. J Clin Microbiol 51: 472-480.
- Makower RU (1970). Extraction and determination of phytic acid in beans (Phaseolus vulgaris). Albany, California: U.S. Department of Agriculture.
- Manger MS, McKenzie JE, Winichagoon P, Gray A, Chavasit V, Pongcharoen T et al (2008). A micronutrient-fortified seasoning powder reduces morbidity and improves short-term cognitive function, but has no effect on anthropometric measures in primary school children in northeast Thailand: a randomized controlled trial. Am J Clin Nutr 87: 1715-1722.

- Maynard CL, Elson CO, Hatton RD, Weaver CT (2012). Reciprocal interactions of the intestinal microbiota and immune system. Nature 489: 231-241.
- Meilgaard MC, Carr BT, Civille GV (2007). Sensory evaluation techniques. (4th ed)Taylor Francis Group, Boca Raton, FL.
- Mevissen-Verhage EA, Marcelis JH, Harmsen-Van Amerongen WC, de Vos NM, Verhoef J (1985). Effect of iron on neonatal gut flora during the first three months of life. European journal of clinical microbiology 4: 273-278.
- Midtvedt AC, Midtvedt T (1992). Production of short chain fatty acids by the intestinal microflora during the first 2 years of human life. J Pediatr Gastroenterol Nutr 15: 395-403.
- Moretti D, Zimmermann MB, Muthayya S, Thankachan P, Lee TC, Kurpad AV et al (2006). Extruded rice fortified with micronized ground ferric pyrophosphate reduces iron deficiency in Indian schoolchildren: a double-blind randomized controlled trial. Am J Clin Nutr 84: 822-829.
- Naikare H, Palyada K, Panciera R, Marlow D, Stintzi A (2006). Major role for FeoB in Campylobacter jejuni ferrous iron acquisition, gut colonization, and intracellular survival. Infection and immunity 74: 5433-5444.
- Nakamura N, Gaskins HR, Collier CT, Nava GM, Rai D, Petschow B et al (2009). Molecular ecological analysis of fecal bacterial populations from term infants fed formula supplemented with selected blends of prebiotics. Appl Environ Microbiol 75: 1121-1128.
- Nemeth E, Tuttle MS, Powelson J, Vaughn MB, Donovan A, Ward DM et al (2004). Hepcidin regulates cellular iron efflux by binding to ferroportin and inducing its internalization. Science 306: 2090-2093.
- Okiro EA, Hay SI, Gikandi PW, Sharif SK, Noor AM, Peshu N et al (2007). The decline in paediatric malaria admissions on the coast of Kenya. Malar J 6: 151.
- Oksanen J, Blanchet FG, Kindt R, Legendre P, Minchin PR, O'Hara RB et al (2012). Vegan: Community Ecology Package. http://CRAN.R-project.org/package=vegan.
- Pandey A, Bringel F, Meyer J-M (1994). Iron requirement and search for siderophores in lactic acid bacteria. Appl Microbiol Biotechnol 40: 735-739.
- Penders J, Thijs C, Vink C, Stelma FF, Snijders B, Kummeling I et al (2006). Factors influencing the composition of the intestinal microbiota in early infancy. Pediatrics 118: 511-521.
- Pereyra LP, Hiibel SR, Prieto Riquelme MV, Reardon KF, Pruden A (2010). Detection and quantification of functional genes of cellulose- degrading, fermentative, and sulfate-reducing bacteria and methanogenic archaea. Appl Environ Microbiol 76: 2192-2202.
- Pfeiffer CM, Cook JD, Mei Z, Cogswell ME, Looker AC, Lacher DA (2007). Evaluation of an automated soluble transferrin receptor (sTfR) assay on the Roche Hitachi analyzer and its comparison to two ELISA assays. Clin Chim Acta 382: 112-116.
- Power HM, Heese HD, Beatty DW, Hughes J, Dempster WS (1991). Iron fortification of infant milk formula: the effect on iron status and immune function. Annals of tropical paediatrics 11: 57-66.
- Qin J, Li R, Raes J, Arumugam M, Burgdorf KS, Manichanh C et al (2010). A human gut microbial gene catalogue established by metagenomic sequencing. Nature 464: 59-65.
- Raffatellu M, George MD, Akiyama Y, Hornsby MJ, Nuccio SP, Paixao TA et al (2009). Lipocalin-2 resistance confers an advantage to Salmonella enterica serotype Typhimurium for growth and survival in the inflamed intestine. Cell host & microbe 5: 476-486.
- Rahn K, De Grandis SA, Clarke RC, McEwen SA, Galan JE, Ginocchio C et al (1992). Amplification of an invA gene sequence of Salmonella typhimurium by polymerase chain reaction as a specific method of detection of Salmonella. Molecular and cellular probes 6: 271-279.

- Ramirez-Farias C, Slezak K, Fuller Z, Duncan A, Holtrop G, Louis P (2008). Effect of inulin on the human gut microbiota: stimulation of Bifidobacterium adolescentis and Faecalibacterium prausnitzii. Br J Nutr 101: 541-550.
- Richard SA, Zavaleta N, Caulfield LE, Black RE, Witzig RS, Shankar AH (2006). Zinc and iron supplementation and malaria, diarrhea, and respiratory infections in children in the Peruvian Amazon. Am J Trop Med Hyg 75: 126-132.
- Ricke SC (2003). Perspectives on the use of organic acids and short chain fatty acids as antimicrobials. Poultry science 82: 632-639.
- Rinttila T, Kassinen A, Malinen E, Krogius L, Palva A (2004). Development of an extensive set of 16S rDNA-targeted primers for quantification of pathogenic and indigenous bacteria in faecal samples by real-time PCR. J Appl Microbiol 97: 1166-1177.
- Sazawal S, Black RE, Ramsan M, Chwaya HM, Stoltzfus RJ, Dutta A et al (2006). Effects of routine prophylactic supplementation with iron and folic acid on admission to hospital and mortality in preschool children in a high malaria transmission setting: community-based, randomised, placebo-controlled trial. Lancet 367: 133-143.
- Scheppach W (1994). Effects of short chain fatty acids on gut morphology and function. Gut 35: S35-38.
- Shannon P, Markiel A, Ozier O, Baliga NS, Wang JT, Ramage D et al (2003). Cytoscape: a software environment for integrated models of biomolecular interaction networks. Genome research 13: 2498-2504.
- Simpson RJ, Sidhar S, Peters TJ (1992). Application of selective extraction to the study of iron species present in diet and rat gastrointestinal tract contents. The British journal of nutrition 67: 437-444.
- Singhal A, Morley R, Abbott R, Fairweather-Tait S, Stephenson T, Lucas A (2000). Clinical safety of iron-fortified formulas. Pediatrics 105: E38.
- Soofi S, Cousens S, Iqbal SP, Akhund T, Khan J, Ahmed I et al (2013). Effect of provision of daily zinc and iron with several micronutrients on growth and morbidity among young children in Pakistan: a cluster-randomised trial. Lancet 382: 29-40.
- Sundquist A, Bigdeli S, Jalili R, Druzin ML, Waller S, Pullen KM et al (2007). Bacterial flora-typing with targeted, chip-based Pyrosequencing. BMC Microbiol 7: 108.
- Tielsch JM, Khatry SK, Stoltzfus RJ, Katz J, LeClerq SC, Adhikari R et al (2006). Effect of routine prophylactic supplementation with iron and folic acid on preschool child mortality in southern Nepal: community-based, cluster-randomised, placebo-controlled trial. Lancet 367: 144-152.
- Tondeur MC, Schauer CS, Christofides AL, Asante KP, Newton S, Serfass RE et al (2004). Determination of iron absorption from intrinsically labeled microencapsulated ferrous fumarate (sprinkles) in infants with different iron and hematologic status by using a dual-stable-isotope method. Am J Clin Nutr 80: 1436-1444.
- Topping DL, Clifton PM (2001). Short-chain fatty acids and human colonic function: roles of resistant starch and nonstarch polysaccharides. Physiological reviews 81: 1031-1064.
- Tsolis RM, Baumler AJ, Heffron F, Stojiljkovic I (1996). Contribution of TonB- and Feo-mediated iron uptake to growth of Salmonella typhimurium in the mouse. Infection and immunity 64: 4549-4556.
- Underwood MA, Salzman NH, Bennett SH, Barman M, Mills DA, Marcobal A et al (2009). A randomized placebo-controlled comparison of 2 prebiotic/probiotic combinations in preterm infants: impact on weight gain, intestinal microbiota, and fecal short-chain fatty acids. J Pediatr Gastroenterol Nutr 48: 216-225.
- UNICEF-CDC (2013). Global Assessment of Home Fortification Interventions, 2011. Geneva: Home Fortification Technical Advisory Group.

- Vael C, Desager K (2009). The importance of the development of the intestinal microbiota in infancy. Current opinion in pediatrics 21: 794-800.
- Van Immerseel F, Russell JB, Flythe MD, I. G, Timbermont L, Pasmans F et al (2006). The use of organic acids to combat Salmonella in poultry: a mechanistic explanantion of the efficacy. Avian Pathology 35: 182-188.
- Vanveldhoven PP, Mannaerts GP (1987). Inorgaic and organic phosphate measurements in the nanomolar range. Anal Biochem 161: 45-48.
- Walker AW, Duncan SH, McWilliam Leitch EC, Child MW, Flint HJ (2005). pH and peptide supply can radically alter bacterial populations and short-chain fatty acid ratios within microbial communities from the human colon. Appl Environ Microbiol 71: 3692-3700.
- Wang RF, Cao WW, Cerniglia CE (1996). PCR detection and quantitation of predominant anaerobic bacteria in human and animal fecal samples. Appl Environ Microbiol 62: 1242-1247.
- Weinberg ED (1997). The Lactobacillus anomaly: total iron abstinence. Perspectives in biology and medicine 40: 578-583.
- WHO (2001). Iron deficiency and anemia: assessment prevention and control. A guide for programme managers. Geneva, Switzerland: World Heatlh Organization.
- WHO (2011). Guideline: Use of multiple micronutrient powders for home fortification of foods consumed by infants and children 6–23 months of age. Geneva, World Health Organization.
- WHO (2013). World Health Statistics 2013.
- Winter SE, Lopez CA, Baumler AJ (2013). The dynamics of gut-associated microbial communities during inflammation. EMBO reports 14: 319-327.
- Yatsunenko (2012). Human gut microbiome viewed across age and geography. Nature.
- Yen CC, Shen CJ, Hsu WH, Chang YH, Lin HT, Chen HL et al (2011). Lactoferrin: an iron-binding antimicrobial protein against Escherichia coli infection. Biometals : an international journal on the role of metal ions in biology, biochemistry, and medicine 24: 585-594.
- Zimmermann MB, Hurrell RF (2007). Nutritional iron deficiency. Lancet 370: 511-520.
- Zimmermann MB, Chassard C, Rohner F, N'Goran E K, Nindjin C, Dostal A et al (2010). The effects of iron fortification on the gut microbiota in African children: a randomized controlled trial in Cote d'Ivoire. The American journal of clinical nutrition 92: 1406-1415.
- Zlotkin S, Newton S, Aimone AM, Azindow I, Amenga-Etego S, Tchum K et al (2013). Effect of iron fortification on malaria incidence in infants and young children in Ghana: a randomized trial. JAMA : the journal of the American Medical Association 310: 938-947.

Chapter 3

Gut microbial metabolism shifts towards a more toxic profile with supplementary iron in a kinetic model of the human large intestine

Guus AM. Kortman[1], Bas E. Dutilh[2,3], Annet JH. Maathuis[4], Udo F. Engelke[1], Jos Boekhorst[5], Kevin P. Keegan[6], Fiona Nielsen[7], Jason Betley[7], Jacqueline C. Weir[7], Zoya Kingsbury[7], Leo AJ. Kluijtmans[1], Dorine W. Swinkels[1], Koen Venema[4†], Harold Tjalsma[1].

[1] Department of Laboratory Medicine – Laboratory of Genetic, Endocrine and Metabolic Diseases. Radboud Institute for Molecular Life Sciences (RIMLS) of the Radboud University Medical Center, P.O. Box 9101, 6500 HB Nijmegen, The Netherlands.

[2] Centre for Molecular and Biomolecular Informatics (CMBI), Radboud Institute for Molecular Life Sciences (RIMLS) of the Radboud University Medical Center, Geert Grooteplein 28, 6525 GA, Nijmegen.

[3] Department of Marine Biology, Institute of Biology, Federal University of Rio de Janeiro, Rio de Janeiro, Brazil.

[4] TNO Healthy Living, PO Box 360, 3700 AJ Zeist, The Netherlands.

[5] NIZO Food Research B.V., Kernhemseweg 2, 6718 ZB, Ede, The Netherlands.

[6] Argonne National Laboratory, 9700 S. Cass Avenue, Argonne, IL 60439, USA.

[7] Illumina Inc., Chesterford Research Park, Little Chesterford, CB10 1XL Essex, UK.

[†] present address: Beneficial Microbes Consultancy; Johan Karschstraat 3, 6709 TN Wageningen

Submitted

Abstract

Oral iron administration in African children has been associated with an increased burden of infectious diseases. However, it remains unclear to what extent oral iron administration affects the intestinal microbiome. We here investigated the impact of multiple iron preparations and doses on growth and metabolism of the human gut microbiota in a well-controlled *in vitro* model of the large intestine. TNO's *in vitro* model of the large intestine (TIM-2) was inoculated with a human microbiota, without supplementary iron, or with 50 or 250 μmol/L ferrous sulfate, 50 or 250 μmol/L ferric citrate, or 50 μmol/L hemin. High resolution responses of the gut microbiota composition and functions were examined by 16S rRNA pyrosequencing, microarray analysis, and metagenomic sequencing. The metabolome was assessed by fatty acid quantification, gas chromatography-mass spectrometry (GC-MS) and ^{1}H-NMR spectroscopy. Cultured intestinal epithelial Caco-2 cells were used to assess fecal water toxicity. Microbiome analysis showed a clear iron-induced decrease of *Bifidobacteriaceae* and *Lactobacillaceae* under iron-rich conditions, paralleled by an increase of *Roseburia* and *Prevotella*. Metagenomic analyses showed an enrichment of microbial motility-chemotaxis systems, while the metabolome markedly changed from a saccharolytic to a proteolytic profile in response to iron. Branched chain fatty acids and ammonia levels increased significantly, in particular with ferrous sulfate, and we also identified siderophoric microbial metabolites that were specifically produced under low-iron conditions. Importantly, we confirmed that the metabolite-containing effluent from iron-rich conditions showed increased cytotoxicity to intestinal epithelial cells. Our data indicate that in the absence of host influences, iron induces a more hostile intestinal environment characterized by i) a reduction of beneficial microbes, ii) increased levels of bacterial metabolites that can impair the barrier function of the gut wall, and iii) an increase of virulence-associated pathways of enteric pathogens. It can be envisaged that these *in vitro* phenomena also contribute to an increased risk for enteric infections *in vivo*.

Introduction

Iron deficiency is the most prevalent nutritional disorder worldwide, mostly affecting infants, young children, and women in developing countries. This deficiency has major health consequences such as poor pregnancy outcome, and impaired physical and cognitive development of children (Muthayya et al 2013, WHO and UNICEF 2004). Several trials have shown that iron deficiency can be effectively controlled by both iron supplementation and fortification programmes (Zimmermann and Hurrell 2007). However, the uptake of iron from the intestines is influenced by many factors and is usually limited (Hurrell and Egli 2010). Iron supplementation or fortification therefore generally results in a large fraction of unabsorbed iron entering the colon, being potentially available for the gut microbiota.

Importantly, oral iron administration is known to increase the incidence of diarrhea and has been associated with increased gut inflammation (Gera and Sachdev 2002, Jaeggi et al 2014, Zimmermann et al 2010). It is therefore highly warranted to investigate the effects of nutritional iron on the gut microbiota composition and metabolic activity. It was recently shown that iron fortification caused a potentially more pathogenic gut microbiota profile (i.e. increased relative abundance of *Enterobacteriaceae* and a decrease of beneficial *Lactobacillaceae* and/or *Bifidobacteriaceae*) in African children and infants (Jaeggi et al 2014, Zimmermann et al 2010) (**Chapter 2**). This increase in the abundance of fecal *Enterobacteriaceae* correlated with an increase of fecal calprotectin (Zimmermann et al 2010), which is a marker of gut inflammation. Notably, the reported effect of oral iron administration on the gut microbiota composition varies between studies. The most consistent outcome of iron supplementation appears to be a decrease in *Lactobacillaceae* and *Bifidobacteriaceae*, and an increase in *Escherichia coli* (Balmer et al 1989, Balmer and Wharton 1991, Benoni et al 1993, Dostal et al 2012a, Dostal et al 2012b, Dostal et al 2014b, Jaeggi et al 2014, Kortman et al 2014a, Krebs et al 2013, Lee et al 2008, Mevissen-Verhage et al 1985, Tompkins et al 2001, Werner et al 2011, Zimmermann et al 2010) (**Chapter 1**). The large variation in the effects of iron on the gut microbiota composition in humans and animals may be explained by differences in host factors such as iron status, intestinal immune function, dietary habits and the environmental setting. Importantly, *in vitro* models for the colon are valuable tools for monitoring microbial composition and metabolism during the passage of food and allow the reproducible testing of the effects of iron on the microbiota in isolation, by eliminating any influence of the host.

Besides the intestinal microbiota composition, the bacterial metabolic activity is also important for gut health. For example, indigestible food components can be metabolized into the main Short Chain Fatty Acids (SCFAs) acetate, propionate and butyrate, which are beneficial for gut health (Macfarlane and Macfarlane 2011). In contrast, protein fermentation can result in toxic or potentially toxic metabolites such as ammonia, Branched Chain Fatty Acids (BCFAs) (e.g. isobutyrate and isovalerate), and phenolic compounds (Macfarlane and Macfarlane 2012, Nyangale et al 2012). It has recently been shown that iron can influence the bacterial metabolic activity (Dostal et al 2012a, Dostal et al 2012b, Dostal et al 2014b). The *in vivo* levels of butyrate and propionate were lower during luminal iron deficiency in rats, which could be restored by iron repletion (Dostal et al 2012a). *In vitro*, butyrate and

propionate production were most clearly impaired during very low iron conditions, while conversely, the production of these SCFAs was not stimulated by high iron conditions. The production of BCFAs as a result of protein fermentation was decreased under low iron conditions, but in contrast to the production of SCFAs, this putrefactive metabolic activity did increase under high iron conditions (Dostal et al 2012b). Interestingly, a recent study in rats showed that supplementary iron increased cecal SCFA levels not only when compared to an iron-deficient diet, but also when compared to a control diet (Dostal et al 2014b). However, the latter rat study did not specifically report on the production of BCFAs. Together, these studies suggest that iron supplementation may have health-promoting effects via an increase in microbial SCFA production, but potentially also deleterious effects by increasing toxic protein fermentation. Thus, the net effects of iron preparations on gut microbial metabolism remain inconclusive and need further exploration with both a targeted and metabolomic approach. In the present study, we investigate the effect of multiple iron sources and doses on the human gut microbiota composition and metabolic activity, for which we use the well suited and highly controlled TNO Intestinal Model 2 (TIM-2) for the human large intestine (Maathuis et al 2009, Minekus et al 1999).

Materials and methods

Origin of the microbiota

The microbiota for the TIM-2 experiments consisted of an active fecal microbiota of 6 Dutch Adult volunteers (male: n= 3, age=32.7 (21-39); female: n=3, age=33.3 (28-41)). Fecal samples were collected, kept in an anaerobic environment and were proceeded quickly to mixing and homogenization in an anaerobic cabinet. Mixed stools were used in a fed batch fermentor simulating the 'cecum' conditions as described by Maathuis *et al.* (Maathuis et al 2012). Briefly, 670 ml medium without the addition of iron and hemin was inoculated with 80 g of mixed stools and incubated for 44h at 37˚C and under anaerobic conditions by a gaseous nitrogen flux, with fed-batch addition of 1250 ml medium over time. The resulting fed-batch culture was aliquoted and snap-frozen in liquid nitrogen and stored at -80˚C before inoculation in TIM-2. It has been shown that these standardized samples are similar in composition and activity of a fresh fecal sample (Kovatcheva-Datchary et al 2009, Minekus et al 1999, Venema et al 2000).

Dynamic *in vitro* model of the large intestine (TIM-2)

TNO's *in vitro* model of the proximal large intestine (TIM-2) accurately simulates the average physical and dynamic conditions in the human proximal colon and has been described in detail before (Maathuis et al 2009, Minekus et al 1999, Rehman et al 2012). TIM-2 is characterized by a physiological water content and constant removal of metabolites via a dialysis system, simulating host uptake, maintaining physiological levels of these metabolites, and allowing metabolite production kinetics to be recorded. This makes the model well suited to study both microbial composition and metabolic activity (Minekus et al 1999, Venema et al 2000). In short, the tightly computer controlled model consisted of glass units with a flexible wall inside through which peristaltic movements were

achieved regularly. This way the lumen was mixed and transported through the system. Temperature was set at 37˚C and the pH at 5.8 (simulating the pH in the proximal colon), controlled with a pH sensor in combination with secretion of sodium hydroxide. A dialysis system consisting of semipermeable hollow fibers was running through the lumen. A flow of dialysis liquid through the dialysis system constantly removed water and fermentation products. Therefore, physiological concentrations of small molecules, such as electrolytes, were retained and accumulation of microbial metabolites was prevented. A constant volume of the luminal content was maintained by water absorption controlled by a level sensor. The system was kept anaerobic by flushing with gaseous nitrogen. This resulted in the growth of a highly active and dense microbiota comparable to that found in the human proximal colon (Kovatcheva-Datchary et al 2009, Minekus et al 1999, Venema et al 2000).

Standard ileal efflux medium (SIEM) and dialysis liquid composition

Standard ileal efflux medium (SIEM) was slightly modified for experiments in TIM-2 compared to the medium which was described by Gibson et al. 1988, mainly concerning a higher concentration of carbohydrates, pepton, casein and Tween 80 (Minekus et al 1999, van Nuenen et al 2003). For the purpose of our experiments we removed the standard iron sources (0.009 g/L $FeSO_4.7H_2O$ and 0.02 g hemin g/L) from the SIEM formulation to produce a non-iron supplemented lowFe control. Notably, it still contained low amounts of endogenous iron sources that come with the other ingredients. SIEM contained the following components (g/L): 9.0 pectin, 9.0 xylan, 9.0 arabinogalactan, 9.0 amylopectin, 43.7 casein, 74.6 starch, 31.5 Tween-80, 43.7 bactopepton, 0.7 ox-bile, 4.7 g $K_2HPO_4.3H_2O$, 8.4 g NaCl, 0.7 g $MgSO_4.7H_2O$, 0.8 g $CaCl_2.2H_2O$ and 0.3 g cysteine.HCl, plus 1.5 ml of a vitamin mixture containing (per litre): 1 mg menadione, 2 mg D-biotin, 0.5 mg vitamin B12, 10 mg pantothenate, 5 mg nicotinamide, 5 mg p-aminobenzoic acid and 4 mg thiamine. The pH was adjusted to 5.8. Low iron dialysis liquid contained (per litre): 2.5 g $K_2HPO_4.3H_2O$, 4.5 g NaCl, 0.5 g $MgSO_4.7H_2O$, 0.45 g $CaCl_2.2H_2O$, 0.05 g bile and 0.4 g cysteine.HCl, plus 1 ml of a vitamin mixture as mentioned above. The pH was adjusted to 5.8. All medium components were provided by Tritium Microbiology (Eindhoven, The Netherlands). SIEM did not require pre-digestion. For the experiments the iron sources as described below were added to the SIEM and dialysis liquid.

TIM-2 experiments and test conditions

Experiments were carried out as described before (Maathuis et al 2012). All experiments were performed in duplicate. At the start of each experiment, the model was inoculated with approximately 30 mL of the standardized microbiota (that was used for each experiment) and 80 mL of dialysate. The microbiota was allowed to adapt to the model conditions for 16h. After this adaptation period there was a 2-hour starvation period to allow the micro-organisms to ferment all available carbohydrates in the system. Then a 3-day experimental period was started. Interventions with iron were as follows:

i) no additional iron (lowFe), ii) 50 µmol/L ferrous sulfate (50FeS), iii) 250 µmol/L ferrous sulfate (250FeS), iv) 50 µmol/L ferric citrate (50FeC), v) 250 µmol/L ferric citrate (250FeC) and vi) 50 µmol/L hemin (FeH). Although hemin will not be used as an oral

iron supplement, we included this iron source as an additional condition to investigate how the gut microbiota responds to a complex iron source that is more difficult to access. Iron was added to the lumen at t=0, 24 and 48h and was also provided in the constant flow of dialysis liquid to prevent loss of iron from the lumen. Samples were taken from the lumen and from the dialysate starting after the adaptation period (set as time point 0) and at 8, 24, 32, 48, 56 and 72h after starting the iron intervention. Samples were used to determine microbiota composition, levels of iron, SCFA, BCFA, ammonia, and to profile the metabolome by ^{1}H-NMR spectroscopy and GC-MS organic acid analysis. After 24h and 48h, a total lumen sample of 25 mL was removed from the system to simulate passage of material from the proximal to the distal colon. This way a physiological colonic transit time of 48h was mimicked. In each experiment, SIEM was fed during the 3-day experimental period with 60 mL/day and contained the iron source and concentration corresponding to the applied conditions.

Iron quantification in the TIM-2 lumen

Iron content of luminal samples was determined by a ferrozine based assay (Iron Reagent, Liquid FerroZine® Method Kit, ThermoScientific). To determine total iron in the lumen, luminal samples were first treated with acid-potassium permanganate to free the iron. Lumen samples were 2x diluted with MilliQ water (MQ) and were 1:1 mixed with freshly prepared iron releasing reagent (1.2 Mol/L HCl:4.5% KMnO4 (1:1)), within a fume hood. Samples were mixed and incubated for 1h at 60˚C, then 1/8 part ascorbic acid (20% w/v) was added to convert all iron to the Fe^{2+} state. After another incubation for 1h at 60˚C samples were cooled to RT and spun for 5 min at 16,100g to pellet stool particles. 32 µl sample was applied to a microplate (in duplicate) and 160 µl reagent A (kit) was added. After a 2 min incubation the absorbance (A) at 560 nm was recorded with a microplate reader. Next 6.4 µl reagent B (kit) was added and samples were incubated for 10 min at 37˚C. Absorbance (B) was recorded at 560 nm and was corrected for absorbance A. The iron content was calculated by comparing the absorbance with a series of ferrous sulfate standards.

DNA extraction from luminal samples for microarray analysis and high throughput sequencing

Total fecal DNA from collected samples was isolated as described previously (Crielaard et al 2011), with some minor adjustments: 200mg of material was initially mixed with 250 µL lysis buffer (Agowa, Berlin,Germany), 250 µL zirconiumbeads (0.1 mm), and 200 µL phenol, before being introduced to a BeadBeater (BioSpec Products, Bartlesville, OK, USA) for two times 2 min. DNA yield was quantified using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies).

Real-time PCR to determine the total number of bacteria in the TIM-2 lumen

DNA extracted from the lumen at 0h and 72h was subjected to quantitative PCR to investigate the variation in the total amount of bacteria after different iron exposures and was performed as described previously (Ladirat et al 2013).

16S rRNA pyrosequencing of the gut microbiome

16S rRNA pyrosequencing was the main method for studying the gut microbiome composition at the 24h and 72h time points. For the preparation of the amplicon pool for pyrosequencing, the following universal primers were applied for amplification of the V3-V6 region of the 16S rRNA gene: a) forward primer , 5'-*CCATCTCATCCCTGCGTGTCTCCGACTAG*NNNN NN**ACTCCTACGGGAGGCAGCAG**-3' (the italicized sequence is 454 Life Sciences primer A, and the bold sequence is the broadly conserved bacterial primer 338F; NNNNNN designates the sample-specific six-base barcode used to tag each PCR product); b) reverse primer 5'-*CCTATCCCCTGTGTGCCTTGGCAGTCTCAG***CRRCACGAGCTGACGAC**-3' (the italicized sequence is 454 Life Sciences primer B, and the bold sequence is the broadly conserved bacterial primer 1061R). PCR amplification mixture contained : 1 µL fecal DNA, 1 µL bar-coded forward primer, 15 µL master mix (1 µL KOD Hot Start DNA Polymerase (1 U/µL; Novagen, Madison, WI, USA), 5 µL KOD-buffer (10×), 3 µL MgSO4 (25 mM), 5 µL dNTP mix (2 mM each), 1 µL (10 µM) reverse primer) and 33 µL sterile water (total volume 50 µL). PCR conditions were: 95°C for 2 minutes followed by 35 cycles of 95°C for 20 s, 55°C for 10 s, and 70°C for 15 s. The approximately 750 bp PCR amplicon was purified using the MSB Spin PCRapace kit (Invitek) and subsequently by using the Purelink PCR Purification kit (Invitrogen), with high-cutoff binding buffer B3. DNA concentration of the purified products was checked with a Nanodrop 1000 spectrophotometer (Thermo Scientific). A composite sample for pyrosequencing was prepared by pooling 200 ng of each purified sample and submitted for pyrosequencing of the V3-V4 region of the 16S rRNA gene on the 454 Life Sciences GS-FLX+ platform using Titanium sequencing chemistry (GATC-Biotech, Germany).

Microarray analysis of the microbiota by I-Chip

Analysis of the microbiota with the Intestinal (I)-Chip was executed as described by Maathuis et al. (Maathuis et al 2012). Briefly, the 'intestinal chip' (I-Chip) has been developed as a fast method to determine the composition of the microbiota. 16S rDNA based sequences of roughly 400 microorganisms have been placed on a DNA micro-array as previously described (Crielaard et al 2011). The DNA was labeled and hybridized to the microarray. After washing the arrays were scanned and analyzed. Analysis of the composition of the microbiota (using I-Chip) indicated the bacterial genera which were selectively stimulated or suppressed by the iron interventions. Changes in the composition of the microbiota in the experiments were analyzed for the 0, 24, 48 and 72h time points as described below. This analysis was included to validate pyrosequencing results, to study additional time points and to study the effect of iron on low-abundant taxa that were below the limit of detection of 16S rRNA pyrosequencing.

Metagenomic sequencing

DNA of the lowFe and 50FeS conditions at the 72h time point was barcoded, the samples pooled, and sequenced using paired 251 bp sequencing on two lanes of an Illumina HiSeq2500 instrument. The pooled samples were applied to both lanes to minimize potential lane-specific biases.

SCFA/BCFA quantification in lumen and dialysate samples

Samples (t = 0, 24, 48 and 72h) were centrifuged as described earlier and a mixture of formic acid (20%), methanol and 2-ethyl butyric acid (internal standard, 2 mg/ml in methanol) was added to the clear supernatant. A 0.5 µl sample was injected on a GC-column (Stabilwax-DA, length 15 m, ID 0.53 mm, film thickness 0.1 mm; Varian Chrompack, Bergen op Zoom, The Netherlands) in a Chrompack CP9001 gas chromatograph using an automatic sampler (Chrompack liquid sampler CP9050; Varian Chrompack). Specifically, concentrations of acetate, propionate, butyrate, valerate, isovalerate and isobutyrate were determined (Maathuis et al 2009).

Ammonia quantification in lumen and dialysate samples

Samples (t = 0, 24, 48 and 72h) were centrifuged for 5 min at 12,000g. Ammonia in the supernatant was determined based on the Berthelot reaction, in which ammonia first reacts with alkaline phenol and then with sodium hypochlorite to form indophenol blue. The absorbance of the indophenol blue is directly proportional to the original ammonia concentration and is measured at 660 nm. The measurement was automated on a Cobas Mira Plus autoanalyzer (Roche, Almere, The Netherlands) and was performed by Bio-aNAlytiX (Mook, the Netherlands). Concentrations in the samples were determined via comparison with a series of standard solutions with known concentrations (Maathuis et al 2009).

^{1}H-NMR spectroscopy of dialysate samples

Dialysate samples of lowFe, 50FeS, 250FeS, FeH and 50FeC at 72h were measured with 1D and 2D COSY NMR spectroscopy. 250FeC was not analyzed because of expected interference of large amounts of H in the citrate. A small volume (20 µl) of 20.2 mM trimethylsilyl-2,2,3,3-tetradeutero-propionic acid (TSP, sodium salt; Aldrich) in D2O was added to 700 µl of the sample, providing a chemical shift reference (0.00 ppm) and a deuterium lock signal. The pH was adjusted to 2.50 and 7.00 using concentrated HCl. Finally, 650 µl of the sample was placed in a 5 mm NMR tube (Wilmad Royal Imperial; Wilmad LabGlass, USA). ^{1}H-NMR spectra were obtained on a Bruker Avance I 500 spectrometer, operating at 11.7 T, with a Broad Band Inverse (BBI) probehead equipped with a actively shielded z-gradient coil. 1D ^{1}H spectra were acquired as 256 transients in 64K data points with a spectral width of 10080 Hz. The sample temperature was 298K; the H2O resonance was presaturated by single-frequency irradiation during a relaxation delay of 4 s; a pulse width of 7 µs was used (corresponded to a 90° excitation pulse). Shimming of the sample was performed automatically using the Topshim shim program (Bruker). The resonance line widths for TSP and all metabolites were <1 Hz. Phase and baseline were corrected manually. All spectra were scaled to TSP and metabolite and TSP resonances were fitted semi-automatically with a Lorentzian line shape. Metabolite concentrations in the sample were calculated to the known concentration of the TSP standard (singlet, 9 protons) and expressed as mmol/l. In the 2D COSY spectra, the spectral widths in the F1 and F2 domains were 6002 Hz. A total of 4K data points were collected in t2, 128 t1 increments with 16 transients per increment were used, and the relaxation delay was set to 2 s. Before the Fourier transformation, a

sine-bell function was applied in both time domains. During the relaxation delay, the water resonance was pre-saturated.

Organic acid analysis of dialysate by GC-MS

Organic acids were analyzed using standard techniques. Briefly, to 0.5 mL of dialysate sample (72h) 2.5 mL SETH buffer (0.25 mol/L sucrose; 2 mmol/L (K)EDTA; 10 mmol/L Tris; $5x10^4$ U heparin (pH 7.4)) was added. The mixture was acidified to pH2 with concentrated HCl (10%), after which the organic acids were extracted by ethylacetate twice, derivatized with trimethylsilyl (TMS), and analyzed on an Agilent 7890A gas chromatograph (GC), coupled to a flame ionisation detector (FID) and an Agilent 5975C inert XL MSD mass spectrometer. Quantification of organic acids was done by calculation of peak areas and comparison with an internal standard (4-phenylbutyric acid). Relative concentrations were expressed relative to the lowFe condition which was set at 100%.

Cell line, media and growth conditions

The colon adenocarcinoma cell line Caco-2 (obtained from the American Type Culture Collection) was cultured under standard conditions (37°C and 5% CO_2) in DMEM (Lonza) supplemented with 10% fetal calf serum (Invitrogen), 20 mmol/L HEPES, 100 nmol/L nonessential amino acids (Invitrogen), 2 mmol/L L-glutamine (Lonza) and Penicillin/Streptomycin (100 U/mL and 100 µg/mL, respectively) (Pen Strep, Invitrogen). The cells were subcultured every 6 days and used between passage numbers 3-16.

Fecal water (dialysate) cytotoxicity to Caco-2 monolayer assays

Caco-2 cells were allowed to grow and differentiate in 22 days to a polarized tight monolayer on the membrane of a Transwell® Permeable Support (24 wells, 6.5 mm insert) with 0.4 µm polycarbonate membrane (Corning) under standard culture conditions.

Dialysis liquid samples (not containing bacterial metabolites) and dialysate samples of the 72h time point were spun for 10 min at 36,000g and 4˚C. Concentrated tannic acid (Riedel-de Haën) was added to the supernatants in a final concentration of 10 µmol/L to precipitate the excess iron. After a 10 min incubation at RT the samples were spun again and the supernatants were filter sterilized by the use of a 0.2 µm filter. Iron removal was confirmed by the ferrozine based assay as described as above (without acid-potassium permanganate treatment). Pre-treated dialysates were warmed to 37˚C and 200 µl was applied in triplicate to the apical side of the Caco-2 monolayers after a wash in Hank's Balanced Salt Solution (HBSS; Invitrogen, Cat. 14025-09). As a control, standard maintaining buffer HBSS with 10 µmol/L tannic acid was used. Basolateral compartments were filled with 0.6 ml HBSS. To check for the monolayer integrity, the Trans Epithelial Electrical Resistance (TEER) was periodically measured with the use of the Millicell®-ERS (Millipore). After incubation the basolateral compartments were sampled for lactate dehydrogenase (LDH) quantification and were replaced with 0.5 ml fresh HBSS. Apical compartments were washed once with HBSS and replaced with 0.2 ml filter sterilized 1 mmol/L phenol red in HBSS. The cells were incubated for 1 h at standard conditions, the TEER was determined and the lower compartment was sampled to quantify phenol red permeability. Next, the cells were washed

twice with PBS and the basolateral compartment once. To quantify cell death 50 µl 0.4% Trypan blue in PBS was applied to the cells and incubated for 2 min at RT. Then, cells (twice) and basolateral chamber (once) were washed with PBS. We want to emphasize that we removed the iron from the dialysate prior to Caco-2 exposure and this way we only examined the effects of the microbial metabolome and excluded potential additional effects towards the cells due to redox activity of iron. We note that the presence of iron may augment the toxic effects of the metabolome as it has previously been shown that iron can have detrimental effects on the intestinal epithelium (Ferruzza et al 2003, Natoli et al 2009).

Phenol red permeability:
Samples from the basolateral compartments were split in technical duplicates of 180 µl and 20 µl 0.1 mol/L NaOH was added. Absorbance was recorded in a microplate reader at 540 nm. The amount of phenol red that was diffused through the monolayers was compared relatively to the control.

LDH quantification:
To investigate the detrimental effect of the dialysates to the Caco-2 monolayers, the LDH release into the basolateral medium was determined. Samples were collected as described and were used in the Cytotox 96® Non-Radioactive Cytotoxicity Assay (Promega) according to the manufacturer's protocol. The LDH concentrations were compared relatively to the control.

Trypan blue-exclusion test:
Washed filters were excised from the insert by scalpel and transferred to a microtube. 200 µl acetone-0.5% (w/v) Na2SO4 (7:3) was added and tubes were sonicated in a water bath to extract the trypan blue from the filters. Tubes were spun for 5 min at 16,100g and the supernatant was transferred to a microplate. Absorbance was recorded at 560 nm with 450 nm as reference wavelength. Trypan blue intensity was compared relatively to the control. Adapted from Sakai *et al.* (Sakai et al 1998).

Statistics and data presentation

Statistical analysis of total bacteria qPCR data, metabolic data and cell cytotoxicity data
TIM-2 experiments were performed in duplicate (n=2) which is a small sample size, but we have previously shown that the variations in the system are small because of the fact that a standardized microbiota is used and because the system is computer controlled (Venema et al 2000). Total bacteria were compared between time points by a paired t-test and among the single iron conditions by a 2-way-ANOVA with repeated measures and Bonferroni's post-hoc test. The effects of iron on the metabolome were compared among the single conditions, but also as pooled data (when appropriate); mediumFe (50 µmol/L ferrous sulfate and 50 µmol/L ferric citrate; n=4) and highFe (250 µmol/L ferrous sulfate and 250 µmol/L ferric citrate; n=4). Data from the hemin condition, a more complex iron source, was

never pooled with another condition. When two doses of the same iron source were pooled this is indicated as FeS (both ferrous sulfate conditions; n=4) and FeC (both ferric citrate conditions; n=4). Metabolite levels, presented as mean+range, were compared by Tukey's multiple comparison post-hoc test. Cytotoxicity of dialysates to Caco-2 monolayers was tested at least in triplicate and results were expressed as mean + SD. The course of TEER was compared by linear regression analysis of the slope and comparison of the mean area under the curve (AUC) by an unpaired t-test. Means of phenol red permeability, LDH-release and trypan blue staining data were compared by an unpaired *t*-test. In case of unequal variances (as assessed by F-test) an unpaired *t*-test with Welch's correction was carried out. Analysis was performed using GraphPad Prism version 5.03 for Windows, GraphPad Software, San Diego California USA. P-values < 0.05 were considered statistically significant and *P*-values < 0.10 were considered as an important trend throughout the paper.

Analysis of 16S rRNA sequencing data

Pyrosequencing data were analyzed with a workflow based on QIIME v1.2 (Caporaso et al 2010) using settings as recommended in the QIIME 1.2 tutorial, with the following exceptions: reads were filtered for chimeric sequences using Chimera Slayer (Haas et al 2011). OTU clustering was performed with settings as recommended in the QIIME newsletter of December 17th 2010 (http://qiime.wordpress.com/2010/12/17/new-default-parameters-for-uclust-otu-pickers/) using an identity threshold of 97%. Diversity metrics were calculated as implemented in QIIME 1.2. Hierarchical clustering of samples was performed using UPGMA with weighted UniFrac as a distance measure as implemented in QIIME 1.2. The RDP classifier version 2.2 was performed for taxonomic classification (Cole et al 2009). Multivariate redundancy analysis (RDA) was done using Canoco 5.0 (Ter Braak and Smilauer 2012), using default settings of the analysis type "Constrained-supplementary". For the permutation tests, 500 randomizations were done, keeping duplicate samples as blocks. Details on the underlying mathematics can be found in (Ter Braak and Smilauer 2012). Visualization of differences in relative abundance of taxa between different study groups was done in Cytoscape (Shannon et al 2003). Statistical analysis of the pyrosequencing data was done with SciPy (www.scipy.org). Differences in relative abundance between groups at a single time point (cross-sectional) were compared by Mann-Whitney U (MWU) testing. Correlations of taxa with metabolite levels in 24h and 72h samples were assessed by Spearman's correlation rank test. Linear regression (GraphPad Prism version 5.03) was used to plot the best-fit line with 95% confidence interval and to determine whether the deviation of the slope was significantly different from zero (when the deviation was not different from zero the correlation was considered not relevant). Medians of phylogenetic diversity and distances were compared by Kruskal-Wallis test with Dunn's post test, or MWU test (GraphPad Prism version 5.03). The effects of iron on the microbiome were compared among the single conditions, but also as pools as described above. P-values for the microbiome data were not corrected for multiple testing as they were analyzed in an explorative manner.

Analysis of microarray (I-Chip) data

Duplicate measurements at four time points (t=0, 24h, 48h, and 72h) from all incubation conditions were analyzed. Intensity measurements of the I-Chip were normalized (normalized - $\log_2$ (x+1)) and scaled between 0 and 1 across all measurements. Next, we calculated Spearman correlations per probe across the time series between all measurements, and calculated permutation p-values by randomizing the measurements for each probe.

Analysis of metagenomic sequencing data

After demultiplexing the metagenomic sequencing reads, stringent Q30 quality trimming was applied using Trimgalore v0.2.4 (www.bioinformatics.babraham.ac.uk/projects/trim_galore/), resulting in 69.9 and 59.8 million quality trimmed read pairs for the lowFe and 50FeS conditions respectively, at an average single ended read length of 207 ± 47 nt. Metagenomic sequencing reads were annotated by aligning them to the human gut gene catalog (Qin et al 2010) with Bowtie using default parameters (Langmead et al 2009). This resulted in an alignment rate of 29.0% and 36.8% for the lowFe and 50FeS conditions respectively. The abundances of KEGG orthologs (Kanehisa et al 2014) and SEED subsystem annotations (level-1 subsystems and the more detailed level-2 subsystems annotation) (Overbeek et al 2005) were normalized by sample size. Enrichment was calculated as the log2 ratio of the average abundance of the function in the iron datasets over the lowFe datasets, plus a small pseudocount of 10^{-9}. Significance was calculated using a two-sided t-test with unequal variance across all the sequenced datasets. P-values for the metagenome data were not corrected for multiple testing as they were analyzed in an explorative manner.

Results

Total iron concentration in the TIM-2 lumen

The total iron content of the TIM-2 lumen was determined over time to study the effect of the different iron sources, iron dose and our dosing schedule on the actual luminal iron content. This also allowed us to compare TIM-2 luminal iron content with the *in situ* iron content of human fecal samples. The iron content of the non-supplemented lowFe condition (see materials and methods) on average (range 8 – 72h) was 1.0 (0.7 – 1.3) mg/L and did not increase over time. Conversely, the iron concentration markedly increased over time in all conditions with supplementary iron (**Supplementary Figure 1**). For the 50FeS and 250FeS conditions (50 and 250 μmol/L ferrous sulfate) the average concentrations were 10.4 (3.7 – 15.4) and 24.3 (9.6 – 34.8) mg/L respectively. In the 50FeC and 250FeC conditions (50 and 250 μmol/L ferric citrate) it was 8.4 (2.8 – 12.7) and 21.0 (10.3 – 32.4) mg/L respectively. In FeH (50 μmol/L hemin; a more complex and less available iron source) the average concentration was 4.2 (1.8 – 6.9) mg/L. The iron concentration in feces of Dutch adult volunteers, extrapolated to the higher water content of the standard ileal efflux medium (SIEM; the water content of SIEM is approximately three times higher than in human feces), was estimated to be 19.5±3.5 μmol/L (**Supplementary Figure 1**). Thus, compared to the total iron concentration in feces, the iron content of the lowFe condition can be described as iron-deficient.

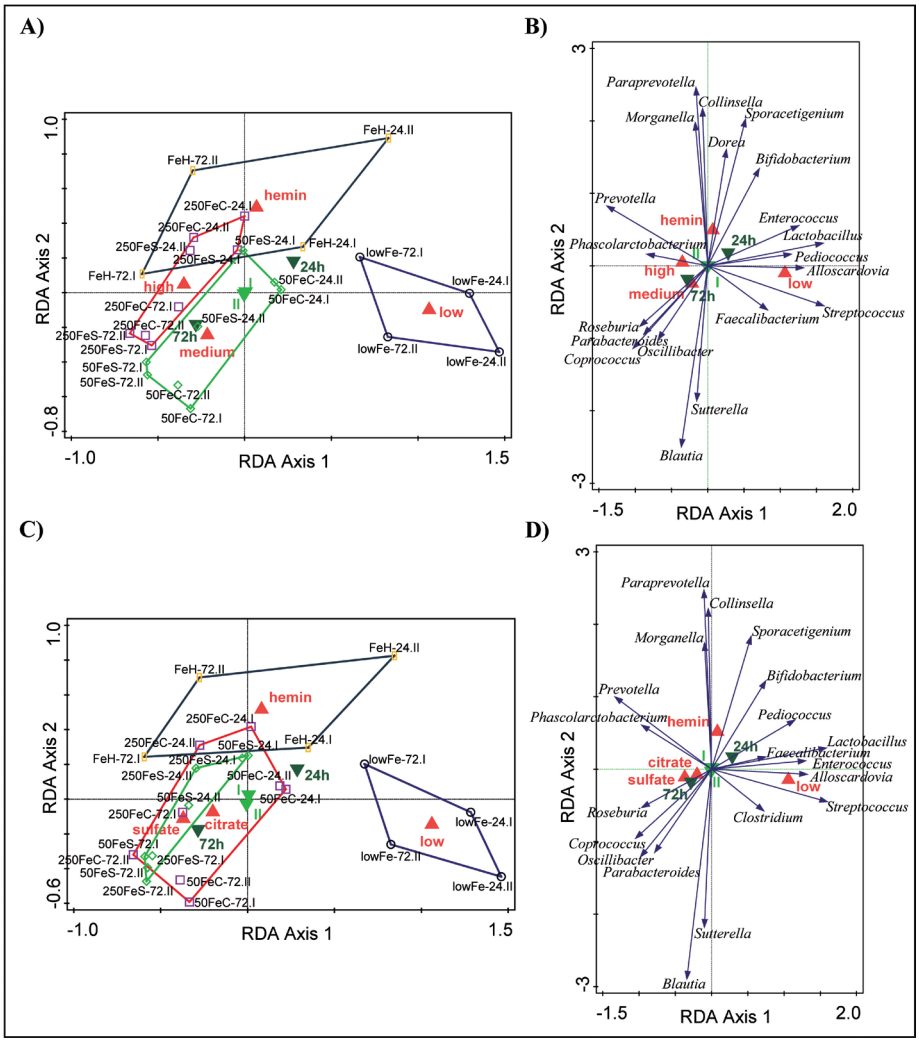


Figure 1. Multivariate redundancy analysis (RDA) of the microbiota composition.

A&B) The conditions were grouped at iron level: low, medium (pooled 50FeS and 50FeC conditions), high (pooled 250FeS and 250FeC conditions) and hemin (50 µmol/L hemin). RDA was performed using Canoco 5.0. Taxonomic composition at the genus level was used as response data, iron level as explanatory variable, time point and experiment as supplementary variables. The variation explained by the ordination axis is significantly higher than random (p = 0.04; permutation test).

C&D) To study differences between iron sources, conditions were grouped at iron source: low (endogenous Fe), sulfate (pooled 50FeS and 250FeS), citrate (pooled 50FeC and 250FeC) and hemin. Taxonomic composition at the genus level was used as response data, iron source as explanatory variable, time point and experiment as supplementary variables. The variation explained by the ordination axis is significantly higher than random (p = 0.032; permutation test). Red symbols represent iron level or iron source classes, light green samples the two replicate experiments and dark green symbols the two time points. Open symbols are the individual samples. The colored lines are envelopes connecting samples of the same iron level or iron source. Length of arrows reflects significance. Classified sample diagrams in **A&C**, Taxa – metadata biplots in **B&D**.

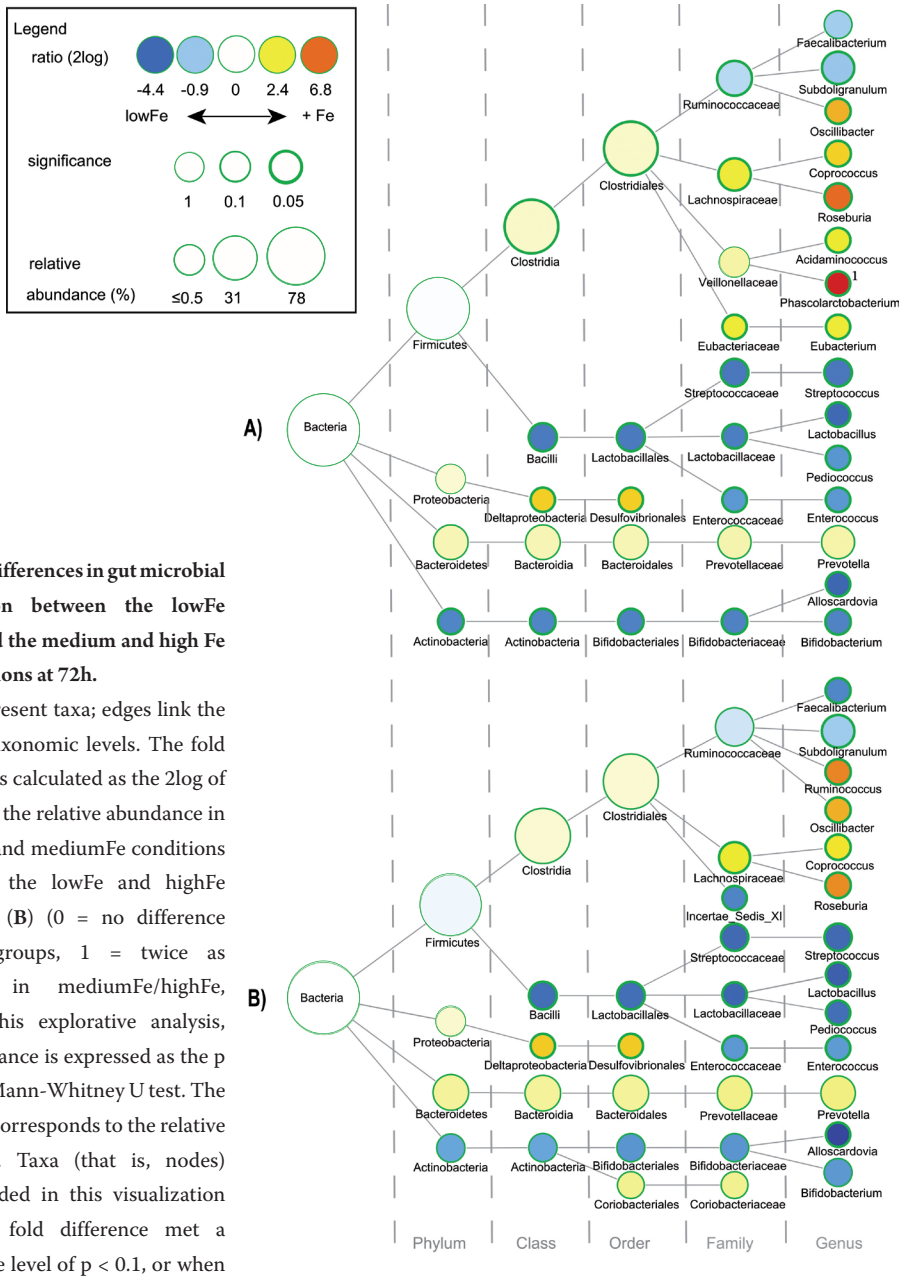


Figure 2. Differences in gut microbial composition between the lowFe control and the medium and high Fe and conditions at 72h.

Nodes represent taxa; edges link the different taxonomic levels. The fold difference is calculated as the 2log of the ratio of the relative abundance in the lowFe and mediumFe conditions (**A**) or in the lowFe and highFe conditions (**B**) (0 = no difference between groups, 1 = twice as abundant in mediumFe/highFe, etc.). In this explorative analysis, the significance is expressed as the p value of a Mann-Whitney U test. The node-size corresponds to the relative abundance. Taxa (that is, nodes) were included in this visualization when the fold difference met a significance level of $p < 0.1$, or when the taxon had a child (that is, more specific taxonomic classification) meeting this criterion.

[1] Phascolarctobacterium was not detected within the lowFe condition and has therefore an estimated 2log fold difference of 10.

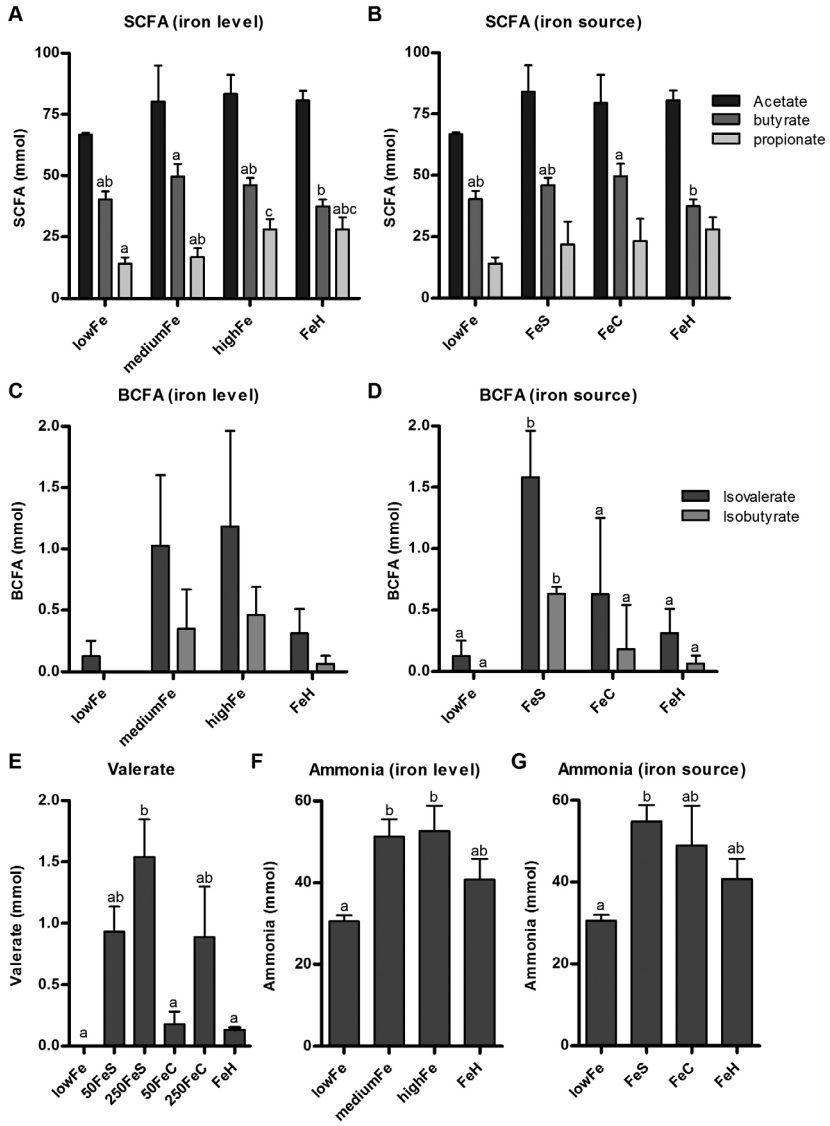


Figure 3. Effect of iron on the gut microbial production of SCFAs, valerate, BCFAs and ammonia.
Cumulative levels at 72h (mean+range) of the main SCFAs acetate, butyrate and propionate are displayed for the pooled mediumFe and highFe conditions in panel **A**. Cumulative levels (mean+range) of the main SCFAs in the pooled FeS and FeC conditions are shown in panel **B**. Valerate levels (mean+range) are displayed for each condition and were not pooled because a pool of iron level or iron source would not very well reflect the differences observed between the individual iron conditions (**E**). Cumulative levels at 72h (mean+range) of the BCFAs isobutyrate and isovalerate are displayed for pooled iron level in panel **C** and for the pooled iron sources in **D**. Cumulative ammonia levels (mean+range) are displayed for the pooled mediumFe and highFe conditions (**F**) and pooled iron sources (**G**). Means of the same metabolite without a common letter differ significantly ($p < 0.05$). Analyses of the single (non-pooled) conditions are shown in **Supplementary Figure 7**.

The influence of iron on the gut microbiota composition

Total bacteria content of the lumen

To study the effect of the various iron sources and concentrations on the total number of bacteria in the lumen of TIM-2, the number of bacteria was determined at t = 0h and 72h by qPCR. Overall, the number of bacteria was higher at 72h compared to 0h ($p = 0.045$), but iron source or concentration had no significant effect on the outcome ($p = 0.14$). Comparisons within the single conditions showed that the total number of bacteria was significantly increased from 0 to 72h in 50FeC only ($p < 0.01$) (**Supplementary Figure 2**).

Microbiome analysis by 16S rRNA sequencing

Next, we determined the microbiome composition by analyzing a total of 82,681 bacterial 16S rRNA sequences obtained by pyrosequencing. Overall, the microbiome (samples taken at 24h and 72h combined) consisted of the phyla Firmicutes (58.4% of the 16S rRNA reads), Bacteroidetes (33.8%), Actinobacteria (6.9%) and Proteobacteria (0.8%) (**Supplementary Figure 3**). At 24h, the phylogenetic diversity index (a measure of α-diversity) was similar among all conditions and was not yet affected by iron. However, at 72h the diversity was different among groups ($p = 0.046$), where the diversity of the lowFe condition was lower compared to the highFe (250FeS and 250FeC combined) conditions ($p < 0.1$) (**Supplementary Figure 4A**). Supplementation of iron as ferrous sulfate (FeS) or ferric citrate (FeC) did not have a significant differential effect on diversity (**Supplementary Figure 4B**).
Multivariate redundancy analysis (RDA) showed that the different iron levels had a distinct non-overlapping microbiota composition ($p = 0.040$) (**Figure 1A**), while iron supplementation as FeS or FeC resulted in a similar microbiota profile, but that was distinct from that of the lowFe and FeH conditions ($p = 0.032$) (**Figure 1C**). The outcome of the RDA was supported by analysis of the phylogenetic distance (Unifrac weighted and unweighted measures for β-diversity) between the samples taken at 72h (**Supplementary Figure 4C-E**). The main differences between the microbiome of the lowFe controls and the conditions with mediumFe and highFe at 72h are summarized in **Figure 2A and B**, respectively. These analyses revealed that the families *Lactobacillaceae, Bifidobacteriaceae* and *Streptococcaceae* were less abundant in the mediumFe and highFe conditions compared to the lowFe controls and that the *Lachnospiraceae*, and to a lesser extent, *Prevotellaceae* families were more abundant in the mediumFe and highFe conditions. This is consistent with the outcome of the RDA, where these same taxa (on the genus level) separated the lowFe from the mediumFe and highFe conditions (**Figure 1B and D**). Differences between the mediumFe and highFe conditions and differences between the lowFe and FeH conditions are shown in **Supplementary Figure 5A and 5B**, respectively.

Microbiome analysis by I-Chip and metagenomic sequencing

To support and validate the data from 16S rRNA sequencing, we investigated the changes of taxa over time and the effects of iron on certain species by using an I-Chip microarray platform for the analysis of the microbial community in the TIM-

2 lumen at every time point (0h, 24h, 48h and 72h). Moreover, shotgun metagenomic sequencing allowed taxonomic analysis of the duplicate samples of lowFe and 50FeS at 72h, this was also used to support the 16S rRNA sequencing data.

In both the I-Chip and metagenomic datasets, *Prevotella* was markedly higher in all conditions with supplementary iron at 72h. I-Chip data furthermore showed that *Prevotella* generally increased in the conditions with iron with a small decrease after 48h, while its levels remained relatively low and stable in the lowFe condition (**Supplementary Table 1 and Supplementary Figure 6A**). Metagenomic analysis also showed that *Prevotella* levels were higher in 50FeS compared to lowFe at 72h (2log ratio: 7.07; p = 0.006) (**Supplementary Figure 6B**). Next, I-Chip analysis showed a gradual increase of *Roseburia* in the50FeS, 250FeS and 50FeC conditions, and metagenomic analysis confirmed that specifically *Roseburia intestinalis* was more abundant at 72h in 50FeS compared to lowFe (2log ratio: 2.01; p = 0.073) (**Supplementary Tables 1 and 2**). 16S rRNA sequencing showed that the sulfate-reducing bacteria (SRB) member *Desulfovibrionales* (order) was more abundant in the conditions with iron at 72h (**Figure 2A, B**). This is consistent with the metagenomic analysis, where the genus *Desulfovibrio* (2log ratio: 3.08; p = 0.042) and specifically the species *D. desulfuricans* (2log ratio: 3.66; p = 0.043) and *D. piger* (2log ratio: 1.33; p = 0.030) were more abundant in the 50FeS condition compared to lowFe (**Supplementary Table 2**). Interestingly, although genus *Bacteroides* as a group was not significantly more abundant in the iron groups, several *Bacteroides* spp. were significantly more abundant in 50FeS compared to lowFe, including the opportunistic pathogen *Bacteroides fragilis* (2log ratio: 1.79; p = 0.029), which was also apparent from the I-Chip analysis, mainly for the FeS conditions (**Supplementary Tables 1 and 2**).

The metagenomic shotgun sequencing also detected sequences from outside the bacterial domain, including Archaea, Eukaryota, and a small minority of viral sequences. On average, Archaea comprised only 0.3% of the metagenomic reads that were annotated to cellular organisms, but Archaea as a group were relatively more abundant in the 50FeS group than in the lowFe group at 72h (2log ratio: 4.90; p = 0.095). Specific Archaea members that were more abundant included *Methanosphaera* (2log ratio: 6.49; p = 0.001), *Methanobrevibacter* (2log ratio: 7.27; p = 0.091) and *Methanobacteria* (2log ratio: 7.24; p = 0.093) (**Supplementary Table 2**).

The influence of iron on the gut microbial metabolome

Iron is involved in many metabolic processes and is indeed known to influence gut bacterial metabolism, as described above. Iron-induced changes in the gut microbial composition may also affect gut microbial metabolic activity. Therefore, it is important to investigate the effects of supplementary iron on the gut microbial metabolome.

Short Chain Fatty Acid and Branched Chain Fatty Acid levels

To study the effect of iron on the production of the main SCFA and BCFA, the cumulative amounts of these metabolites were determined in the lumen and dialysate. Analysis of the individual SFCAs showed no significant effect of iron supplementation on acetate production. However, propionate production was doubled in highFe compared to lowFe ($p < 0.05$) and

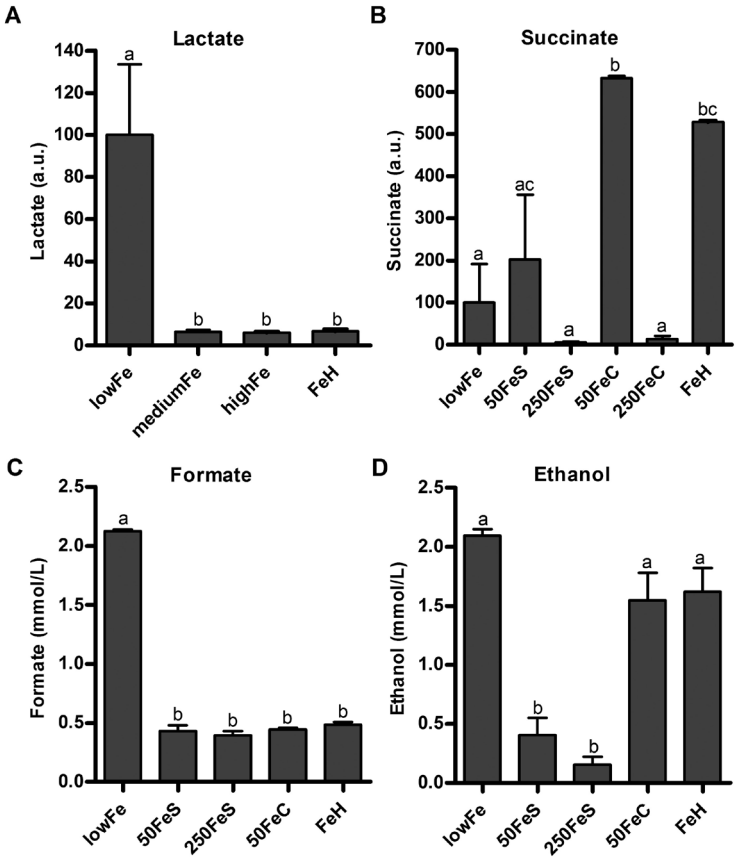


Figure 4. Effect of iron on metabolite levels associated with saccharolytic activity.

Lactate levels (mean+range) at 72h are shown relative to the lowFe control which was set at 100%; the average concentration of lactate in the lowFe condition was 3.3 mmol/L as quantified by ^{1}H-NMR spectroscopy (**A**). Iron source and level had marked effects on relative succinate levels (mean+range) at 72h (lowFe set at 100%) and are therefore shown for every single condition. The average concentration of succinate in the lowFe condition was 0.29 mmol/L as quantified by ^{1}H-NMR spectroscopy (**B**). Formate levels (**C**) and ethanol levels (**D**) (mean+range) at 72h as determined by ^{1}H-NMR spectroscopy are not shown for pooled conditions because the 250 µmol/L ferric citrate condition was not analyzed by ^{1}H-NMR spectroscopy. Means without a common letter differ significantly ($p < 0.05$).

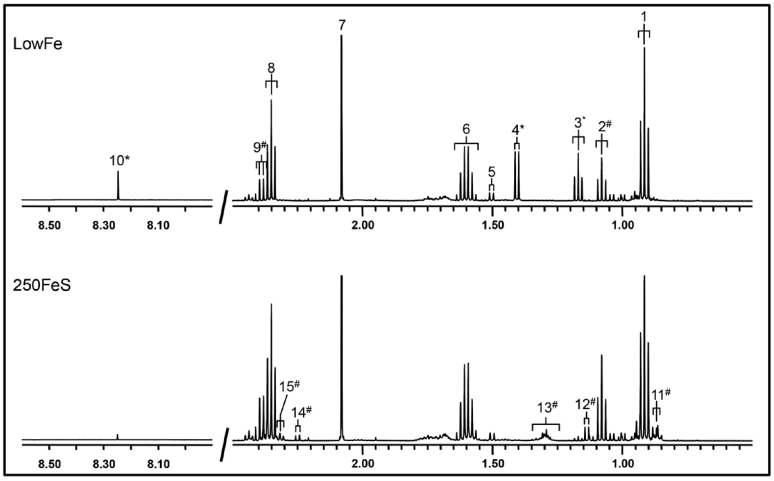


Figure 5. 500 MHz 1H-NMR spectra (regions 2.7-0.5 ppm and 8.5-8.0 ppm) of dialysate samples.
1H-NMR spectroscopy at pH 2.50 of the lowFe condition (upper panel) and 250FeS condition (lower panel) at 72h showed that the SCFAs butyrate and acetate were similar in both conditions, while propionate content was slightly higher in 250FeS. Lactate and ethanol disappeared in the iron condition, while valerate content showed the opposite effect (see also Figure 4 and 5). Peak assignments: butyrate (1, 6 and 8), propionate (2 and 9), ethanol (3), lactate (4), alanine (5), acetate (7), formate (10), valerate (11, 13 and 15) isobutyrate (12) and isovalerate (14). # increased in the condition with iron; * decreased in the condition with iron. Of both conditions 1 replicate is shown, which were representative for their duplicate.

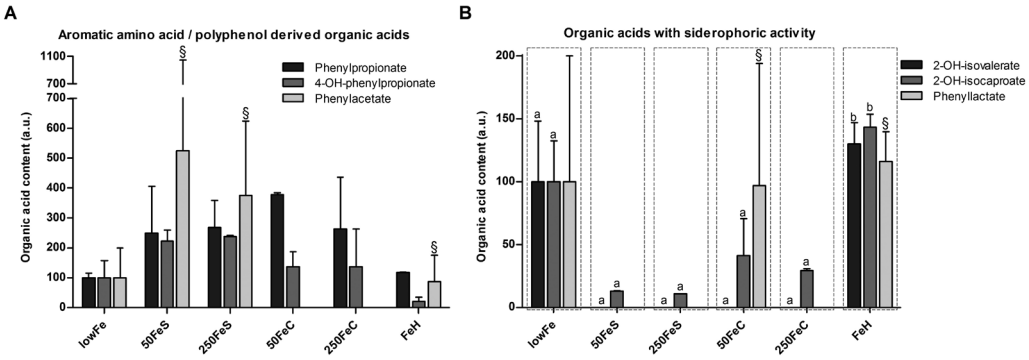


Figure 6. Effect of iron on organic acid levels associated with proteolytic or polyphenol metabolism and on organic acids with siderophoric activity.
Concentrations (mean+range) of phenylpropionate, 4-hydroxy-phenylpropionate and phenylacetate at 72h are displayed relative to the lowFe condition which was set at 100%. §We note that phenylacetate levels included traces of glycerol in 1 biological replicate of 50FeS, 250FeS and FeH, but which were negligible amounts relative to the phenylacetate levels (**A**). Concentrations (mean+range) of metabolites with siderophoric activity; 2-hydroxy-isovalerate, 2-hydroxy-isocaproate and phenyllactate at 72h are displayed relative to the lowFe condition which was set at 100%. §We note that phenyllactate levels included negligible traces of a unknown metabolite in 1 biological replicate of 50FeC and FeH. Means of the same metabolite without a common letter differ significantly ($p < 0.05$) (**B**).

propionate levels were also significantly higher in highFe compared to mediumFe ($p < 0.05$). Butyrate production was similar in all conditions, but was lowest in FeH, which was different from mediumFe ($p < 0.05$) (**Figure 3A**). Iron source had no significant impact on SCFA levels, except for a lower butyrate level in FeH compared to FeC (**Figure 3B**). Although the absolute valerate content was low compared to the other SCFAs, it showed a significant increase under 250FeS compared to lowFe ($p < 0.05$). The valerate level in 250FeS was also significantly higher than in FeH and 50FeC ($p < 0.05$ for both) (**Figure 3E**). Total BCFA production was markedly increased in both ferrous sulfate conditions compared to lowFe, being significantly different for 250FeS ($p < 0.05$). Also the FeS pool showed significantly higher BCFA levels compared to lowFe, FeH, and to the FeC conditions (all $p < 0.05$), which implies that ferrous sulfate stronger stimulated protein fermentation compared to ferric citrate. (**Figure 3D**). Interestingly, iron level (medium vs high) had no significant impact on BCFA levels (**Figure 3C**). Analyses of the single (non-pooled) conditions are shown in **Supplementary Figure 7.**

Ammonia levels

Ammonia, a toxic metabolite that originates from protein breakdown, was determined in the lumen and dialysate of TIM-2. Similar to BCFAs, the cumulative ammonia level was increased in all iron conditions and most prominently in the ferrous sulfate conditions. When iron levels were pooled, the ammonia level was higher in both mediumFe and highFe than in lowFe ($p < 0.05$) (**Figure 3F**). Interestingly, when the iron sources were pooled, only the ferrous sulfate pool showed a significantly higher ammonia level than lowFe ($p < 0.05$), but the ferrous sulfate pool was not significantly different from the ferric citrate pool (**Figure 3G**).

^{1}H-NMR-spectroscopy and organic acid analysis

^{1}H-NMR spectroscopy and GC-MS organic acid analysis were used to explore the gut microbial metabolome in the dialysate. 1H-NMR spectroscopy detected a total of 26 metabolites, of which 20 could be identified and quantified (**Supplementary Table 3**). Moreover, 9 organic acid metabolites could be identified and relatively quantified by GC-MS organic acid analysis. A main effect of iron supplementation was the sharp decrease of lactate compared to the lowFe control ($p < 0.05$), which was detected with both GC-MS and ^{1}H-NMR techniques (**Figure 4A**). Another abundant metabolite that mainly derives from carbohydrate fermentation, was succinate. Compared to lowFe, succinate content was higher in FeH and 50FeC ($p < 0.05$ for both). Remarkably, the succinate level was higher in 50FeC compared to 50FeS ($p < 0.05$), but was almost absent in both high-iron conditions, with a large difference between 50FeC and 250FeC ($p < 0.01$) (**Figure 4B**).

Part of a ^{1}H-NMR spectrum is depicted in **Figure 5** where lowFe and 250FeS are compared. ^{1}H-NMR spectroscopy thus confirms the increase in valerate as mentioned above. This technique also revealed an almost equal decrease of approximately four fold in formate content for all iron-supplemented conditions (250FeC not assessed) compared to lowFe ($p < 0.001$) (**Figure 4C**). Further, metabolite profiling showed a decrease in ethanol content in both ferrous sulfate conditions compared to lowFe (both $p < 0.01$) and also when compared to FeH and 50FeC (both $p < 0.05$) (**Figure 4D**).

Among the organic acids identified, most were amino acid or polyphenol-derived and they generally showed an increase upon iron supplementation. Phenylpropionate content tended to be higher in both ferrous sulfate conditions, and also in the ferric citrate conditions (not significant) (**Figure 6A**). Phenylacetate levels appeared to be higher 50FeS and 250FeS compared to lowFe, but it was not detected in 50FeC and 250FeC (**Figure 6A**). 4-Hydroxy-phenylpropionate content was mainly increased in both ferrous sulfate conditions (not significant) (**Figure 6A**). Although only in small amounts, 3-hydroxy-phenylpropionate was exclusively detected in 250FeS and was considered to be absent in all other conditions (data not shown). Although none of these effects were statistically significant, they support our findings that proteolytic activity is stimulated by iron supplementation.

Siderophoric metabolites detected by organic acid analysis

Other amino acid-derived metabolites were categorized as compounds with siderophore activity. 2-Hydroxy-isovalerate was only detected in substantial amounts in the dialysate of lowFe and FeH (**Figure 6B**). 2-Hydroxy-isocaproate content showed a similar pattern and was significantly lower in the pooled ferrous sulfate, the pooled ferric citrate, the pooled mediumFe and the pooled highFe conditions compared to lowFe and FeH (all $p < 0.05$). Phenyllactate content was relatively variable within the biological duplicates, nevertheless it was exclusively below the detection limit in all ferrous sulfate replicates and also in 250FeC (**Figure 6B**). These data support a potential siderophore function of these metabolite in a low-iron environment or in an environment with a complex iron source that is poorly available for microbes.

Correlations of metabolite levels with the gut microbiome

It is conceivable that the changes in gut microbial metabolism can result from an altered gut microbiome composition. However, gut microbial metabolism may, in theory, also respond to iron without a change in abundances of certain taxa. As a first attempt to link changes in the gut microbiome composition to the marked changes in microbial metabolism we aimed to correlate SCFA, butyrate, propionate, acetate, BCFA and ammonia levels with the relative abundance of bacterial taxa of the microbiome as analyzed by 16S rRNA pyrosequencing.

Short Chain Fatty Acids

The sum of luminal butyrate, propionate and acetate levels in the lumen at 24h and 72h correlated positively with *Roseburia*, and was inversely correlated with *Bifidobacteriaceae* (**Supplementary Figure 8A-B**). Butyrate levels were positively correlated with *Roseburia* and *Coprococcus*, which both are known for the production of butyrate, and with *Clostridiales*, while *Bifidobacterium* correlated negatively (**Figure 7A-D**). Propionate correlated with *Prevotella* and *Dialister*, and was inversely correlated with *Pediococcus* and *Lactobacillaceae* (**Figure 7E-H**). Acetate levels could be correlated to *Bacteroidales* and were inversely correlated to *Desulfovibrionales, Clostridiales* and *Enterobacteriaceae* (**Figure 7I-L**). Other correlations of SCFA levels with the microbiome are displayed in **Supplementary Figure 8C-L**. Spearman r and p-value of a Spearman rank test are depicted within the figures.

Branched Chain Fatty Acids and ammonia

The level of the proteolytic metabolites ammonia and BCFA could be correlated to the following taxa. Lachnospiraceae (among which were *Roseburia* and *Coprococcus*) were strongly correlated with BCFA levels, and also *Oscillibacter, Clostridiales, Desulfovibrionales* and *Enterobacteriaceae* correlated positively with BCFA levels. *Conversely, Pediococcus, Lactobacillaceae* and *Enterococcaceae* were inversely correlated (**Figure 8**). Similar results were found for cumulative ammonia levels as they strongly correlated with *Lachnospiraceae* (among which were *Roseburia* and *Coprococcus*) , and also with *Clostridiales, Desulfovibrionales, Oscillibacter, Enterobacteriaceae* and *Dialister*. Furthermore, cumulative ammonia levels were inversely correlated with *Pediococcus, Bacteroides* and *Lactobacillaceae* (**Supplementary Figure 9A-K**). Other correlations of BCFA and ammonia with the microbiome are displayed in **Supplementary Figure 9L-T**.

Metagenomic analysis: mapping the metabolome to the metagenome

To deduce where the changes in microbial metabolism may originate from, we correlated the relative abundance of bacterial taxa to metabolite levels, and moreover, we linked the metabolite levels to metabolic pathways identified in the metagenome. Furthermore, metagenomic analysis allowed us to explore what other functions and hallmarks were over- or underrepresented in the lowFe and 50FeS microbial communities.

General metagenomic annotations

To investigate the effect of iron on the functional potential of the gut microbial community, we analyzed a total of 69.9 and 59.8 million quality trimmed metagenomic sequencing read pairs (average single end read length: 207 base pairs) for the lowFe and 50FeS replicates at 72h, respectively. A total of 43.2 million sequences (32.5%) were aligned to the human gut microbial gene catalogue (Qin et al 2010) allowing them to be functionally characterized in terms of the SEED subsystems and KEGG orthology annotations An overview of the functional composition (SEED subsystems) of the lowFe and 50FeS conditions is shown in **Supplementary Figure 10**.

The functions that were most strongly affected by the iron treatment were related to the 'Motility & Chemotaxis' level-1 subsystem (**Supplementary Table 4**), that was overrepresented in 50FeS compared to lowFe (2log ratio: 0.40; p = 0.044). The more detailed annotations provided by the SEED level-2 subsystems showed that this could be attributed to the categories 'Flagellar Motility' (2log ratio: 0.80; p = 0.053), 'Flagellum' (2log ratio: 0.67; p = 0.054) and 'Bacterial Chemotaxis' (2log ratio: 0.19; p = 0.091) (**Supplementary Table 4**). Moreover, this was also supported by the many flagellar and chemotaxis related KEGG orthologs that were significantly enriched in 50FeS (**Supplementary Table 5**). One subsystem of special interest in the context of this study was 'Iron Acquisition & Metabolism'. Although there was no net difference found of the level-1 subsystem, we found that the level-2 subsystem 'Transport of Iron' was enriched in the 50FeS condition (2log ratio: 0.34; p = 0.001) (**Supplementary Table 4**), as may be expected for the condition with supplementary iron.

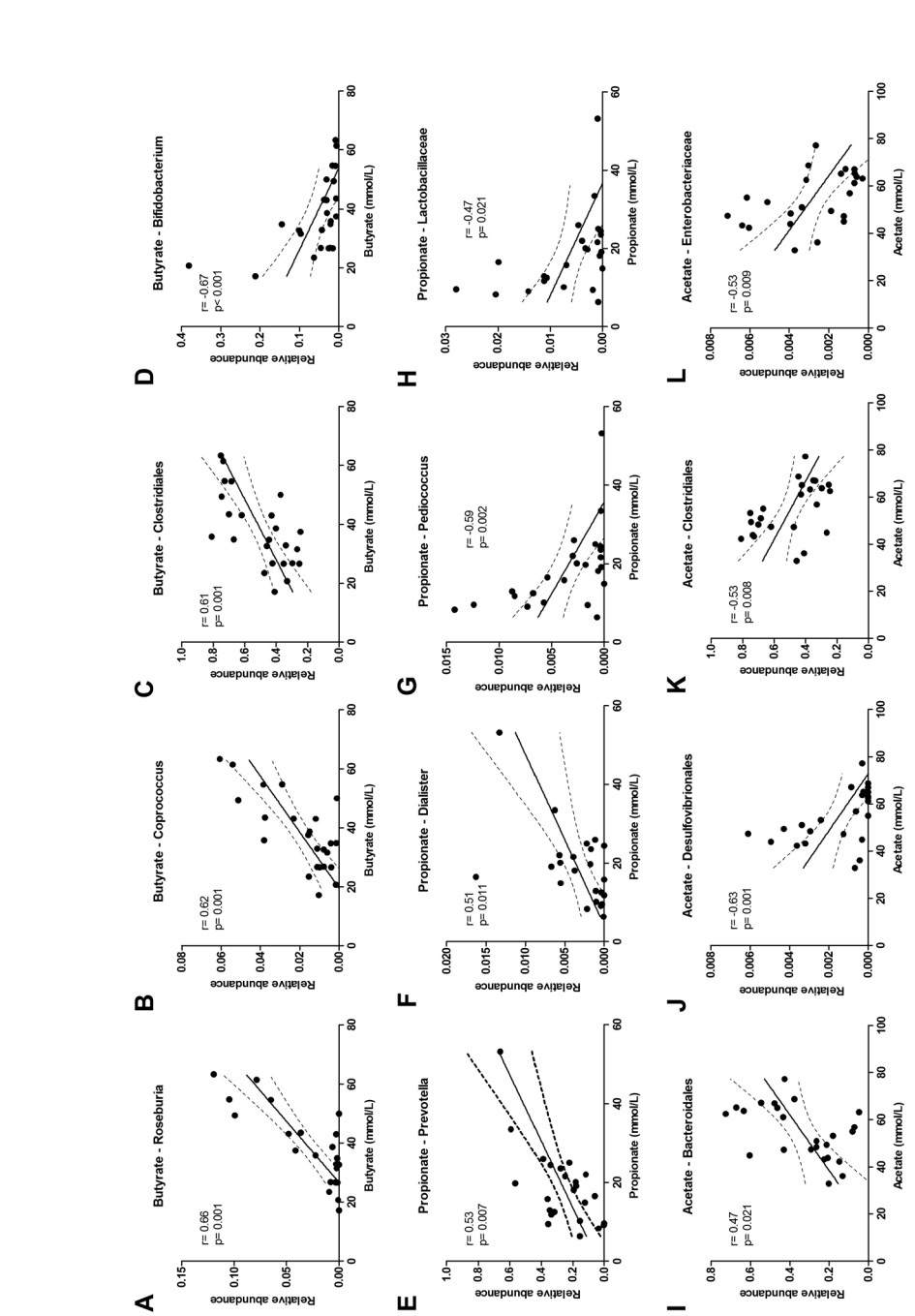


Figure 7. Bacterial taxa that correlate with luminal acetate, butyrate and propionate levels.
The relative abundance (fraction of the total 16S rRNA; e.g. 0.01 = 1%) of bacterial taxa in all samples at 24h and 72h (as determined by 16S rRNA pyrosequencing) was correlated with luminal butyrate (**A-D**), propionate (**E-H**) and acetate (**I-L**) levels in the same samples. Best fit lines with 95% confidence bands were generated by linear regression analysis. Spearman r and p-value are displayed for each graph. This figure shows the taxa that correlated best, additional taxa are shown in **Supplementary Figure 8**.

Saccharolytic and proteolytic related subsystems

With metagenome analysis we aimed to link the marked metabolic changes described above to the enrichment or depletion of SEED subsystems and KEGG orthologs to find out which mechanisms were possibly responsible for the metabolic changes. This is rather complex as many enzymatic systems can work in two directions and because the balance in microbial production and utilization of metabolites is often difficult to predict. This analysis nevertheless provided some clues which might explain part of the metabolic pattern as we observed in the lumen/dialysate. Metabolite analysis pointed at a shift from carbohydrate fermentation towards protein fermentation upon iron supplementation. Subsystem data showed that the level-2 subsystem 'Protein Degradation' was enriched in 50FeS (2log ratio: 0.39; p = 0.008). This involved enrichment of aminopeptidases (EC 3.4.11.x) (KEGG orthologs; **Supplementary Table 5**), which may be involved in exogenous protein fermentation. Carbohydrate related subsystems that may support a decreased carbohydrate fermentation include level-2 subsystems 'Fructooligosaccharides (FOS) & Raffinose Utilization' (2log ratio: -0.20; p = 0.071) and 'Beta-glucoside Metabolism' (2log ratio: -0.64; p = 0.022), both of which were reduced in 50FeS (**Supplementary Table 4**). The apparent shift towards a proteolytic profile was characterized by an increase in ammonia, BCFA and aromatic metabolites. We attempted to link these specific changes to the subsystems and KEGG orthologs as specified below.

Ammonia related subsystems

Level-1 subsystem 'Amino Acids and Derivatives' showed that its level-2 subsystems 'Lysine Degradation' (2log ratio: -0.25; p = 0.045) was underrepresented in 50FeS, while 'Tryptophan Synthesis' and 'Cysteine Biosynthesis' were enriched (2log ratio: 0.41; p = 0.005 and 0.36; p = 0.012 respectively). This would typically lead to decreased amounts of ammonia, but in contrast, 'Glutamate Dehydrogenase' (2log ratio: -0.28; p = 0.061) and 'Glutamine Synthetases' (2log ratio: -0.36; p = 0.106), which may assimilate ammonia, were underrepresented in 50FeS. Additionally, subsystem 'Ammonia Assimilation' was underrepresented in 50FeS (2log ratio: -0.24; p = 0.037) which could fit with the increase of ammonia in this condition (**Supplementary Table 4**). Annotation of the metagenomic sequencing reads to KEGG orthologs showed that nitrogen fixation genes *NifA* and *NifB* were enriched in 50Fe, which also fits with increased levels of ammonia (**Supplementary Table 5**).

Branched Chain Fatty Acid-related subsystems

Level-1 subsystem 'Amino Acids and Derivatives' showed that its level-2 subsystem 'Ketoisovalerate Oxidoreductase' was enriched in 50FeS (2log ratio: 0.45; p = 0.050) (**Supplementary Table 4**). This ferredoxin-type enzyme is involved in the degradation of valine, leucine and isoleucine degradation which can result in the production of BCFAs. Annotation of the sequences to KEGG orthologs also showed that ketoisovalerate oxidoreductase (EC 1.2.7.7) was enriched in the 50FeS condition compared to lowFe (**Supplementary Table 5**).

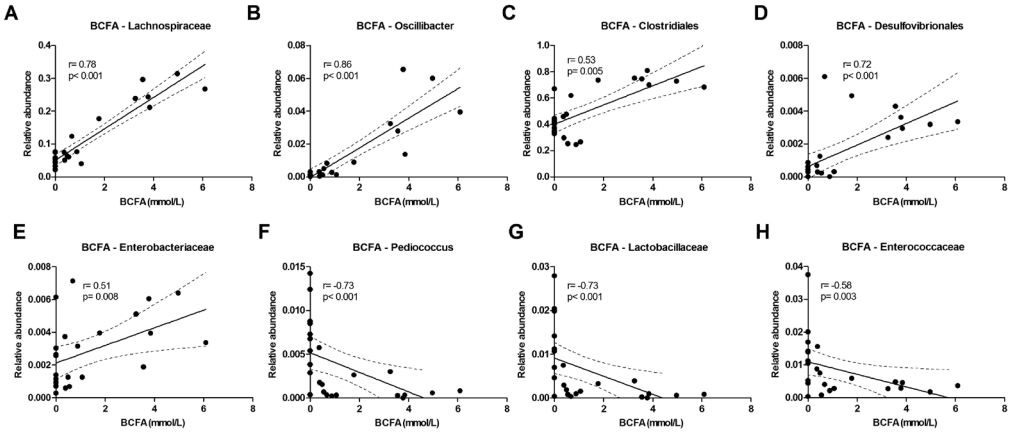


Figure 8. Bacterial taxa that correlate with BCFA levels.

The relative abundance (fraction of the total 16S rRNA; e.g. 0.01 = 1%) of bacterial taxa in all samples at 24h and 72h (as determined by 16S rRNA pyrosequencing) was correlated with the sum of isobutyrate and isovalerate (collectively called BCFAs) levels in the same samples. Panels **A-E** show positive correlations while panels **F-H** show inverse correlations. Best fit lines with 95% confidence bands were generated by linear regression analysis. Spearman r and p-value are displayed for each graph. This figure shows the taxa that correlated best, additional taxa are shown in **Supplementary Figure 9**.

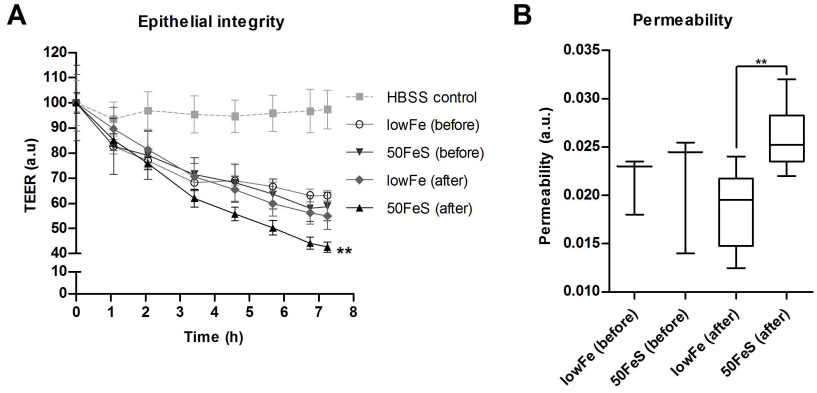


Figure 9. Toxicity of the microbial metabolome for an intestinal monolayer.

Effect of dialysis liquids (before exchange in TIM-2; not containing microbial metabolites) and dialysates (after exchange in TIM-2; at 72h) of the lowFe condition and 50FeS condition on the integrity and permeability of a Caco-2 monolayer. Epithelial integrity is displayed as relative transepithelial electrical resistance (TEER) which was regularly recorded during 7h incubation. HBSS was used as a standard maintaining buffer which should keep the TEER at a constant high level. The decrease in epithelial integrity of the 50FeS dialysate was significantly faster compared to the lowFe dialysate as analyzed by linear regression. We note that iron was removed from the dialysis liquids and dialysates prior to applying them to the epithelial monolayers (**A**). Epithelial permeability as determined by the flux of phenol red from the apical side to the basolateral side (**B**). ** $p < 0.01$. n=3 for the dialysis liquids before exchange in TIM-2 and n=6 for the dialysates after exchange; at 72h in TIM-2.

Aromatic compounds related subsystems

Level-1 subsystem 'Amino Acids and Derivatives' showed that its level-2 subsystem 'Aromatic Amino Acid Interconversions with Aryl Acids' was enriched in 50FeS (2log ratio: 0.76; p = 0.040). This ferredoxin-type system converts aromatic amino acids to aryl acids, among which is phenylacetate and possibly also other phenylalanine derivatives like phenyllactate and phenylpropionic acid. Similarly, level-1 subsystem 'Metabolism of Aromatic Compounds' showed an enrichment of the level-2 subsystem 'Aromatic Amin Catabolism' (2log ratio: 0.55; p = 0.088) which may have contributed to the higher levels of phenyl derivatives in the 50FeS condition. In contrast, subsystem 'Homogentisate Pathway of Aromatic Compound Degradation' was mildly underrepresented in 50FeS (2log ratio: -0.27; p = 0.014) (**Supplementary Table 4**).

Cytotoxicity of the fecal water

Based on the metabolome data, the toxicity of the fecal water was increased with iron, especially in the ferrous sulfate conditions. To confirm this, the cytotoxicity of the 50FeS dialysate towards a tight monolayer of intestinal epithelial Caco-2 cells was assessed. These cytotoxicity tests comprised multiple readouts, i.e. monitoring of the trans epithelial electrical resistance (TEER) (cellular monolayer integrity), phenol red permeability (cellular monolayer permeability), lactate dehydrogenase (LDH) release (cell cytotoxicity) and trypan blue staining (cell death). To exclude the effect of iron itself on the cells, iron was removed from all dialysates prior to the cytotoxicity analyses. Monitoring of TEER showed a deteriorating effect of the dialysis liquids (before exchange in TIM-2) and dialysates (after exchange; sampled at 72h) in the TIM-2 system when compared to a standard maintaining buffer. Remarkably, the 50FeS dialysate at 72h showed a larger relative TEER decrease compared to the lowFe dialysate at 72h (**Figure 9A**). The slopes were significantly different (p = 0.002), and also the area under the curve (AUC) of 50FeS was smaller compared to lowFe (p = 0.021). Phenol red permeability confirmed these findings as the permeability of the Caco-2 monolayer was shown to be increased after exposure to 50FeS dialysate at 72h compared to lowFe dialysate at 72h (p = 0.008) (**Figure 9B**). LDH release and trypan blue staining showed the same trend, as 50FeS dialysate evoked increased cell cytotoxicity and cell death (p = 0.081 and p = 0.067 respectively) (**Supplementary Figure 11A and B**).

Discussion

To find solutions for the unwanted side effects of oral iron administration, such as diarrhea and increased gut inflammation, it is important to investigate the effects of iron on the gut microbiota composition and metabolic activity. Although several studies have reported on the effects of iron on the gut microbiota composition, the effects of iron on gut microbial metabolism and its functional potential has remained largely unexplored. The present study is the first to report on the effect of multiple iron sources and doses on the gut microbiome composition, metagenome, and that included profiling of the effects of these iron interventions on the gut microbial metabolome in both an explorative and targeted manner.

As expected, iron had a marked effect on the gut microbiome composition. Our data indicate that the high iron doses had an additional effect on the gut microbiome composition compared to the medium iron doses. Interestingly, the ferrous sulfate and ferric citrate conditions had a very similar effect on the gut microbiota composition. Comparison of the relative abundance of single bacterial taxa between groups showed that the most apparent differences were the lower abundance of *Streptococcus, Lactobacillus* and *Bifidobacterium* with supplementary iron, which is roughly in line with previous studies for at least the latter two taxa (Kortman et al 2014a) (**Chapter 1**). We did however not observe an increase in *Enterobacteriaceae* or closely related taxa, which was frequently observed in former *in vivo* studies (Balmer et al 1989, Benoni et al 1993, Jaeggi et al 2014, Lee et al 2008, Mevissen-Verhage et al 1985, Zimmermann et al 2010). This could suggest that an increase in *Enterobacteriaceae* depends on host inflammatory responses which they can exploit to their own benefit (Winter et al 2013). Another explanation that we cannot exclude, is that the specific composition of the here used adult gut microbiota of Dutch volunteers, which is different from the above mentioned infant or animal microbiota in previous studies, might inhibit overgrowth of this taxon. Instead, we found increased levels of the genera *Roseburia, Coprococcus, Oscillibacter, Prevotella* and *Desulfovibrio*. So far, reported effects of iron on *Roseburia* abundance have been disperse (Dostal et al 2012a, Dostal et al 2012b, Dostal et al 2014b). We here report an increase in *Roseburia intestinalis* in response to iron in both the mediumFe and highFe conditions compared to lowFe. Consistent with our findings, Dostal *et al.* showed an increase of *Coprococcus* during iron supplementation (Dostal et al 2014b). In contrast to our findings on *Prevotella*, one study that reported on the effect of iron on *Prevotella* abundance showed a lower abundance in mice fed with an iron-supplemented diet compared to an iron-deficient diet (Werner et al 2011). Similar to our findings, *Desulfovibrio* has previously been found to increase in mice on an iron-supplemented diet, but it was also shown that iron did not change *Desulfovibrio* abundance in rats despite an increase in the sulfate reducing bacteria (SRB) group (Dostal et al 2014b, Werner et al 2011), of which *Desulfovibrio* is the most dominant genus. *Desulfovibrio* are known for the production of toxic H2S from sulfate and sulfur containing amino acids (Blaut and Clavel 2007, Davila et al 2013, Nyangale et al 2012). Interestingly, iron appears to induce the activity of certain SRB, while increases in both SRB and in H_2S have been associated with inflammatory bowel disease (IBD) (Carbonero et al 2012, Dinh et al 2004). An increase in *Desulfovibrio* might thus be undesirable. Moreover, we show that several bacteria of the lactic acid bacteria group, including *Lactobacillus* (as mentioned above), *Streptococcus, Pediococcus* and *Enterococcus* showed a decreased relative abundance in response to iron. *Enterococci* have been described in relation to iron supplementation before. Previous studies showed inconsistent outcomes, but like in our study, most studies also showed a lower abundance of *Enterococci* with iron (Balmer and Wharton 1991, Benoni et al 1993, Dostal et al 2012b, Dostal et al 2014b, Tompkins et al 2001, Werner et al 2011). Next, previous studies differ in the reported effects of iron on the abundance of *Bacteroides* (Balmer et al 1989, Balmer and Wharton 1991, Benoni et al 1993, Dostal et al 2012a, Krebs et al 2013, Mevissen-Verhage et al 1985, Werner et al 2011). We here report that the relative abundance of certain *Bacteroides* species, among which is the opportunistic and potent pathogen *B. fragilis* (Wexler 2007), was increased

with 50FeS. Finally, it appeared from our study that Archaea tend to increase with ferrous sulfate, with *Methanosphaera* being the most significant taxon. The role of Archaea in the human gut microbiota is rather unexplored. They could be involved in the pathogenesis of intestinal diseases, but it was also proposed that their presence is a sign of a healthy gut, because they can thrive on health promoting bacterial metabolites such as SCFAs and formate (Jarrell et al 2011, Matarazzo et al 2012). The relative increase in Archaea may thus have contributed to the changed metabolite levels as we observed, either in the utilization of bacterial metabolites and/or the production of other metabolites (discussed below).

The gut microbiome composition moved thus slightly towards a less favorable profile, but the most prominent effect under iron-rich conditions was the change of the gut microbial metabolome from a saccharolytic to a proteolytic profile. Lactate and formate levels were much lower in all conditions with supplementary iron compared to lowFe, also ethanol levels were decreased and succinate levels were decreased in the highFe conditions. These metabolites are primarily derived from carbohydrate, and levels of lactate and formate being the highest in the lowFe condition are consistent with previous findings of an *in vitro* study of Dostal *et al.* under extremely low iron conditions (Dostal et al 2012b). Despite the apparent decrease in saccharolytic activity with iron in our model, the production of the mainly carbohydrate derived SCFAs was not decreased, probably due to the fact that the main SCFAs can also be produced from protein (Macfarlane and Macfarlane 2012). More specifically, acetate and butyrate levels did not differ significantly compared to lowFe, but propionate levels were higher in the highFe conditions. This is partially in line with previous *in vivo* and *in vitro* findings of Dostal *et al.*, although their data from different models were not always consistent at this point (Dostal et al 2012a, Dostal et al 2012b, Dostal et al 2014b).

In our model, the increased proteolytic activity of the gut microbiota under iron-rich conditions was evident by the increased levels of ammonia (a toxic metabolite), BCFAs and valerate, which are all protein fermentation-related metabolites (Hoyles and Wallace 2010, Hughes et al 2008, Macfarlane and Macfarlane 2012, Nery et al 2012, Nyangale et al 2012, Smith and Macfarlane 1997). The iron-induced switch towards a more proteolytic profile is further supported by the tendency that aromatic metabolites were increased. These metabolites are degradation products of aromatic amino acids and polyphenols and it is known that phenylacetate levels can increase upon a high protein diet (Beloborodova et al 2012, Davila et al 2013, Smith and Macfarlane 1997). Little is known about the toxicity of these specific compounds, but phenolic compounds are generally considered to be unbeneficial for human health (Nyangale et al 2012, Smith and Macfarlane 1997, Windey et al 2012b). Importantly, it is well known that protein fermentation in general results in the formation of a putrefactive and toxic environment (Macfarlane and Macfarlane 2012, Nyangale et al 2012).

Our present study shows for the first time that the gut bacterial metabolites that are produced under iron-supplemented (50FeS) conditions are indeed harmful to a human intestinal epithelial monolayer. This was not evident from previously described in vitro studies, but which may be explained by a different design and the use of different gut-model and cell-systems (Dostal et al 2012b, Dostal et al 2014a). In addition, we want to emphasize

that our *in vitro* toxicity findings cannot directly be translated to *in vivo* situations (Dostal et al 2012a, Dostal et al 2014b, Windey et al 2012a), but our data nevertheless indicate that similar effects on gut bacterial metabolism *in vivo* could have significant implications in infection endemic regions as an impaired barrier function of the gut wall could play a pivotal role in the initiation of infections by (opportunistic) enteric pathogens.

Increased iron availability has been associated with an increase in virulence of bacterial pathogens (Bullen et al 2005, Kortman et al 2012). This inspired us to look into the abundance of virulence associated subsystems in our metagenome data. Flagella enable microbes to swim and swarm over surfaces and can be involved in adhesion to epithelial cells, which make them an important virulence factor (Moens and Vanderleyden 1996). Intriguingly, our results show that the level-1 subsystem 'Motility and Chemotaxis' was enriched in the 50FeS condition. Level-2 subsystems not only showed that flagella related subsystems were enriched in 50FeS, but also bacterial chemotaxis. This could suggest that ferrous sulfate selected for bacterial groups that use flagella and chemotaxis to seek for nutrient-rich niches (Rivera-Chavez et al 2013, Stecher et al 2008). Thus, iron could provide motile and chemotactic enteric pathogens with a competitive advantage over other members of the microbiota under iron-rich conditions. Overall, our data provide indications that in the absence of host influences, iron creates a more hostile intestinal environment which can be characterized by i) a reduction in beneficial microbes and an increase of certain bacterial species with pathogenic potential, ii) increased levels of metabolites that can impair the barrier function of the gut wall, and iii) an increase of virulence-associated pathways of enteric pathogens. The combination of these three effects could very well contribute to the increased risk for enteric infections. *In vivo*, the host immune status and the composition of the gut microbiota will largely determine whether or not an intestinal infection will become apparent.

Our metabolome data represent a steady state situation that is the result of both metabolite production and consumption. Cross-feeding of microbial metabolites is anticipated to have a large influence on the metabolome composition (Nyangale et al 2012, Rajilic-Stojanovic 2013). Based on our 16S rRNA pyrosequencing and metagenomic data and literature we can however provide specific leads for the explanation of the iron-induced changes in gut microbial metabolic activity. First of all, many metabolic pathways rely on the action of iron-dependent enzymes (ferredoxin-type enzymes with iron-sulfur clusters), for instance in the Wood-Ljungdahl pathway, and may thus be influenced by iron supplementation (Hood and Skaar 2012, Ragsdale and Pierce 2008). Secondly, redox balance needs to be maintained and microbial fermentation is regulated by this balance (Macfarlane and Macfarlane 2003). It can be envisaged that supplementary iron has a large impact on the redox balance as this transition metal is able to function as an electron donor and electron acceptor. Importantly, also the availability of carbohydrates has a large impact on the type of metabolites that will ultimately be produced. Interestingly, when sufficient carbohydrate is present there is a decreased need to decarboxylate succinate to produce propionate, as has been observed for certain *Bacteroides* spp. (Macfarlane and Macfarlane 2003). This fits with our findings that levels of succinate decreased while propionate levels increased in the high-iron conditions, indicating that carbohydrate sources were depleted early in the

iron-rich conditions. Early depletion of carbohydrate sources by the gut microbiota in the conditions with supplementary iron may also clarify the increase in proteolytic activity. Furthermore, it may explain the low levels of lactate in the iron-supplemented conditions, but this might also be attributed to the lower abundance of lactic acid bacteria in the same conditions.

Correlations of metabolite levels with the relative abundance of bacterial taxa do not provide a causal relationship but may provide some indication which taxa are responsible for the differences observed. It is interesting to see that *Prevotella*, whose growth was stimulated with iron, was positively correlated with propionate levels. *Prevotella* may drive the conversion of succinate to propionate (Nyangale et al 2012), especially when carbon sources are limiting (as discussed above), which fits with the finding that propionate levels were increased in the highFe conditions while succinate was decreased in those conditions. From literature it is well-known that *Roseburia* and *Coprococcus* (both belong to the *Lachnospiraceae* family) and *Faecalibacterium* are producers of butyrate (Louis and Flint 2009). Indeed we found a strong positive correlation of *Roseburia* and *Coprococcus* with butyrate levels, but not with *Faecalibacterium*. So far, very little is known about gut microbiota members that convert amino acids to BCFA. Suspected members are *Clostridium* spp. and *Atopobium*, but only *Clostridium coccoides* has previously been correlated with fecal BCFA levels (Rajilic-Stojanovic 2013, Thompson-Chagoyan et al 2011). Two groups of bacteria that are generally associated with proteolytic activity are *Bacteroides* and *Clostridium* (Cummings and Englyst 1987, Nyangale et al 2012), and although iron may theoretically have induced their metabolic activity, their abundance did not significantly change in our system. Candidates that were identified in this study and that may be involved in BCFA and ammonia production are *Lachnospiraceae, Oscillibacter, Clostridiales* and *Desulfovibrionales* as they all positively correlated with BCFA and ammonia levels. Thus, *Lachnospiraceae* not only correlated with (beneficial) butyrate levels, but also strongly correlated with the (unbeneficial) BCFA and ammonia levels in our study, which has not been described before. Enrichment or depletion of certain genetic characteristics in the metagenomic data also provided clues about the microbial pathways involved in the iron-induced changes in gut microbial metabolism. The most obvious findings involved the protein- and amino acid related subsystems, such as 'Protein Degradation', 'Ketoisovalerate Oxidoreductase', 'Aromatic Amino Acid Interconversions with Aryl Acids' and 'Aromatic Amin Catabolism' which were enriched in the 50FeS condition compared to the lowFe condition. It is interesting to note that the enzyme ketoisovalerate oxidoreductase needs iron as a cofactor, and is not only present in bacteria, but also in Archaea (Heider et al 1996). This may implicate that not only bacteria, but also Archaea contributed to the iron-induced production of BCFAs.

Another fascinating aspect of our findings concerned the identification of the bacterial metabolites 2-hydroxy-isovalerate, 2-hydroxy-isocaproate and phenyllactate that have siderophoric activity (Drechsel et al 1993). Indeed, levels of these iron-binding acids were relatively high in the lowFe condition and the hemin (a more complex and less available iron source, and which in TIM-2 resulted in a lower total iron content compared to the mediumFe and highFe conditions) condition, while they were low or absent in the medium and high-iron conditions. This confirms the idea that bacteria only produce siderophores

when readily available iron is scarce. We cannot be sure that these siderophoric metabolites indeed acted as siderophores, but as the levels of these amino acid derived siderophores oppose the trend of the other amino acid derived metabolites levels, our results strongly suggest that limitation of iron has been the trigger to produce these acids for at least a subset of the gut microbiota. This also implies that iron availability was limited in both the lowFe and hemin conditions. Notably, the production of siderophoric α-ketoacids has previously been shown to be upregulated in *Salmonella enterica* serovar Typhimurium in response to iron-limiting conditions, underlining that the production of such acids responds to iron deprivation (Reissbrodt et al 1997). Despite the relatively low affinity for iron compared to conventional siderophores (Kingsley et al 1996, Reissbrodt et al 1997) these acids may play an important role in iron scavenging and iron cross-feeding in the colon lumen under iron limiting conditions.

Although the iron sources ferrous sulfate and ferric citrate differed in their counter-anion and initial valence state of the iron, they appeared not to cause marked differences in microbiome composition. However, differential results were observed on gut microbial activity and it appeared that the metabolic profile of the ferric citrate conditions was less proteolytic compared to the ferrous sulfate conditions. This indicates that ferric citrate may be preferred as an iron source to be used in human food supplements, we however did not compare the *in vitro* cytotoxicity of the ferric citrate conditions with the ferrous sulfate conditions. More research is needed to reveal whether ferric citrate is indeed preferred over ferrous sulfate as a more beneficial source of dietary iron, from both a gut microbial and host perspective. In a previous study we observed good bioavailability of ferric citrate in a Caco-2 model (Kortman et al 2014b), but it is also required to study the effects of gastrointestinal passage on iron preparations as this will affect iron speciation and possibly also the effects on the gut microbiota. We note that inorganic anions such as sulfate can affect microbial fermentation as these anions can function as an electron sink (Macfarlane and Macfarlane 2003). Although the contribution of sulfate in the condition with ferrous sulfate was relatively small compared to the amount of sulfate already present in the SIEM and dialysis liquid (2.5-12.3% of the amount of sulfate present as magnesium sulfate), the additional sulfate may have influenced microbial metabolism. The citrate in the ferric citrate condition might have formed an alternative carbon source and could have contributed to differential microbial activity compared to the ferrous sulfate conditions, but the contribution of citrate to the amount of carbon sources already present is also relatively small. Notwithstanding their biochemical differences, both ferrous sulfate and ferric citrate caused a shift towards an unbeneficial proteolytic profile, which appeared not to be sufficiently compensated by the increase in beneficial propionate levels.

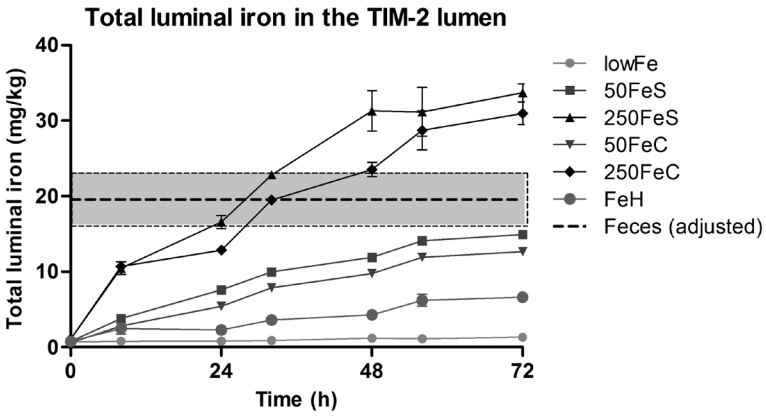
In conclusion, this study shows that the provision of iron (provided as ferrous sulfate or as ferric citrate) to a human gut microbiota resulted in a lower abundance of beneficial bacteria, an increase in toxic metabolites and an increase in bacterial virulence-associated pathways. Thus, supplementation of these forms of iron creates a more hostile intestinal environment which is likely to increase the risk of enteric infection. Our data also show that iron limitation increases the production of bacterial metabolites with iron-binding features. If and how these metabolites act as siderophores and mediate bacterial iron uptake

under these conditions remains to be determined. One future challenge that remains, is to pinpoint metabolic changes to particular microbes, for which we provide novel leads. A metatranscriptomic approach is needed to provide better insight in the effects of iron on the actual microbial metabolism and can help to pinpoint these metabolic properties to certain taxa. This may be complemented with e.g. a LC-MS, HPLC, and/or NMR spectroscopy metabolomics approach to identify and quantify additional microbial volatile and non-volatile metabolites (Nyangale et al 2012). This will aid in the development of an iron preparation that is highly available to humans, but has little to no impact on the gut microbiota composition and metabolism. To reduce the risk for unwanted side effects this is an important challenge that remains in combating anemia in underdeveloped infection endemic regions.

Data access

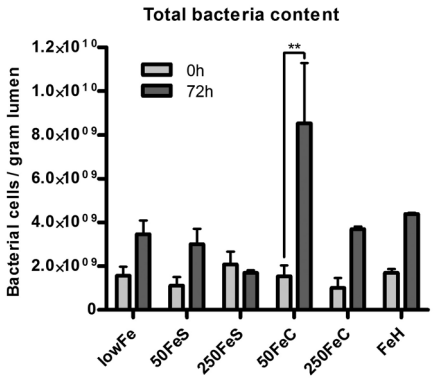
The sequencing datasets accompanying this study are available in the European Nucleotide Archive with the study accession number [ENA: PRJEB6542]. Supplementary Tables 1, 2, 4 and 5 will provisionally be available as online supplementary information, upon publication of the manuscript.

Supplementary Figures and Legends



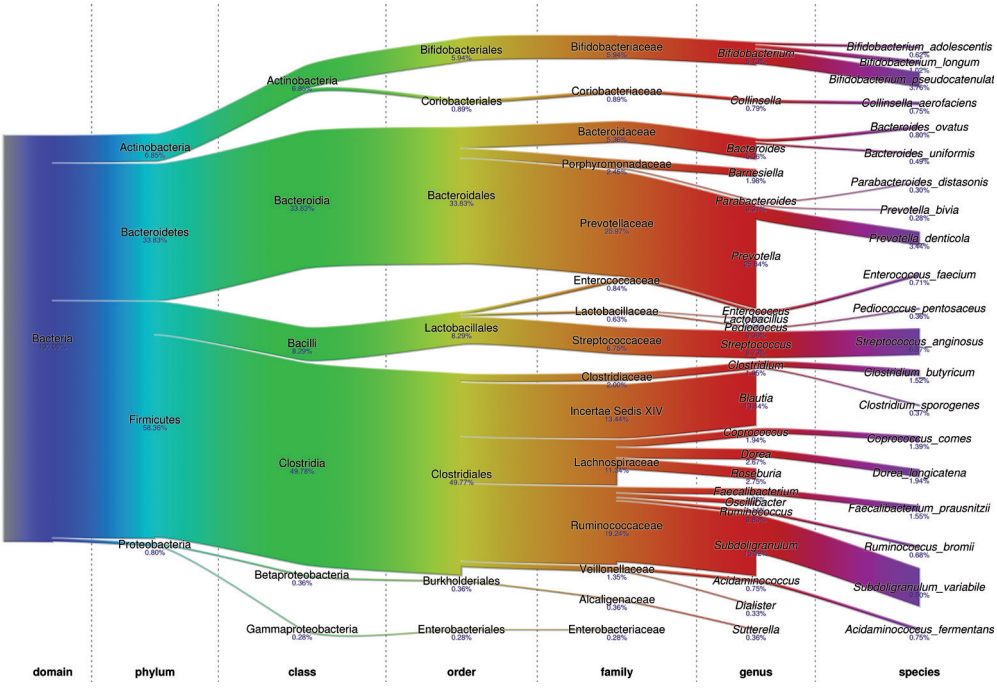
Supplementary Figure 1. Iron content of the TIM-2 lumen in the various iron conditions.
Average total iron concentration in the TIM-2 lumen over time (mean±range; n = 2 for each time point). Supplementary iron accumulated in the lumen during the 72h incubation. As a reference, the total iron content measured in feces of Dutch adult volunteers (n=3) is indicated by the dashed line (mean±SD). The feces was diluted three times to obtain a similar consistency as the TIM-2 lumen which has a higher water content as compared to human feces.

It should be noted that the majority of the supplemented iron was bound to solid matter of the SIEM, or was precipitated in the TIM-2 lumen, and that iron concentration in the 'fecal water' (lumen supernatant) was much lower and relatively stable over time compared to the total lumen sample (data not shown). Iron could accumulate via influx of iron from the dialysis liquid and feeding the system with fresh SIEM. The measured fecal iron concentration of 59 mg Fe/kg wet weight feces (undiluted) reflected the fecal iron content of healthy Dutch human adults. This is somewhat lower compared to the approximate 100 mg Fe/kg wet weight feces that was found in British adults in a study of Lund et al. (Lund et al 1999). Remarkably, Chile infants fed with unfortified cow's milk formula had an average fecal iron content of 60 mg Fe/kg, which is higher than expected for such a diet and a group with an iron deficiency prevalence of 25.7%. The iron content even increased to 100 mg Fe/kg feces when solid foods were introduced at the age of 7 months (Pizarro et al 1987). Based on these studies our highFe condition would reflect a rather normal iron content, even when compared to the fecal iron content of infants. However, iron supplemented as ferrous sulfate or ferric citrate is very different from less readily available endogenous iron sources such as present in grains and vegetables. Therefore our mediumFe and highFe conditions can be best described as iron-supplemented conditions. Readily (freely) available iron concentrations and iron speciation in the colon lumen remain however very difficult to predict and to measure.



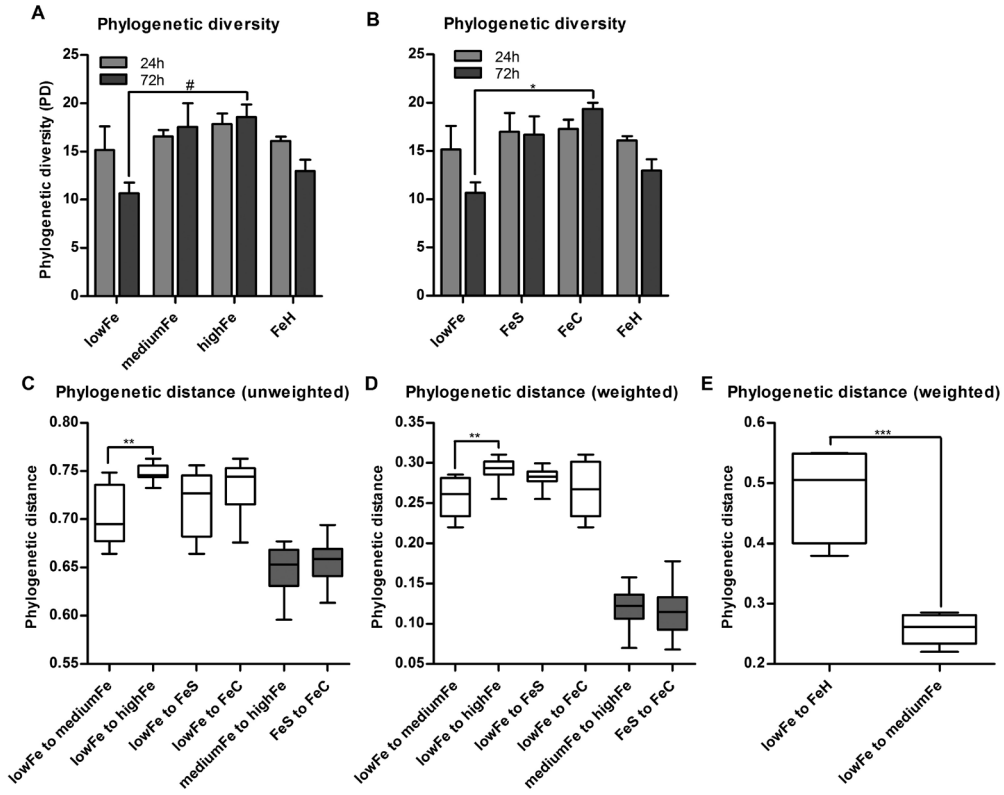
Supplementary Figure 2. Total bacterial cells in the TIM-2 lumen at baseline and at 72h.

Total number of bacteria (mean+range; n = 2) as determined by qPCR in the TIM-2 lumen was similar at 0h (baseline) and was generally increased at 72h (p = 0.045). This increase was significant for the 50FeC condition only when the single conditions were analyzed. Overall, iron source or concentration had no effect on the outcome on the change in total bacteria from baseline to 72h. ** $p < 0.01$.



Supplementary Figure 3. Overall gut microbiome as determined by 16S rRNA pyrosequencing.

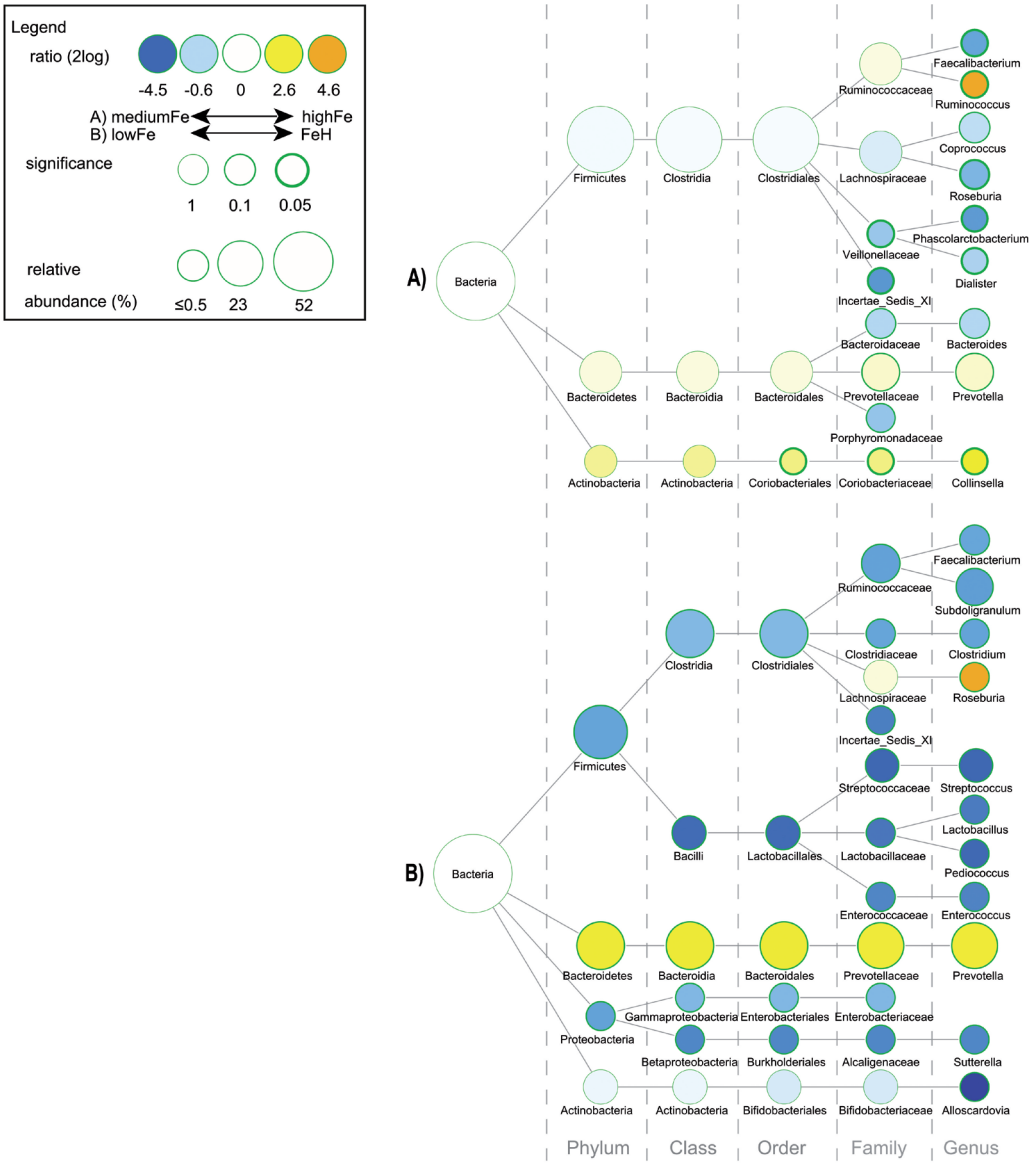
This visualization shows the average abundance (in %) of 16S rRNA reads attributed to the specific taxonomic level and represents all samples in the 16S rRNA pyrosequencing analysis (all conditions at 24h and at 72h).



Supplementary Figure 4. Effect of iron on the phylogenetic diversity and phylogenetic distance.

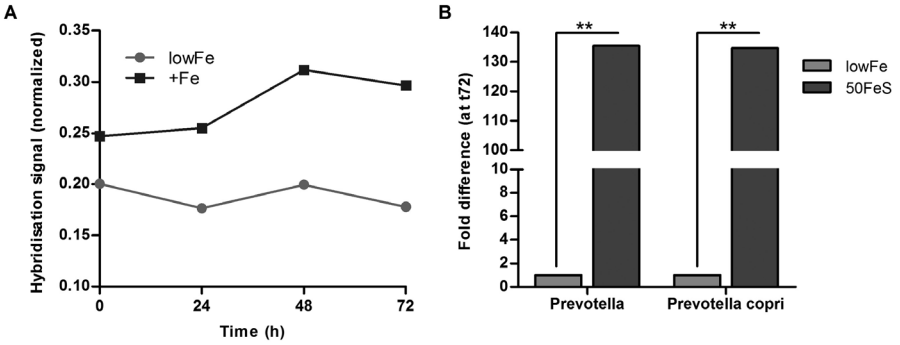
The phylogenetic diversity (PD) index of the lowFe, mediumFe (pooled 50FeS and 50FeC conditions), highFe (pooled 250FeS and 250FeC conditions), FeH (50 µmol/L hemin) is given at 24h and 72h (median + range; n = 2-4). At 24h medians were similar, but at 72h medians differed significantly (P = 0.046) and the diversity of lowFe was lower compared to highFe (**A**).The phylogenetic diversity (PD) index of the lowFe, FeS (pooled 50FeS and 250FeS conditions), FeC (pooled 50FeC and 250FeC conditions), and FeH at 24h and 72h (median + range; n = 2) is given in panel **B**. At 72h, medians tended to differ significantly (P = 0.023), and the diversity of lowFe was lower compared to FeC (**B**).

The phylogenetic distance (min-max whiskers with median) between the various (pooled) conditions at 72h for the unweighted (**C**) and weighted index (**D**) are given. Both showed that the phylogenetic distance between the lowFe and highFe conditions was significantly larger compared to the distance between the lowFe and mediumFe conditions (p = 0.003 and p = 0.005 for weighted and unweighted respectively). This analysis further showed that the phylogenetic distance between the ferrous sulfate conditions and the lowFe condition was not different compared to the distance between the ferric citrate conditions and the lowFe condition, for both the weighted and unweighted measures (**C** and **D**). The phylogenetic distance (min-max whiskers with median) at 72h between the lowFe and FeH was significantly higher compared to the distance between lowFe and mediumFe for the weighted Unifrac index only, suggesting that the high abundance of Prevotella in the hemin condition accounts for this difference in phylogenetics (**E**). All indices were calculated from the 16S rRNA sequencing data. # p< 0.1, * p < 0.05, ** p < 0.01, *** p < 0.001.

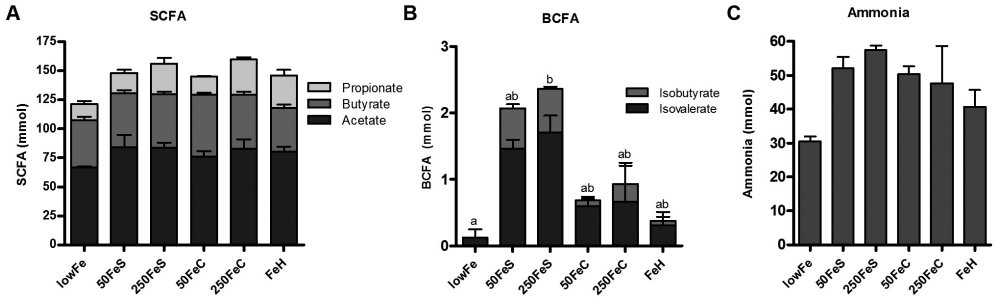


Supplementary Figure 5. Differences in gut microbial composition between the mediumFe and the highFe conditions and differences between the lowFe and FeH condition at 72h.

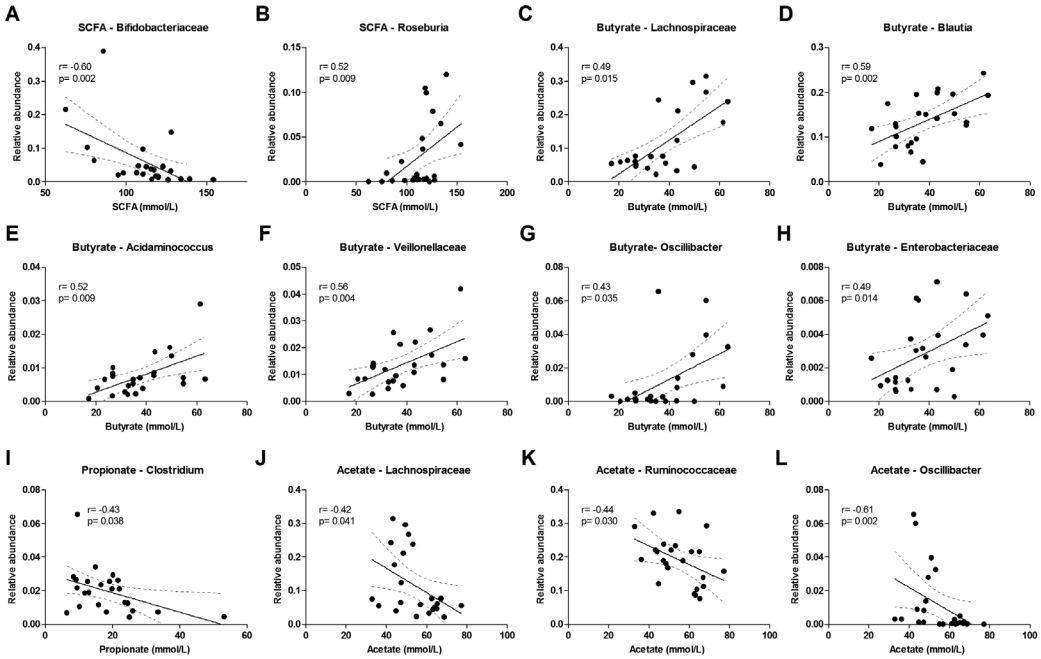
Nodes represent taxa; edges link the different taxonomic levels. The fold difference is calculated as the 2log of the ratio of the relative abundance in the mediumFe and highFe conditions (**A**) or as the 2log ratio of the relative abundance in the lowFe and FeH conditions (**B**) (0 = no difference between groups, 1 = twice as abundant in mediumFe/highFe, etc.). In this explorative analysis, the significance is expressed as the p value of a Mann-Whitney U test. The node-size corresponds to the relative abundance. Taxa (that is, nodes) were included in this visualization when the fold difference met a significance level of $p < 0.1$, or when the taxon had a child (that is, more specific taxonomic classification) meeting this criterion.



Supplementary Figure 6. The effect of supplementary iron on *Prevotella* abundance.
I-Chip analysis showed that the *Prevotella* level (all *Prevotella* probes on the chip taken together; n=9) was fairly constant in the lowFe condition, while there was a visible increase over time in the conditions with iron (pool of all iron conditions; +Fe) (**A**). Metagenomic analysis showed a large fold difference in genus *Prevotella* at 72h between the lowFe (set to 1) and 50FeS condition (2log ratio: 7.07; p = 0.006). For the majority this appeared to be attributable to the species *Prevotella copri* (p = 0.006), but which was also the only *Prevotella* species that was found in the metagenomic database that we used for annotation (Qin et al 2010) (**B**).
Interestingly, we here show that supplementary iron increased the abundance of *Prevotella*, while a relatively high *Prevotella* abundance has been associated with carbohydrate rich plant based diets and has been found to be more abundant in African children compared to European children (De Filippo et al 2010, Wu et al 2011, Yatsunenko et al 2012). It is however difficult to speculate how these finding may relate to the iron-induced increase in *Prevotella* in TIM-2.

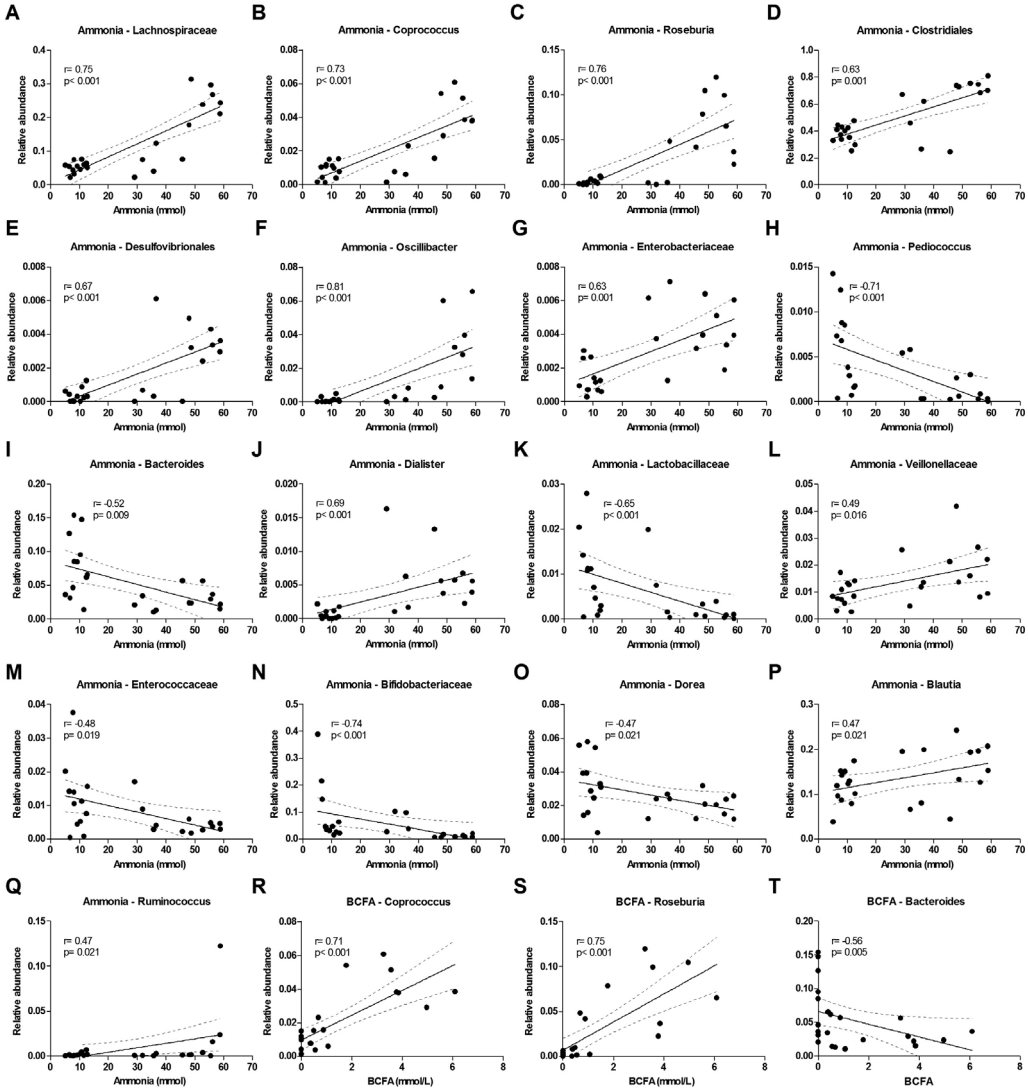


Supplementary Figure 7. Effect of supplementary iron on SCFA, BCFA, and ammonia levels.
Cumulative levels at 72h (mean + range) of the main SCFAs acetate, butyrate and propionate are displayed for the single conditions in panel **A**. Cumulative levels at 72h (mean + range) of the BCFAs isovalerate and isobutyrate are displayed for the single conditions in panel **B**. Means of the total BCFA without a common letter differ significantly ($p < 0.05$). Cumulative levels at 72h (mean + range) of ammonia are displayed for the single conditions in panel **C**.



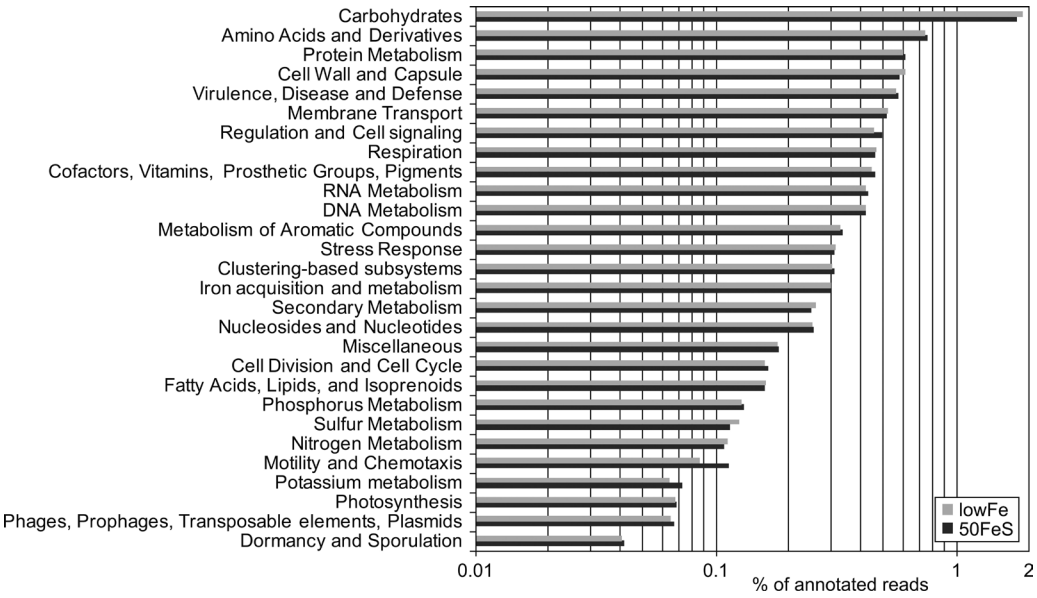
Supplementary Figure 8. Overview of additional bacterial taxa that correlate with luminal SCFA, acetate, butyrate and propionate levels.

The relative abundance (fraction of the total 16S rRNA) of bacterial taxa in all samples at 24h and 72h (as determined by 16S rRNA pyrosequencing) was correlated with luminal SCFA (**A-B**), butyrate (**C-H**) propionate (**I**) and acetate (**J-L**) levels in the same samples. Best fit lines with 95% confidence bands were generated by linear regression analysis. Spearman r and p-value are displayed for each graph.

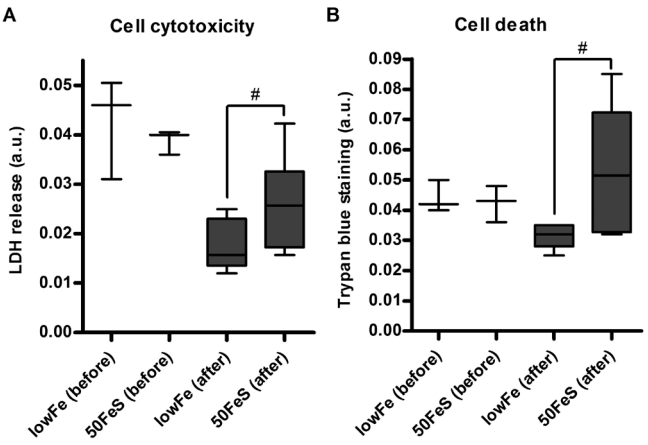


Supplementary Figure 9. Overview of bacterial taxa that correlate with cumulative ammonia levels and additional taxa that correlate with luminal BCFA levels.

The relative abundance (fraction of the total 16S rRNA) of bacterial taxa in all samples at 24h and 72h (as determined by 16S rRNA pyrosequencing) was correlated with luminal ammonia levels in the same samples at 24h and cumulative ammonia levels at 72h (**A-Q**). Panels R-T show additional (to the main article) correlations of the relative abundance of bacterial taxa at 24h and 72h with luminal BCFA levels in the same samples. Best fit lines with 95% confidence bands were generated by linear regression analysis. Spearman r and p-value are displayed for each graph.



Supplementary Figure 10. Functional analysis of metagenomic sequencing data.
Overview of the functions that were present in the lowFe and 50FeS conditions (Level-1 SEED subsystems) and the proportion of reads that were annotated to these functions.



Supplementary Figure 11. Effect of iron on the microbial metabolome toxicity towards a Caco-2 intestinal monolayer.
Effect of dialysis liquids (before exchange in TIM-2; not containing microbial metabolites) and dialysates (after exchange in TIM-2; at 72h) of the lowFe condition and 50FeS condition on cell cytotoxicity and cell death of a Caco-2 monolayer. Cell cytotoxicity as determined by LDH-release (whiskers min to max) tended to be higher in the 50FeS dialysate compared to the lowFe dialysate (n=6) (**A**). Cell death as determined by trypan blue staining (whiskers min to max) tended to be higher in the 50FeS dialysate compared to the lowFe dialysate (n=6) (**B**). # $p < 0.1$.

Supplementary Table 3. 1H-NMR resonances from metabolites observed in dialysate samples at 72h (measured at pH=2.50)

Metabolite	CS [splitting] (ppm)			
Acetate	2.08 [si]			
Alanine	1.50 [do]	3.87 [qu]		
Butyrate	0.91 [tr]	1.60 [sx]	2.35 [tr]	
Cholate†	0.71 [si]			
Ethanol	1.17 [tr]	3.65 [qu]		
Formate	8.24 [si]			
Isobutyrate	1.14 [do]	2.60 [mu]		
Isoleucine	0.94 [tr]	1.02 [do]		
Isovalerate	0.94 [do]	2.02 [mu]	2.26 [do]	
Lactate	1.41 [do]	4.36 [qu]		
Leucine	0.95 [tr]	0.97 [do]		
Methanol	3.35 [si]			
Phenylalanine	7.35 [mu]			
Phenylpropionate	2.71 [tr]	2.94 [tr]	7.32 [mu]	
Propionate	1.06 [tr]	2.20 [qu]		
Succinate	2.66 [si]			
Trimethylamine	2.89 [do]			
Tyrosine	6.89 [do]	7.19 [do]		
Valerate	0.88[tr]	1.32 [mu]	1.56 [mu]	2.32 [tr]
Valine	0.99 [do]	1.05 [do]	2.28 [mu]	3.62 [do]
X1	2.66 [tr]	2.86 [tr]		
X2	1.74 [mu]	3.28 [mu]		
X3	3.55 [tr]			
X4	3.60 [tr]			
X5	6.84 [do]	7.17 [do]		
X6	2.44 [tr]			

† Tentative assignment based on Jacobs et al. (Jacobs et al 2008)

X1-X6 could not with certainty or tentatively be assigned to a known metabolite

References

- Balmer SE, Scott PH, Wharton BA (1989). Diet and faecal flora in the newborn: lactoferrin. Archives of disease in childhood 64: 1685-1690.
- Balmer SE, Wharton BA (1991). Diet and faecal flora in the newborn: iron. Archives of disease in childhood 66: 1390-1394.
- Beloborodova N, Bairamov I, Olenin A, Shubina V, Teplova V, Fedotcheva N (2012). Effect of phenolic acids of microbial origin on production of reactive oxygen species in mitochondria and neutrophils. Journal of biomedical science 19: 89.
- Benoni G, Cuzzolin L, Zambreri D, Donini M, Del Soldato P, Caramazza I (1993). Gastrointestinal effects of single and repeated doses of ferrous sulphate in rats. Pharmacological research : the official journal of the Italian Pharmacological Society 27: 73-80.
- Blaut M, Clavel T (2007). Metabolic diversity of the intestinal microbiota: implications for health and disease. The Journal of nutrition 137: 751S-755S.
- Bullen JJ, Rogers HJ, Spalding PB, Ward CG (2005). Iron and infection: the heart of the matter. FEMS immunology and medical microbiology 43: 325-330.
- Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK et al (2010). QIIME allows analysis of high-throughput community sequencing data. Nature methods 7: 335-336.
- Carbonero F, Benefiel AC, Alizadeh-Ghamsari AH, Gaskins HR (2012). Microbial pathways in colonic sulfur metabolism and links with health and disease. Frontiers in physiology 3: 448.
- Cole JR, Wang Q, Cardenas E, Fish J, Chai B, Farris RJ et al (2009). The Ribosomal Database Project: improved alignments and new tools for rRNA analysis. Nucleic acids research 37: D141-145.
- Crielaard W, Zaura E, Schuller AA, Huse SM, Montijn RC, Keijser BJ (2011). Exploring the oral microbiota of children at various developmental stages of their dentition in the relation to their oral health. BMC medical genomics 4: 22.
- Cummings JH, Englyst HN (1987). Fermentation in the human large intestine and the available substrates. The American journal of clinical nutrition 45: 1243-1255.
- Davila AM, Blachier F, Gotteland M, Andriamihaja M, Benetti PH, Sanz Y et al (2013). Intestinal luminal nitrogen metabolism: role of the gut microbiota and consequences for the host. Pharmacological research : the official journal of the Italian Pharmacological Society 68: 95-107.
- De Filippo C, Cavalieri D, Di Paola M, Ramazzotti M, Poullet JB, Massart S et al (2010). Impact of diet in shaping gut microbiota revealed by a comparative study in children from Europe and rural Africa. Proc Natl Acad Sci U S A 107: 14691-14696.
- Dinh HT, Kuever J, Mussmann M, Hassel AW, Stratmann M, Widdel F (2004). Iron corrosion by novel anaerobic microorganisms. Nature 427: 829-832.
- Dostal A, Chassard C, Hilty FM, Zimmermann MB, Jaeggi T, Rossi S et al (2012a). Iron depletion and repletion with ferrous sulfate or electrolytic iron modifies the composition and metabolic activity of the gut microbiota in rats. The Journal of nutrition 142: 271-277.
- Dostal A, Fehlbaum S, Chassard C, Zimmermann MB, Lacroix C (2012b). Low iron availability in continuous in vitro colonic fermentations induces strong dysbiosis of the child gut microbial consortium and a decrease in main metabolites. FEMS microbiology ecology 83: 161-175.

- Dostal A, Gagnon M, Chassard C, Zimmermann MB, O'Mahony L, Lacroix C (2014a). Salmonella adhesion, invasion and cellular immune responses are differentially affected by iron concentrations in a combined in vitro gut fermentation-cell model. PloS one 9: e93549.
- Dostal A, Lacroix C, Pham VT, Zimmermann MB, Del'homme C, Bernalier-Donadille A et al (2014b). Iron supplementation promotes gut microbiota metabolic activity but not colitis markers in human gut microbiota-associated rats. The British journal of nutrition 111: 2135-2145.
- Drechsel H, Thieken A, Reissbrodt R, Jung G, Winkelmann G (1993). Alpha-keto acids are novel siderophores in the genera Proteus, Providencia, and Morganella and are produced by amino acid deaminases. Journal of bacteriology 175: 2727-2733.
- Ferruzza S, Scarino ML, Gambling L, Natella F, Sambuy Y (2003). Biphasic effect of iron on human intestinal Caco-2 cells: early effect on tight junction permeability with delayed onset of oxidative cytotoxic damage. Cellular and molecular biology (Noisy-le-Grand, France) 49: 89-99.
- Gera T, Sachdev HP (2002). Effect of iron supplementation on incidence of infectious illness in children: systematic review. BMJ (Clinical research ed 325: 1142.
- Haas BJ, Gevers D, Earl AM, Feldgarden M, Ward DV, Giannoukos G et al (2011). Chimeric 16S rRNA sequence formation and detection in Sanger and 454-pyrosequenced PCR amplicons. Genome research 21: 494-504.
- Heider J, Mai X, Adams MW (1996). Characterization of 2-ketoisovalerate ferredoxin oxidoreductase, a new and reversible coenzyme A-dependent enzyme involved in peptide fermentation by hyperthermophilic archaea. Journal of bacteriology 178: 780-787.
- Hood MI, Skaar EP (2012). Nutritional immunity: transition metals at the pathogen-host interface. Nature reviews 10: 525-537.
- Hoyles L, Wallace RJ (2010). Gastrointestinal tract: intestinal fatty acid metabolism and implications for health. In: Timmis K (ed). Handbook of hydrocarbon and lipid microbiology. Springer: Berlin. pp 3119-3132.
- Hughes R, Kurth MJ, McGilligan V, McGlynn H, Rowland I (2008). Effect of colonic bacterial metabolites on Caco-2 cell paracellular permeability in vitro. Nutrition and cancer 60: 259-266.
- Hurrell R, Egli I (2010). Iron bioavailability and dietary reference values. The American journal of clinical nutrition 91: 1461S-1467S.
- Jacobs DM, Deltimple N, van Velzen E, van Dorsten FA, Bingham M, Vaughan EE et al (2008). (1)H NMR metabolite profiling of feces as a tool to assess the impact of nutrition on the human microbiome. NMR in biomedicine 21: 615-626.
- Jaeggi T, Kortman GA, Moretti D, Chassard C, Holding P, Dostal A et al (2014). Iron fortification adversely affects the gut microbiome, increases pathogen abundance and induces intestinal inflammation in Kenyan infants. Gut.
- Jarrell KF, Walters AD, Bochiwal C, Borgia JM, Dickinson T, Chong JP (2011). Major players on the microbial stage: why archaea are important. Microbiology (Reading, England) 157: 919-936.
- Kanehisa M, Goto S, Sato Y, Kawashima M, Furumichi M, Tanabe M (2014). Data, information, knowledge and principle: back to metabolism in KEGG. Nucleic acids research 42: D199-205.
- Kingsley R, Rabsch W, Roberts M, Reissbrodt R, Williams PH (1996). TonB-dependent iron supply in Salmonella by alpha-ketoacids and alpha-hydroxyacids. FEMS microbiology letters 140: 65-70.

- Kortman GA, Boleij A, Swinkels DW, Tjalsma H (2012). Iron availability increases the pathogenic potential of Salmonella typhimurium and other enteric pathogens at the intestinal epithelial interface. PloS one 7: e29968.
- Kortman GA, Raffatellu M, Swinkels DW, Tjalsma H (2014a). Nutritional iron turned inside out: intestinal stress from a gut microbial perspective. FEMS microbiology reviews.
- Kortman GA, Roelofs RW, Swinkels DW, de Jonge MI, Burt SA, Tjalsma H (2014b). Iron-Induced Virulence of Salmonella enterica Serovar Typhimurium at the Intestinal Epithelial Interface Can Be Suppressed by Carvacrol. Antimicrobial agents and chemotherapy 58: 1664-1670.
- Kovatcheva-Datchary P, Egert M, Maathuis A, Rajilic-Stojanovic M, de Graaf AA, Smidt H et al (2009). Linking phylogenetic identities of bacteria to starch fermentation in an in vitro model of the large intestine by RNA-based stable isotope probing. Environmental microbiology 11: 914-926.
- Krebs NF, Sherlock LG, Westcott J, Culbertson D, Hambidge KM, Feazel LM et al (2013). Effects of different complementary feeding regimens on iron status and enteric microbiota in breastfed infants. The Journal of pediatrics 163: 416-423.
- Ladirat SE, Schols HA, Nauta A, Schoterman MH, Keijser BJ, Montijn RC et al (2013). High-throughput analysis of the impact of antibiotics on the human intestinal microbiota composition. Journal of microbiological methods 92: 387-397.
- Langmead B, Trapnell C, Pop M, Salzberg SL (2009). Ultrafast and memory-efficient alignment of short DNA sequences to the human genome. Genome biology 10: R25.
- Lee SH, Shinde P, Choi J, Park M, Ohh S, Kwon IK et al (2008). Effects of dietary iron levels on growth performance, hematological status, liver mineral concentration, fecal microflora, and diarrhea incidence in weanling pigs. Biological trace element research 126 Suppl 1: S57-68.
- Louis P, Flint HJ (2009). Diversity, metabolism and microbial ecology of butyrate-producing bacteria from the human large intestine. FEMS microbiology letters 294: 1-8.
- Lund EK, Wharf SG, Fairweather-Tait SJ, Johnson IT (1999). Oral ferrous sulfate supplements increase the free radical-generating capacity of feces from healthy volunteers. The American journal of clinical nutrition 69: 250-255.
- Maathuis A, Hoffman A, Evans A, Sanders L, Venema K (2009). The effect of the undigested fraction of maize products on the activity and composition of the microbiota determined in a dynamic in vitro model of the human proximal large intestine. Journal of the American College of Nutrition 28: 657-666.
- Maathuis AJ, van den Heuvel EG, Schoterman MH, Venema K (2012). Galacto-oligosaccharides have prebiotic activity in a dynamic in vitro colon model using a (13)C-labeling technique. The Journal of nutrition 142: 1205-1212.
- Macfarlane GT, Macfarlane S (2011). Fermentation in the human large intestine: its physiologic consequences and the potential contribution of prebiotics. Journal of clinical gastroenterology 45 Suppl: S120-127.
- Macfarlane GT, Macfarlane S (2012). Bacteria, colonic fermentation, and gastrointestinal health. Journal of AOAC International 95: 50-60.
- Macfarlane S, Macfarlane GT (2003). Regulation of short-chain fatty acid production. The Proceedings of the Nutrition Society 62: 67-72.
- Matarazzo F, Ribeiro AC, Faveri M, Taddei C, Martinez MB, Mayer MP (2012). The domain Archaea in human mucosal surfaces. Clinical microbiology and infection : the official publication of the European Society of Clinical Microbiology and Infectious Diseases 18: 834-840.

- Mevissen-Verhage EA, Marcelis JH, Harmsen-Van Amerongen WC, de Vos NM, Verhoef J (1985). Effect of iron on neonatal gut flora during the first three months of life. European journal of clinical microbiology 4: 273-278.
- Minekus M, Smeets-Peeters M, Bernalier A, Marol-Bonnin S, Havenaar R, Marteau P et al (1999). A computer-controlled system to simulate conditions of the large intestine with peristaltic mixing, water absorption and absorption of fermentation products. Appl Microbiol Biotechnol 53: 108-114.
- Moens S, Vanderleyden J (1996). Functions of bacterial flagella. Critical reviews in microbiology 22: 67-100.
- Muthayya S, Rah JH, Sugimoto JD, Roos FF, Kraemer K, Black RE (2013). The global hidden hunger indices and maps: an advocacy tool for action. PloS one 8: e67860.
- Natoli M, Felsani A, Ferruzza S, Sambuy Y, Canali R, Scarino ML (2009). Mechanisms of defence from Fe(II) toxicity in human intestinal Caco-2 cells. Toxicol In Vitro 23: 1510-1515.
- Nery J, Goudez R, Biourge V, Tournier C, Leray V, Martin L et al (2012). Influence of dietary protein content and source on colonic fermentative activity in dogs differing in body size and digestive tolerance. Journal of animal science 90: 2570-2580.
- Nyangale EP, Mottram DS, Gibson GR (2012). Gut microbial activity, implications for health and disease: the potential role of metabolite analysis. Journal of proteome research 11: 5573-5585.
- Overbeek R, Begley T, Butler RM, Choudhuri JV, Chuang HY, Cohoon M et al (2005). The subsystems approach to genome annotation and its use in the project to annotate 1000 genomes. Nucleic acids research 33: 5691-5702.
- Pizarro F, Amar M, Stekel A (1987). Determination of iron in stools as a method to monitor consumption of iron-fortified products in infants. The American journal of clinical nutrition 45: 484-487.
- Qin J, Li R, Raes J, Arumugam M, Burgdorf KS, Manichanh C et al (2010). A human gut microbial gene catalogue established by metagenomic sequencing. Nature 464: 59-65.
- Ragsdale SW, Pierce E (2008). Acetogenesis and the Wood-Ljungdahl pathway of CO(2) fixation. Biochimica et biophysica acta 1784: 1873-1898.
- Rajilic-Stojanovic M (2013). Function of the microbiota. Best practice & research Clinical gastroenterology 27: 5-16.
- Rehman A, Heinsen FA, Koenen ME, Venema K, Knecht H, Hellmig S et al (2012). Effects of probiotics and antibiotics on the intestinal homeostasis in a computer controlled model of the large intestine. BMC microbiology 12: 47.
- Reissbrodt R, Kingsley R, Rabsch W, Beer W, Roberts M, Williams PH (1997). Iron-regulated excretion of alpha-keto acids by Salmonella typhimurium. Journal of bacteriology 179: 4538-4544.
- Rivera-Chavez F, Winter SE, Lopez CA, Xavier MN, Winter MG, Nuccio SP et al (2013). Salmonella uses energy taxis to benefit from intestinal inflammation. PLoS pathogens 9: e1003267.
- Sakai M, Imai T, Ohtake H, Otagiri M (1998). Cytotoxicity of absorption enhancers in Caco-2 cell monolayers. The Journal of pharmacy and pharmacology 50: 1101-1108.
- Shannon P, Markiel A, Ozier O, Baliga NS, Wang JT, Ramage D et al (2003). Cytoscape: a software environment for integrated models of biomolecular interaction networks. Genome research 13: 2498-2504.
- Smith EA, Macfarlane GT (1997). Dissimilatory amino Acid metabolism in human colonic bacteria. Anaerobe 3: 327-337.
- Stecher B, Barthel M, Schlumberger MC, Haberli L, Rabsch W, Kremer M et al (2008). Motility allows S. Typhimurium to benefit from the mucosal defence. Cellular microbiology 10: 1166-1180.

- Ter Braak CJF, Smilauer P (2012). Canoco reference manual and user's guide: software for ordination, version 5.0. Microcomputer Power: Ithaca, USA.
- Thompson-Chagoyan OC, Fallani M, Maldonado J, Vieites JM, Khanna S, Edwards C et al (2011). Faecal microbiota and short-chain fatty acid levels in faeces from infants with cow's milk protein allergy. International archives of allergy and immunology 156: 325-332.
- Tompkins GR, O'Dell NL, Bryson IT, Pennington CB (2001). The effects of dietary ferric iron and iron deprivation on the bacterial composition of the mouse intestine. Current microbiology 43: 38-42.
- van Nuenen MHMC, Diederick Meyer P, Venema K (2003). The Effect of Various Inulins and Clostridium difficile on the Metabolic Activity of the Human Colonic Microbiota in vitro. Microbial Ecology in Health and Disease 15: 137-144.
- Venema K, van Nuenen MHMC, Smeets-Peeters M, Minekus M, Havenaar R (2000). TNO's in vitro large intestinal model: an excellent screening tool for functional food and pharmaceutical research. Ernährung 24: 558-564
- Werner T, Wagner SJ, Martinez I, Walter J, Chang JS, Clavel T et al (2011). Depletion of luminal iron alters the gut microbiota and prevents Crohn's disease-like ileitis. Gut 60: 325-333.
- Wexler HM (2007). Bacteroides: the good, the bad, and the nitty-gritty. Clinical microbiology reviews 20: 593-621.
- WHO, UNICEF (2004). Focusing on anaemia: Towards an integrated approach for effective anaemia control.
- Windey K, De Preter V, Louat T, Schuit F, Herman J, Vansant G et al (2012a). Modulation of protein fermentation does not affect fecal water toxicity: a randomized cross-over study in healthy subjects. PloS one 7: e52387.
- Windey K, De Preter V, Verbeke K (2012b). Relevance of protein fermentation to gut health. Molecular nutrition & food research 56: 184-196.
- Winter SE, Lopez CA, Baumler AJ (2013). The dynamics of gut-associated microbial communities during inflammation. EMBO reports 14: 319-327.
- Wu GD, Chen J, Hoffmann C, Bittinger K, Chen YY, Keilbaugh SA et al (2011). Linking long-term dietary patterns with gut microbial enterotypes. Science (New York, NY 334: 105-108.
- Yatsunenko T, Rey FE, Manary MJ, Trehan I, Dominguez-Bello MG, Contreras M et al (2012). Human gut microbiome viewed across age and geography. Nature 486: 222-227.
- Zimmermann MB, Hurrell RF (2007). Nutritional iron deficiency. Lancet 370: 511-520.
- Zimmermann MB, Chassard C, Rohner F, N'Goran E K, Nindjin C, Dostal A et al (2010). The effects of iron fortification on the gut microbiota in African children: a randomized controlled trial in Cote d'Ivoire. The American journal of clinical nutrition 92: 1406-1415.

Chapter 4

Low dietary iron intake restrains the intestinal inflammatory response and pathology of enteric infection by food-borne bacterial pathogens

Guus AM Kortman[1], Michelle LM Mulder[1,2#], Thijs JW Richters[1,2#], Nanda KN Shanmugam[2], Estela Trebicka[2], Jos Boekhorst[3], Harro M Timmerman[3], Rian Roelofs[1], Erwin T Wiegerinck[1], Coby M Laarakkers[1], Dorine W Swinkels[1], Albert Bolhuis[4], Bobby J Cherayil[2¡], Harold Tjalsma[1¡]

[1] Department of Laboratory Medicine – Laboratory of Genetic, Endocrine and Metabolic Diseases. Radboud Institute for Molecular Life Sciences (RIMLS) of the Radboud University Medical Center, P.O. Box 9101, 6500 HB Nijmegen, The Netherlands.

[2] Mucosal Immunology and Biology Research Center, Massachusetts General Hospital, Boston, Massachusetts, United States of America.

[3] NIZO Food Research B.V., Kernhemseweg 2, 6718 ZB, Ede, The Netherlands.

[4] Department of Pharmacy and Pharmacology, University of Bath, Claverton Down, Bath, BA2 7AY United Kingdom.

[#]These authors contributed equally

[¡]These authors contributed equally

Submitted

Abstract

Orally administrated iron is suspected to increase susceptibility to enteric infections among children in infection endemic regions. Here we investigated the effect of dietary iron depletion and supplementation on the pathology and local immune responses in intestinal infection models. Mice were held on iron-deficient, normal-iron, or high-iron diets and after two weeks they were orally challenged with the mouse pathogen *Citrobacter rodentium*. Dietary intervention significantly altered iron stores in liver and spleen. Microbiome analysis by pyrosequencing revealed profound iron- and infection-induced shifts in microbiota composition. Two weeks after infection, animals on the iron-deficient diet had a gut microbiota that was dominated by *Parabacteroides*, while animals on the medium/high-iron containing diets had an *Allobaculum* dominated microbiota profile. Remarkably, fecal levels of the innate defensive molecules and markers of inflammation lipocalin-2 and calprotectin were not influenced by dietary iron intervention alone, but were markedly lower in mice on the iron-deficient diet after infection. Furthermore, mice on the iron-deficient diet tended to have a lower grade of colon pathology and to gain more weight. Complementary experiments showed that iron-deprivation was associated with prolonged survival of the nematode *Caenorhabditis elegans* after infection with *Salmonella enterica* serovar Typhimurium and, importantly, that iron increased the pathogenicity of this pathogen. Together, these data show that iron limitation restricts disease pathology upon bacterial infection in two different animal models. However, our data also showed decreased intestinal inflammatory responses of mice fed on high-iron diets. Thus additionally, our study indicates that iron influences several processes at the intestinal host-pathogen interface and that the clinical outcome of oral iron administration is difficult to predict as this may highly depend on host iron status, immune status and the gut microbiota composition.

Introduction

Iron deficiency is highly prevalent among the world and has major health consequences (Muthayya et al 2013, WHO and UNICEF 2004). Oral iron administration programmes can effectively correct iron deficiency (Zimmermann and Hurrell 2007), but concerns have been raised regarding the safety of iron supplementation as there is evidence suggesting that untargeted oral iron supplementation in regions with high prevalence of malaria transmission and infectious diseases can cause an increase in infections, hospital admission and mortality in young children (Oppenheimer 2001, Sazawal et al 2006, WHO and UNICEF 2006). This might be at least partly ascribed to iron also being an essential requirement for the growth of most bacterial species (Andrews et al 2003). Importantly, iron uptake by the upper intestine is generally limited (Hurrell and Egli 2010), which results in a large fraction of unabsorbed iron entering the colon, being potentially available for the gut microbiota. It is therefore not surprising that iron has been shown to influence the gut microbiota composition in a number of studies, among which were two studies among African children and infants which showed that iron fortification caused a potentially more pathogenic gut microbiota profile (Jaeggi et al 2014, Kortman et al 2014, Zimmermann et al 2010) (**Chapters 1 & 2**).

In the past few years it thus became apparent that supplementary iron can have a large impact on the gut microbiota composition, but the potential effects on host immune responses remained largely unexplored. Given the importance of the gut microbiota in shaping the host intestinal immune system (Hooper et al 2012) this issue however deserves further investigation as dietary iron could have an indirect effect on the responsiveness of the immune system via alteration of the gut microbiota (Cherayil et al 2011). Furthermore, host iron metabolism is largely intertwined with host immunity and it is known that host iron status affects the inflammatory response to pathogenic invaders (Cherayil 2010). We previously showed that the dysregulated iron metabolism in a mouse model of type I hemochromatosis resulted in an attenuated host immune response against *Salmonella enterica* serovar Typhimurium in the gastrointestinal tract (Wang et al 2008). Importantly, also iron deficiency is associated with an impaired immune response, but may remarkably increase the resistance against intracellular pathogens, probably due to increased nutritional immunity (Oppenheimer 2001, Puschmann and Ganzoni 1977, Weiss 2005).

As iron status can affect the immune response it is likely that also the array of antimicrobial defenses that is secreted from the intestinal mucosa gets affected. Enterocytes and Paneth cells secrete antimicrobial peptides (AMPs) such as defensins, cathelicidins and lipocalin-2 (a.k.a. neutrophil gelatinase-associated lipocalin (NGAL) or siderocalin). Lipocalin-2 is a molecule of our special interest as it is involved in host iron homeostasis and because it can prevent bacterial iron uptake via iron-scavenging siderophores, which they produce under iron-limiting conditions (Philpott 2010). The importance of lipocalin-2 based defense is demonstrated by a study showing that lipocalin-2 knockout mice had an increased susceptibility to bacterial infection (Liu et al 2013). In the intestine, lipocalin-2 is only weakly expressed under normal conditions, but increases markedly during intestinal inflammation mainly due to the influx of neutrophils which secrete large amounts of lipocalin-2 (Blaschitz

and Raffatellu 2010, Cowland and Borregaard 1997, Nielsen et al 1996, Raffatellu et al 2009). The effects of supplementary iron have not yet been investigated during gastroenteritis caused by a bacterial pathogen in an animal model. This is now warranted as the last few years it became apparent that intestinal inflammation can be exploited by specific enteric pathogens, such as *Salmonella* spp. and *Citrobacter* spp., a process leading to dysbiosis (Winter and Baumler 2011) and which may be influenced by supplementary iron (Kortman et al 2014) (**Chapter 1**). To get more insight in the effects of iron during gastroenteritis we here for the first time examined the effects of dietary iron depletion and supplementation on the mouse gut microbiome and on intestinal immunity and pathology. We focused on the expression of intestinal lipocalin-2, which may be affected either through direct effects of iron on mammalian cells or indirectly through an altered gut microbiota. To investigate the effects of these iron-related modulations on the outcome during gastroenteritis we orally challenged mice with the mouse pathogen *Citrobacter rodentium*. This well-established model for infectious gastroenteritis closely mimics the pathology caused by human food-borne bacterial pathogens (Borenshtein et al 2008, Mundy et al 2005, Mundy et al 2006). We show that mice on an iron-deficient diet as well as mice on a high-iron supplemented diet showed a decrease in fecal lipocalin-2 levels during intestinal inflammation, which may have result in impaired host defense against siderophilic pathogens in these mice. We also show that iron limitation restrains the pathology of enteric infection in a simple gut nematode model.

Materials and methods

Animals, iron diets and *Citrobacter rodentium* challenge

The mouse trials were performed in four separate experiments, allowing the analysis of an increased number of different parameters. For all experiments, female, 4-6 weeks old C57BL/6 mice from Jackson Laboratories were group-housed and placed on diets with different concentrations of iron (iron-deficient, normal-iron, and high-iron). The iron-deficient diet (Harlan laboratories) contained 2-6 mg of iron per kg chow. The normal-iron diet (control diet; 45 mg/kg iron) was either obtained pre-prepared from Harlan (experiments 1-3), or was prepared by adding ferrous sulfate to the iron-deficient diet to give a total of 45 mg/kg iron (experiment 4). To obtain the high-iron diet, enough ferrous sulfate was added to either the pre-prepared normal-iron diet (experiments 1-3) or iron-deficient diet (experiment 4) to provide a total of 225 mg iron per kg chow. Mice in experiments 1 (n=3 per group) and 2 (n=3-4 per group) were placed on the iron diets for 2 weeks after which they were euthanized to investigate the local and systemic effects of dietary iron intervention alone. For animals in experiment 3 (n=5 per group) and experiment 4 (n=5 per group) these two weeks were followed by a challenge with *C. rodentium* (strain DBS100; from the American Type Culture Collection) through oral gavage. To this purpose, bacteria were grown overnight in Luria broth (LB) and resuspended in PBS before administration to the mice (0.2 mL/mouse; ~$5*10^8$ CFUs). Diets were continued during infection. Body weights were monitored during the course of infection and stool samples were sampled at appropriate time points. Animals were euthanized 2 weeks after starting the *C. rodentium*

challenge after which colon, liver, spleen, serum/plasma and fecal samples were collected for several analyses. The design of these experiments is depicted in **Figure 1**.

Colon histopathology

At necropsy, the entire colon was removed and flushed gently with PBS. An approximately 0.5 cm long piece was excised from the junction of the middle and distal thirds of the colon, placed in a tissue mould containing Tissue-Tek Cryo-OCT compound (Thermo Fisher Scientific, Waltham, MA) and quick frozen in liquid nitrogen. Five µm sections were cut on a Leica CM1850 cryostat (Leica Biosystems, Buffalo Grove, IL) and stained with hemotoxylin and eosin following standard protocols. The severity of *C. rodentium*-induced colitis was examined in a blinded fashion by two independent investigators, and graded using a histopathological scoring system (more severe colitis is indicated by a higher score) as published previously (Chen et al 2005).

Iron measurements

Description available in the **Supplementary materials and methods.**

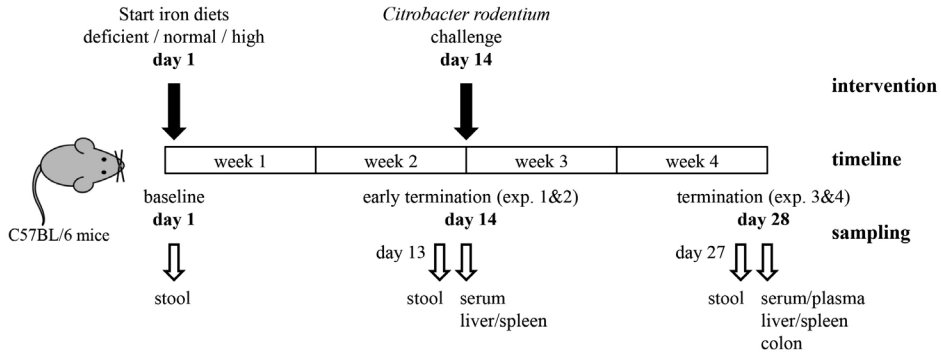


Figure 1. Trial profile.

Mouse trials which were performed in four separate experiments. 4-6 weeks old , female, C57BL6 mice in experiment 1 (n = 3 per group) and 2 (n = 3-4) were sacrificed after 14 days of dietary iron intervention and were analyzed for serum / stool / tissue iron content, or lipocalin-2. Mice in experiment 3 (n = 5) and 4 (n = 5) were orally infected with *C. rodentium*, while the diets were continued for another 14 days. Samples were analyzed for stool / tissue iron content, hepcidin, lipocalin-2, calprotectin, or colon histopathology. Gut microbiome analysis was performed on mice in experiment 4.

ELISA measurements

Fecal and plasma/serum lipocalin-2

Lipocalin-2 levels were quantified in stool and plasma/serum samples using the Duoset murine Lcn-2 ELISA kit (R&D Systems) with the following adaptations. The capture antibody and detection antibody were used at a concentration of 2 µg/mL and 250 ng/mL respectively. Serum and plasma were 2000 times diluted in reagent diluent (1.0% BSA in PBS). Fecal samples were reconstituted in 250 µL PBS containing 0.1% Tween 20 (100 mg/mL) and protease inhibitors (Complete Mini cocktail tablets, Roche). Samples were vortexed for 20 min at 4°C to obtain a homogenous suspension. Debris was pelleted and the supernatant was stored at -80°C until use. Samples were measured in a 50-2000 times dilution. Protein concentrations in the fecal samples were estimated by using the PierceTM BCA Protein Assay Kit (Thermo Scientific) and lipocalin-2 levels were normalized for total protein content. Lipocalin-2 levels in plasma/serum samples were expressed as ng/mL.

Fecal calprotectin

Protein from mouse fecal samples was extracted as described for Lipocalin-2 ELISA and a 2 times diluted extract was applied to the S100A8/S100A9 ELISA Kit (Immundiagnostik AG) according to the manufacturer's instructions. Calprotectin levels were normalized for total protein content.

Colon cytokine secretion

At necropsy, the entire colon was removed and flushed gently with PBS. An approximately 0.5 cm long piece was excised from the junction of the middle and distal thirds of the colon, placed in 1 mL of complete Dulbecco's Modified Eagle Medium with 10% heat-inactivated fetal bovine serum, and incubated overnight at 37°C in a tissue culture incubator. The supernatants were collected and used to estimate IL-6, IL-17 and TNFα concentrations by ELISA using the relevant Duo-set capture and detection antibodies (R&D Systems). The colon tissue was homogenized in lysis buffer containing 1% Triton X-100 in 10 mM Tris-HCl, pH 8.0; 150 mM NaCl and the protein concentrations of the homogenates used to normalize the cytokine concentrations.

Hepcidin quantification

Hepcidin-1 (Hep-1) levels in mouse serum/plasma samples from C. rodentium-infected mice that were held on different iron diets and reference Hep-1 values from an independent group of (uninfected) C57BL/6 mice fed on a standard diet were determined as described previously by a combination of hydrophobic extraction and time-of-flight mass spectrometry (TOF MS) using a synthetic internal standard (rabbit Hep-25; Peptides International) for normalization (Tjalsma et al 2011). Peptide spectra were generated on a Microflex LT matrix-enhanced laser desorption/ionisation TOF MS platform (Bruker Daltonics). Mouse Hep-1 levels were expressed in nM relative to the known concentration of the internal standard.

Fecal DNA extraction

Fecal DNA was extracted by using the FastDNA® SPIN Kit for Feces (Mpbio) according to the manufacturer's instruction but with the following adaptations. The fecal pellets were resuspended in 500 µL of sodium phosphate buffer, after which the sample was transferred to the lysing matrix. Lysis by bead-beating was performed in a MagNa Lyser instrument (Roche) for 2 times 20 sec at 6500 rpm.

Mouse gut microbiome 16S rRNA pyrosequencing

Mouse fecal microbiomes were determined at baseline, at 13 days (before infection) and at 27 days (after infection; end point). Pyrosequencing was performed as described previously (Jaeggi et al 2014), (**Chapter 2**) with the exception that all barcoded amplicons were separately purified by using the Purelink Purification kit (Invitrogen), before pooling them. The amplicon pool was submitted for pyrosequencing of the V3-V4 region of the 16S rRNA gene on the 454 Life Sciences GS-FLX+ platform using Titanium sequencing chemistry (GATC-Biotech, Germany).

Quantitative real-time PCR to determine fecal *C. rodentium* abundance

qPCR of *C. rodentium* was performed as follows. The PCR amplification mixture contained 1 µL fecal DNA, 1 µL forward primer, 1µL reverse primer, 10 µL of Power SYBR® green PCR Master Mix (Applied Biosystems) and 7 µL of sterile water (total volume 20 µL). PCR conditions were: 95°C for 10 min followed by 40 cycles of 95°C for 15 sec and 60°C for 60 sec. The quantification of *C. rodentium* was normalized for total bacteria in the sample. qPCR for total bacteria was performed according to the same protocol with the exception that a 100-fold dilution of the fecal DNA was used. Primers for the detection of *C. rodentium* were Sig70S: TCCAGCGTAGAGTCCGAAATC and Sig70A: TGCCCATTTCGCGCATAT (Cordone et al 2005) and for total bacteria P891F: TGGAGCATGTGGTTTAATTCGA and P1033R: TGCGGGACTTAACCCAACA (Yang et al 2002).

Nematode viability assay

For nematode infection assays, *Caenorhabditis elegans glp-4(bn2) sek-1(km4)*, a pathogen-sensitive strain with temperature-sensitive sterility (ref Moy et al., 2006) was maintained at 15°C on nematode growth medium, using *E. coli* DH5α (Life Technologies Inc.) as a source of food. Nematodes were age-synchronised by isolating eggs through treatment with hypochlorite/NaOH, and L1 hatchlings were deposited on lawns of *E. coli* DH5a grown on NGM agar. Plates were incubated at 25°C (at this temperature adult nematodes of the strain used do not produce any progeny), and when the nematodes reached the L4 stage they were collected from the plates and washed at least three times using M9 buffer (3 g/L KH_2PO_4, 6 g/L Na_2HPO_4, 5 g/L NaCl and 0.25 g/L $MgSO_4{\cdot}7H_2O$). Agar plates of Iscove's Modified Dulbecco's Medium (does not contain iron in its formulation) (IMDM, Invitrogen) were prepared as follows. Firstly, IMDM medium was briefly warmed to 45°C and mixed with 5% (in water) melted ultra pure agar (Difco Noble Agar) that was cooled to 45°C, to give a final concentration of 1.2% agar. Ferric ammonium citrate to final concentrations of 0, 1, 10 or 100 µmol/L were added and plates (55 mm) were poured immediately. Cultures

of *S.* Typhimurium NTB6 (Kortman et al 2012) or *E. coli* DH5α were grown in IMDM medium with 0.5 μmol/L ferric ammonium citrate until the end of the exponential growth phase. 10 μl of these cultures was used to seed the IMDM-agar plates, which were then incubated for 18 hours at 37°C. 30-40 L4 stage nematodes were deposited on the lawns of *S.* Typhimurium or *E. coli* DH5α. 5 plates were used for each iron concentration. Survival of nematodes during co-incubation was scored regularly during 13 days and was expressed as the LT50 (defined as the time to kill 50% of the population) and the area under the curve (AUC) was determined as another measure for survival time. Observations were carried out using a standard dissecting microscope; nematodes were scored as dead when they lost their normal sigmoidal shape and failed to move in response to gentle touch with a platinum wire. Viability of the bacterial lawns was not affected by iron concentration (data not shown).

Statistics and data representation

Analysis of mouse responses and nematode survival

To compare means, one-way ANOVA with Tukey's post-hoc test (to compare all means) or with Bonferroni's post-hoc test (for comparison of selected means) was performed. To analyze the effect of dietary iron intervention and intestinal infection on body weights, the area under the curve (AUC) for each mouse was determined, after which groups were compared as described above. The correlation between fecal lipocalin-2 and calprotectin levels was assessed by Pearson correlation test and linear regression was used to plot the best-fit line (with 95% confidence interval). Statistical tests were performed using GraphPad Prism version 5.03 for Windows, GraphPad Software, San Diego California USA. P-values < 0.05 were considered statistically significant and P-values < 0.10 were considered as an important significance level.

Analysis of 16S rRNA pyrosequencing data

Pyrosequencing data were analyzed with a workflow based on QIIME v1.2 (Caporaso et al 2010) using settings as recommended in the QIIME 1.2 tutorial, with the following exceptions: reads were filtered for chimeric sequences using Chimera Slayer (Haas et al 2011). OTU clustering was performed with settings as recommended in the QIIME newsletter of December 17th 2010 (http://qiime.wordpress.com/2010/12/17/new-default-parameters-for-uclust-otu-pickers/) using an identity threshold of 97%. Diversity metrics were calculated as implemented in QIIME 1.2. Hierarchical clustering of samples was performed using UPGMA with weighted UniFrac as a distance measure as implemented in QIIME 1.2. The RDP classifier version 2.2 was performed for taxonomic classification (Cole et al 2009). Multivariate redundancy analysis (RDA) was done using Canoco 5.0 (Ter Braak and Smilauer 2012), using default settings of the analysis type "Constrained-supplementary". Visualization of differences in relative abundance of taxa between different study groups was done in Cytoscape (Shannon et al 2003). Statistical analysis of the pyrosequencing data was done with SciPy (www.scipy.org). Differences in relative abundance between groups at a single time point (cross-sectional) were compared by Mann-Whitney U (MWU) testing.

Medians of phylogenetic diversity were compared by Kruskal-Wallis test with Dunn's post test (GraphPad Prism version 5.03). The effects of iron on the microbiome were compared among the single conditions, but also as pools as described above. P-values for the microbiome data were not corrected for multiple testing as they were analyzed in an explorative manner.

Ethics Statement

All mouse studies were carried out in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was reviewed and approved by the Institutional Animal Care and Use Committee of Massachusetts General Hospital (protocol number 2008N000061, animal welfare assurance number A3596-01).

Results

Systemic and local responses to dietary iron intervention

Effects of dietary iron intervention on systemic and intestinal parameters

Previous studies have shown that the intestinal infection after ingestion of *C. rodentium* by mice closely mimics human colitis caused by food-borne bacterial pathogens, such as enteropathogenic and enterohemorrhagic *Escherichia coli* (EPEC and EHEC respectively) (Borenshtein et al 2008, Mundy et al 2005, Mundy et al 2006). Here we used this mouse model to investigate the effect of dietary iron levels on disease pathology by first placing mice for 14 days on diets with three different iron levels (deficient, normal, high) (**Figure 1**). During this period, body weight as a measure for general health was similar for all three groups (**Figure 2**). Also serum iron (**Supplementary Figure 1A**) and serum lipocalin-2 levels (**Figure 3A**) were not different among groups. Furthermore, fecal lipocalin-2 and calprotectin levels were not different in the three groups (**Figure 3B-D**). In contrast, fecal iron concentrations and liver/spleen iron levels were significantly different among the three groups at day 14 (**Supplementary Figure 1B-C**), showing that the iron diets were, as expected, effective in changing luminal iron concentrations as well as body iron stores. Together, these data show that the iron-deficient and the high-iron dietary interventions for 2 weeks changed luminal iron content and body iron stores, but also indicate that there was no clear effect on intestinal and general health at this point.

Effects of dietary iron intervention on the gut microbiome composition

In total, 214,520 bacterial 16S rRNA sequences were analyzed by pyrosequencing. At baseline, the mouse gut microbiome consisted of the phyla Bacteroidetes (71.2% of the 16S rRNA reads), Firmicutes (27.3%), Tenericutes (0.8%) and Actinobacteria (0.6%). After 2 weeks of dietary intervention the phylogenetic diversity index (a measure of α-diversity) was decreased in the iron-deficient group compared to baseline ($p < 0.05$). The same effect was observed in the high-iron group, although not significantly different from baseline, the diversity was lower in the high-iron group compared to the normal-iron group at day 13 (p

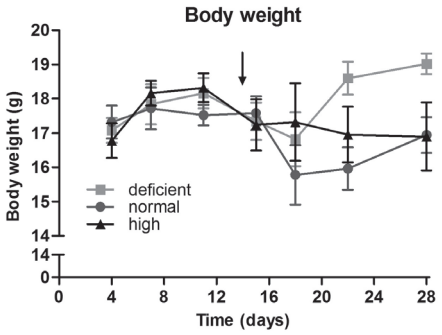


Figure 2. Body weight of the mice during the time course of the experiment.

Body weights (mean ±SE) of mice in experiment 4 (n = 5) were monitored during 28 days. Until infection at day 14 (indicated by the arrow) body weights were similar, but tended to differ among the dietary groups after infection.

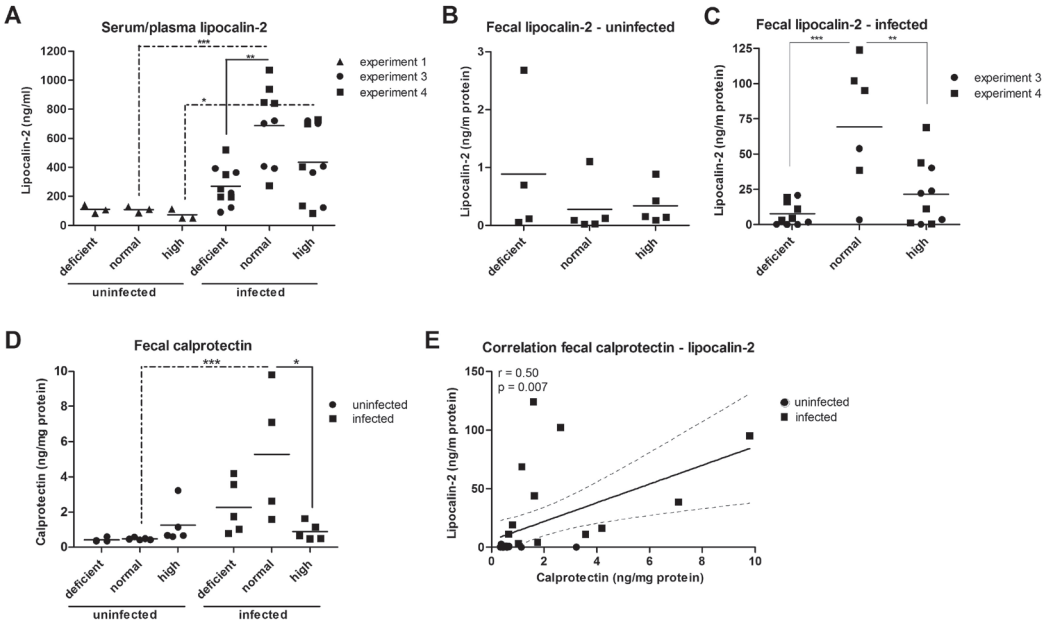


Figure 3. Lipocalin-2 and calprotectin levels in uninfected and infected mice.

Systemic lipocalin-2 levels in uninfected mice of experiment 1 (serum), or in infected mice of experiments 3 (serum) and 4 (plasma) are depicted in panel **A**. Fecal lipocalin-2 levels in uninfected mice of experiment 3 (day 13), or in infected mice of experiment 3 and 4 (day 27) are depicted in panel **B** and **C** respectively. Note, the different scale of the y-axis in these panels. Panel **D** shows fecal calprotectin levels in uninfected (day 13) and infected (day 27) mice of experiment 4. The (Pearson) correlation between fecal calprotectin and lipocalin-2 levels in mice of experiment 4 (day 13 and day 27), plotted by linear regression (best-fit line with 95% confidence interval), is shown in panel **E**. # $p < 0.1$; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. Solid lines indicate comparisons with significant outcome between dietary groups at one time point. Dashed lines indicate significant comparisons between uninfected and infected mice (only mice of the same dietary intervention group were compared).

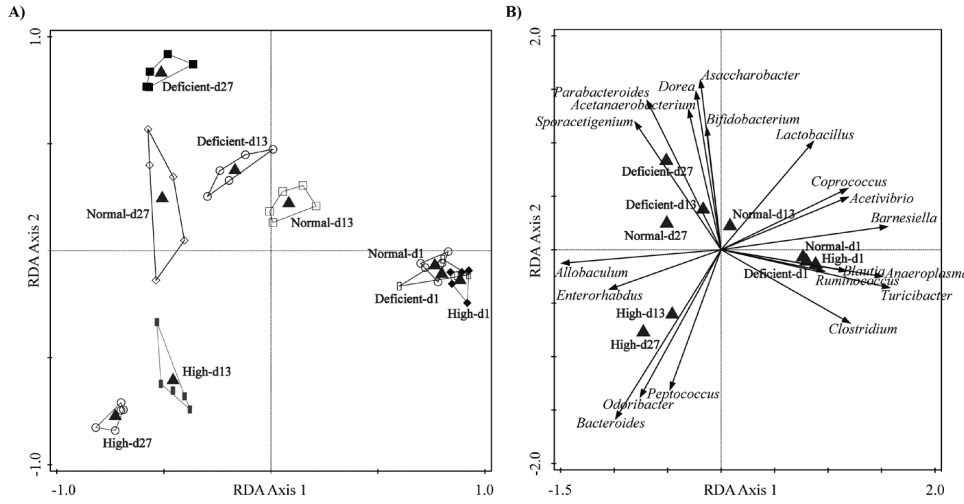


Figure 4. Multivariate redundancy analysis (RDA) of the microbiota composition.

RDA was performed using Canoco 5.0. Taxonomic composition at the genus level was used as response data and dietary iron groups over time as explanatory variable. Triangles represent dietary iron intervention groups at day 1, 13 and 27 (experiment 4). Other symbols are the individual samples, which are connected with lines to form envelopes of the same group. Length of arrows reflects significance. **A**: Classified sample diagram. **B**: Taxa – metadata biplot. To test the significance of the links between dietary intervention and sample clustering, separate RDAs were done for each individual time point. Clustering by treatment was not significant at baseline ($p = 0.422$), while they significantly differed at day 13 ($p = 0.002$) and at day 27 ($p = 0.002$).

4

< 0.05) (**Supplementary Figure 2**). Multivariate Redundancy Analysis (RDA) shows that the gut microbiome of mice at baseline was similar for all dietary iron groups ($p = 0.422$), as expected. Clearly, after two weeks of dietary iron intervention, the mice had a distinct non-overlapping gut microbiome composition ($p = 0.002$)(**Figure 4A**). Hierarchical clustering analysis clearly clustered the baseline microbiomes together and separated them from the later time points (**Figure 5**). It also showed that the microbiome of the mice on the high-iron diet had most extensively changed. The most prominent change appeared to be the shift from a *Barnesiella* dominated profile to an *Allobaculum* dominated profile.

Next the relative abundances of all detected bacterial taxa among groups at day 13 were compared in an explorative manner. The overall main differences between diets with a higher iron level compared to diets with a lower iron level were an increased abundance of *Clostridium* and a lower abundance of *Dorea* (**Figure 6**). Although the normal-iron group did not show a lower abundance of *Lactobacillus* and *Bifidobacterium* compared to the iron-deficient group, in the high-iron group there was a consistent relative lower abundance of these taxa compared to the iron-deficient and normal-iron groups. Furthermore, *Peptococcus, Bacteroides* and *Allobaculum* were consistently more abundant in the high-iron group (**Figure 6**), which is also indicated by multivariate RDA (**Figure 4B**).

Responses to intestinal bacterial infection during dietary iron intervention

Effects of dietary iron intervention and colitis on the gut microbiome composition

As mentioned above, the phylogenetic diversity index decreased over time in all groups. This was strongest for the high-iron group, which ended up significantly lower at day 27 compared to the iron-deficient group ($p < 0.05$) (**Supplementary Figure 2**). This analysis shows that during infection mice that were held on the iron-deficient diet were able to maintain part of their gut microbial diversity. Hierarchical clustering analysis roughly grouped the normal-iron and the high-iron group together at day 27, while it separated these groups from the iron-deficient group at day 27, which clustered more close to the baseline situation (**Figure 5**). It also indicates that the microbiomes of the mice on the normal-iron and high-iron diets had most extensively changed. Again, the most prominent change was a shift from a *Barnesiella* dominated profile to an *Allobaculum* dominated profile. The microbiome of the mice on the iron-deficient diet also changed and was dominated by *Parabacteroides* at day 27. The difference in gut microbiome composition among groups at day 27 is strongly supported by the RDA, which shows that the dietary iron intervention caused a distinct non-overlapping gut microbiome profile ($p = 0.002$). Additionally, the gut microbiome profile of each group at day 27 appeared not to overlap with the groups at day 13 (**Figure 4A**). Together, these analyses show that dietary iron had a large impact on the mouse gut microbiome, especially during intestinal inflammation. In the subsequent paragraph the differences among groups in both the dominant and subdominant taxa are shown.

To reveal differences, the relative abundances of all detected bacterial taxa were compared among groups at day 27 in an explorative manner. After infection, main differences were a higher abundance of *Allobaculum* and *Enterorhabdus* in the normal/high-iron groups compared to the iron-deficient group ($p = 0.005$ and $p = 0.004$ respectively for both the normal-iron and high-iron groups vs the iron-deficient group). In addition, in the high-iron group a higher abundance of *Bacteroides* was found compared to both the iron-deficient and the normal-iron groups ($p = 0.004$ for both). For the taxa *Bifidobacterium, Lactobacillales/Lactobacillus* and *Parabacteroides* we consistently found a lower abundance in all comparisons, meaning that that their abundance was lower in the normal/high-iron groups compared to the iron-deficient group, as well as in the high-iron group compared to the normal-iron group (**Figure 7**). Multivariate RDA also generally associates the taxa mentioned in this paragraph with the respective dietary iron groups (**Figure 4B**).

The course of C. rodentium infection during dietary iron intervention

Citrobacter was lowly abundant relative to all 16S rRNA reads at day 27, but was not detected at all in samples from baseline and day 13 by pyrosequencing. We therefore performed a qPCR specific for *C. rodentium* to be able to quantify this low-abundant pathogen which initiated the intestinal inflammation. As expected, this analysis showed that there was much more *C. rodentium* present in the stools at day 27 compared to baseline and day 13 (approx. $1*10^5$ fold difference), but there were no differences observed among the dietary groups (data not shown). This suggests that *in vivo C. rodentium* colonization was not influenced

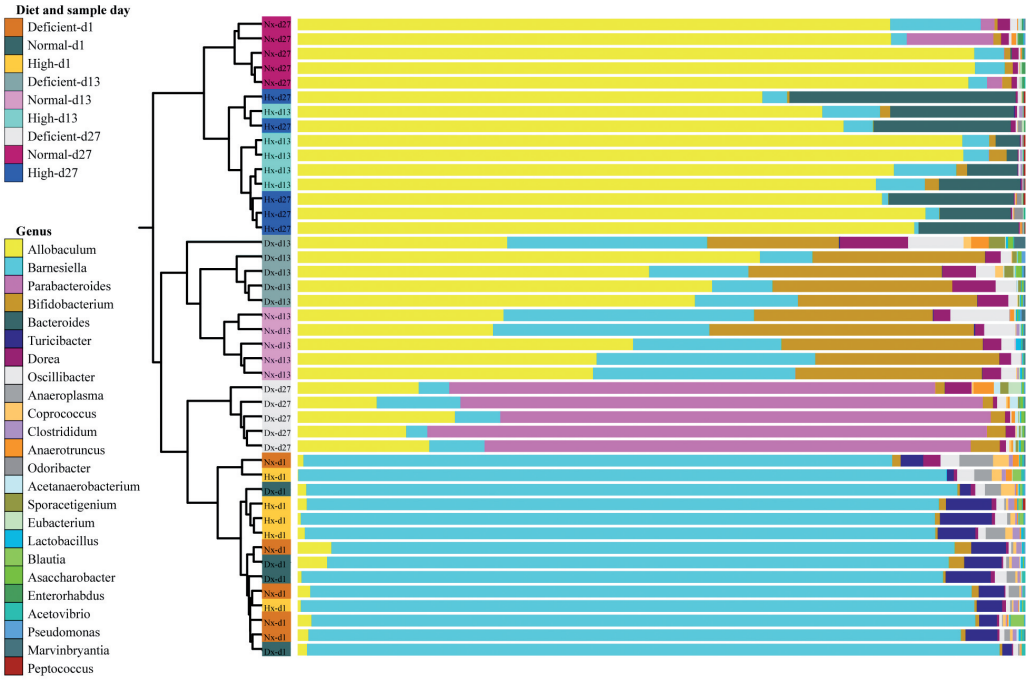


Figure 5. Hierarchical microbiome clustering of the dietary groups over time.

The microbiomes of fecal samples at day 1, day 13 and day 27 (experiment 4) were clustered using UPGMA with weighted UniFrac as a distance measure. The figure was generated using iTOL (Letunic and Bork 2011). Sample names with the same color are within the same dietary group at the same time point. Colored bars represent the relative abundance of a bacterial genus (the number of reads assigned to a genus divided by the total number of reads assigned up to the phylum level) in the sample. Mice within a group had no individual ID (indicated as `x`).

by the iron diets at this point, although differences may have existed at an earlier time point after infection, which was not assessed in this study.

The body weights of mice tended to differ rapidly after initiation of infection at day 14 among groups ($p = 0.085$) (**Figure 2**). Interestingly, mice on the iron-deficient diet seemed to recover most quickly as only this group had at day 28 an average body weight that was above that of the day of infection, while mice on a diet with normal iron content tended to suffer most from the colitis. Like uninfected mice fed on the iron diets for 2 weeks, also infected mice fed on the diets for 4 weeks had altered tissue iron stores (**Supplementary Figure 1D**). Histological examination of colon sections at day 28, showed a non-significant tendency towards a lower average histopathology score for mice on the iron-deficient diet compared to the normal and high-iron diets, with a large within-group variation (**Figure 8A**). Notably, this observation could fit with the observation that mice on the iron-deficient diet appeared to suffer less from the colitis as indicated by the outcome on body weights. However, based

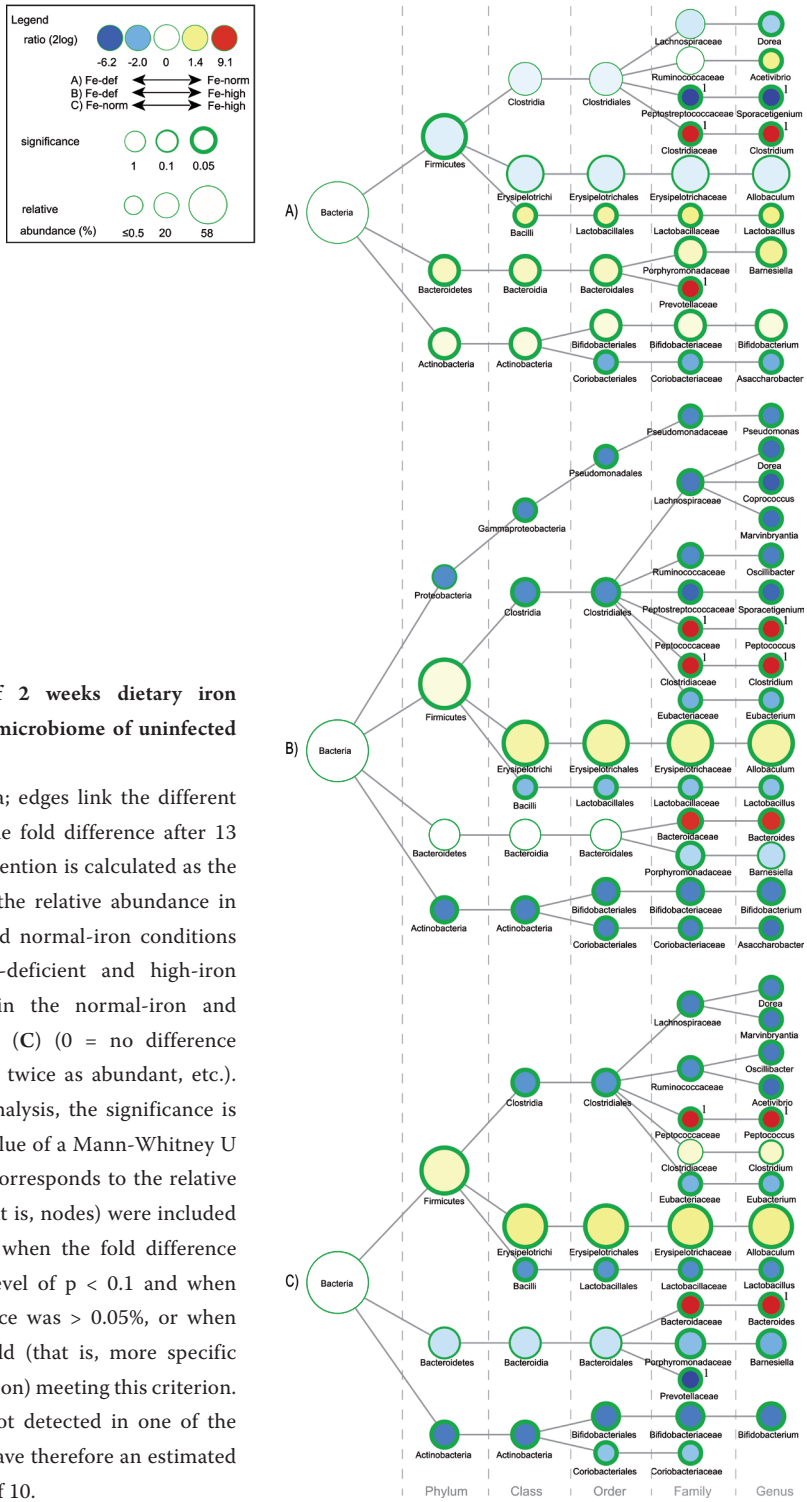


Figure 6. Effect of 2 weeks dietary iron intervention on the microbiome of uninfected mice.

Nodes represent taxa; edges link the different taxonomic levels. The fold difference after 13 days of dietary intervention is calculated as the 2log of the ratio of the relative abundance in the iron-deficient and normal-iron conditions (**A**) or in the iron-deficient and high-iron conditions (**B**) or in the normal-iron and high-iron conditions (**C**) (0 = no difference between groups, 1 = twice as abundant, etc.). In this explorative analysis, the significance is expressed as the p value of a Mann-Whitney U test. The node-size corresponds to the relative abundance. Taxa (that is, nodes) were included in this visualization when the fold difference met a significance level of $p < 0.1$ and when the relative abundance was > 0.05%, or when the taxon had a child (that is, more specific taxonomic classification) meeting this criterion. [1] These taxa were not detected in one of the dietary groups and have therefore an estimated 2log fold difference of 10.

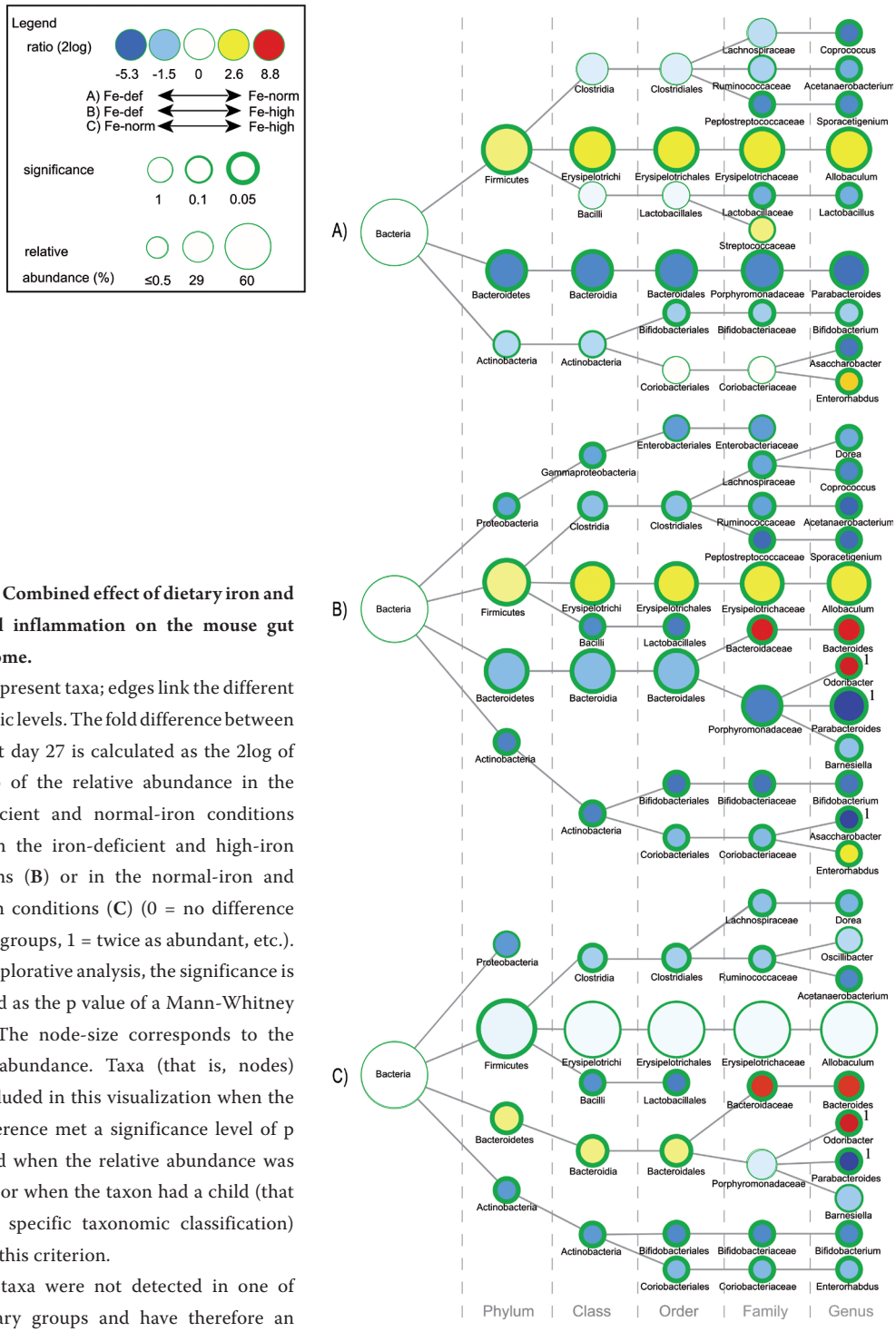


Figure 7. Combined effect of dietary iron and intestinal inflammation on the mouse gut microbiome.

Nodes represent taxa; edges link the different taxonomic levels. The fold difference between groups at day 27 is calculated as the 2log of the ratio of the relative abundance in the iron-deficient and normal-iron conditions (**A**) or in the iron-deficient and high-iron conditions (**B**) or in the normal-iron and high-iron conditions (**C**) (0 = no difference between groups, 1 = twice as abundant, etc.). In this explorative analysis, the significance is expressed as the p value of a Mann-Whitney U test. The node-size corresponds to the relative abundance. Taxa (that is, nodes) were included in this visualization when the fold difference met a significance level of $p < 0.1$ and when the relative abundance was > 0.05%, or when the taxon had a child (that is, more specific taxonomic classification) meeting this criterion.

[1] These taxa were not detected in one of the dietary groups and have therefore an estimated 2log fold difference of 10.

on the body weights, mice on a normal-iron diet seemed to suffer more from colitis than mice on the high-iron diet, something which is not evident from the histopathological examination.

Intestinal responses to *C. rodentium* challenge during dietary iron intervention

Colon pro-inflammatory cytokine production ex vivo

To evaluate the effects of intestinal infection during dietary iron intervention on the intestinal cytokine response, colon sections were sampled at day 28 (14 days after *C. rodentium* challenge) and *ex vivo* secreted levels of IL-6, TNF-α and IL-17 were measured by ELISA. **Figure 8B** shows that secreted levels from animals on the iron-deficient diet were lowest for all three pro-inflammatory cytokines. For IL-17 this was significantly different from the normal-iron diet ($p < 0.01$), but secreted IL-17 was also significantly lower in the high-iron group ($p < 0.05$). Secreted IL-6 levels showed a similar tendency. TNF-α tended to increase with increasing amounts of iron in the diet (not significantly), but the sum of the three pro-inflammatory cytokines per mouse showed the same trend as for IL-6 and IL-17. The sum of secreted levels was different among groups ($p = 0.040$) and was significantly lower for the iron-deficient diet compared to the normal-iron diet ($p < 0.05$) (**Supplementary Figure 3**). Together, these findings suggest that the intestinal cytokine response is about maximum at normal dietary iron levels, and which generally appears diminished at both low and high dietary iron levels.

Fecal lipocalin-2 and calprotectin levels upon intestinal inflammation

ELISA measurements of lipocalin-2 in feces showed that lipocalin-2 levels were low at day 13, prior to infection. Lipocalin-2 levels were however markedly increased after infection at day 27, most prominently in the feces of mice on the normal-iron diet. After infection, fecal lipocalin-2 levels were significantly higher in the normal-iron group compared to both the iron-deficient and high-iron group ($p < 0.01$ and $p < 0.05$ respectively) (**Figure 3C**).

Fecal calprotectin is a common and validated marker for intestinal inflammation (Kopylov et al 2014). We therefore measured fecal calprotectin next to fecal lipocalin-2, to examine whether the effect of dietary iron on lipocalin-2 levels were also found on calprotectin levels and to examine the grade of inflammation. Interestingly, like lipocalin-2 levels, fecal calprotectin levels were increased at day 27 compared to day 13 and most prominently in the normal-iron diet group, which was significantly different from the high-iron group ($p < 0.05$) (**Figure 3D**). Notably, although levels of lipocalin-2 were generally still higher in inflamed mice compared to non-inflamed mice, fecal calprotectin levels in inflamed mice on the high-iron diet were mostly not elevated compared to levels in non-inflamed mice. The similar response of lipocalin-2 and calprotectin is underscored by the finding that their fecal levels had a strong correlation (Pearson $r = 0.50$; $p = 0.007$) (**Figure 3E**). Together, these findings suggest that the intestinal inflammatory response was blunted in both the iron-deficient and high-iron diets.

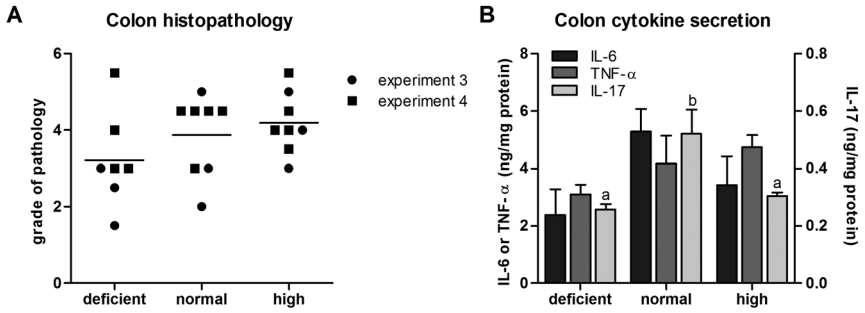


Figure 8. Colon histopathology and Ex vivo secretion of pro-inflammatory cytokines by mouse colon explants.
A) Histopathological score of colon sections of infected mice in experiment 3 (n = 3) and experiment 4 (n = 4 - 5) at day 28.
B) Colon explants of infected mice (day 28) in experiment 4 (n = 3 - 5) that were on the different iron-diets and were challenged with *C. rodentium*, were incubated overnight in culture medium, after which secreted pro-inflammatory cytokines IL-6, TNF-α and IL-17 were measured by ELISA. Cytokine levels were normalized for total colon protein. Levels (mean + SE) of IL-6 and TNF-α are on the left y-axis, and IL-17 levels on the right y-axis. Means without a common letter of the same cytokine differ significantly, $p < 0.05$.

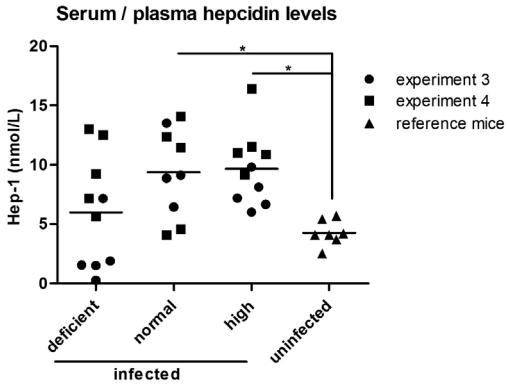


Figure 9. Systemic hepcidin (Hep-1) levels in infected mice and uninfected reference mice.
Hepcidin levels in infected mice (day 28) in serum of mice in experiment 3 (n = 4 - 5) and in plasma of mice in experiment 4 (n = 5). Hepcidin levels in plasma of an independent group of (uninfected) control mice (C57BL/6 mice on a standard diet) were included to show reference Hep-1 levels in healthy mice (n = 7). * $p < 0.05$.

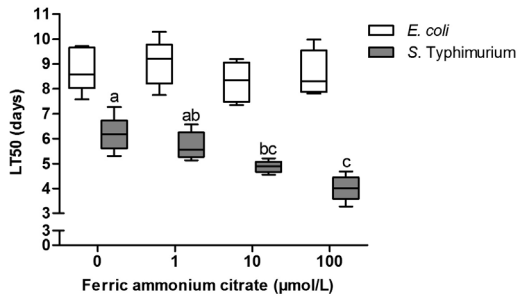


Figure 10. Survival of C. elegans decreases upon bacterial infection with increasing iron levels.
Survival of the nematode *C. elegans*, which was used as a simple *in vivo* gut model, was monitored during 13 days and the LT50 (defined as the time to kill 50% of the population) was determined. Whiskers are displayed with median and min to max. Whiskers without a common letter and representing nematode survival on *S.* Typhimurium differ significantly, $p < 0.01$.

Systemic responses to C. rodentium challenges during dietary iron intervention

Analogous to the findings in feces, systemic lipocalin-2 levels were markedly higher in the infected mice at day 28 compared to uninfected mice at day 14 (**Figure 3A**). Intestinal inflammation thus induces production of lipocalin-2 which can be detected systemically. In addition, systemic lipocalin-2 levels were different among groups ($p = 0.002$) and highest in the mice on the normal-iron diet, which was significantly different from mice on the iron-deficient diet ($p < 0.05$) (**Figure 3A**).

As mentioned above, the iron diets did not affect systemic iron levels. Systemic iron levels are mediated by the key iron-regulatory peptide hormone hepcidin (Hep-1 in mice), which blocks the ferroportin transporter in macrophages and the duodenum. Among others, hepcidin expression is regulated by host iron status and inflammation (Kroot et al 2011). We here aimed to assess the effect of dietary iron on Hep-1 induction by colitis. As shown in **Figure 9**, nearly all mice on the normal and high-iron diets had plasma Hep-1 levels that were clearly above those found in an independent group of uninfected reference control mice (Tjalsma et al 2011) on a standard diet ($p < 0.05$ for both comparisons), but were not markedly different from each other. In contrast, a subset of mice on the iron-deficient diet had very low Hep-1 levels that were just below the hepcidin levels found in uninfected control mice.

Iron limitation prolongs survival of *Salmonella*-infected nematodes

To confirm the potential protective effect of iron limitation on the pathology of enteric infection as observed in our mouse model, we investigated the effect of iron on the pathogenicity of the human gut pathogen *S.* Typhimurium in a live nematode gut model. This has previously been shown to be a suitable model host for *S.* Typhimurium infection (Labrousse et al 2000). Survival of *C. elegans* that forages on *S.* Typhimurium decreased in the presence of increasing concentrations of iron. This was reflected in the nematode survival time (LT50), which was on average decreased by 1.3 and 2 days in the 10 and 100 µmol/L ferric ammonium citrate conditions respectively, compared to the no-iron condition ($p < 0.01$ and $p < 0.001$, respectively) (**Figure 10**). Furthermore, the AUCs of the survival curves of the conditions with 10 and 100 µmol/L ferric ammonium citrate were significantly lower compared to the no-iron condition over the course of the experiment ($p < 0.05$ and $p < 0.001$ respectively) (**Supplementary Figure 4**). At the same time this iron-dependency was absent when *C. elegans* foraged on a *E. coli* control strain. These data confirm a subtle, but reproducible, protective effect of low dietary iron intake on the pathology of enteric infection. Importantly, these data also confirm that iron can increase the virulence of an enteric pathogen as we previously showed *in vitro* (Kortman et al 2012) (**Chapter 5**).

Discussion

It is well known that both oral iron administration and intestinal inflammation can alter the gut microbiota composition and that host iron status influences the inflammatory response (Kortman et al 2014) (**Chapter 1**). Furthermore, oral iron administration has been associated with increased levels of fecal calprotectin (indicating increased gut inflammation) and with

an increased incidence of diarrhea (Gera and Sachdev 2002, Jaeggi et al 2014, Zimmermann et al 2010). Nonetheless, little is known about the effects of nutritional iron on the gut microbiota composition and the host immunological response during periods of intestinal inflammation. We therefore investigated the effects of iron on pathology, gut microbiota composition and host intestinal immune responses in the non-inflamed and inflamed colon and we here for the first time show that dietary iron has profound effects on the gut microbiome composition and on the host immune response during colonic infection by common food-borne bacterial pathogens in a mouse model.

Although iron diets by themselves had clearly effect on luminal iron content and tissue iron stores as expected, we found no effects on general health as reflected by body weights and the immunological parameters lipocalin-2 and calprotectin that we measured at this point. Remarkably, there were profound effects on the gut microbiome composition. Compared to the composition at baseline, all dietary groups had a changed microbiota after 2 weeks, which can probably be explained by the change in diet at the start of the intervention. The gut microbiomes shifted towards an *Allobaculum* dominated profile, which was most apparent for the high-iron diet group. This group also showed a lower relative abundance of the beneficial *Bifidobacteriaceae* and *Lactobacillaceae* families compared to the normal-iron and iron-deficient diets, which is one of the most consistent findings of dietary iron intervention studies so far (Kortman et al 2014) (**Chapter 1**). As expected, *C. rodentium* challenge clearly resulted in colonic inflammation as observed by colon histopathology and determination of the inflammatory parameters lipocalin-2 and calprotectin. Dietary iron intervention had limited effect on the grade of inflammation as determined by histopathological examination. However, there appeared to be a minor trend towards a higher grade of inflammation with supplementary iron. This fits with previous studies showing that supplementary iron during IBD exaggerates colitis in animals and gastrointestinal complaints in IBD patients (Carrier et al 2006, Lee et al 2012, Reifen et al 2000, Seril et al 2002, Zhu et al 2010). Our experiments also indicate that mice on the iron-deficient diet suffered slightly less from the colitis compared to the other groups as reflected by mouse body weights. Interestingly, previous studies showed that non-infected rats on an iron-deficient diet for 5 weeks gained less weight and ate less, while also mice on an iron-deficient diet had a lower body weight compared to control mice after 12 weeks of intervention (Dostal et al 2012, Tompkins et al 2001). This suggests that an iron-deficient diet in a non-inflamed situation tends to decrease weight gain on the longer term, while it may prevent weight loss during intestinal inflammation.

Another systemic effect of the colonic inflammation was an increase in hepcidin levels. Importantly, several infected mice on the iron-deficient diet had lower hepcidin levels compared to uninfected reference mice. These findings are in-line with previous human studies showing that severely anemic infants and children in Africa with elevated inflammatory markers did not always have concurrent elevated hepcidin levels (Jaeggi et al 2013, Jonker et al 2013). This suggests that also in mice, a threshold body iron level is required for a physiological Hep-1 upregulation upon intestinal infection. If body iron levels are below this threshold, the iron demand, which asks for low hepcidin, renders these animal non-responsive to the infection stimulus. Analogous to hepcidin, lipocalin-2 has

a role in iron homeostasis and is involved in innate immunity by withholding iron from bacterial pathogens (Malyszko et al 2010). Iron withholding by lipocalin-2 is established by the direct sequestering of iron-containing bacterial siderophores, for example in the gut lumen (Kortman et al 2014) (**Chapter 1**). It is known that systemic lipocalin-2 levels are elevated in patients with IBD, but the origin of circulating lipocalin-2 is not clear and could be attributed to the activation of both local and distant immune cells (Cayatte et al 2012, Janas et al 2014, Oikonomou et al 2012). Notably, levels of lipocalin-2 (secreted by intestinal epithelial cells and infiltrating immune cells) have also been shown to increase in the inflamed gut (Chassaing et al 2012, Nielsen et al 1999, Vijay-Kumar et al 2007). It has therefore been suggested that fecal lipocalin-2 can be used as a non-invasive marker for gut inflammation (Chassaing et al 2012). Remarkably, our study shows that both the iron-deficient diet and the high-iron diet lowered lipocalin-2 production compared to the normal-iron diet and that the effects were very similar on the local and systemic level. Fecal calprotectin has been known as a marker for gut inflammation for a long time (Berni Canani et al 2004, Kopylov et al 2014) and contributes to innate immunity by binding of zinc and manganese, hereby preventing uptake of these micronutrients by bacteria (Hood and Skaar 2012). We here show that the calprotectin response of infected mice on the high-iron diet was blunted, which was in line with fecal lipocalin-2 levels. As the histopathological score of these mice appeared not to be lower, these findings suggest that calprotectin and lipocalin-2 as a marker for gut inflammation may be less reliable during the provision of a high-iron-supplemented diet. The lower levels of these inflammatory markers may also indicate a reduced innate immune defense. From a functional point-of-view, the consequences of lower lipocalin-2 levels during high-iron conditions might be limited as it is likely that no or less siderophores are produced by intestinal pathogens in that case. In contrast, during iron-limiting conditions, when there likely is siderophore production, it may be advantageous to intestinal pathogens that lipocalin-2 levels are lower. Notably, certain intestinal pathogens can exploit the host lipocalin-2 and calprotectin based defense to their own benefit (Liu et al 2012, Raffatellu et al 2009). It may be envisaged that lower levels of lipocalin-2 and calprotectin abate these exploitation mechanisms. This is however subject for further investigations.

It is difficult to explain why both the iron-deficient diet and the high-iron diet lowered lipocalin-2 production compared to the normal-iron diet. We hypothesized that local lipocalin-2 production could be influenced by a combination of luminal iron concentration and bacterial siderophore production as it would make sense that expression of lipocalin-2 is highest when readily available iron is low and when siderophores are being produced. This does however not fit with the lower levels of lipocalin-2 in the iron-deficient group compared to the normal-iron group. In addition, pro-inflammatory cytokine secretion and fecal calprotectin levels generally showed the same trend, pointing at a more general mechanism. As already mentioned, both host iron deficiency and iron overload have been associated with an attenuated immune response (Oppenheimer 2001, Puschmann and Ganzoni 1977, Wang et al 2008, Weiss 2005) and it might thus very well be that the iron status of mice contributed to a decrease in levels of pro-inflammatory cytokines, lipocalin-2 and calprotectin. Other explanations could lie in the effects of dietary iron and host iron

status on the gut microbiome and whether *C. rodentium* and other intestinal pathogens were able to thrive in the intestinal lumen and trigger the host immune response. Interestingly, it has recently been described that the gut microbiota composition is an important factor in the susceptibility to *C. rodentium* infection and the subsequent immune response (Collins et al 2014).

After 2 weeks of infection, *C. rodentium* abundance in the feces was not different among groups, but we cannot exclude that earlier during infection *C. rodentium* thrived best in the colonic lumen of mice on the normal-iron diet. We nevertheless found profound effects of dietary iron and colitis on the gut microbiome composition, which may have contributed to the effects on the host inflammatory response. Of note, *Enterorhabdus* virtually only appeared after infection and its relative abundance was highest in the normal-iron group, in which also fecal lipocalin-2 and calprotectin levels were highest. Although further research to a potential causal relationship is needed, it could imply that *Enterorhabdus* spp., of which little is known, contributed to the effects on the host inflammatory response. It may be hypothesized that the gut microbiome profile of the mice on the iron-deficient diet was potentially less pathogenic compared to the diets with iron as the relative abundance of the beneficial *Bifidobacteriaceae* and *Lactobacillaceae* was highest in the iron-deficient group. It has previously been shown that *C. rodentium* infection in mice caused a temporal increase in the relative abundance of *Enterobacteriaceae* (of which *C. rodentium* itself is a member) 1 week after initiation of infection, with a decrease to 2 weeks after infection (Lupp et al 2007). However, a later study could not confirm this temporary generalized bloom of *Enterobacteriaceae* in the intestinal lumen, possibly due to a different microbiota profile prior to infection and the provision of different diets (Hoffmann et al 2009). In the present study, we only analyzed the fecal gut microbiome 2 weeks after initiation of infection and found a very low relative abundance of *Enterobacteriaceae*. These were however exclusively detected after infection and tended to be relatively more abundant in the gut microbiome of mice on the high-iron diet.

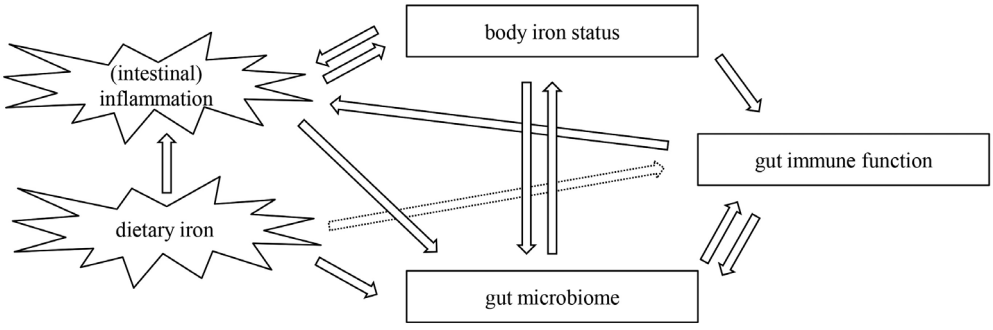
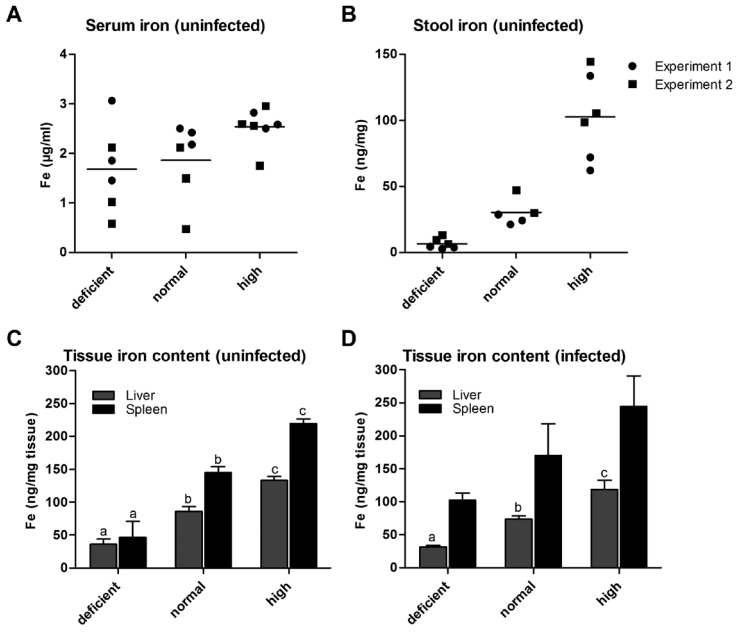


Figure 11. Complex interplay of iron, host immunity and the gut microbiome.
Schematic overview of the interactions between dietary iron, inflammation, host iron status, gut immune function and the gut microbiome.

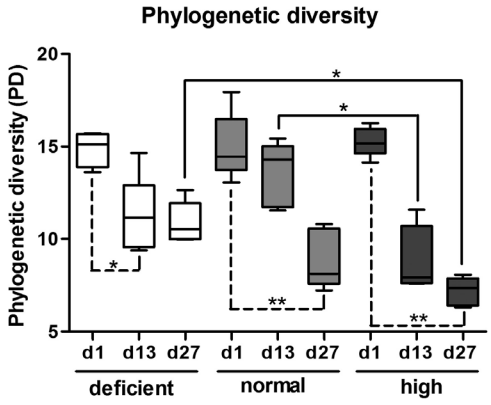
Although our experiments provide leads for the mechanisms behind, the design did not allow to dissect the complex interplay of dietary iron, host iron status and the gut microbiome on the host intestinal immune response (**Figure 11**). Future studies are needed to dissect the underlying mechanisms further. A similar mouse study, but with a larger number of mice to increase power, could be complemented with non-infected control mice and with mice that receive oral iron treatment during infection only, to allow exclusion of host iron status effects. Our analyses can be extended with measurements of other immune factors such as expression of AMPs, a larger panel of cytokines, and analysis of host gene expression by e.g. microarray. It remains however difficult to investigate the interplay between the gut microbiome and host iron status as both host iron status and dietary iron content have been reported to affect the gut microbiome composition, making it very hard to unravel the effects of host iron status on the gut microbiome composition and vice versa (Kortman et al 2014) (**Chapter 1**).

In summary, our data suggest the existence of a maximum in the inflammatory response with regard to dietary iron content, with the peak lying around the normal daily intake of iron, something which has not previously been described in literature. Mainly the iron-deficient diet dampened the intestinal inflammatory response, and which also seemed to lower intestinal pathology. The latter was however more evident in our simple nematode gut model, in which iron-limitation restrained pathology as reflected by a prolonged survival of *C. elegans* that foraged on the intestinal pathogen *S.* Typhimurium under iron-limitation. Furthermore, it confirms that supplementary iron can enhance the virulence of *S.* Typhimurium as we previously have shown *in vitro* (Kortman et al 2012) (**Chapter 5**). These findings underscore the paradox that oral administration may increase the virulence of intestinal pathogens, while decreasing the host intestinal defense at the same time. This undesired combination may provide intestinal pathogens with increased opportunities to evade the host immune response during oral iron therapy and strengthens the idea that oral iron administration programs in developing countries need to be set up with the highest amount of care, with close monitoring until the remaining questions about the actual effect of iron at the intestinal host-microbiota interface have been unraveled. Future research should also be directed at finding iron formulations that do not affect the gut microbiome to a large extent. Together, our data support the hypothesis that low iron intake is predictive against intestinal infection and inflammation, but also suggest that the clinical outcomes of oral iron administration may highly depend on the iron status, immune status and the gut microbiota composition of children that receive oral iron treatment.

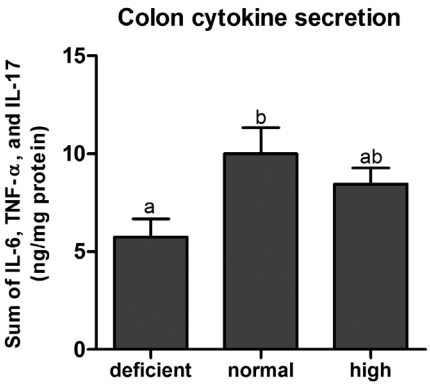
Supplementary Figures and legends



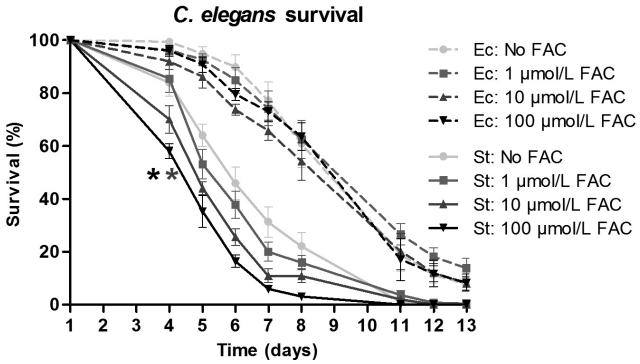
Supplementary Figure 1. Iron content of serum, stools and tissues during the intervention.
Serum iron (**A**) and stool iron (**B**) in uninfected mice of experiment 1 and 2 after 14 days of dietary iron intervention. Tissue iron stores (mean + SE) of uninfected mice of experiment 2 after 14 days of dietary iron intervention (**C**) and of infected mice of experiment 4 after 28 days of dietary intervention (**D**). Means without a common letter differ significantly, $p < 0.05$



Supplementary Figure 2. Effect of dietary iron and intestinal inflammation on the mouse gut microbiome.
The phylogenetic diversity (PD) index of the microbiomes of the dietary groups over time are given (min-max whiskers with median). Differences between groups at the same time point are indicated with solid lines (comparisons between groups were made at the same time point only). Differences within groups over time are indicated with dashed lines (**A**).



Supplementary Figure 3. Colon secreted pro-inflammatory cytokines.
Colon explants of infected mice (day 28) in experiment 4 (n = 3 - 5) that were on the different iron-diets and were challenged with *C. rodentium*, were incubated overnight in culture medium, after which secreted pro-inflammatory cytokines IL-6, TNF-α and IL-17 were measured by ELISA. Cytokine levels were normalized for total colon protein. Displayed is the sum of IL-6, TNF-α and IL-17 secretion. Means without a common letter of the same cytokine differ significantly, $p < 0.05$.



Supplementary Figure 4. Nematode survival on pathogenic *S.* Typhimurium and non-pathogenic *E. coli*.
Survival of the nematode *C. elegans*, which was used as a simple *in vivo* gut model, was monitored during 13 days (mean±SE). The AUC of both the 100 µmol/L and 10 µmol/L ferric ammonium citrate (FAC) was significantly lower ($p < 0.05$) compared to the iron-limited (no FAC) condition when the nematode foraged on *S.* Typhimurium.

Supplementary materials and methods

Iron measurements

Serum iron concentration was determined by the use of the Trace DMA Kit (Thermo Scientific) according to the manufacturer's instruction. Spleen, liver, and stool iron concentrations were measured by the tissue non-heme iron assay protocol as described by Torrance and Bothwell (Torrance and Bothwell 1968). Briefly, 500 µL of acid reagent (3 mol/L HCl containing 10% trichloroacetic acid) was added to approximately 75 mg tissue and incubated at 65˚C for 20 h. After incubation, samples were centrifuged to obtain the supernatant and 20 µL was applied to a microplate. Then, 200 µL chromogenic reagent containing 127 mg/L bathophenanthroline sulphonate disodium salt (Fluka), 936 mg/L thioglycolic acid sodium salt (Sigma-Aldrich) and sodium acetate (2.2 times diluted from a saturated solution) was added to the samples. After 10 min incubation at room temperature (RT) color formation was measured at 560 nm. Iron concentration was determined by comparison to a standard curve made of ferrous sulfate and was corrected for tissue weight.

References

- Andrews SC, Robinson AK, Rodriguez-Quinones F (2003). Bacterial iron homeostasis. FEMS microbiology reviews 27: 215-237.
- Berni Canani R, Rapacciuolo L, Romano MT, Tanturri de Horatio L, Terrin G, Manguso F et al (2004). Diagnostic value of faecal calprotectin in paediatric gastroenterology clinical practice. Digestive and Liver Disease 36: 467-470.
- Blaschitz C, Raffatellu M (2010). Th17 cytokines and the gut mucosal barrier. Journal of clinical immunology 30: 196-203.
- Borenshtein D, McBee ME, Schauer DB (2008). Utility of the Citrobacter rodentium infection model in laboratory mice. Current opinion in gastroenterology 24: 32-37.
- Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK et al (2010). QIIME allows analysis of high-throughput community sequencing data. Nature methods 7: 335-336.
- Carrier JC, Aghdassi E, Jeejeebhoy K, Allard JP (2006). Exacerbation of dextran sulfate sodium-induced colitis by dietary iron supplementation: role of NF-kappaB. International journal of colorectal disease 21: 381-387.
- Cayatte C, Joyce-Shaikh B, Vega F, Boniface K, Grein J, Murphy E et al (2012). Biomarkers of Therapeutic Response in the IL-23 Pathway in Inflammatory Bowel Disease. Clinical and translational gastroenterology 3: e10.
- Chassaing B, Srinivasan G, Delgado MA, Young AN, Gewirtz AT, Vijay-Kumar M (2012). Fecal lipocalin 2, a sensitive and broadly dynamic non-invasive biomarker for intestinal inflammation. PloS one 7: e44328.
- Chen CC, Louie S, McCormick B, Walker WA, Shi HN (2005). Concurrent infection with an intestinal helminth parasite impairs host resistance to enteric Citrobacter rodentium and enhances Citrobacter-induced colitis in mice. Infection and immunity 73: 5468-5481.
- Cherayil BJ (2010). Iron and immunity: immunological consequences of iron deficiency and overload. Archivum immunologiae et therapiae experimentalis 58: 407-415.
- Cherayil BJ, Ellenbogen S, Shanmugam NN (2011). Iron and intestinal immunity. Current opinion in gastroenterology 27: 523-528.
- Cole JR, Wang Q, Cardenas E, Fish J, Chai B, Farris RJ et al (2009). The Ribosomal Database Project: improved alignments and new tools for rRNA analysis. Nucleic acids research 37: D141-145.
- Collins JW, Keeney KM, Crepin VF, Rathinam VA, Fitzgerald KA, Finlay BB et al (2014). Citrobacter rodentium: infection, inflammation and the microbiota. Nature reviews 12: 612-623.
- Cordone A, Mauriello EM, Pickard DJ, Dougan G, De Felice M, Ricca E (2005). The lrp gene and its role in type I fimbriation in Citrobacter rodentium. Journal of bacteriology 187: 7009-7017.
- Cowland JB, Borregaard N (1997). Molecular characterization and pattern of tissue expression of the gene for neutrophil gelatinase-associated lipocalin from humans. Genomics 45: 17-23.
- Dostal A, Chassard C, Hilty FM, Zimmermann MB, Jaeggi T, Rossi S et al (2012). Iron depletion and repletion with ferrous sulfate or electrolytic iron modifies the composition and metabolic activity of the gut microbiota in rats. The Journal of nutrition 142: 271-277.
- Gera T, Sachdev HP (2002). Effect of iron supplementation on incidence of infectious illness in children: systematic review. BMJ (Clinical research ed 325: 1142.

- Haas BJ, Gevers D, Earl AM, Feldgarden M, Ward DV, Giannoukos G et al (2011). Chimeric 16S rRNA sequence formation and detection in Sanger and 454-pyrosequenced PCR amplicons. Genome research 21: 494-504.
- Hoffmann C, Hill DA, Minkah N, Kirn T, Troy A, Artis D et al (2009). Community-wide response of the gut microbiota to enteropathogenic Citrobacter rodentium infection revealed by deep sequencing. Infection and immunity 77: 4668-4678.
- Hood MI, Skaar EP (2012). Nutritional immunity: transition metals at the pathogen-host interface. Nature reviews 10: 525-537.
- Hooper LV, Littman DR, Macpherson AJ (2012). Interactions between the microbiota and the immune system. Science (New York, NY 336: 1268-1273.
- Hurrell R, Egli I (2010). Iron bioavailability and dietary reference values. The American journal of clinical nutrition 91: 1461S-1467S.
- Jaeggi T, Moretti D, Kvalsvig J, Holding PA, Tjalsma H, Kortman GAM et al (2013). Iron Status and Systemic Inflammation, but Not Gut Inflammation, Strongly Predict Gender-Specific Concentrations of Serum Hepcidin in Infants in Rural Kenya. PloS one 8: e57513.
- Jaeggi T, Kortman GA, Moretti D, Chassard C, Holding P, Dostal A et al (2014). Iron fortification adversely affects the gut microbiome, increases pathogen abundance and induces intestinal inflammation in Kenyan infants. Gut.
- Janas RM, Ochocinska A, Snitko R, Dudka D, Kierkus J, Teisseyre M et al (2014). Neutrophil gelatinase-associated lipocalin (NGAL) in blood in children with inflammatory bowel disease. Journal of gastroenterology and hepatology.
- Jonker FAM, Calis JCJ, Phiri K, Kraaijenhagen RJ, Brabin BJ, Faragher B et al (2013). Low Hepcidin Levels in Severely Anemic Malawian Children with High Incidence of Infectious Diseases and Bone Marrow Iron Deficiency. PloS one 8: e78964.
- Kopylov U, Rosenfeld G, Bressler B, Seidman E (2014). Clinical utility of fecal biomarkers for the diagnosis and management of inflammatory bowel disease. Inflammatory bowel diseases 20: 742-756.
- Kortman GA, Boleij A, Swinkels DW, Tjalsma H (2012). Iron availability increases the pathogenic potential of Salmonella typhimurium and other enteric pathogens at the intestinal epithelial interface. PloS one 7: e29968.
- Kortman GA, Raffatellu M, Swinkels DW, Tjalsma H (2014). Nutritional iron turned inside out: intestinal stress from a gut microbial perspective. FEMS microbiology reviews.
- Kroot JJ, Tjalsma H, Fleming RE, Swinkels DW (2011). Hepcidin in human iron disorders: diagnostic implications. Clinical chemistry 57: 1650-1669.
- Labrousse A, Chauvet S, Couillault C, Kurz CL, Ewbank JJ (2000). Caenorhabditis elegans is a model host for Salmonella typhimurium. Current biology : CB 10: 1543-1545.
- Lee TW, Kolber MR, Fedorak RN, van Zanten SV (2012). Iron replacement therapy in inflammatory bowel disease patients with iron deficiency anemia: a systematic review and meta-analysis. Journal of Crohn's & colitis 6: 267-275.
- Letunic I, Bork P (2011). Interactive Tree Of Life v2: online annotation and display of phylogenetic trees made easy. Nucleic acids research 39: W475-478.

- Liu JZ, Jellbauer S, Poe AJ, Ton V, Pesciaroli M, Kehl-Fie TE et al (2012). Zinc sequestration by the neutrophil protein calprotectin enhances Salmonella growth in the inflamed gut. Cell host & microbe 11: 227-239.
- Liu Z, Petersen R, Devireddy L (2013). Impaired neutrophil function in 24p3 null mice contributes to enhanced susceptibility to bacterial infections. Journal of immunology 190: 4692-4706.
- Lupp C, Robertson ML, Wickham ME, Sekirov I, Champion OL, Gaynor EC et al (2007). Host-mediated inflammation disrupts the intestinal microbiota and promotes the overgrowth of Enterobacteriaceae. Cell host & microbe 2: 204.
- Malyszko J, Tesar V, Macdougall IC (2010). Neutrophil gelatinase-associated lipocalin and hepcidin: what do they have in common and is there a potential interaction? Kidney & blood pressure research 33: 157-165.
- Mundy R, MacDonald TT, Dougan G, Frankel G, Wiles S (2005). Citrobacter rodentium of mice and man. Cellular microbiology 7: 1697-1706.
- Mundy R, Girard F, FitzGerald AJ, Frankel G (2006). Comparison of colonization dynamics and pathology of mice infected with enteropathogenic Escherichia coli, enterohaemorrhagic E. coli and Citrobacter rodentium. FEMS microbiology letters 265: 126-132.
- Muthayya S, Rah JH, Sugimoto JD, Roos FF, Kraemer K, Black RE (2013). The global hidden hunger indices and maps: an advocacy tool for action. PloS one 8: e67860.
- Nielsen BS, Borregaard N, Bundgaard JR, Timshel S, Sehested M, Kjeldsen L (1996). Induction of NGAL synthesis in epithelial cells of human colorectal neoplasia and inflammatory bowel diseases. Gut 38: 414-420.
- Nielsen OH, Gionchetti P, Ainsworth M, Vainer B, Campieri M, Borregaard N et al (1999). Rectal dialysate and fecal concentrations of neutrophil gelatinase-associated lipocalin, interleukin-8, and tumor necrosis factor-alpha in ulcerative colitis. The American journal of gastroenterology 94: 2923-2928.
- Oikonomou KA, Kapsoritakis AN, Theodoridou C, Karangelis D, Germenis A, Stefanidis I et al (2012). Neutrophil gelatinase-associated lipocalin (NGAL) in inflammatory bowel disease: association with pathophysiology of inflammation, established markers, and disease activity. Journal of gastroenterology 47: 519-530.
- Oppenheimer SJ (2001). Iron and its relation to immunity and infectious disease. The Journal of nutrition 131: 616S-633S; discussion 633S-635S.
- Philpott C (2010). Bioinorganic chemistry: Getting a grip on iron. Nature chemical biology 6: 568-570.
- Puschmann M, Ganzoni AM (1977). Increased resistance of iron-deficient mice to salmonella infection. Infection and immunity 17: 663-664.
- Raffatellu M, George MD, Akiyama Y, Hornsby MJ, Nuccio SP, Paixao TA et al (2009). Lipocalin-2 resistance confers an advantage to Salmonella enterica serotype Typhimurium for growth and survival in the inflamed intestine. Cell host & microbe 5: 476-486.
- Reifen R, Matas Z, Zeidel L, Berkovitch Z, Bujanover Y (2000). Iron supplementation may aggravate inflammatory status of colitis in a rat model. Digestive diseases and sciences 45: 394-397.
- Sazawal S, Black RE, Ramsan M, Chwaya HM, Stoltzfus RJ, Dutta A et al (2006). Effects of routine prophylactic supplementation with iron and folic acid on admission to hospital and mortality in preschool children in a high malaria transmission setting: community-based, randomised, placebo-controlled trial. Lancet 367: 133-143.

- Seril DN, Liao J, Ho KL, Warsi A, Yang CS, Yang GY (2002). Dietary iron supplementation enhances DSS-induced colitis and associated colorectal carcinoma development in mice. Digestive diseases and sciences 47: 1266-1278.
- Shannon P, Markiel A, Ozier O, Baliga NS, Wang JT, Ramage D et al (2003). Cytoscape: a software environment for integrated models of biomolecular interaction networks. Genome research 13: 2498-2504.
- Ter Braak CJF, Smilauer P (2012). Canoco reference manual and user's guide: software for ordination, version 5.0. Microcomputer Power: Ithaca, USA.
- Tjalsma H, Laarakkers CM, van Swelm RP, Theurl M, Theurl I, Kemna EH et al (2011). Mass spectrometry analysis of hepcidin peptides in experimental mouse models. PloS one 6: e16762.
- Tompkins GR, O'Dell NL, Bryson IT, Pennington CB (2001). The effects of dietary ferric iron and iron deprivation on the bacterial composition of the mouse intestine. Current microbiology 43: 38-42.
- Torrance JD, Bothwell TH (1968). A simple technique for measuring storage iron concentrations in formalinised liver samples. The South African journal of medical sciences 33: 9-11.
- Vijay-Kumar M, Sanders CJ, Taylor RT, Kumar A, Aitken JD, Sitaraman SV et al (2007). Deletion of TLR5 results in spontaneous colitis in mice. The Journal of clinical investigation 117: 3909-3921.
- Wang L, Johnson EE, Shi HN, Walker WA, Wessling-Resnick M, Cherayil BJ (2008). Attenuated inflammatory responses in hemochromatosis reveal a role for iron in the regulation of macrophage cytokine translation. Journal of immunology 181: 2723-2731.
- Weiss G (2005). Modification of iron regulation by the inflammatory response. Best practice & research Clinical haematology 18: 183-201.
- WHO, UNICEF (2004). Focusing on anaemia: Towards an integrated approach for effective anaemia control.
- WHO, UNICEF (2006). Joint Statement: Iron supplementation of young children in regions where malaria transmission is intense and infectious disease highly prevalent.
- Winter SE, Baumler AJ (2011). A breathtaking feat: to compete with the gut microbiota, Salmonella drives its host to provide a respiratory electron acceptor. Gut microbes 2: 58-60.
- Yang S, Lin S, Kelen GD, Quinn TC, Dick JD, Gaydos CA et al (2002). Quantitative multiprobe PCR assay for simultaneous detection and identification to species level of bacterial pathogens. Journal of clinical microbiology 40: 3449-3454.
- Zhu A, Kaneshiro M, Kaunitz JD (2010). Evaluation and treatment of iron deficiency anemia: a gastroenterological perspective. Digestive diseases and sciences 55: 548-559.
- Zimmermann MB, Hurrell RF (2007). Nutritional iron deficiency. Lancet 370: 511-520.
- Zimmermann MB, Chassard C, Rohner F, N'Goran E K, Nindjin C, Dostal A et al (2010). The effects of iron fortification on the gut microbiota in African children: a randomized controlled trial in Cote d'Ivoire. The American journal of clinical nutrition 92: 1406-1415.

Chapter 5

Iron availability increases the pathogenic potential of Salmonella Typhimurium and other enteric pathogens at the intestinal epithelial interface

Guus AM Kortman, Annemarie Boleij, Dorine W Swinkels, Harold Tjalsma

Department of Laboratory Medicine – Laboratory of Genetic, Endocrine and Metabolic Diseases. Radboud Institute for Molecular Life Sciences (RIMLS) of the Radboud University Medical Center, P.O. Box 9101, 6500 HB Nijmegen, The Netherlands

This chapter is published in PLoS One. 2012;7(1):e2996

Abstract

Recent trials have questioned the safety of untargeted oral iron supplementation in developing regions. Excess of luminal iron could select for enteric pathogens at the expense of beneficial commensals in the human gut microbiota, thereby increasing the incidence of infectious diseases. The objective of the current study was to determine the effect of high iron availability on virulence traits of prevalent enteric pathogens at the host-microbe interface.

A panel of enteric bacteria was cultured under iron-limiting conditions and in the presence of increasing concentrations of ferric citrate to assess the effect on bacterial growth, epithelial adhesion, invasion, translocation and epithelial damage *in vitro*. Translocation and epithelial integrity experiments were performed using a transwell system in which Caco-2 cells were allowed to differentiate to a tight epithelial monolayer mimicking the intestinal epithelial barrier. Growth of *Salmonella* Typhimurium and other enteric pathogens was increased in response to iron. Adhesion of *S.* Typhimurium to epithelial cells markedly increased when these bacteria were pre-incubated with increasing iron concentration ($p = 0.0001$), whereas this was not the case for the non-pathogenic *Lactobacillus plantarum* ($p = 0.42$). Cellular invasion and epithelial translocation of *S.* Typhimurium followed the trend of increased adhesion. *Epithelial* damage was increased upon incubation with *S.* Typhimurium or *Citrobacter freundii* that were pre-incubated under iron-rich conditions. In conclusion, our data fit with the consensus that oral iron supplementation is not without risk as iron could, in addition to inducing pathogenic overgrowth, also increase the virulence of prevalent enteric pathogens.

Introduction

Iron is a highly abundant metal on earth and is vital for virtually all organisms. Despite its abundance, iron deficiency is the most prevalent nutrition disorder worldwide. It mostly affects infants, young children and women in developing countries. Iron deficiency has major health consequences such as infection, poor pregnancy outcome, and impaired physical and cognitive development (WHO and UNICEF 2004). Several trials have shown that iron deficiency can be effectively controlled by both iron supplementation and fortification programmes (Zimmermann and Hurrell 2007). However, safety of iron supplementation has been questioned and there is evidence suggesting that untargeted oral iron supplementation in regions with high prevalence of malaria transmission and infectious diseases can cause an increase in infections, hospital admission and mortality in young children (Oppenheimer 2001, Sazawal et al 2006, WHO 2007). This might be at least partly ascribed to iron also being an essential requirement for the growth of most bacterial species. Importantly, iron availability is frequently involved in the expression of virulence-associated properties in pathogenic bacteria (Andrews et al 2003, Bullen et al 2005).

The human gut is the natural habitat for a large and dynamic bacterial community. Major functions of the gut microbiota include important trophic effects on intestinal epithelia, on immune structure and function, and protection of the colonized host against invasion by pathogenic microbes (Flint et al 2007). It has been described that dietary ferric iron and iron deprivation can influence the microbiota composition of the mouse intestine (Tompkins et al 2001, Werner et al 2011). Very recently, Zimmermann et al. showed in a study among African children that iron fortification caused a potentially more pathogenic gut microbiota profile (i.e. increased relative abundance of pathogenic species) (Zimmermann et al 2010). This was predominantly the case for *Salmonella* spp., which are capable of invading human epithelial cells, translocate across the colonic wall, and subsequently can cause systemic disease (Gordon et al 2008, Ibarra and Steele-Mortimer 2009, Zimmermann et al 2010). The increase in infections upon oral iron supplementation that was reported by Sazawal et al. (Sazawal et al 2006) might partly originate from such pathogenic shifts in the colon microbiota due to high concentrations of unabsorbed iron during treatment. Abundance of pathogenic enterobacteria after oral iron supplementation might cause diarrhea and a systematic review of Gera and Sachdev indeed reported a slight increase in the risk of developing diarrhea upon oral iron administration (Gera and Sachdev 2002). Importantly, diarrhea is most often a sign of gastrointestinal infection and is a major cause of morbidity and mortality among young children worldwide (WHO and UNICEF 2009). Therefore, a prior recommendation of both the WHO and the U.S. National Institutes of Health Technical Working Group (NIH TWG) is to investigate the impact of iron preparations on the gut microbiota (WHO 2006).

Roughly, there are two factors - directly driven by luminal iron - which may act together in gut borne infections: i) decrease in epithelial integrity and ii) increase in pathogen growth and virulence. Decrease in epithelial integrity has been reported *in vitro* and *ex vivo* and the corresponding increased permeability of the intestine may provide a portal of entry for opportunistic enteric pathogens (Ferruzza et al 2003, Hansen et al 2009, Nchito et al 2006).

However, little is known about the direct effects of luminal iron on the growth and virulence of enteric pathogens. Therefore, the aim of this study was to investigate how the pathogenic potential of gut bacteria is modulated by iron *in vitro*. To this purpose, bacterial adhesion, invasion and translocation characteristics of a panel of enteric pathogens was investigated using differentiated monolayers of the intestinal epithelial cell lines Caco-2 and E12 as a model for the gut epithelium.

Materials and methods

Bacterial strains, media and growth conditions

The strains used in this study were: *Salmonella* Typhimurium NTB6, *Escherichia coli NTB5, Enterococcus faecalis ATCC 19433, Lactobacillus plantarum WCFS1* (Boleij et al 2011) and *Citrobacter freundii NTBK1*. These bacteria were cultured at 37˚C/5%CO_2 in Iscove's Modified Dulbecco's Medium (IMDM, Invitrogen). This chemically defined medium does not contain iron in its formulation. For *L. plantarum* the medium was supplemented with 30 mg/L $MnCl_2{\cdot}4H_2O$, 1 g/L sodium acetate and 10 mmol/L HCl. To determine the effect of iron on bacterial growth, fresh IMDM medium with increasing ferric citrate (Sigma-Aldrich) concentrations (0-1000 µmol/L) was inoculated with a fresh overnight culture. To monitor bacterial growth, the optical density was periodically measured at 620 nm (OD_{620}).

Cell line, media and growth conditions

The colon adenocarcinoma cell line Caco-2 (obtained from the American Type Culture Collection) was cultured under standard conditions in DMEM (Lonza) supplemented with 10% heat-inactivated FBS (Invitrogen), 20 mmol/L HEPES, 100 nmol/L nonessential amino acids (Invitrogen), and 2 mmol/L L-glutamine (Lonza). The cells were subcultured every 6 days and used between passage numbers 2-25. A description of the culturing of mucus producing E12 cells is available from the Online Supporting Material and methods.

Bacterial adhesion and invasion assay

The influence of iron on adhesion and invasion of the mentioned bacterial strains was studied by the use of the following adhesion assay.

Pre-incubation of bacteria with ferric citrate: IMDM with increasing ferric citrate concentrations (0-50 µmol/L) was inoculated with overnight cultures and grown to exponential phase. The bacterial cells were pelleted, resuspended and concentrated in IMDM with 10% glycerol for storage at -80˚C until use. Serial dilutions of thawed stocks were transferred to blood agar plates and incubated overnight to determine the colony forming units (CFU).

Culturing of Caco-2 cells: Caco-2 cells were subcultured in a 24-well plate and maintained until use in adhesion and invasion assays. The assays were performed on confluent monolayers between 13-21 days after seeding the cells.

Bacterial adhesion and invasion assay: The Caco-2 monolayers were washed once with PBS. The stocks of bacterial strains that were grown in IMDM with or without ferric citrate were pelleted and resuspended in IMDM. Next, bacteria were added to the monolayers at a

multiplicity of infection (MOI) of 10:1 in IMDM followed by incubation for 2 h at standard conditions. To determine the number of adherent bacteria, monolayers were washed three times with PBS, cells were trypsinized and lysed with ice-cold PBS containing 0.025% Triton X-100. Serial dilutions of cell lysates were transferred to blood-agar plates for CFU counting. To determine the number of invaded bacteria, monolayers were washed three times with PBS, incubated for another 1.5 h and subsequently incubated with 200 mg/L gentamycin (Invitrogen) + 50 mg/L ampicillin (Calbiochem) for 1 h at standard conditions to kill extracellular bacteria. Subsequently, the monolayers were washed twice with PBS, trypsinized and lysed for CFU counting as described above.

Bacterial translocation assay

Caco-2 cells were allowed to grow and differentiate in 21 days to a polarized tight monolayer on the membrane of a Transwell® Permeable Support (12 wells, 12 mm insert) with 3.0 μm polycarbonate membrane (Corning) under standard culture conditions. At day 21, the wells and inserts were washed once with PBS and IMDM was added. To check for the monolayer integrity, the trans epithelial electrical resistance (TEER) was measured with the use of the Millicell®-ERS (Millipore). Bacteria that were or were not pre-loaded with iron (as described above) were apically added to the monolayers at a MOI of 10:1 in IMDM. Infected cells were incubated at standard conditions and monolayer integrity was monitored by periodical TEER measurements. After 2 h both compartments were washed three times with PBS and fresh IMDM was added. The incubation of infected cells was continued for 2 h after which a sample of the lower compartment was taken to determine the amount of translocated bacteria by CFU counting.

Determination of LDH-release of epithelial cells upon bacterial infection.

To investigate the detrimental effect of enteric bacteria to Caco-2 monolayers, the lactate dehydrogenase (LDH) release into the growth medium was determined. Media from adhesion experiments were collected after 2 h of incubation. Samples were spun for 15 min at 16,100 × g at 4˚C. Supernatants were used in the Cytotox 96® Non-Radioactive Cytotoxicity Assay (Promega) according to the manufacturer's protocol.

Statistical analysis

Each adhesion/invasion and translocation experiment was performed with 2-3 biological replicates, repeated up to 3 times on separate days and results were expressed as mean + SD. To compare the means, one-way ANOVA with Tukey's post-hoc test (for comparison of > 2 means with equal variances, as assessed by Bartlett's test or F-test) or an unpaired t-test (2-tailed) (for comparison of 2 means) was used. In case of unequal variances (as assessed by F-test), unpaired t-test with Welch's correction was carried out. Analysis was performed using GraphPad Prism version 4.00 for Windows, GraphPad Software, San Diego California USA. P-values < 0.05 were considered statistically significant and P-values < 0.10 were considered as an important significance level.

Results

In vitro growth of enteric bacteria

To investigate whether pathogenic bacteria have a potential growth advantage over non-pathogenic bacteria in an iron-rich environment, the effect of ferric citrate on growth of a selection of enteric bacteria was tested *in vitro* (**Figure 1**). These experiments revealed a clear concentration-dependent growth stimulatory effect in iron-supplemented growth medium for the pathogen *S.* Typhimurium and the opportunistic pathogens *C. freundii* and *E. coli* (**Figure 1A-C**). Only a small beneficial effect was noted for the opportunistic pathogen *E. faecalis*, while iron did not influence growth of the non-pathogenic commensal *L. plantarum* (**Figure 1D and E**).

Adhesion of enteric bacteria to an epithelial monolayer

Adhesion to host epithelial cells is an important virulence characteristic for pathogenic bacteria. To test the contribution of iron availability to bacterial adhesion, bacteria were pre-incubated with ferric citrate and added to epithelial monolayers under iron-limiting conditions, after which the percentage adhesion of the inoculum was determined. As shown in **Figure 2A**, adhesion of S. Typhimurium significantly increased with increasing iron concentration (one-way ANOVA: $p = 0.0001$, 0 µmol/L vs. 10 µmol/L: $p < 0.05$). This increase was not due to differential growth since the CFU did not differ among the 1-50 µmol/L ferric citrate conditions during the time course of this experiment. The opportunistic pathogen *C. freundii* tended to adhere more ($p = 0.097$) and *E. coli* adhered more ($p = 0.014$) after pre-incubation with 10 µmol/L ferric citrate compared to bacteria pre-incubated under iron-limiting conditions (**Figure 2B and C**). Adhesion of the opportunistic pathogen *E. faecalis* was not influenced by pre-incubation with iron (**Figure 2D**). Interestingly, the non pathogenic commensal *L. plantarum* (**Figure 2E**) displayed a slight, but non-significant decrease in adhesion characteristics in response to an increase in iron availability.

Mucus, covering the human intestinal epithelium, could influence bacterial adhesion. Therefore, we investigated whether iron availability could also increase the adhesion of *S.* Typhimurium to mucus producing E12 cells. The latter cell line is derived from HT29-MTX and capable of forming tight monolayers covered with an adherent mucus layer (Behrens et al 2001). For all conditions the adhesion of S. Typhimurium to E12 cells was much higher compared to Caco-2 cells (data not shown). Importantly, also in this case the tendency towards increased adhesion of bacteria that were pre-incubated with increased iron concentrations was found to be statistically significant ($p < 0.05$; Supplementary **Figure 1A**). The latter observation indicates that a protective mucus layer does not prevent the increased adhesion of iron-loaded bacteria to intestinal epithelial cells.

Invasion of enteric bacteria into epithelial cells

During the infectious process, bacterial adhesion can be followed by invasion into intestinal epithelial cells. Therefore, the effect of pre-incubation with ferric citrate on cell invasion was assessed for *S.* Typhimurium, *E. faecalis* and *L. plantarum*. Of these strains, only *S.* Typhimurium was able to substantially invade a differentiated monolayer of epithelial

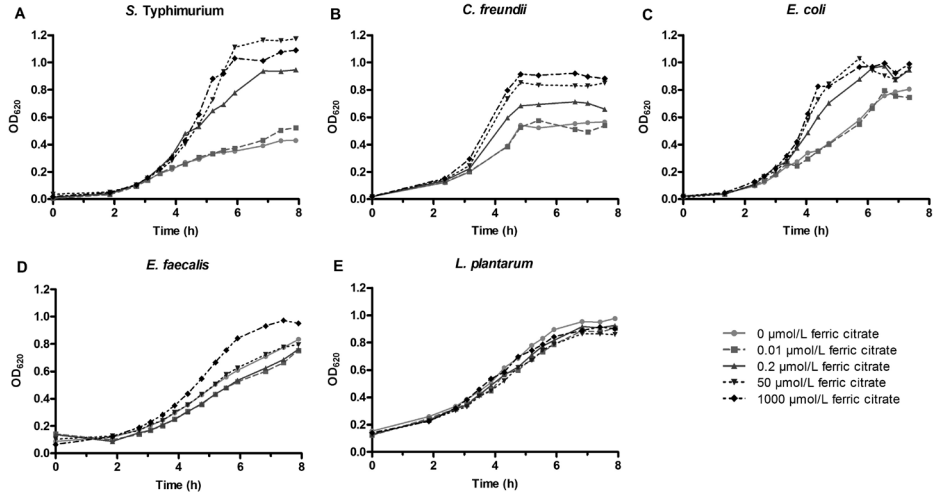


Figure 1. Effect of iron on growth of enteric bacteria.

Effect of various concentrations of ferric citrate on in vitro growth of (**A**) *S.* Typhimurium, (**B**) *C. freundii*, (**C**) *E. coli*, (**D**) *E. faecalis* and (**E**) *L. plantarum.*

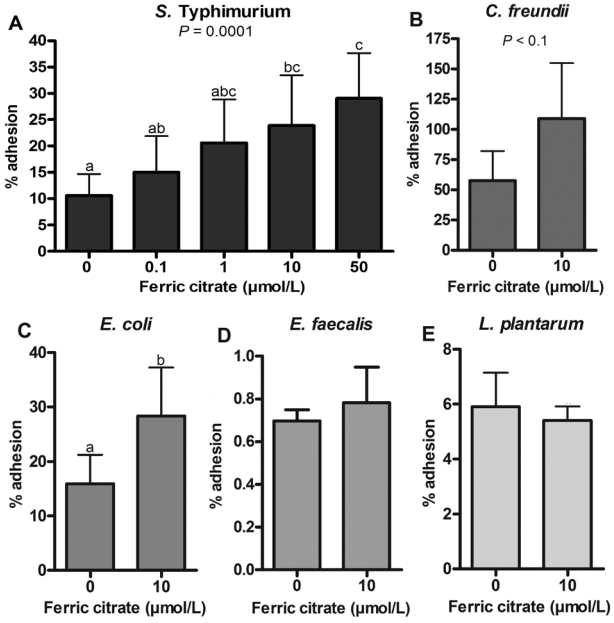


Figure 2. Effect of iron on bacterial adhesion to an epithelial monolayer.

Adhesion (mean + SD) of enteric bacteria to a monolayer of Caco-2 cells is given as percentage of the inoculum. **A**: *S.* Typhimurium, n=8. **B**: *C. freundii*, n=4. **C**: *E. coli*, n=6. **D**: *E. faecalis*, n=6. **E**: *L. plantarum*, n=5. Means without a common letter differ, $p < 0.05$. Notably, adhesion data of *S.* Typhimurium were derived from 4 separate experiments performed at 13, 15, 18 and 21 days post-seeding of Caco-2 cells. The fact that each experiment revealed the same trend is indicative for similar physiochemical properties of the monolayer at these time points.

cells. Similar to adhesion, invasion of *S.* Typhimurium tended to increase with increasing iron concentration up to 10 µmol/L ferric citrate (One-way ANOVA, p = 0.09; **Figure 3**). However, this trend disappeared when the percentage bacterial invasion was calculated as a function of the adherent bacteria (**Supplementary Figure 2**). This indicates that the increase in invasion is merely a consequence of increased adhesion of *S.* Typhimurium, and that invasion itself is not largely influenced by iron availability. Notably, the invasion of bacteria pre-incubated with 50 µmol/L ferric citrate was unexpectedly low in relation to the increased adhesion under these conditions.

Translocation of enteric bacteria across an epithelial monolayer

To cause a systemic infection, enteric bacteria first need to breach the intestinal epithelial barrier. Therefore, the ability of *S.* Typhimurium to translocate across an epithelial monolayer was investigated as a function of iron availability. As shown in **Figure 4A**, translocation of *S.* Typhimurium clearly increased with increasing iron availability up to 10 µmol/L ferric citrate (10 µmol/L vs. iron deplete control p = 0.06). Interestingly, translocation sharply decreased when bacteria were pre-incubated with 50 µmol/L ferric citrate (**Figure 4A**). As a control experiment, the translocation of L. plantarum was assessed. Translocation of this bacterium was neglectably low and not stimulated by increased iron availability (data not shown).

The ability of *S.* Typhimurium to translocate across a mucus covered epithelial layer of E12 cells was also investigated. These experiments showed that also in this case, the translocation of *S.* Typhimurium across E12 cells increased ($p < 0.05$) when the bacteria were pre-incubated with increasing concentrations of ferric citrate (**Supplementary Figure 1B**). These data indicate that a protective mucus layer does not abolish the iron-dependent increase in translocation efficiency of *S.* Typhimurium across an epithelial monolayer (**Figure 4A**).

Effect of iron-loaded bacteria on epithelial integrity and cell cytotoxicity

To monitor epithelial integrity during the translocation experiments, the electrical resistance of the monolayer was monitored by TEER measurements at regular intervals. These experiments showed that incubation with *S.* Typhimurium resulted in decreased TEER values in time (**Figure 4B**). As expected, the deterioration rate of the epithelial integrity increased when *S.* Typhimurium was pre-incubated with iron as depicted for the 10 µmol/L ferric citrate condition. In contrast, the integrity of the monolayer remained unaffected upon incubation with other tested bacteria, irrespective of a pre-incubation step with ferric citrate, as illustrated for *L. plantarum* (**Figure 4B**). As a second indicator of epithelial damage, the cellular LDH release was determined after enteric bacteria were allowed to adhere to the monolayer for 2 hours. As shown in **Figure 5**, epithelial cells tended to release more LDH in response to *S.* Typhimurium that was pre-incubated with 10 µmol/L ferric citrate compared to the 0 µmol/L condition (p = 0.09). This is in line with the increased deterioration rate under these conditions as monitored by TEER measurements. Interestingly, the LDH release in response to *C. freundii* pre-incubated with 10 µmol/L ferric citrate was also greater compared to *C. freundii* grown in iron deplete medium

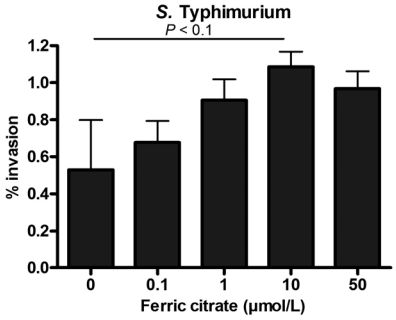


Figure 3. Effect of iron on invasion of *S.* Typhimurium into epithelial cells.

Invasion (mean + SD) of *S.* Typhimurium into Caco-2 cells, n=2. Invasion after 3.5 h is given as percentage of the inoculum. The inoculum was removed after 2 hours of adhesion time. Means of 0 – 10 µmol/L ferric citrate were compared by one-way ANOVA.

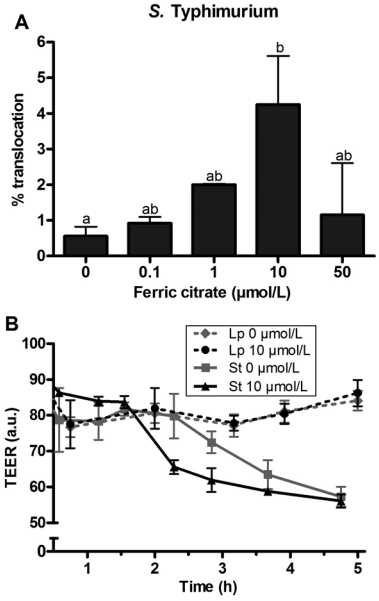


Figure 4. Effect of iron on the ability of *S.* Typhimurium to cross and deteriorate an epithelial monolayer.

Effect of iron on the ability of *S.* Typhimurium to cross an epithelial monolayer of Caco-2 cells and the integrity of this monolayer. **A**: The translocation is given as percentage of the inoculum (mean + SD), n=2. Means without a common letter differ, $p < 0.07$. **B**: The integrity of the Caco-2 monolayer during *S.* Typhimurium (St) and L. plantarum (Lp) translocation, monitored by TEER measurements.

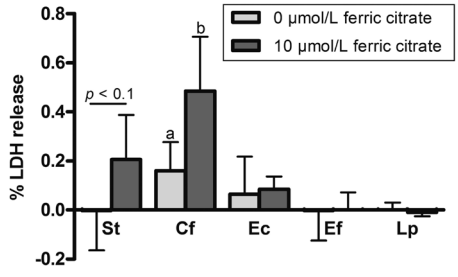


Figure 5. Effect of iron on the ability of enteric bacteria to induce cell damage.

LDH-release (mean + SD) as a measure of cell damage of Caco-2 cells upon co-incubation with *S.* Typhimurium (St, n=5), *C. freundii* (Cf, n=4), *E. coli* (Ec, n=4), *E. faecalis* (Ef, n=4), and *L. plantarum* (Lp, n=2) pre-incubated with or without ferric citrate. The percentage LDH release compared to the control (no bacteria) was corrected for the number of bacteria in the medium (average between t=0 and t=2 h). Means within a group and without a common letter differ significantly, $p < 0.05$.

($p = 0.018$), despite the fact that the TEER did not drop under these conditions (data not shown). The latter observation implies that LDH release is an early marker for epithelial damage, which may correlate with adhesion of pathogenic bacteria (see **Figure 2B**). In this respect, the *E. coli* strain used in this study seems not very hostile since the TEER did not drop and LDH release did not increase despite an increased adhesion after pre-incubation with 10 µmol/L ferric citrate. Contrarily, the LDH release in response to *E. faecalis* and *L. plantarum* was low compared to the release in response to the other bacteria, and unaffected by pre-incubation of these bacteria with ferric citrate. The latter observation is consistent with the fact that adhesion of these bacteria remained unaffected under iron rich conditions (**Figure 2D and E**).

Discussion

The safety of iron supplementation and fortification programmes in developing countries has been questioned (WHO 2007). Very recently it has been shown that biscuits fortified with low bioavailable electrolytic iron caused an increase in fecal enterobacteria (predominately *Salmonella* spp.) in African children (Zimmermann et al 2010). This shift towards a more unfavorable number of enteric pathogens might be one of the origins of the increase in infections, hospital admissions and mortality which was found by Sazawal et al. (Sazawal et al 2006). Here we investigated the relative effects of iron on growth and virulence traits of enteric pathogens and commensals.

Our *in vitro* experiments showed that the switch from iron-limiting to iron rich conditions resulted in an enhanced growth of *S.* Typhimurium and other enteric pathogens. On the other hand, growth of *L. plantarum* that is not strictly dependent on iron (Pandey et al 1994), was not enhanced by iron. These results fit with the finding that enteric pathogens have the potential to outgrow the commensal population when large amounts of unabsorbed dietary iron enter the colon *in vivo* (Zimmermann et al 2010).

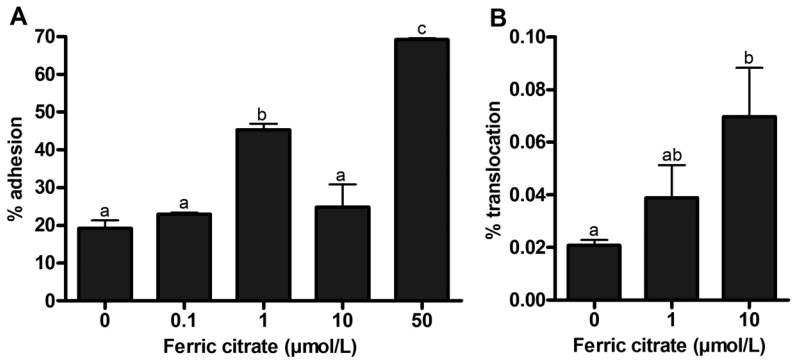
The ability to replicate is important for all bacteria, but to establish an infection, pathogens first need to adhere to the colonic wall. Our current study clearly showed that iron availability increased adhesion of enteric pathogens to intestinal epithelial cells *in vitro*, which was most prominently observed for *S.* Typhimurium. Importantly, the increased adhesion of *S.* Typhimurium as a function of iron was not only observed with Caco-2 cells, but also in case of mucus producing intestinal epithelial E12 cells that may more closely resemble the *in vivo* situation. In contrast, iron did not affect adhesion of *E. faecalis* and even seemed to slightly reduce the adhesion of *L. plantarum* to epithelial cells. Importantly, these results indicate that in addition to a growth advantage of enteric pathogens, iron also has the potential to contribute to increased colonization of these enteric pathogens to the colonic wall. *In vivo*, colonization of enteric pathogens depends on many factors, among which the colonization resistance of the resident commensal population. In this respect, Stecher et al. postulated the interesting "*like will to like*" concept, based on the observation that mice with relatively high *E. coli* densities in their intrinsic intestinal population were more susceptible to *Salmonella* infections (Stecher et al 2010). It may therefore be envisaged that iron can also indirectly enhance *S.* Typhimurium infections *in vivo* by increasing intestinal colonization with related commensals.

Translocation across the colonic wall is the third step in establishing a gut-borne infection. Our current *in vitro* data show that iron availability increases the cell invasion and epithelial translocation potential of *S.* Typhimurium. However, the increase in invasion seems to be a direct consequence of the increased adhesion, which implies that there is no stimulatory effect of iron on invasion itself. This could indicate that the type III secretion system (T3SS) of *S.* Typhimurium, which is directly involved in the invasion of epithelial cells, was not further induced by ferric citrate under the applied experimental conditions. Nevertheless, other studies have shown that T3SS is induced by iron through the ferric uptake regulator (Fur) (Ellermeier and Slauch 2008, Janakiraman and Slauch 2000, Teixido et al 2011). Interestingly, invasion even appeared slightly less efficient when *S.* Typhimurium was pre-incubated in 50 µmol/L ferric citrate, suggesting that certain invasion-specific factors become affected under excessive iron conditions. *S.* Typhimurium initially invades directly into host cells and can hereby affect tight junction complex proteins (Ibarra and Steele-Mortimer 2009, Jepson et al 1995, Kohler et al 2007). This affects epithelial integrity and subsequently provides the opportunity for *S.* Typhimurium to cross the epithelium via the paracellular route (Kohler et al 2007). The reduced translocation efficacy at excessive iron conditions could therefore fit with impaired cell invasion of *S.* Typhimurium during the initial phases of infection. Besides enteroinvasive strains like *S.* Typhimurium, there are many other enteric pathogens that do not translocate across the bowel wall, but can cause severe intestinal inflammation. The virulence of such pathogens is also likely to be positively influenced by increased iron availability.

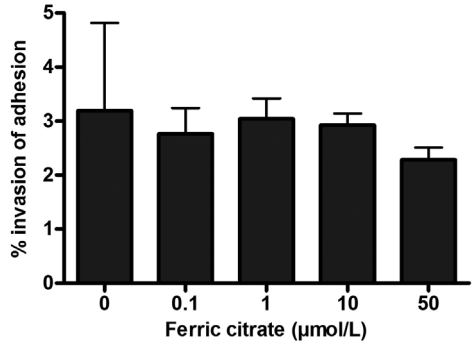
Our experiments showed that pre-incubation of the enterobacteria *S.* Typhimurium and *C. freundii* with iron increased damage to epithelial cells as measured by LDH release. This may very well be associated with the increased adhesion of these bacteria under these conditions. However, the release of bacterial products such as toxins, which was not assessed in this study, could differ between high and low iron conditions and can also play a role in the increased epithelial cell damage. Furthermore, iron-loading of *S.* Typhimurium resulted in faster deterioration the epithelial integrity of a monolayer (TEER measurement), which was associated with increased bacterial translocation up to 10 µmol/L ferric citrate. Since high TEER values mainly represent the existence of tight junctions (Kohler et al 2007), this is in line with a hypothetical model in which *S.* Typhimurium actively affects tight-junction complexes, which is stimulated upon increased invasion of epithelial cells under iron-rich conditions. We want to emphasize that all adhesion, invasion and translocation assays were performed with bacteria that were pre-loaded with iron, but that the experiments themselves were performed under iron-limiting conditions. This was important to prevent bias in our data, as it has been reported that iron itself has the potential to damage the gut wall directly via generation of oxygen radicals (Ferruzza et al 2003, Natoli et al 2009). In our current study, Caco-2 cells and E12 cells were maintained under standard condition without any additional iron, which is different from the study design of Foster et al. who showed that elevated iron status of enterocytes increased bacterial invasion (Foster et al 2001). Clearly, our study adds that enteric pathogens themselves have the potential to benefit from increased luminal iron availability, already during the initial phases of infection (i.e. cell adhesion and cell damage).

In summary, our *in vitro* data support the hypothesis that luminal iron from oral iron supplementation or fortification can increase growth and virulence of enteric pathogens. It goes without saying that an animal infection model is required for future validation of our data in a more complex situation. Nevertheless, our study clearly supports the current idea that nutritionist should be aware of the potential harmful effects of oral iron supplementation in areas with high infection pressure, as present in developing countries. The ideal safe iron preparation should be low dose and highly bioavailable for humans, while it is difficult to access for enteric pathogens. Research to the latter part is currently underexposed, but certainly deserves more attention in the light of the recent awareness of the risk of general iron supplementation programmes. In addition, it is important to develop point-of-care diagnostic tools to discriminate between individuals with iron deficiency anemia (IDA) who will directly benefit from iron supplementation and those with anemia due to chronic infection (ACD). In the latter case, anemia is (also) a result from the human iron-withdrawal strategy mediated by increased circulating levels of the iron-regulatory hormone hepcidin upon (malarial) infection (de Mast et al 2009, Kemna et al 2008). Subsequently, hepcidin not only blocks iron release from the reticulo-endothelial macrophages but it also inhibits absorption from the intestine (Cercamondi et al 2010, Kemna et al 2008), meaning that luminal pathogens will benefit more from dietary iron supplementation than the host itself. Reliable on-site discrimination between ACD and IDA may contribute to the safe supplementation of iron. From a mechanistic point of view, several questions remain to be answered, such as the identification of the enterobacterial factors that are responsible for the increased adhesion under iron-rich conditions. This and other questions will be subject of our ongoing investigations.

Supplementary Figures and legends



Supplementary Figure 1. Effect of iron on the ability of *S.* Typhimurium to adhere to, and translocate across, an epithelial monolayer of E12 cells covered with mucus. *In vitro* adhesion (mean + SD) to E12 cells, and translocation (mean + SD) of *S.* Typhimurium across a monolayer of E12 cells. **A:** Because adhesion to E12 cells was much higher than adhesion to Caco-2 cells, the number of adherent bacteria to E12 cells was expressed as percentage of the average CFU (CFU at start – CFU after 2h) in the culture medium, n=2. **B:** Translocation is given as percentage of the average CFU (CFU at start – CFU after 2.5h) in the culture medium, n=3. Means without a common letter differ $p < 0.05$.



Supplementary Figure 2. Effect of iron on invasion of *S.* Typhimurium into Caco-2 epithelial cells. Invasion (mean + SD) of *S.* Typhimurium into Caco-2 epithelial cells. Invasion after 3.5 hours is given as percentage invaded bacteria of the adherent bacteria at the 2 hour time point, n=2.

References

- Andrews SC, Robinson AK, Rodriguez-Quinones F (2003). Bacterial iron homeostasis. FEMS microbiology reviews 27: 215-237.
- Behrens I, Stenberg P, Artursson P, Kissel T (2001). Transport of lipophilic drug molecules in a new mucus-secreting cell culture model based on HT29-MTX cells. Pharm Res 18: 1138-1145.
- Boleij A, Muytjens CM, Bukhari SI, Cayet N, Glaser P, Hermans PW et al (2011). Novel clues on the specific association of Streptococcus gallolyticus subsp gallolyticus with colorectal cancer. The Journal of infectious diseases 203: 1101-1109.
- Bullen JJ, Rogers HJ, Spalding PB, Ward CG (2005). Iron and infection: the heart of the matter. FEMS immunology and medical microbiology 43: 325-330.
- Cercamondi CI, Egli IM, Ahouandjinou E, Dossa R, Zeder C, Salami L et al (2010). Afebrile Plasmodium falciparum parasitemia decreases absorption of fortification iron but does not affect systemic iron utilization: a double stable-isotope study in young Beninese women. The American journal of clinical nutrition 92: 1385-1392.
- de Mast Q, Nadjm B, Reyburn H, Kemna EH, Amos B, Laarakkers CM et al (2009). Assessment of urinary concentrations of hepcidin provides novel insight into disturbances in iron homeostasis during malarial infection. The Journal of infectious diseases 199: 253-262.
- Ellermeier JR, Slauch JM (2008). Fur regulates expression of the Salmonella pathogenicity island 1 type III secretion system through HilD. Journal of bacteriology 190: 476-486.
- Ferruzza S, Scarino ML, Gambling L, Natella F, Sambuy Y (2003). Biphasic effect of iron on human intestinal Caco-2 cells: early effect on tight junction permeability with delayed onset of oxidative cytotoxic damage. Cellular and molecular biology (Noisy-le-Grand, France) 49: 89-99.
- Flint HJ, Duncan SH, Scott KP, Louis P (2007). Interactions and competition within the microbial community of the human colon: links between diet and health. Environmental microbiology 9: 1101-1111.
- Foster SL, Richardson SH, Failla ML (2001). Elevated iron status increases bacterial invasion and survival and alters cytokine/chemokine mRNA expression in Caco-2 human intestinal cells. The Journal of nutrition 131: 1452-1458.
- Gera T, Sachdev HP (2002). Effect of iron supplementation on incidence of infectious illness in children: systematic review. BMJ (Clinical research ed 325: 1142.
- Gordon MA, Graham SM, Walsh AL, Wilson L, Phiri A, Molyneux E et al (2008). Epidemics of invasive Salmonella enterica serovar enteritidis and S. enterica Serovar typhimurium infection associated with multidrug resistance among adults and children in Malawi. Clin Infect Dis 46: 963-969.
- Hansen SL, Ashwell MS, Moeser AJ, Fry RS, Knutson MD, Spears JW (2009). High dietary iron reduces transporters involved in iron and manganese metabolism and increases intestinal permeability in calves. Journal of dairy science 93: 656-665.
- Ibarra JA, Steele-Mortimer O (2009). Salmonella--the ultimate insider. Salmonella virulence factors that modulate intracellular survival. Cellular microbiology 11: 1579-1586.
- Janakiraman A, Slauch JM (2000). The putative iron transport system SitABCD encoded on SPI1 is required for full virulence of Salmonella typhimurium. Mol Microbiol 35: 1146-1155.
- Jepson MA, Collares-Buzato CB, Clark MA, Hirst BH, Simmons NL (1995). Rapid disruption of epithelial barrier function by Salmonella typhimurium is associated with structural modification of intercellular junctions. Infection and immunity 63: 356-359.

- Kemna EH, Tjalsma H, Willems HL, Swinkels DW (2008). Hepcidin: from discovery to differential diagnosis. Haematologica 93: 90-97.
- Kohler H, Sakaguchi T, Hurley BP, Kase BA, Reinecker HC, McCormick BA (2007). Salmonella enterica serovar Typhimurium regulates intercellular junction proteins and facilitates transepithelial neutrophil and bacterial passage. American journal of physiology 293: G178-187.
- Natoli M, Felsani A, Ferruzza S, Sambuy Y, Canali R, Scarino ML (2009). Mechanisms of defence from Fe(II) toxicity in human intestinal Caco-2 cells. Toxicol In Vitro 23: 1510-1515.
- Nchito M, Friis H, Michaelsen KF, Mubila L, Olsen A (2006). Iron supplementation increases small intestine permeability in primary schoolchildren in Lusaka, Zambia. Transactions of the Royal Society of Tropical Medicine and Hygiene 100: 791-794.
- Oppenheimer SJ (2001). Iron and its relation to immunity and infectious disease. The Journal of nutrition 131: 616S-633S; discussion 633S-635S.
- Pandey A, Bringel F, Meyer J-M (1994). Iron requirement and search for siderophores in lactic acid bacteria. Applied Microbiology and Biotechnology 40: 735-739.
- Sazawal S, Black RE, Ramsan M, Chwaya HM, Stoltzfus RJ, Dutta A et al (2006). Effects of routine prophylactic supplementation with iron and folic acid on admission to hospital and mortality in preschool children in a high malaria transmission setting: community-based, randomised, placebo-controlled trial. Lancet 367: 133-143.
- Stecher B, Chaffron S, Kappeli R, Hapfelmeier S, Freedrich S, Weber TC et al (2010). Like will to like: abundances of closely related species can predict susceptibility to intestinal colonization by pathogenic and commensal bacteria. PLoS pathogens 6: e1000711.
- Teixido L, Carrasco B, Alonso JC, Barbe J, Campoy S (2011). Fur activates the expression of Salmonella enterica pathogenicity island 1 by directly interacting with the hilD operator in vivo and in vitro. PloS one 6: e19711.
- Tompkins GR, O'Dell NL, Bryson IT, Pennington CB (2001). The effects of dietary ferric iron and iron deprivation on the bacterial composition of the mouse intestine. Current microbiology 43: 38-42.
- Werner T, Wagner SJ, Martinez I, Walter J, Chang JS, Clavel T et al (2011). Depletion of luminal iron alters the gut microbiota and prevents Crohn's disease-like ileitis. Gut.
- WHO, UNICEF (2004). Focusing on anaemia: Towards an integrated approach for effective anaemia control.
- WHO (2006). Conclusions and recommendations of the WHO Consultation on prevention and control of iron deficiency in infants and young children in malaria-endemic areas.
- WHO (2007). Conclusions and recommendations of the WHO Consultation on prevention and control of iron deficiency in infants and young children in malaria-endemic areas. Food Nutr Bull 28: S621-627.
- WHO, UNICEF (2009). Diarrhoea: why children are still dying and what can be done.
- Zimmermann MB, Hurrell RF (2007). Nutritional iron deficiency. Lancet 370: 511-520.
- Zimmermann MB, Chassard C, Rohner F, N'Goran E K, Nindjin C, Dostal A et al (2010). The effects of iron fortification on the gut microbiota in African children: a randomized controlled trial in Cote d'Ivoire. The American journal of clinical nutrition 92: 1406-1415.

Chapter 6

Iron induced virulence of Salmonella Typhimurium at the intestinal epithelial interface can be suppressed by carvacrol

Guus AM Kortman[1], Rian WHM Roelofs[1], Dorine W Swinkels[1], Marien I de Jonge[1,2], Sara A Burt[3], Harold Tjalsma[1]

[1] Department of Laboratory Medicine – Laboratory of Genetic, Endocrine and Metabolic Diseases. Radboud Institute for Molecular Life Sciences (RIMLS) of the Radboud University Medical Center, P.O. Box 9101, 6500 HB Nijmegen, The Netherlands

[2] Department of Pediatrics – Laboratory of Pediatric Infectious Diseases of the Radboud University Medical Center, P.O. Box 9101, 6500 HB Nijmegen, The Netherlands

[3] Institute for Risk Assessment Sciences, Veterinary Public Health Division, Faculty of Veterinary Medicine, Utrecht University, P.O. Box 80175, 3508 TD Utrecht, The Netherlands

This chapter is published in Antimicrobial Agents and Chemotherapy. 2014 Mar;58(3):1664-70

Abstract

Oral iron therapy can increase the abundance of bacterial pathogens, e.g. *Salmonella* spp. in the large intestine of African children. Carvacrol is a natural compound with antimicrobial activity against various intestinal bacterial pathogens, among which is the highly prevalent *Salmonella enterica* serovar Typhimurium. This study aimed to explore a presumed interaction between carvacrol and bacterial iron handling and to assess the potential of carvacrol in preventing the increase of bacterial pathogenicity during high iron availability. *S.* Typhimurium was cultured with increasing concentrations of iron and carvacrol to study the effects of these combined interventions on growth, adhesion to intestinal epithelial cells and iron uptake/influx in both bacterial and epithelial cells. In addition, the ability of carvacrol to remove iron from the high-affinity ligand transferrin and a Fe-dye complex was examined. Carvacrol retarded growth of *S.* Typhimurium at all iron conditions. Furthermore, iron-induced epithelial adhesion was effectively reduced by carvacrol at high iron concentrations. The reduction of growth and virulence by carvacrol was not paralleled by a change in iron uptake or influx into *S.* Typhimurium. In contrast, bioavailability of iron for epithelial cells was moderately decreased under these conditions. Further, carvacrol was shown to lack the properties of an iron-binding molecule, however, it was able to weaken iron-ligand interactions by which it may possibly interfere with bacterial virulence. In conclusion, our *in vitro* data suggest that carvacrol has the potential to serve as a novel dietary supplement to prevent pathogenic overgrowth and colonization in the large intestine during oral iron therapy.

Introduction

Salmonella enterica serovar Typhimurium is a major cause of gastroenteritis and invasive disease in humans, particularly in susceptible children in tropical Africa (Graham 2010). Concurrently, there is a high prevalence of iron deficiency among children in developing countries. These children require oral iron therapy to prevent developmental impairment (WHO and UNICEF 2004). Precariously, growth and colonization of *S.* Typhimurium appears to be stimulated by increased luminal iron availability *in vitro*, as well as *in vivo*, in several but not all published studies (Dostal et al 2012a, Dostal et al 2012b, Kortman et al 2012, Zimmermann et al 2010). Thus, there is an unfulfilled need to suppress this common intestinal pathogen.

Carvacrol is a monoterpenoid phenol present in essential oils, it has antimicrobial activity and is one of the main components in oregano and thyme oil (Burt 2004). It has been shown to be active against various intestinal pathogens, including *S.* Typhimurium (Burt 2004). Carvacrol appears to target the outer membrane of gram-negative bacteria and increases membrane permeability, hereby causing depletion of the cellular ATP pool, leakage of other cytoplasmic constituents and outer membrane-associated material (Burt 2004, Helander et al 1998). However, this process is currently not fully understood and additional modes of action might exist. It has previously been shown that subinhibitory concentrations of carvacrol can affect virulence traits as it slightly reduced adhesion of *S.* Typhimurium to intestinal epithelial cells and significantly reduced invasion into these cells (Inamuco et al 2012). In contrast, it was shown that increased iron availability promoted *S.* Typhimurium adhesion to, and invasion into, intestinal epithelial cells (Kortman et al 2012) (**Chapter 5**). Further, carvacrol was shown to increase expression of the heat shock protein GroEL and inhibit the synthesis of flagellin in *Escherichia coli* O157:H7 when grown in a standard rich medium (Burt et al 2007). On the other hand, moderate to high iron availability reduced GroEL expression and increased flagellin synthesis in *S.* Typhimurium compared to low iron availability (Kortman et al, unpublished data). The above findings inspired us to speculate that carvacrol may exert (in part) its antimicrobial mode of action through interference with pathways for bacterial iron handling. The present study aimed to investigate this hypothesis and to explore the potential of carvacrol to serve as a food additive to prevent pathogenic overgrowth and colonization in the large intestine during oral iron therapy, by an *in vitro* approach.

Materials and methods

Bacterial strain, media and growth conditions

The strain used in this study was *Salmonella* Typhimurium *NTB6* (Kortman et al 2012). This bacterium was cultured at 37˚C and 5% CO_2 (standard conditions) in Iscove's Modified Dulbecco's Medium (IMDM, Invitrogen). This chemically defined medium does not contain iron in its formulation.

Growth curves and determination of the Minimal Inhibitory Concentration (MIC)

To determine the effects of iron and carvacrol on growth of *S.* Typhimurium, IMDM medium with increasing ferric citrate (Sigma-Aldrich) and carvacrol (Sigma-Aldrich) concentrations was inoculated with a fresh bacterial culture after overnight growth. A stock solution of ferric citrate was made in MilliQ water (MQ) and a 0.1 mol/L stock of carvacrol was made in absolute ethanol. Final concentrations of ethanol were kept constant at 2.5% (v/v) for each condition. Portions of 200 µL culture medium were inoculated in a microplate (in duplicate) and incubated statically at 37˚C within a plate reader. To monitor bacterial growth, the optical density was automatically measured at 620 nm (OD_{620}) every 20 min.

To determine the lowest concentration of carvacrol that inhibits the visible growth of *S.* Typhimurium *NTB6* in IMDM, a serial dilution of carvacrol (0, 0.75, 1.0, 1.25, 1.5, 1.75, 2.0 and 2.5 mmol/L) in IMDM in the presence of 0, 1, 10, or 100 µmol/L ferric citrate was tested. 2 ml media in sterile 75x12 mm capped tubes was inoculated with a fresh bacterial culture and the OD_{620} was assessed after overnight incubation at 37˚C. A negative control containing no bacteria was included. The MIC value, for the specific incubation conditions applied in the present study, was defined as the lowest concentration where no increase in optical density was observed and concentrations of carvacrol below the MIC (and above 0 mmol/L) that still supported growth were defined as subinhibitory. It should be noted that we chose to determine the MIC values for the growth medium that was used in this study to sustain consistent growth conditions. If desired in future studies, the MIC should be determined in a more standard reference growth medium (see e.g. CLSI or EUCAST recommendations) (CLSI , EUCAST).

Cell line, media and growth conditions

The human colorectal adenocarcinoma cell line Caco-2 (obtained from the American Type Culture Collection) was cultured at standard conditions in DMEM (Lonza) supplemented with 10% heat-inactivated FBS (Invitrogen), 20 mmol/L HEPES, 100 nmol/L nonessential amino acids (Invitrogen), and 2 mmol/L L-glutamine (Lonza). Cells were subcultured every 6 days and used between passage numbers 3-6.

Bacterial adhesion assay

The influence of iron and carvacrol on adhesion of S. Typhimurium to Caco-2 cells was studied by the use of the following adhesion assay.

Pre-incubation of bacteria with ferric citrate and carvacrol: IMDM with increasing ferric citrate concentrations (0-50 µmol/L) and carvacrol concentrations (0-0.5 mmol/L) was inoculated with *S.* Typhimurium (overnight culture) and grown to exponential phase. The bacterial cells were pelleted, resuspended and concentrated in IMDM with 10% glycerol for storage at -80˚C until use. Serial dilutions of thawed stocks were transferred to blood agar plates and incubated overnight to determine the colony forming units (CFU).

Culturing of Caco-2 cells: Caco-2 cells were subcultured in a 24-well plate and maintained until use in adhesion assays. The assays were performed on confluent monolayers between 14-16 days after seeding the cells.

Bacterial adhesion assay: The Caco-2 monolayers were washed once with PBS. The pre-

incubated stocks of *S.* Typhimurium were pelleted and resuspended in IMDM. Next, bacteria were added to the monolayers at a multiplicity of infection (MOI) of 10:1 in IMDM followed by incubation for 2 h at standard conditions. To determine the number of adherent bacteria, monolayers were washed three times with PBS, cells were trypsinized and lysed with ice-cold PBS containing 0.025% Triton X-100. Serial dilutions of cell lysates were transferred to blood-agar plates for CFU counting.

Determination of iron uptake by *S.* Typhimurium under influence of carvacrol

To determine the effect of carvacrol on iron uptake by *S.* Typhimurium, bacterial cells were grown with iron and carvacrol after which total iron content of the bacteria was determined. 10 mL cultures of *S.* Typhimurium were incubated with ferric citrate (0-50 µmol/L) and carvacrol (0-0.5 mmol/L) under standard conditions. Bacteria were harvested by centrifugation for 5 min at 3220 x g and pellets were resuspended in 5 mL PBS containing 1 mmol/L deferoxamine (DFO; Sigma-Aldrich) to sequester iron that is loosely bound to the outer membrane of the bacteria. After another centrifugation step, pellets were resuspended in 1.5 mL PBS and transferred to fresh 2-mL tubes. Tubes were centrifuged for 5 min at 10,000 x g and the supernatant was removed. Pellets were snap frozen in liquid nitrogen (3x), wet pellet weight was noted and iron content was determined by acid digestion of the bacteria and use of the chromogen bathophenanthroline as described by Torrance and Bothwell (Torrance and Bothwell 1968). To this purpose, bacterial pellets were resupended in 80 µL of acid reagent (3 mol/L HCl containing 10% trichloroacetic acid) and incubated at 65˚C for 20 h with continuous mixing. After incubation, samples were centrifuged to obtain the supernatant and 20 µL was applied to a microplate. Then, 200 µL chromogenic reagent containing 127 mg/L bathophenanthroline sulphonate disodium salt (Fluka), 936 mg/l thioglycolic acid sodium salt (Sigma-Aldrich) and sodium acetate (2.2 times diluted from a saturated solution) was added to the samples. After 10 min incubation at room temperature (RT) color formation was measured at 540-560 nm. Iron concentration was determined by comparison to a standard curve made of ferrous sulfate and was corrected for wet pellet weight.

Determination of iron bioavailability to Caco-2 cells

To examine the effect of carvacrol on the uptake of iron by enterocytes, Caco-2 cells were exposed to iron and carvacrol. Iron uptake was quantified by measurement of intracellular ferritin levels.

Culturing of Caco-2 cells: As described above, with the exception that the assays were performed between 13-14 days after seeding the cells.

Exposure of Caco-2 cells to iron and carvacrol: 2 days before incubation of the cells with iron and carvacrol the standard culture medium was replaced by IMDM. Both ferric citrate and ferrous sulfate (20 µmol/L Fe) in combination with carvacrol (0-0.3 mmol/L) or ascorbic acid (200 µmol/L) and tannic acid (2 µmol/L) as positive and negative control respectively, were applied to the cells in fresh IMDM. Higher concentrations of carvacrol could not be tested because of cytotoxic effects to the cells. The cells were incubated for 20 h at standard conditions. At harvesting cells were washed once with 1 mL PBS containing 1 mmol/L DFO

and washed once with 1 mL PBS. Cells were frozen on dry ice and after thawing 100 µL of 5 mmol/L NaOH was added. Cells were then lysed by sonication for 15 min in a sonication bath containing ice water. To neutralize, 50 µL of 10 mmol/L HCl was added and cells were loosened by scraping. Suspensions were sonicated 5 min and then transferred to fresh tubes. Debris was pelleted by centrifugation and the supernatant was used for quantification of total protein and ferritin.

Quantification of ferritin levels: Samples were applied to the ferritin ELISA (Spectro Ferritin, Ramco) and the assay was performed according to the manufacturer's instructions. Ferritin levels were normalized for total protein content. Total protein was measured by the use of a Bradford protein assay (Biorad) according to the manufacturer's instructions.

Iron removal from transferrin assay

To examine the potential of carvacrol to sequester iron, the ability of carvacrol to remove iron from transferrin was investigated. Incubation buffer containing 100 mmol/L Tris-HCl (pH 7.5) and 10% glycerol (v/v) was depleted of trace iron by pre-treatment with Chelex-100 resin (Sigma-Aldrich). Then, 50 µg of holo-transferrin (hTf) from human (Sigma-Aldrich) was dissolved in 50 µL incubation buffer and hTf was incubated with carvacrol (0-250 µmol/L) and positive controls DFO (500 µmol/L) and tannic acid (Sigma-Aldrich; 100 µmol/L) for 18 h at 37˚C. The iron binding status of transferrin (Tf) was investigated by polyacrylamide gel electrophoresis according to the method described by Makey and Seal, 1976 (Makey and Seal 1976) with a few adaptations. Briefly, a freshly prepared solution containing 6% acrylamide (37.5:1 with bis-acrylamide), 6 mol/L urea and TBE buffer (0.1 mol/L Tris; 0.01 mol/L boric acid; 0.05 mol/L EDTA, pH 8.4) was pre-treated with chelex-100 resin and then polymerized in a BioRad Protean II vertical minigel system. The gel was pre-run at 70V for 20 min, and 20 µg of the Tf samples was loaded. Partially saturated human transferrin (Sigma-Aldrich) was used as a marker standard (20 µg). The gel tray was placed in ice water and electrophoresis was performed at 100 V for 7 h. After electrophoresis the gel was fixed for 15 min in fixative containing 10% (v/v) acetic acid, 50% (v/v) methanol and 40% demineralized water (v/v). To visualize forms of Tf, it was stained with Coommassie Brilliant Blue.

Iron removal from CAS assay

A universal chemical assay for the detection of iron acquisition siderophores was used to examine the capability of carvacrol to remove iron from a dye-Fe complex. Chrome Azurol S (CAS) in complex with ferric iron and hexadecyltrimethylammonium bromide (HDTMA) has a blue color, which turns orange when iron is removed from the dye by a stronger ligand. The method used was originally described by Schwyn & Neilands (1986) (Schwyn and Neilands 1987). Briefly, a 2 mmol/L CAS (Tokyo Chemical Industry) solution was prepared and 1875 µL was mixed with 375 µL ferric iron solution (1 mmol/L FeCl3 (Merck) in 10 mmol/L HCl). Separately, 1.5 mL of 10 mmol/L HDTMA (Sigma-Aldrich) was placed in an acid cleaned volumetric flask of 25 mL and the CAS-iron mixture was slowly added with simultaneous mixing. Then 1.077 g of anhydrous piperazine (Fluka) was dissolved in 7 mL MQ and 1560 µL of 12 mol/L HCl was added. This buffer was rinsed into the volumetric

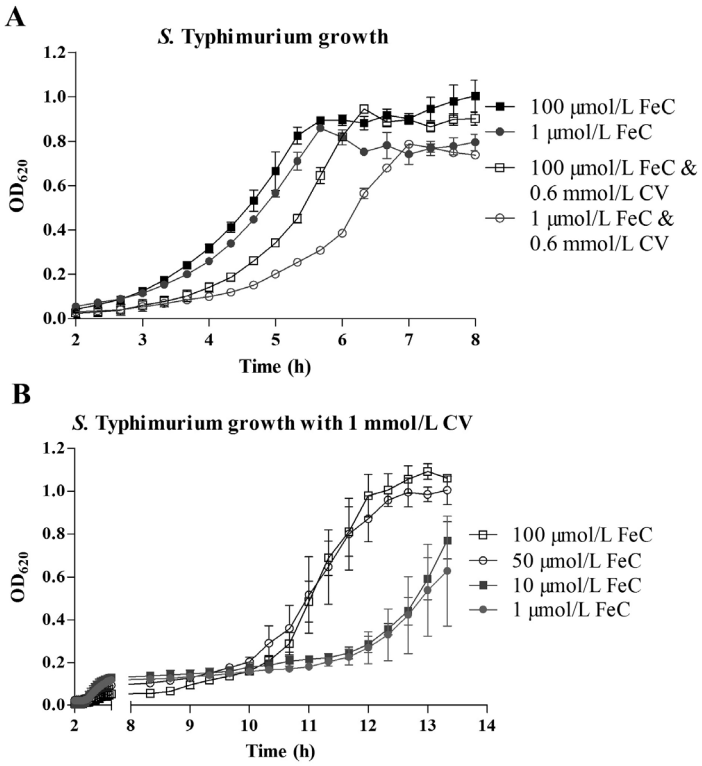


Figure 1. Effect of iron and subinhibitory concentrations of carvacrol on growth of *S.* Typhimurium.

(**A**) Effect of 0.6 mmol/L carvacrol (CV) on *S.* Typhimurium growth (mean ± range) during 8 h in medium with 1 µmol/L (open circles) or 100 µmol/L ferric citrate (FeC) (open squares) compared to the same conditions without carvacrol (filled circles and squares, respectively) (n = 2; duplicate wells). (**B**) Effect of 1mmol/L carvacrol on growth of *S.* Typhimurium during 13.3 h with 100 (open squares), 50 (open circles), 10 (filled squares), or 1 µmol/L ferric citrate (filled circles), n = 2. Without addition of any iron growth is more hampered as the trace amount of iron in the IMDM limits growth of *S.* Typhimurium (Kortman et al 2012).

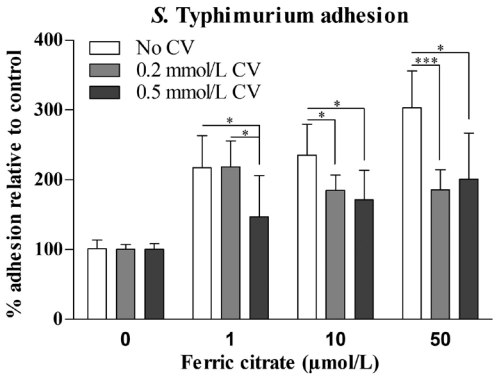


Figure 2. Effect of carvacrol on iron-induced adhesion of *S.* Typhimurium to an epithelial monolayer.

Adhesion (mean + SD) of *S.* Typhimurium to a monolayer of Caco-2 cells is given as percentage of the inoculum and expressed relative to the conditions without iron during pre-incubation. Data consists of 3 separate experiments in which adherent colony forming units were determined on different days with duplicate wells, n = 6. * $p < 0.05$; *** $p < 0.001$.

flask and MQ was added to 25 mL to complete the assay solution. The solution was stored in a plastic tube in the dark. To establish increased speed in iron exchange, a shuttle solution was prepared which involved the addition of 25.42 mg 5-sulfosycilic acid dihydrate (Sigma-Aldrich) to the assay solution before adding the final MQ to a total of 25 mL.
Iron removal capacity and speed of iron removal was tested in a microplate. 100 µL carvacrol (0-1 mmol/L in IMDM or MQ), DFO (0-20 µmol/L in IMDM or MQ), or tannic acid (0-5.9 µmol/L in IMDM or MQ) was mixed with 100 µL CAS assay (shuttle) solution. Absorption was periodically monitored at 620 nm.

Statistical analysis

To compare means, an unpaired t-test (2-tailed) was used. In case of unequal variances (as assessed by F-test), an unpaired t-test with Welch's correction was carried out. To assess the slope of iron dependent adhesion, linear regression analysis was applied. For comparison of iron uptake by *S.* Typhimurium under influence of carvacrol, two-way ANOVA was performed. All analyses were performed using GraphPad Prism version 5.00 for Windows, GraphPad Software, San Diego California USA. p-values < 0.05 were considered statistically significant.

Results

Growth of S. Typhimurium in response to iron and carvacrol

To evaluate the possibility that the antimicrobial effect of carvacrol can be explained by its interference with bacterial iron handling, the effect of carvacrol on growth of *S.* Typhimurium was tested under moderate iron and iron rich conditions. In general, these experiments showed that carvacrol can delay the onset of growth of this bacterium. Growth delay was established under both moderate iron concentrations and high iron conditions (**Figure 1A and supplementary Figure 1**). In the absence of carvacrol the high iron conditions were not influencing growth as compared to the moderate iron concentrations. However, in the presence of carvacrol a shorter lag phase is observed with culture medium containing high iron concentrations as compared to growth with moderate iron concentrations. This suggests that high iron availability partly compensates the postponement effect of carvacrol. Notably, increased carvacrol concentration of 1 mmol/L strongly retarded growth, but growth was strikingly less retarded in 50-100 µmol/L ferric citrate as compared to 1-10 µmol/L ferric citrate (**Figure 1B**). Levels of carvacrol ≤ 1 mmol/L were found to be the subinhibitory range under these conditions as 1 mmol/L carvacrol was the highest concentration tested that still supported growth under all iron conditions. A slightly higher concentration of 1.25 mmol/L effectively prevented an increase in optical density after overnight growth (MIC).

Adhesion of *S.* Typhimurium to an epithelial monolayer

Adhesion to host epithelial cells is an important virulence characteristic for pathogenic bacteria. To test whether iron-induced adhesion, as observed previously (Kortman et al 2012), can be counteracted by carvacrol, *S.* Typhimurium was first pre-incubated with

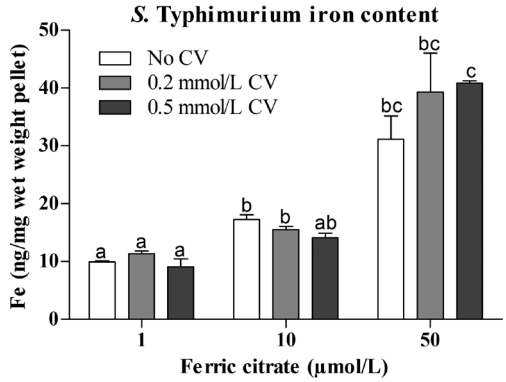


Figure 3. Total iron content of *S.* Typhimurium after growth with iron and carvacrol.
S. Typhimurium was grown to mid-log phase with increasing concentrations of iron and carvacrol. Iron content (mean + range) is given as ng per mg wet biomass, n = 2. Means without a common letter differ significantly, $p < 0.05$.

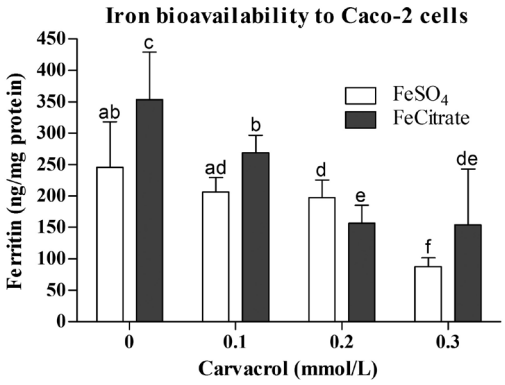


Figure 4. Influence of carvacrol on the bioavailability of iron to an epithelial monolayer.
Caco-2 cells were exposed to equimolar concentrations of iron and increasing concentrations of carvacrol. Bioavailability of iron is expressed as total intracellular ferritin content, corrected for total protein (mean + SD). Data consists of 2 separate experiments with measurements performed in triplicate, n = 6. Means without a common letter differ significantly, $p < 0.05$.

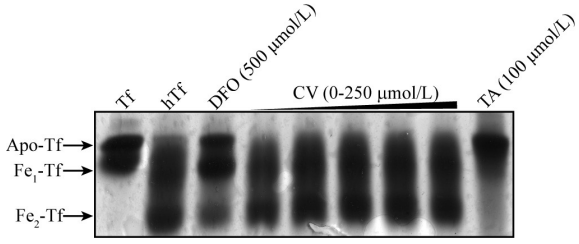


Figure 5. Iron from transferrin removal assay hTf was incubated with increasing concentrations of carvacrol and the positive controls DFO and tannic acid (TA) to assess iron removal from hTf. Lane 1: partially saturated Tf, lane 2: hTf control, lane 3: 500 µmol/L DFO, lane 4: 0 µmol/L carvacrol (CV) solvent control, lane 5: 1 µmol/L CV, lane 6: 10 µmol/L CV, lane 7: 100 µmol/L CV, lane 8: 250 µmol/L CV, lane 9: 100 µmol/L TA. Upper arrow indicates Tf without iron bound to it, middle arrow indicates Tf with 1 Fe-atom bound, lower arrow indicates hTf with 2 Fe-atoms bound to the protein.

increasing concentrations of ferric citrate and carvacrol. To determine the adhesion, pre-incubated bacteria were added to intestinal epithelial monolayers (Caco-2) under iron limiting conditions and without carvacrol. Adhesion was expressed as the percentage of the bacterial inoculum and relative to the conditions without iron addition during pre-incubation. As shown in **Figure 2**, iron stimulated the adhesion of *S.* Typhimurium, however this effect was significantly reduced by carvacrol, especially at the highest iron concentration tested. It should be noted that carvacrol did not significantly affect adhesion in the condition without iron (**Supplementary Figure 2**). Linear regression analysis revealed that the slope of the control conditions without carvacrol (2.79 ± 0.50) was significantly steeper than the slope of the 0.2 mmol/L carvacrol conditions (0.51 ± 0.20) ($p = 0.013$). Slope of the conditions with 0.5 mmol/L carvacrol (1.47 ± 0.48) was lower compared to the controls, although not significantly ($p = 0.129$). Taken together, these experiments show that subinhibitory levels of carvacrol can reduce iron-induced adhesion of *S.* Typhimurium to an epithelial monolayer.

Iron uptake by *S.* Typhimurium under influence of carvacrol

One of the mechanisms by which carvacrol could influence bacterial adhesion is the inhibition of iron uptake by *S.* Typhimurium, which consequently would affect adhesion traits. To investigate this, we measured total iron content of *S.* Typhimurium cells which were grown in the presence of increasing concentrations of ferric citrate and carvacrol. The iron content of the bacteria grown without carvacrol was significantly increased in the 10 µmol/L ferric citrate condition compared to the 1 µmol/L ferric citrate condition ($p = 0.012$) and further increased with 50 µmol/L ferric citrate ($p = 0.034$ compared to 1 µmol/L ferric citrate), indicating increased iron uptake at increasing iron concentration as expected (**Figure 3**). Carvacrol appeared not to influence iron uptake or influx significantly at all iron concentrations tested (two-way ANOVA). As carvacrol did not reduce the uptake of iron by *S.* Typhimurium, the inhibiting effect on adhesion can probably not be explained by altered iron uptake.

Bioavailability of iron to intestinal epithelial cells under influence of carvacrol

To examine the effect of carvacrol on intestinal iron uptake *in vitro*, iron together with nontoxic concentrations of carvacrol were applied to Caco-2 monolayers. Next to ferric citrate, ferrous sulfate was tested as this form of iron is widely used in iron supplementation studies. Cytoplasmic levels of ferritin were used as a measure for cellular iron uptake. As shown in **Figure 4**, in the absence of carvacrol bioavailability of ferric citrate was higher compared to ferrous sulfate ($p = 0.004$). Bioavailability of both iron sources was considerably reduced by carvacrol; ferritin formation was about 2.5 times less with 0.3 mmol/L carvacrol compared to the no carvacrol controls ($p < 0.0002$ for both iron sources). Notably, carvacrol did not abolish iron uptake, while the addition of the food derived and strong iron binding tannic acid at 2 µmol/L resulted in a near complete block of iron uptake (**Supplementary Figure 3A**). We note that higher concentrations of carvacrol could not be tested due to toxic effects to the cells, as determined by a LDH-release assay (**Supplementary Figure 4**). In contrast, 200 µmol/L ascorbic acid as a known promoter of iron uptake enhanced iron uptake of both iron sources, especially of

ferric citrate ($p = 0.003$ and $p < 0.0001$ for ferrous sulfate and ferric citrate respectively) (**Supplementary Figure 3B**).

Iron binding capacity of carvacrol

The potential iron binding capacity of carvacrol, or its ability to dissociate iron from iron binding ligands, was investigated in an iron from transferrin removal assay and a universal siderophore CAS assay. The iron from transferrin removal assay revealed that carvacrol up to 0.25 mmol/L could not take away iron from transferrin, indicating that carvacrol is not a high-affinity iron binding molecule and cannot dissociate the transferrin-iron complex (**Figure 5**). In contrast, use of the strong iron-binding molecules DFO and tannic acid that served as positive controls in this assay, clearly showed their ability to remove of 1 or 2 iron atoms from the transferrin molecule.

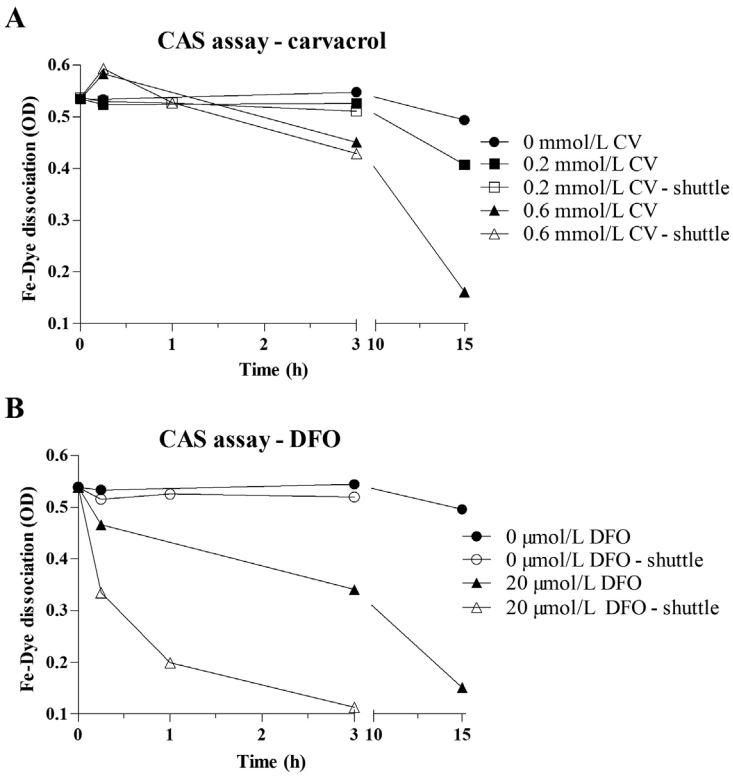


Figure 6. Effect of carvacrol on the stability of the CAS-Fe complex.

Removal of iron from the colored complex is displayed as a decrease in optical density. Carvacrol or DFO was incubated with the CAS-Fe complex without and with shuttle solution (filled and open symbols respectively). (**A**) Dissociation of the Fe-dye complex in time was monitored without addition of carvacrol (filled circles) and with addition of 0.2 mmol/L (filled and open squares) or 0.6 mmol/L carvacrol (filled and open triangles). (**B**) As control conditions, the CAS-Fe complex was incubated without DFO (filled and open circles) and with addition of 20 µmol/L DFO (filled and open triangles).

As shown in **Figure 6A**, the siderophore-CAS assay revealed that carvacrol can dissociate the Fe-dye complex partly (0.2 mmol/L) or completely (0.6 mmol/L), however this was only observed after a long incubation of 15 hours, while dissociation of the Fe-dye complex was already evident after only 3 hours for the positive controls DFO (**Figure 6B**) and tannic acid (not shown) at low concentrations. CAS-shuttle solution increased the transfer speed of Fe from the dye to DFO, but this was not true for carvacrol (**Figure 6 A&B**). Together, these results indicate that carvacrol does not bind iron strongly, but it may be able to disturb iron-ligand complexes under certain conditions.

S. Typhimurium global LPS composition during growth with iron and carvacrol

As LPS is an important factor in bacterial adhesion (Bravo et al 2011, Kong et al 2011) and carvacrol is known to induce the release of LPS from *E. coli O157:H7* (Helander et al 1998), carvacrol could affect adhesion characteristics of *S.* Typhimurium by removing LPS from the cell surface. We therefore examined the effect of carvacrol on LPS content and global LPS composition during growth under moderate to high iron conditions. As shown in **Supplementary Figure 5**, carvacrol up to 0.5 mmol/L did not detectably influence LPS quantity or global LPS composition. It should be noted that this general analysis cannot visualize LPS in great detail and only indicates that carvacrol did not alter global LPS status of *S.* Typhimurium during growth, which makes it less likely that iron-induced adhesion was reduced by modulating LPS integrity at the bacterial surface.

Discussion

Oral iron supplementation has potential harmful side effects on gastrointestinal health as a consequence of increased colonization and growth of bacterial pathogens (Kortman et al 2012, Zimmermann et al 2010) (**Chapter 5**). As a result there is a demand for safer iron supplementation programmes. Therefore, we here assessed by an *in vitro* approach whether the simultaneous addition of carvacrol as an antimicrobial agent could counteract the potential adverse effect of iron on the prevalent intestinal pathogen *S.* Typhimurium.
In the present study, we showed that subinhibitory concentrations of carvacrol retarded bacterial growth under all tested conditions. Notwithstanding the presence of sufficient amount of iron in all conditions, the growth-retardation effect of carvacrol was most pronounced under conditions of moderate iron availability. This suggested that its antimicrobial activity is partly related to an iron scavenging effect that is less effectual under high iron conditions. Nevertheless, we want to emphasize that subinhibitory concentrations of carvacrol delayed growth of *S.* Typhimurium under all conditions. Hence, the previously described antimicrobial activity of carvacrol also applies for conditions with high iron availability (Burt 2004). Although the ability to replicate is important for bacterial pathogens, the adhesion to host tissues or epithelium is pivotal to establish an infection. Our current data confirm previous findings that iron induces adhesion of *S.* Typhimurium to intestinal epithelial cells *in vitro* (Kortman et al 2012) (**Chapter 5**). Importantly, pre-incubation of *S.* Typhimurium with subinhibitory concentrations of carvacrol clearly reduced adhesion induced by moderate to high iron concentrations. Together, these observations support

the use of carvacrol as a potential additive in oral iron preparations to prevent pathogenic overgrowth and colonization during iron supplementation.

An antimicrobial additive in an iron preparation should not decrease iron uptake from the intestinal lumen or, ideally, should increase it. We therefore investigated the effect of carvacrol on the uptake of iron from different sources by intestinal epithelial Caco-2 cells. Expression of ferritin was used as a read-out, which is commonly used as a gold standard to study bioavailability of iron preparations (Glahn et al 1998). Although carvacrol clearly restricted iron uptake of both ferrous sulfate and ferric citrate, it did not block iron uptake completely like tannic acid did, a well-known iron binder (South and Miller 1998). In general, the bioavailability of ferrous iron is better than ferric iron (Hernandez et al 2003, Santiago 2012, Yokoi et al 2009), but with ferric citrate higher amounts of ferritin were measured as compared to ferrous sulfate, indicating that in this system the bioavailability of ferric citrate is better. The higher solubility and stability of ferric citrate compared to ferrous sulfate is probably the explanation for this observation. Thus, carvacrol seems not to meet the ideal criteria of an iron additive as it impairs iron uptake by intestinal cells *in vitro*. Nevertheless, this decreased level of iron bioavailability could still suffice for adequate iron uptake and healthy iron homeostasis in vivo, whereas it may be accompanied by a desirable decrease in virulence of pathogenic gut bacteria.

Iron scavenging by carvacrol could have explained the growth delay and decreased adhesion of *S.* Typhimurium under moderate iron conditions and the decreased bioavailability of iron to Caco-2 cells in the presence of carvacrol. However, based on the molecular structure of carvacrol it seems unlikely that it can bind iron ions. Additionally, in the present study we showed that carvacrol could not remove iron from the high affinity protein transferrin, but was able to dissociate a dye-Fe complex during prolonged incubations. Together, this indicates that carvacrol seems not to bind iron directly, but is able to disturb iron-ligand complexes with lower affinity. It can therefore be envisaged that carvacrol may interfere with iron uptake pathways of intestinal epithelial cells rather than making it less bioavailable by scavenging of iron ions. However, it remains to be elucidated by which molecular mechanisms carvacrol could affect cellular iron uptake systems. In contrast to the reduced iron uptake by intestinal epithelial cells, we were not able to show a significant effect of carvacrol on iron uptake or influx into *S.* Typhimurium. Consequently, the observed effects of subinhibitory concentrations of carvacrol on iron-induced growth and adhesion can probably not be explained by interference with bacterial iron uptake or increased influx, but instead seems predominantly caused by other (iron-independent) activities of carvacrol.

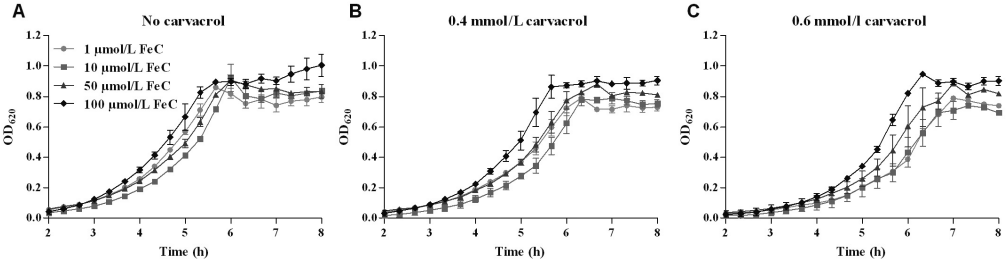
Although total iron content of *S.* Typhimurium was not altered by carvacrol, the free intracellular iron pool balance could be distorted. A potential elevation of the intracellular "free" iron pool may enhance cell stress via generation of oxygen radicals (Fenton chemistry). This increased iron toxicity may contribute to an overall reduced fitness of the bacteria, which could very well result in the observed adhesion defects under iron-replete conditions in the presence of carvacrol. If true it remains difficult to explain why *S.* Typhimurium grows slightly better in medium with carvacrol and high iron concentrations, but adheres less well under these conditions when compared to bacteria grown under intermediate iron conditions with carvacrol. Another possible explanation for the carvacrol-induced

delay in growth and decreased adhesion of *S.* Typhimurium involves a decrease in outer membrane integrity/stability or an altered surface structure. Carvacrol is known to induce release of LPS from *E. coli O157:H7* (Helander et al 1998). Similarly, iron depletion is associated with reduced LPS content in Helicobacter pylori (Keenan et al 2008). As LPS is a known modulator of bacterial adhesion, both iron concentration and addition of carvacrol could therefore theoretically affect *S.* Typhimurium adhesion. However, the outcome of our first LPS characterization experiments did not show a clear alteration of LPS content or structure when *S.* Typhimurium was grown in the presence of iron and carvacrol. Nevertheless, carvacrol and high concentrations of iron could still destabilize the bacterial outer membrane through interference with divalent cations such as Mg^{2+} and Ca^{2+} that bind to the anionic LPS and hereby stabilize the outer membrane (Nikaido 2003). Carvacrol and iron could somehow distort this delicate balance and thereby indirectly influence LPS-mediated adhesion. To asses this hypothesis, the potential interaction of iron and carvacrol with LPS needs to be investigated further in greater detail. This could involve the detection and characterization of LPS shed into the medium using additional LPS characterization techniques.

In summary, the natural antimicrobial carvacrol can potentially be used as an additive during oral iron therapy to prevent pathogenic overgrowth and colonization in the large intestine. Future *in vivo* research should reveal whether carvacrol can indeed improve safety of oral iron therapy. The success of this approach will depend on defining an optimal dose of carvacrol which exerts its antimicrobial effect in the host's intestines, but does not inhibit improvement of host iron status. One challenge in dosing is the absorption of carvacrol by the host upper GI-tract which likely results in low concentrations in the colon and reduced effectiveness in this target organ. This is probably the reason that oral carvacrol administration was previously shown to have little effect on colonic microbiota composition in piglets (Michiels et al 2010). Nevertheless, gastrointestinal health in these piglets was improved which could for instance relate to a reduction in virulence of low abundant gut pathogens, whereas other modes of carvacrol delivery, e.g.in encapsulated form, still need to be examined (Michiels et al 2010). Importantly, the latter approach could be used for the targeted delivery of antimicrobial activity in the human colon during oral iron administration with minimal effect on iron uptake in the ileum of the host. Subinhibitory concentrations of carvacrol are nevertheless likely to occur in the colon. Nonetheless, subinhibitory concentrations are effective in reducing bacterial virulence as we show in the present study and which is supported by previous findings (Inamuco et al 2012, van Alphen et al 2012). Under the experimental culture conditions of our study a concentration of carvacrol $\geq$ 0.2 mmol/L was already effective in reducing iron induced adhesion, while concentrations $\leq$ 0.3 mmol/L were well tolerated by the differentiated intestinal epithelial cell line Caco-2. The latter is supported by previous findings (Fabian et al 2006) and previous *in vivo* studies have shown that carvacrol as a feed additive is well tolerated and does not show signs of great cytotoxicity (Michiels et al 2010, Windisch et al 2008). Another challenge is to simultaneously find the optimal iron preparation and dose for effective improvement of host iron status, with minimal effect on the gut microbiota. Although we did not test the effect of iron carrying substances other than ferric citrate on bacterial virulence, we

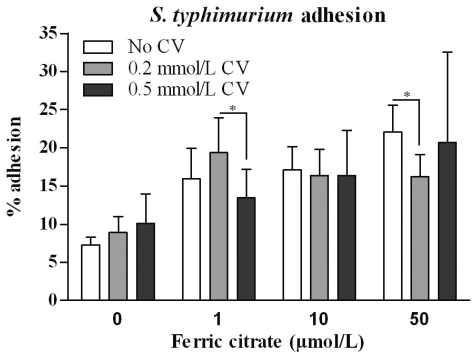
believe that most soluble iron sources will enhance the virulence of intestinal pathogens as we previously showed for ferric citrate (Kortman et al 2012). Taken together, carvacrol is a known natural dietary additive, with the potential to prevent gastrointestinal side effects during oral iron therapy in infection endemic regions. Nevertheless, it goes without saying that future in vivo studies are required to further evaluate the real therapeutic utility of carvacrol as anti-infective agent in combination with dietary iron supplements.

Supplementary Figures and legends



Supplementary Figure 1. Effect of iron and subinhibitory concentrations of carvacrol on growth of *S.* Typhimurium.

Effect of carvacrol on *S.* Typhimurium growth (mean ± range) in medium with 1 µmol/L (grey circles), 10 µmol/L (squares), 50 µmol/L (triangles), or 100 µmol/L ferric citrate (black circles), n = 2. (**A**) control conditions without addition of carvacrol. (**B**) With addition of 0.4 mmol/L carvacrol. (**C**) With addition of 0.6 mmol/L carvacrol.



Supplementary Figure 2. Effect of carvacrol on iron induced adhesion of *S.* Typhimurium to an epithelial monolayer.

Adhesion (mean + SD) of *S.* Typhimurium to a monolayer of Caco-2 cells is given as percentage of the inoculum and is not normalized for the adhesion at the conditions without iron during pre-incubation. Data consists of 3 separate experiments in which adherent colony forming units were determined on different days with duplicate wells, n = 6. * $p < 0.05$.

References

- Bravo D, Hoare A, Silipo A, Valenzuela C, Salinas C, Alvarez SA et al (2011). Different sugar residues of the lipopolysaccharide outer core are required for early interactions of Salmonella enterica serovars Typhi and Typhimurium with epithelial cells. Microbial pathogenesis 50: 70-80.
- Burt S (2004). Essential oils: their antibacterial properties and potential applications in foods--a review. International journal of food microbiology 94: 223-253.
- Burt SA, van der Zee R, Koets AP, de Graaff AM, van Knapen F, Gaastra W et al (2007). Carvacrol Induces Heat Shock Protein 60 and Inhibits Synthesis of Flagellin in Escherichia coli O157:H7. Applied and Environmental Microbiology 73: 4484-4490.
- CLSI http://www.clsi.org/standards/.
- Dostal A, Chassard C, Hilty FM, Zimmermann MB, Jaeggi T, Rossi S et al (2012a). Iron depletion and repletion with ferrous sulfate or electrolytic iron modifies the composition and metabolic activity of the gut microbiota in rats. The Journal of nutrition 142: 271-277.
- Dostal A, Fehlbaum S, Chassard C, Zimmermann MB, Lacroix C (2012b). Low iron availability in continuous in vitro colonic fermentations induces strong dysbiosis of the child gut microbial consortium and a decrease in main metabolites. FEMS microbiology ecology.
- EUCAST http://www.eucast.org/antimicrobial_susceptibility_testing/mic_determination.
- Fabian D, Sabol M, Domaracka K, Bujnakova D (2006). Essential oils--their antimicrobial activity against Escherichia coli and effect on intestinal cell viability. Toxicol In Vitro 20: 1435-1445.
- Glahn RP, Lee OA, Yeung A, Goldman MI, Miller DD (1998). Caco-2 cell ferritin formation predicts nonradiolabeled food iron availability in an in vitro digestion/Caco-2 cell culture model. The Journal of nutrition 128: 1555-1561.
- Graham SM (2010). Nontyphoidal salmonellosis in Africa. Current opinion in infectious diseases 23: 409-414.
- Helander IM, Alakomi H-L, Latva-Kala K, Mattila-Sandholm T, Pol I, Smid EJ et al (1998). Characterization of the Action of Selected Essential Oil Components on Gram-Negative Bacteria. Journal of agricultural and food chemistry 46: 3590-3595.
- Hernandez M, Sousa V, Moreno A, Villapando S, Lopez-Alarcon M (2003). Iron bioavailability and utilization in rats are lower from lime-treated corn flour than from wheat flour when they are fortified with different sources of iron. The Journal of nutrition 133: 154-159.
- Inamuco J, Veenendaal AK, Burt SA, Post JA, Tjeerdsma-van Bokhoven JL, Haagsman HP et al (2012). Sub-lethal levels of carvacrol reduce Salmonella Typhimurium motility and invasion of porcine epithelial cells. Veterinary microbiology 157: 200-207.
- Keenan JI, Davis KA, Beaugie CR, McGovern JJ, Moran AP (2008). Alterations in Helicobacter pylori outer membrane and outer membrane vesicle-associated lipopolysaccharides under iron-limiting growth conditions. Innate immunity 14: 279-290.
- Kittelberger R, Hilbink F (1993). Sensitive silver-staining detection of bacterial lipopolysaccharides in polyacrylamide gels. Journal of biochemical and biophysical methods 26: 81-86.
- Kong Q, Yang J, Liu Q, Alamuri P, Roland KL, Curtiss R, 3rd (2011). Effect of deletion of genes involved in lipopolysaccharide core and O-antigen synthesis on virulence and immunogenicity of Salmonella enterica serovar typhimurium. Infection and immunity 79: 4227-4239.

- Kortman GA, Boleij A, Swinkels DW, Tjalsma H (2012). Iron availability increases the pathogenic potential of Salmonella typhimurium and other enteric pathogens at the intestinal epithelial interface. PloS one 7: e29968.
- Makey DG, Seal US (1976). The detection of four molecular forms of human transferrin during the iron binding process. Biochimica et biophysica acta 453: 250-256.
- Michiels J, Missotten J, Van Hoorick A, Ovyn A, Fremaut D, De Smet S et al (2010). Effects of dose and formulation of carvacrol and thymol on bacteria and some functional traits of the gut in piglets after weaning. Archives of animal nutrition 64: 136-154.
- Nikaido H (2003). Molecular basis of bacterial outer membrane permeability revisited. Microbiol Mol Biol Rev 67: 593-656.
- Santiago P (2012). Ferrous versus ferric oral iron formulations for the treatment of iron deficiency: a clinical overview. TheScientificWorldJournal 2012: 846824.
- Schwyn B, Neilands JB (1987). Universal chemical assay for the detection and determination of siderophores. Analytical biochemistry 160: 47-56.
- South PK, Miller DD (1998). Iron binding by tannic acid: Effects of selected ligands. Food Chemistry 63: 167-172.
- Torrance JD, Bothwell TH (1968). A simple technique for measuring storage iron concentrations in formalinised liver samples. The South African journal of medical sciences 33: 9-11.
- van Alphen LB, Burt SA, Veenendaal AK, Bleumink-Pluym NM, van Putten JP (2012). The natural antimicrobial carvacrol inhibits Campylobacter jejuni motility and infection of epithelial cells. PloS one 7: e45343.
- WHO, UNICEF (2004). Focusing on anaemia: Towards an integrated approach for effective anaemia control.
- Windisch W, Schedle K, Plitzner C, Kroismayr A (2008). Use of phytogenic products as feed additives for swine and poultry. Journal of animal science 86: E140-148.
- Yokoi K, Konomi A, Otagi M (2009). Iron bioavailability of cocoa powder as determined by the Hb regeneration efficiency method. The British journal of nutrition 102: 215-220.
- Zimmermann MB, Chassard C, Rohner F, N'Goran E K, Nindjin C, Dostal A et al (2010). The effects of iron fortification on the gut microbiota in African children: a randomized controlled trial in Cote d'Ivoire. The American journal of clinical nutrition 92: 1406-1415.

Chapter 7

Summary

General Discussion

Introduction (Chapter 1)

Iron is a highly abundant metal on earth and is vital for virtually all organisms, including most bacterial species. Nonetheless, iron deficiency is the most prevalent human nutrition disorder worldwide and is generally treated by oral iron administration. In particular infants and young children in tropical underdeveloped countries with high infection pressure are vulnerable to iron deficiency. Ironically, iron supplementation has been associated with an increased burden of infectious diseases and accumulating evidence suggests that unabsorbed iron can stimulate growth and virulence of bacterial pathogens in the intestinal environment. Simultaneously, host iron status influences the intestinal defense against pathogens.

The human gut is the natural habitat for a unique large and dynamic bacterial community. Major functions include important trophic effects on intestinal epithelia, on immune structure and function, and protection of the colonized host against invasion by pathogenic microbes via the production of trophic metabolites and immunostimulatory molecules. The healthy gut microbiota is a balanced community that is shaped by host immunity and metabolism and environmental factors. Perturbations of the balanced gut microbiota have been associated with gastrointestinal disorders such as diarrhea, gastroenteritis and chronic inflammatory bowel disease. Importantly, oral iron administration has been associated with a potentially more pathogenic gut microbiota profile and an increase in diarrhea and gut inflammation. This thesis covers the multifaceted aspects of nutritional iron with respect to growth, composition, metabolism and pathogenicity of the gut microbiota in relation to human health.

Clinical outcomes of oral iron fortification and the effects on the gut microbiome in Kenyan infants

Chapter 2 describes the clinical outcomes of oral iron fortification and the effects on the gut microbiome in a clinically very relevant target population; Kenyan infants living in a rural area where infections are endemic. One group of infants received a micronutrient powder (MNP) containing 2.5 mg iron and another group received a different MNP containing 12.5 mg iron, the control groups received a MNP without iron. Remarkably, after 4 months the iron-fortified groups showed a lower relative abundance of beneficial *Bifidobacteriaceae* and a higher relative abundance of potentially pathogenic *Enterobacteriaceae*, while also the ratio of *Enterobacteriaceae* to *Lactobacillaceae* was increased. Importantly, iron fortification increased the abundance of pathogenic *E. coli* strains and caused elevated fecal calprotectin levels, indicating increased intestinal inflammation. In this setting, provision of iron-containing MNPs to weaning infants thus adversely affects the gut microbiome and appears to promote gut inflammation. This study underscores the importance of further basic research on the effects of iron on the gut microbiota for which both *in vitro* as animal models were exploited as outlined below.

Iron-induced changes at the host-microbial interface in experimental models: gut microbiota composition, metabolism and host responses

Chapter 3 extensively describes the effects of supplementary iron in different formulations and doses on the human gut microbiota. This was studied in the well suited and highly controlled TNO's Intestinal Model for the human large intestine (TIM-2). Microbiome analysis showed clear iron-induced changes and prominent shifts included a relative decrease of beneficial *Bifidobacteriaceae* and *Lactobacillaceae* under iron-rich conditions. Metagenomic analyses showed a general enrichment of bacterial motility-chemotaxis systems under iron-rich conditions. Metabolome profiling showed that gut microbial activity markedly shifted from a saccharolytic to a proteolytic profile in response to iron, in particular with ferrous sulfate. Importantly, cell viability tests showed increased cytotoxicity of metabolite-containing effluent from iron-rich conditions. These data indicate that iron preparations induce a more hostile intestinal environment characterized by i) reduction of beneficial microbes, ii) increased levels of bacterial metabolites that can impair the barrier function of the gut wall, and iii) increase of virulence-associated pathways of enteric pathogens.

Both the *in vivo* Kenyan field study (**Chapter 2**) as well as the experiments with the in vitro model for the large intestine (**Chapter 3**) show that supplementary iron can change the gut microbiota composition towards a potentially more pathogenic profile. In combination with an increased toxicity *in vitro* (**Chapter 3**) and increased gut inflammation *in vivo* (**Chapter 2**) this may be a bad recipe for the human host. Intestinal inflammation is highly associated with dysbiosis of the gut microbiota and we hypothesized that oral iron administration exaggerates intestinal inflammation caused by bacterial infection, via promoting pathogenic growth and virulence or via effects on the host inflammatory response. These hypotheses were tested in a mouse model as well as in a simple nematode model for intestinal infection, and are described in **Chapter 4**. Dietary intervention (iron-deficient, normal-iron, or high-iron diet) significantly altered tissue iron stores in uninfected mice and in mice infected with *Citrobacter rodentium*. Microbiome analysis, like in the human and *in vitro* studies, revealed a lower relative abundance of *Bifidobacteriaceae* and *Lactobacillaceae* in iron-supplemented mice compared to mice on a iron-deficient diet. Fecal levels of the innate defensive molecules and markers of inflammation lipocalin-2 and calprotectin were lower in iron-deprived infected mice. These mice also tended to have a lower grade of colon pathology and to gain more weight. Surprisingly, similar to animals on the iron-deficient diet, mice on the high-iron diet also showed decreased intestinal inflammatory responses compared to the normal-iron diet. Notably, iron-deprivation was associated with prolonged survival of the nematode *Caenorhabditis elegans* after infection with *Salmonella enterica* serovar Typhimurium, while supplementary iron increased the pathogenicity of this pathogen (see also **Chapter 5**; described below). Together, these experiments support the notion that low iron intake is beneficial in case of intestinal infection and inflammation, but also suggest that the clinical outcomes of oral iron administration may highly depend on the iron status, immune status and the gut microbiota composition of children that receive oral iron treatment.

Iron-induced virulence of intestinal pathogens at the intestinal epithelial interface *in vitro*

In **Chapter 5** we investigated the effect of iron on bacterial growth, bacterial attachment to intestinal epithelial cells, and the ability of bacteria to break through an intestinal epithelial cell layer *in vitro*. Our results clearly show that supplementary iron can promote growth and adhesion of intestinal pathogens, which potentially plays an important role during the initial and succeeding stages of gut-borne infections in the human body. These data fit with the consensus that oral iron administration in underdeveloped tropical countries is not without risk as iron could, in addition to inducing pathogenic overgrowth, also increase the virulence of prevalent enteric pathogens.

The outcomes of these experiments raised the question which bacterial mechanisms are responsible for the increased ability to adhere to intestinal epithelial cells. This motivated us to explore which iron-induced bacterial factors were involved as understanding of iron-induced adhesion mechanisms may provide leads for preventive interventions. Explorations at the surface of the model organism *S.* Typhimurium suggested that iron-induced adhesion is rather due to a change in surface LPS expression and composition than to a change in the expression of surface adhesion proteins (data not shown and ongoing investigations).

In **Chapter 6** we explored the potential of carvacrol, a natural compound with antimicrobial activity against various intestinal bacterial pathogens, in preventing iron-induced pathogenicity of *S.* Typhimurium. This study investigated a presumed interaction between carvacrol, bacterial adhesion and iron handling, and the ability of carvacrol to interfere with LPS structure at the surface of *S.* Typhimurium. Carvacrol retarded growth of *S.* Typhimurium at any iron concentration. Furthermore, iron-induced epithelial adhesion was effectively reduced by carvacrol at high iron concentrations. With our experiments we could not show that carvacrol altered LPS abundance or structure at the bacterial surface, but carvacrol was able to weaken iron-ligand interactions by which it may possibly interfere with bacterial virulence. In conclusion, our *in vitro* data suggest that carvacrol has the potential to serve as a novel dietary supplement to prevent pathogenic overgrowth and colonization in the large intestine during oral iron therapy, but for which further *in vivo* research is warranted.

Conclusion

Together, this thesis exemplifies that oral administration can increase the abundance of intestinal pathogens and may increase their pathogenicity, while it may decrease the host intestinal defense (i.e. via: iron-induced oxidative damage to the gut wall, increased production of toxic microbial metabolites affecting the gut wall, or a decreased host innate defense) at the same time. This undesired combination may provide intestinal pathogens with increased opportunities to evade the host defense during oral iron therapy. This strengthens the idea that oral iron administration programs in developing countries with high infection pressure need to be set up with the highest amount of care, and should be accompanied with close health monitoring until the remaining questions about the actual effect of iron at the intestinal host-microbiota interface have been unraveled.

Future research should also be directed at finding iron formulations that minimize the negative impact on the gut microbiome. This thesis exposes health problems and potential threats with regarding to oral iron administration and it increases the understanding of iron-driven alterations of host-pathogen interactions, which is indispensible in the design of safe iron formulations.

General discussion

This thesis provides a comprehensive view on the effects of oral iron treatment on the gut microbiota. As described in the summary, it documents novel findings that show profound effects of iron supplements on microbiota composition, metabolism and virulence. A burning question that remains to be answered is however whether these generally adverse effects are indeed a threat for human health.

Adverse effects of oral iron administration on the gut microbiota and clinical relevance

Iron deficiency is widespread among the world, even in industrialized countries, and treatment with orally administrated iron is commonly associated with gastrointestinal complaints, such as diarrhea, constipation and nausea (Schumann et al 2007). Notably, iron deficiency is much more prevalent in developing countries with a high burden of infectious diseases and, importantly, in these areas oral iron administration has been associated with an increased morbidity and a tendency towards increased mortality among African children (Sazawal et al 2006). Especially iron-replete children that received extra iron appeared to suffer from this treatment, suggesting that iron treatment should be targeted at iron-deficient individuals only. In this study most adverse events appeared to be due to malaria, but also other infections, possibly in part derived from invading intestinal pathogens, were among the adverse events (Sazawal et al 2006).

As a large part of orally administrated iron is not taken up by the upper intestine, most iron ends up in the colon were it, at least partly, becomes available to the large and diverse community of gut microbes. In the past few years multiple studies (including studies in this thesis) have shown that iron indeed can alter the gut microbiota composition, can influence gut microbial metabolism, and can enhance the virulence of intestinal pathogens *in vitro* (**Chapters 1-5**). In the aforementioned study of Sazawal et al., iron supplementation was not associated with an increased incidence of diarrhea, but it has been systematically reviewed in 2002 that oral iron administration causes a slight increase in the risk for diarrhea (Gera and Sachdev 2002), which was corroborated by additional recent studies (Soofi et al 2013, Zlotkin et al 2013) (**Chapter 2**). The effect of oral iron administration on diarrhea outcome is a major concern as diarrhea is a common symptom of intestinal infection, and is an important cause of death among African children (WHO and UNICEF 2009, WHO 2013). One study among Pakistani children showed that *Aeromonas* spp. were increasingly detected in diarrhea samples from children on micronutrient fortification (including iron) compared to children on placebo treatment (Soofi et al 2013). *Aeromonas* spp. are a known cause of diarrhea in Asia (Kotloff et al 2014) and this is so far the first intestinal pathogen that has been shown to be associated with micronutrient-induced diarrhea. It should be noted that micronutrients other than iron might have contributed to the effect in the Pakistan study. Two recent studies among African children and infants showed clear iron-induced adverse effects on the microbiome composition (Zimmermann et al 2010) (**Chapter 2**) and showed a higher level of gut inflammation (as reflected by fecal calprotectin levels) in the groups treated with iron. In the first study performed in Ivory Coast, gut inflammation was correlated with an

increase in *Enterobacteriaceae* (Zimmermann et al 2010). However, an iron fortification study performed in Kenya did not yield gut microbiome related associations for the increased level of gut inflammation (**Chapter 2**). Overall, these latter two studies did not report any study related serious adverse effects. The number of individuals in these studies might have been too small to detect a significant increase in morbidity, but it should be noted that the Kenyan infants in the subgroup with a dose of 12.5 mg iron per day tended to require treatment for diarrhea more often compared to infants in the control group. Together, these African field studies clearly show an undesirable shift of the gut microbiome towards one with an increased pathogenic potential, which goes together with a higher level of gut inflammation. The clinical implications of this shift and potential effects on the long term are nevertheless not yet clear and require further investigations.

As described in **Chapter 1**, various studies have described differential outcomes of the effects of iron fortification/supplementation on the gut microbiota composition. We note that, among other influences, the dose of iron and the length of treatment will have contributed to the differential findings so far. This is underscored by findings in **Chapter 2**. In this chapter, the low and medium iron doses had only a slightly different effect on the gut microbiota composition of (mainly breast-fed) Kenyan infants. However, the ratio of *Enterobacteriaceae* to *Lactobacillaceae* or *Bifidobacteriaceae* was only higher in the iron groups after 4 months of fortification, while this was not the case after 3 weeks. Even the opposite was found for the *Enterobacteriaceae* to *Lactobacillaceae* ratio at 3 weeks, pointing at a temporary potentially beneficial effect. It should however be noted that rapid fluctuations in the gut microbiome at 3 weeks, caused by the recent introduction of complementary feeding, may have contributed to this effect. This indicates it is important to allow the gut microbiome time to adapt to changes in diet when evaluating an intervention and comparing different trials. Overall, fortification/supplementation studies nevertheless support the notion of the studies in Africa that *Lactobacillaceae* and *Bifidobacteriaceae* decrease, while members of the *Enterobacteriaceae* tend to increase upon supplementary iron (**Chapter 1**). It is yet unknown if, and to what extent this effect can persist after discontinuation of iron treatment.

It is not only the effect of iron on the gut microbiota composition that raises concerns with regard to safety, also the combination with other (potential) effects of supplementary iron on the gut microbiota and the host intestine indicate that prudency is needed when supplementing iron in regions with high infection pressure and low hygiene standards. First of all, as outlined in **Chapter 1**, iron may elicit direct harm to the gut by the generation of oxidative stress, thereby increasing the permeability of the gut wall. Secondly, this effect may be enhanced by the increased production of toxic metabolic products associated with proteolytic activity of the gut microbiota (**Chapter 3**). It should be noted that this effect has so far only be demonstrated *in vitro*, and which thus requires further investigations. In the third place, this thesis also shows that iron can induce the virulence of intestinal pathogens *in vitro* (**Chapter 5**) and in a simple in vivo intestinal infection model (**Chapter 4**). Furthermore, it can increase the relative abundance of virulence associated pathways within the gut microbiota in an *in vitro* model for the human large intestine (**Chapter 3**). The effects of oral iron treatment on the virulence of intestinal pathogens and its implications have however not yet been investigated in human individuals. Finally, host

iron status and the gut microbiota composition influence the host immune response. Vice versa, host iron status and immune responses influence the gut microbiota composition. It is therefore difficult to predict what the effects of oral iron administration are on the inflammatory response of iron deficient and iron replete individuals. This thesis shows that oral iron administration appears to attenuate the intestinal inflammatory response in mice (**Chapter 4**), which is in particular undesired in combination with the aforementioned adverse effects of iron on gut microbiota composition, metabolism and virulence. Also this issue needs further investigation to unravel the underlying mechanisms of this attenuated inflammatory response and to find out how this is related to the increase in gut inflammation and infectious disease that has been observed in the field studies in Africa. More insight in this complex interplay may help us to prevent potential attenuating effects of supplementary iron on the host immune system.

Taken together, a tempting question is whether the adverse effects of oral iron administration on the gut microbiota and intestinal defense can contribute to an increased incidence of infectious diseases which has previously been shown in the larger clinical trials and meta-analyses (Gera and Sachdev 2002, Iannotti et al 2006, Oppenheimer 2001, Sazawal et al 2006, Zlotkin et al 2013). This question is difficult to answer due to lack of direct evidence, but it is conceivable that part of the iron-induced reported infections may have originated from the gut. Although this question remains to be answered, the reported increase in diarrhea and gut inflammation upon oral iron treatment is nonetheless worrisome and clinically very important. The current data points at a role of the gut microbiota in this matter and implicates that the negative impact of oral iron administration on the gut microbiota needs to be minimized.

Future perspectives

Future studies should aim at gaining mechanistic insights in the effects of oral iron administration on the gut microbiota and host responses, which will aid in the development of safe iron administration programmes. Simultaneously, future research should already be directed at finding ways of iron administration that prevent adverse effects on the gut microbiota. Both types of research should be done in experimental models that reflect better the clinically relevant human situation. Until safe approaches have been found, iron administration programmes in infection endemic regions should be executed according to the recommendations of the World Health Organization (WHO) as described below.

Development of experimental models that better reflect the clinically relevant human situation

As indicated above, the effects of iron on gut microbial virulence and metabolism need to be investigated in more relevant *in vivo* situations. *In vivo* studies should in particular reveal whether oral iron administration can indeed enhance the virulence of intestinal pathogens, whether it indeed stimulates gut microbial proteolytic activity in the human intestine, and which clinical consequences this might have. Past studies can help us in finding the most optimal design of future studies, as they revealed the limitations of study models and gaps in knowledge of how iron behaves in the colon. The most clinically relevant setting for

this research is in developing countries where iron deficiency and infections are highly prevalent. This setting is difficult to simulate *in vitro* and *in vivo* models, but an advantage is that these models can be better controlled. Studies in industrialized countries so far not only involved *in vitro* and *in vivo* models, but were also performed with infants from these countries. Although similar studies can still be very useful in finding oral iron preparations with less impact on the gut microbiota, we should realize that they do not totally reflect the clinically most relevant situation. Other past studies involved rats and mice with a very different gut microbiota profile and dietary intake compared to humans, or *in vitro* fermentation models with a microbiota from healthy 'Western' individuals. Notably, iron concentrations and availability *in vitro* do not necessarily reflect the *in vivo* situation and animal iron depletion-repletion models may not accurately represent the relevant human situation (**Chapter 1**). Several measures could however bring these models closer to the clinical relevant setting, which will be discussed below.

Animal models can possibly be improved by establishing a humanized gut microbiota first, which so far has been done for one iron-related study (Dostal et al 2014). To get even more close to the most relevant situation this humanized microbiota should ideally be derived from (anemic) infant/child stools from a rural setting with high infection pressure. Most relevant for studying both iron bioavailability to the host and the effects on the gut microbiota are iron-deficient animals. Notably, past studies did establish iron deficiency by feeding the animals an iron-deficient diet, but other ways such as iron chelation or phlebotomy might have been better alternatives as an iron-deficient diet not only lowers host iron status, it also directly alters gut microbiota composition and activity. Hence, the gut microbiota had already changed before an iron preparation was administrated, which makes it difficult to distinguish the effects of initial iron depletion from the effects of consequent iron supplementation on the gut microbiota. Even though iron deficient individuals have a low iron status, their diet usually is not that much depleted from iron (though it is likely to be lowly bioavailable). This is underscored by iron measurements in feces of iron deficient weaning infants fed with complementary solid food, which show that their total fecal iron content is comparable to that of adults consuming western type diets (**Chapter 1**). This also appeared from our preliminary iron measurements in feces of Kenyan infants (data not shown).

The relevance of *in vitro* gut models, which are in particular useful to study gut microbial metabolic activity, can also be improved. Also in this case fecal samples which more closely resemble the gut microbiota of infants/children from rural areas with high infection pressure may be used. In combination with mammalian cell models these gut models can be very valuable tools to study the effects of iron on both the gut microbiota and the host. Also here the iron concentrations applied are important and should closely resemble the fecal iron concentration of infants/children. By determining iron speciation *in vitro* gut models and comparison with the *in vivo* situation, better insight in the relevance of these *in vitro* models can be obtained. By simulation of the digestive tract from the mouth onwards an iron speciation profile more similar to the *in vivo* situation can possibly be attained. Achieving this can however in particular be very challenging in cell culture models as iron added to culture media will have a

completely different availability and speciation profile compared to iron in feces, or iron in fermentation media (used in *in vitro* gut models) that more closely resemble feces.

Gaining mechanistic insights in the utilization of iron by gut microbiota members

As described above, iron measurements in feces can help to find the most relevant fecal iron concentration to simulate in the experimental models. It is pivotal to also look into the speciation of iron and the ability of gut microbes to utilize this iron, as total fecal iron concentration cannot predict the availability to the gut microbiota. This is not only relevant for experimental models, but also in the human situation as increased insights in the iron speciation and availability can help us in the design of oral iron administration approaches that do not increase the availability of iron to the gut microbiota. Notably, when the speciation of supplementary iron in the colon has been analyzed, it will still be difficult to evaluate the bioavailability of the various iron species. A good alternative indicator for iron availability to the gut microbiota is potentially siderophore production. If iron bioavailability to the microbes in the colon lumen is high, siderophore production will likely be low and vice versa. Levels of bacterial siderophores in the colon lumen or feces may therefore predict iron availability to the gut microbiota. In case an oral iron preparation results in lower siderophore levels, this indicates that the iron becomes available to the gut microbiota. However, practical assays to measure siderophores in human and animal feces need to be developed.

A methodology to actually pinpoint the bacteria that take up the supplemented iron can be the Halogen In Situ Hybridization-Secondary Ion Mass Spectroscopy (HISH-SIMS). With this technique stable iron isotopes can be quantified within bacterial cells, and by staining the same cells with specific probes (on the genus or family level) it can be deduced which bacteria take up the extra iron. Next, by applying metabolomic, metatranscriptomic and metaproteomics analyses a much better view on the actual metabolic and pathogenic activity of the gut microbiota in response to iron can be obtained. These approaches will gain mechanistic insights that aid in the design of more safe oral iron administration programmes.

Prevention of iron-induced adverse effects on the gut microbiota

To minimize the impact of oral iron administration on the gut microbiota, while maintaining or increasing bioavailability to humans, strategies to prevent undesired effects of iron on the gut microbiota need to be developed. Although this is most important in developing countries where infections are highly endemic, it is conceivable that such strategies also increase the tolerance of the gastrointestinal tract for oral iron in western society. **Chapter 1** describes various approaches to achieve this, but most have not yet been tested for their effects on the gut microbiome. Successful strategies of universal administration with a minimal impact on the gut microbiota depend on the combination of successful improvement of host iron status of the person in need with i) the prevention of gut microbiota iron uptake, or ii) the simultaneous suppression of intestinal pathogens, or iii) the stimulation of beneficial gut microbiota members such as *Bifidobacteriaceae* and *Lactobacillaceae*, hereby restraining pathogenic growth and/or improving gut health.

One approach that has been tested in this thesis is the provision of iron in a low dose, but highly bioavailable preparation, to prevent large amounts of iron entering the colon. However, as outlined in **Chapter 2**, in Kenyan infants this approach was not successful as their gut microbiota still shifted towards a more pathogenic profile, similar to children that received a 5 times higher dose. Importantly, the low dose approach did not yield a better iron status, while the higher dose approach did successfully improve infant iron levels. This also questions the approach of universal iron fortification programmes without screening individuals for actual iron deficiency, while screening was already recommended by the WHO (WHO 2007). If adverse effects on the gut microbiota in iron deficient individuals are accompanied by an improved body iron status, the possible adverse effects may be acceptable as long as health is closely monitored. In contrast, adverse effects on the gut microbiome are surely not acceptable in iron replete infants, because they do not benefit from the iron fortificant. Screening of individuals for their iron status in underdeveloped, rural and infection endemic regions is however highly challenging and costly, while proper biomarkers to this purpose have not yet been found. The peptide hormone hepcidin, that regulates body iron homeostasis, has been proposed as a promising biomarker to this purpose (Prentice et al 2012). Hepcidin levels usually decrease when the iron requirement of the body is high, and increase upon infection. Notably, in severely anemic individuals, hepcidin levels can be low despite infection, which limits its value as a biomarker to guide safe iron administration (Jaeggi et al 2013, Jonker et al 2014, Jonker et al 2013) (**Chapter 1**). Notably, iron treatment to combat iron deficiency is needed because iron deficiency has major health consequences such as impaired physical and cognitive development of children (McCann and Ames 2007, WHO and UNICEF 2004). The benefits are thus substantial and not treating iron deficiency to prevent adverse effects is therefore not under consideration, but sufficient health care to combat adverse effects and to monitor health during oral iron treatment should be available (WHO 2007).

In this thesis, next to the provision of a highly bioavailable low dose iron preparation, one other approach of preventing an increase in pathogenic gut microbiota members during oral iron administration was explored. This is the provision of the antimicrobial compound carvacrol, as described in **Chapter 6**. It is interesting to note that carvacrol may not only prevent iron-induced virulence of intestinal pathogens, but also inhibit the microbial production of (proteolytic activity related) isobutyrate, isovalerate and valerate. It has previously been shown that carvacrol can reduce the production of these compounds in swine waste, while lactate accumulated when carvacrol was added (Michiels 2009). Next to its inhibiting effects on growth and virulence of pathogens as described above, carvacrol may thus have the potential to prevent the iron-induced undesired metabolic changes that were described in **Chapter 3**, but this issue is subject to further investigations. In addition, future research could aim at finding other antimicrobial compounds with a similar potential.

Future directions

To further investigate the clinical relevance, future studies should be directed to:

I. further assess the potential involvement of iron-induced changes in the gut microbiota (composition, metabolism, virulence) in the development of:
   a. diarrhea,
   b. intestinal inflammation,
   c. and invasive disease;

To increase the understanding of iron-host-microbiota interactions and thereby aid in the design of more safe oral iron preparations, future studies should be performed in experimental models that closely mimic the human in vivo situation and should aim at elucidating:

II. iron speciation and availability in the colon;

III. the effect of oral iron administration on gut microbial siderophore production;

IV. which members of the gut microbiota:
   a. utilize the orally administrated iron,
   b. are responsible for the undesired metabolic shift in response to iron,
   c. get more virulent in response to iron;

V. the complex interplay between host iron status, host immunity and gut microbiota;

Furthermore, future research should be directed at:

VI. developing a point of care test that easily discriminates between iron-deficient and iron-replete individuals. This would allow to selectively target the iron to those in need, until safe formulations for universal use have been developed.

Concluding remarks

This thesis provides multiple novel insights in the effects of iron on the gut microbiota and its pathogenic members; it becomes clear that iron has profound effects on the gut microbiota composition, metabolism, and virulence of intestinal pathogens. Together, this points at unbeneficial effects from the host' perspective. Although these unwanted side-effects need to be examined in closer detail, it is anyhow desired to reduce the risk for dysbiosis, intestinal infection and diarrhea. More knowledge on the responses of gut bacteria to supplementary iron will aid in the design of novel iron supplementation strategies that improve host iron status while minimizing the negative impact on the gut microbiota and gut health.

References

- Dostal A, Lacroix C, Pham VT, Zimmermann MB, Del'homme C, Bernalier-Donadille A et al (2014). Iron supplementation promotes gut microbiota metabolic activity but not colitis markers in human gut microbiota-associated rats. The British journal of nutrition: 1-11.
- Gera T, Sachdev HP (2002). Effect of iron supplementation on incidence of infectious illness in children: systematic review. BMJ (Clinical research ed 325: 1142.
- Iannotti LL, Tielsch JM, Black MM, Black RE (2006). Iron supplementation in early childhood: health benefits and risks. The American journal of clinical nutrition 84: 1261-1276.
- Jaeggi T, Moretti D, Kvalsvig J, Holding PA, Tjalsma H, Kortman GAM et al (2013). Iron Status and Systemic Inflammation, but Not Gut Inflammation, Strongly Predict Gender-Specific Concentrations of Serum Hepcidin in Infants in Rural Kenya. PloS one 8: e57513.
- Jonker FA, Boele van Hensbroek M, Leenstra T, Vet RJ, Brabin BJ, Maseko N et al (2014). Conventional and novel peripheral blood iron markers compared against bone marrow in Malawian children. Journal of clinical pathology.
- Jonker FAM, Calis JCJ, Phiri K, Kraaijenhagen RJ, Brabin BJ, Faragher B et al (2013). Low Hepcidin Levels in Severely Anemic Malawian Children with High Incidence of Infectious Diseases and Bone Marrow Iron Deficiency. PloS one 8: e78964.
- Kotloff KL, Nataro JP, Blackwelder WC, Nasrin D, Farag TH, Panchalingam S et al (2014). Burden and aetiology of diarrhoeal disease in infants and young children in developing countries (the Global Enteric Multicenter Study, GEMS): a prospective, case-control study. The Lancet 382: 209-222.
- McCann JC, Ames BN (2007). An overview of evidence for a causal relation between iron deficiency during development and deficits in cognitive or behavioral function. The American journal of clinical nutrition 85: 931-945.
- Michiels J (2009). Effect of essential oils on gut bacteria and functionality in the pig. PhD Thesis, Ghent University, Belgium, 282p.
- Oppenheimer SJ (2001). Iron and its relation to immunity and infectious disease. The Journal of nutrition 131: 616S-633S; discussion 633S-635S.
- Prentice AM, Doherty CP, Abrams SA, Cox SE, Atkinson SH, Verhoef H et al (2012). Hepcidin is the major predictor of erythrocyte iron incorporation in anemic African children. Blood 119: 1922-1928.
- Sazawal S, Black RE, Ramsan M, Chwaya HM, Stoltzfus RJ, Dutta A et al (2006). Effects of routine prophylactic supplementation with iron and folic acid on admission to hospital and mortality in preschool children in a high malaria transmission setting: community-based, randomised, placebo-controlled trial. Lancet 367: 133-143.
- Schumann K, Ettle T, Szegner B, Elsenhans B, Solomons NW (2007). On risks and benefits of iron supplementation recommendations for iron intake revisited. J Trace Elem Med Biol 21: 147-168.
- Soofi S, Cousens S, Iqbal SP, Akhund T, Khan J, Ahmed I et al (2013). Effect of provision of daily zinc and iron with several micronutrients on growth and morbidity among young children in Pakistan: a cluster-randomised trial. Lancet 382: 29-40.
- WHO, UNICEF (2004). Focusing on anaemia: Towards an integrated approach for effective anaemia control.
- WHO (2007). Conclusions and recommendations of the WHO Consultation on prevention and control of iron deficiency in infants and young children in malaria-endemic areas. Food Nutr Bull 28: S621-627.
- WHO, UNICEF (2009). Diarrhoea: why children are still dying and what can be done.

- WHO (2013). World Health Statistics 2013.
- Zimmermann MB, Chassard C, Rohner F, N'Goran E K, Nindjin C, Dostal A et al (2010). The effects of iron fortification on the gut microbiota in African children: a randomized controlled trial in Cote d'Ivoire. The American journal of clinical nutrition 92: 1406-1415.
- Zlotkin S, Newton S, Aimone AM, Azindow I, Amenga-Etego S, Tchum K et al (2013). Effect of iron fortification on malaria incidence in infants and young children in Ghana: a randomized trial. JAMA : the journal of the American Medical Association 310: 938-947.

Chapter 8

Nederlandse samenvatting

Dankwoord

Curriculum Vitae

List of publications

Nederlandse samenvatting

Inleiding (Hoofdstuk 1)

IJzer is één van de meest voorkomende metalen op aarde en is essentieel voor nagenoeg alle levende wezens, waaronder de meeste soorten bacteriën. Ondanks de grote hoeveelheid ijzer op onze aardbol is ijzertekort bij mensen wereldwijd de meest voorkomende voedingsgerelateerde stoornis. Vooral baby's en jonge kinderen in tropische ontwikkelingslanden waar veel infecties voorkomen, zijn vatbaar en hebben vaak ijzertekort. IJzertekort wordt meestal behandeld door ijzersupplementatie in de vorm van tabletten of verrijking van voedsel met ijzer, maar uit onderzoek blijkt dat dat ervoor kan zorgen dat kinderen in die landen meer infecties krijgen.

Niet al het ijzer uit supplementen of voeding wordt opgenomen in het eerste deel van de darm, en komt in de dikke darm terecht. De menselijke dikke darm is de natuurlijke leefomgeving van een unieke, grote en dynamische darmflora (darmmicrobiota, ofwel alle bacteriën en andere micro-organismen in de darm samen). Het heeft een belangrijke positieve invloed op ons immuunsysteem en de gezonde darmmicrobiota beschermt onze darm tegen invasie van pathogene (ziekteverwekkende) bacteriën. De gezonde darmmicrobiota is in balans en wordt gevormd door ons immuunsysteem, onze stofwisseling en omgevingsfactoren zoals voeding. Onder andere de ijzerstatus (de hoeveelheid ijzer in het menselijk lichaam) is van belang voor een goede werking van de afweer in de darm. De gebalanceerde microbiota kan verstoord raken en dit komt voor bij darmziekten zoals diarree, darminfectie en chronische inflammatoire darmziekten. De afgelopen jaren is steeds meer bewijs gevonden dat ijzersupplementatie en ijzerverrijking de groei en pathogeniciteit (het vermogen van bacteriën om infecties te veroorzaken) van bacteriën in de dikke darm kan stimuleren. Daarnaast is het geassocieerd met een toename van diarree en ontsteking van de darm. Dit proefschrift beschrijft verscheidene aspecten van ijzertoediening in relatie tot de groei, samenstelling, metabolisme en pathogeniciteit van de darmmicrobiota en de effecten op menselijke gezondheid.

Klinische resultaten van ijzerverrijking en effecten op de darmmicrobiota in Keniaanse baby's

In **Hoofdstuk 2** beschrijven we de uitkomsten van ijzerverrijking en de effecten op de darmmicrobiota in een klinisch relevante doelpopulatie; Keniaanse baby's die opgroeien in een plattelandsgebied waar infecties veel voorkomen. Eén groep baby's in deze studie gaven we dagelijks een micronutriëntenpoeder (MNP) dat 2,5 mg ijzer bevatte en een andere groep kreeg een ander MNP met 12,5 mg ijzer. De controlegroepen kregen een identiek MNP, maar zonder ijzer. Na 4 maanden was er in de groep die ijzerverrijking kreeg een lagere relatieve hoeveelheid van gezonde *Bifidobacteriaceae* te zien, terwijl de relatieve hoeveelheid van potentieel pathogene *Enterobacteriaceae* hoger was dan in de controlegroep. Ook was de verhouding tussen *Enterobacteriaceae* en gezonde *Lactobacillaceae* significant toegenomen. Daarnaast zorgde ijzerverrijking voor een toename van pathogene *E. coli* soorten en een verhoging van de ontstekingsmarker calprotectine, wat een toename in darmontsteking aangeeft. In deze studie zorgt de toediening van ijzerbevattende MNPs aan baby's dus

voor een negatief effect op de darmmicrobiota en bevordert het darmontsteking. Dit onderstreept het belang van het doen van meer onderzoek naar de effecten van ijzer op de darmmicrobiota, waarvoor in dit proefschrift zowel *in vitro* modellen als diermodellen werden toegepast en zoals hieronder beschreven wordt.

Veranderingen veroorzaakt door ijzer op het grensvlak van gastheer en bacterie: samenstelling van de darmmicrobiota, metabolisme en reacties van de gastheer

Hoofdstuk 3 beschrijft uitgebreid de effecten van verschillende ijzerpreparaten en hoeveelheden op de darmmicrobiota van de mens. We hebben dit bestudeerd in een sterk gereguleerd en computergestuurd model voor de humane dikke darm; TNO's Intestinal Model (TIM-2). Analyse van de samenstelling van de darmmicrobiota liet zien dat er duidelijke veranderingen waren in de testcondities waarbij ijzer aan de darminhoud was toegevoegd, met onder andere een afname van de gunstige *Bifidobacteriaceae* en *Lactobacillaceae*. Analyse van het microbiële metagenoom (genetische eigenschappen van de darmmicrobiota) liet een verrijking van mobiliteit- en chemotaxissystemen zien in ijzerrijke condities, wat mogelijk het veroorzaken van infecties stimuleert. Het metabolisme van de darmmicrobiota verschoof duidelijk van een saccharolytisch (afbreken van koolhydraten) naar een proteolytisch (afbreken van eiwitten) profiel, vooral in de aanwezigheid van ijzersulfaat. Dat bleek bovendien meer toxische producten te geven die schadelijk waren voor gekweekte darmcellen. Samengenomen wijzen deze data erop dat ijzerpreparaten een nadelige omgeving in de darm veroorzaken die gekenmerkt wordt door i) een afname van gunstige darmbacteriën, ii) een toename van microbiële metabolieten die de barrièrefunctie van de darmwand aantasten, en iii) een toename van pathogeniciteits-geassocieerde eigenschappen van de darmmicrobiota.

Zowel de *in vivo* veldstudie in Kenia (**Hoofdstuk 2**) als de experimenten met het in vitro darmmodel (**Hoofdstuk 3**) laten zien dat door toediening van ijzer de samenstelling van de darmmicrobiota potentieel meer pathogeen wordt. In combinatie met een toegenomen toxiciteit (*in vitro*; **Hoofdstuk 3**) en een toename van darmontsteking (*in vivo*; **Hoofdstuk 2**) zou dit een slechte mix kunnen zijn voor de menselijke gastheer. Darmontsteking is sterk geassocieerd met een disbalans van de darmmicrobiota en onze hypothese in **Hoofdstuk 4** was dat orale ijzertoediening bacteriële darmontsteking verergert, door het stimuleren van de groei en pathogeniciteit van pathogene bacteriën, of door effecten op de immuunrespons van de gastheer. Dit werd getest in een muismodel en in een eenvoudig wormmodel voor darminfectie. Interventie met ijzer via de voeding (laag-ijzer, normaal-ijzer, of hoog-ijzer) zorgde voor een verandering in de hoeveelheid ijzer die opgeslagen is in de lever en de milt van niet-geïnfecteerde muizen en muizen die geïnfecteerd waren met de knaagdier- en darmpathogeen *Citrobacter rodentium*. Analyse van de samenstelling van de darmmicrobiota liet, net als in de humane en *in vitro* studies, zien dat er een lagere relatieve hoeveelheid van gunstige *Bifidobacteriaceae* en *Lactobacillaceae* was in de muizen met ijzer in de voeding ten opzichte van muizen op een dieet met een laag ijzergehalte. Daarnaast waren de waarden van de afweermoleculen en markers van ontsteking lipocalin-2 en calprotectine in de ontlasting verlaagd in ijzerarme en geïnfecteerde muizen. Deze muizen hadden tevens een iets lagere graad van darmschade/infectie, zoals bekeken met microscopie, en hun

lichaamsgewicht leek minder te lijden te hebben onder de darminfectie. Verrassend genoeg hadden de muizen op een hoog-ijzerdieet, net als de muizen op een laag-ijzerdieet, een verlaagde ontstekingsreactie vergeleken met muizen op het normaal-ijzerdieet. Een andere belangrijke bevinding was dat het weghouden van ijzer bij de darmpathogeen *Salmonella enterica* serovar Typhimurium ervoor zorgt dat de overleving van *Caenorhabditis elegans* wormen, die geïnfecteerd zijn met *S.* Typhimurium, verlengd wordt. Tegelijkertijd maakte dit duidelijk dat ijzer de pathogeniciteit van deze bacterie verhoogt (zie ook **Hoofdstuk 5**; hieronder beschreven). Samen doen deze resultaten vermoeden dat een lage ijzerinname gunstig is tijdens darminfectie en ontsteking, maar ze suggereren ook dat de klinische uitkomsten van ijzersupplementatie sterk afhankelijk zijn van de ijzerstatus, immuunstatus en samenstelling van de darmmicrobiota van individuen die behandeld worden.

IJzergeïnduceerde pathogeniciteit van pathogene darmbacteriën bij het darmepitheel *in vitro*

In **Hoofdstuk 5** hebben we het effect van ijzer op de bacteriegroei, het vermogen van bacteriën om aan darmcellen te hechten en het vermogen van bacteriën om door het darmepitheel heen te breken, onderzocht. De resultaten maken duidelijk dat extra ijzer de groei en hechting van pathogene darmbacteriën aan darmcellen stimuleert. Dit speelt mogelijk een belangrijke rol tijdens het vroege stadium van infecties die ontstaan in de darm. Deze bevindingen passen bij de algemene gedachte dat ijzersupplementatie in ontwikkelingslanden niet zonder risico is, omdat ijzer niet alleen overgroei van pathogene darmbacteriën, maar ook de pathogeniciteit van veel voorkomende pathogene darmbacteriën zou kunnen stimuleren.

De bevindingen van deze experimenten roepen de vraag op welke bacteriële mechanismen betrokken zijn bij de toename in hechting aan darmcellen. Daarom hebben we gezocht naar ijzergeïnduceerde bacteriële factoren die hierbij betrokken zijn, wat mogelijk aanknopingspunten biedt voor het nemen van preventieve maatregelen tijdens ijzersupplementatie. Onderzoek naar het oppervlak van de pathogene darmbacterie *S.* Typhimurium suggereerde dat ijzergeïnduceerde hechting aan darmcellen mogelijk komt door een verandering in de hoeveelheid en samenstelling van het lipopolysaccharide (LPS; suikerstructuren met vetketens die in de bacteriemembraan zitten), terwijl een verandering in de hoeveelheid van hechtingseiwitten aan het bacterieoppervlak minder waarschijnlijk is (deze data staan niet in dit proefschrift).

In **Hoofdstuk 6** hebben we onderzocht of carvacrol, een natuurlijke stof met antimicrobiële werking tegen verscheidene pathogene darmbacteriën, ijzergeïnduceerde hechting van *S.* Typhimurium aan darmcellen kan voorkomen. Deze studie toetste hiermee een veronderstelde interactie tussen carvacrol, bacteriële ijzerhuishouding en hechting aan darmcellen, en het vermogen van carvacrol om de structuur van LPS op het bacteriële oppervlak te beïnvloeden. Carvacrol vertraagde de groei van *S.* Typhimurium bij elke ijzerconcentratie en het was effectief in het verminderen van ijzergeïnduceerde hechting bij hoge ijzerconcentraties. Met onze experimenten hebben we niet kunnen aantonen dat dit komt doordat carvacrol de LPS op het bacterieoppervlak beïnvloedt. Wel vonden we dat carvacrol de binding van ijzer aan een ijzerbindende stof kan verzwakken, een

mechanisme waarmee het mogelijk de pathogeniciteit van bacteriën kan beïnvloeden. We concluderen dat deze *in vitro* data erop wijst dat carvacrol de potentie heeft om als een nieuw voedingssupplement te dienen om overgroei en kolonisatie van pathogene bacteriën in de dikke darm tijdens ijzersupplementatie te voorkomen. Meer onderzoek hiernaar, met name *in vivo*, is echter vereist om te testen of toediening van carvacrol dat inderdaad kan bereiken.

Conclusie

Samenvattend demonstreert dit proefschrift dat ijzersupplementatie de groei en pathogeniciteit van pathogene darmbacteriën kan stimuleren, terwijl het tegelijkertijd mogelijk ook de barrièrefunctie en immunologische afweer van de darm kan verzwakken. Dat is een ongewenste combinatie die pathogene darmbacteriën mogelijk in staat stelt om de afweer van de gastheer tijdens ijzersupplementatie beter te ontwijken. Dit versterkt de gedachte dat ijzersupplementatieprogramma's in tropische ontwikkelingslanden opgezet moeten worden met de hoogst mogelijke hoeveelheid zorg. IJzersupplementatie zou dus vergezeld moeten gaan met het goed in de gaten houden van de gezondheid totdat de opengebleven vragen over het effect van ijzer op de darmmicrobiota en de darm zijn beantwoord. Verder onderzoek moet dit uitwijzen en zou ook gericht moeten zijn op het ontwikkelen van ijzerpreparaten die weinig of geen negatieve effecten op de darmmicrobiota hebben. Dit proefschrift laat zien welke gezondheidsproblemen en potentiële gevaren er zijn met betrekking tot ijzersupplementatie. Daarnaast draagt het bij aan de kennis over ijzergeïnduceerde veranderingen in de interactie tussen gastheer en pathogene bacteriën, die onmisbaar is bij de ontwikkeling van veilige ijzerpreparaten.

Dankwoord (acknowledgements)

Het is pas af als je ermee ophoudt. Met het schrijven van dit laatste, maar belangrijke, onderdeel kan ik er bijna mee ophouden en vormt mijn proefschrift een geheel. Dit proefschrift is het resultaat van ruim 4 jaar werk, maar was niet geworden tot wat het nu is zonder de hulp van vele anderen en waardevolle samenwerkingen. In dit dankwoord wil ik daarom graag iedereen bedanken die op de een of andere manier heeft bijgedragen en waarbij ik een aantal mensen in het bijzonder wil noemen.

Als eerste wil ik graag mijn promotor Dorine Swinkels bedanken voor het faciliteren en begeleiden van mijn promotietraject. Beste Dorine, wat een tekort of een overschot aan ijzer met het menselijke lichaam kan doen was jou aan het begin van mijn onderzoek natuurlijk al als geen ander al bekend, maar hoe reageren darmbacteriën daar eigenlijk op? Ik vond het erg leuk en boeiend dat twee onderzoeksonderwerpen binnen de onderzoeksgroep, 'ijzer' en 'darmbacteriën', bij elkaar kwamen in mijn project. Binnen dit project was je enthousiasme stimulerend, hebben je 'so-what vragen' me aan het denken gezet en hadden we waardevolle overlegmomenten waarin we het totaalplaatje duidelijk kregen. "De aanhouder wint", dat komt ook bij me op en dit is inderdaad meerdere keren gebleken. Je advies en steun bij het uitstippelen van mijn vervolgcarrière is erg fijn. Bedankt voor dit alles!

Beste Harold, als copromotor was je het nauwst betrokken bij mijn hele promotietraject. Je dagelijkse begeleiding, waarbij je altijd tijd had voor een vraag of discussie, was onmisbaar. Meestal leidde dat een pragmatische oplossing waarmee we verder konden en wat me het vertrouwen gaf dat ik goed bezig was. Op het gebied van het schrijven van een wetenschappelijk artikel, en het gepubliceerd krijgen daarvan, heb ik ook veel van je geleerd. Je goede verbeteringen en mooie zinnen hebben een grote bijdrage geleverd aan de inhoud van dit proefschrift. We hebben meerdere onderzoekslijntjes uitgezet en samenwerkingen gezocht, waarvan de meeste goed zijn uitgepakt en erg leerzaam waren. Ter ontspanning waren de gezamenlijke lunchwandelingen erg prettig en zo ook de talrijke woordgrapjes. Die woordgrapjes of andere droge humor zorgden ook voor de nodige lach als het onderzoek even, of langer, tegenzat. Ongeveer anderhalf jaar geleden is het alweer dat je aankondigde het lab te gaan verlaten om een moeder-kind huis te beginnen samen met je vrouw, een bijzonder en mooi plan. Je zei meteen dat je de begeleiding van mijn promotie zeker af zou maken en dat was voor mij natuurlijk erg fijn. Veel succes met jullie onderneming en bedankt voor alles!

Het spreekt voor zich dat ik nu uitkom bij mijn andere directe collega's binnen de IJzergroep. Annemarie, je hebt me vooral in het begin goed op weg geholpen door me wegwijs te maken op de verschillende labs en bij het opzetten van experimenten. Ik vond het ook erg leuk om met je samen te werken en het was fijn om promotieperikelen te delen. Rian, wij hebben veel experimenten samen gedaan, vooral in de zoektocht naar een antwoord op de vraag hoe het komt dat bacteriën beter aan darmcellen hechten als we ze extra ijzer geven. Het kostte veel herhalen van experimenten, het doen van vele blotjes en kleuringen, en zelfs een flinke

krachtinspanning bij het filter-steriliseren van groeimedia met Salmonella. Uiteindelijk hebben de blotjes het net niet gered tot in dit proefschrift, maar ik ga ervan uit dat we ze nog gaan publiceren. Wel zien we de LPS-kleuringen, ijzermetingen en calprotectinemetingen terug. Erwin, wij hebben vooral op het darmmicrobioomgebied samengewerkt. Je hebt veel PCR's gedaan om darmmicrobiomen voor de verschillende projecten in kaart te kunnen brengen. Het duurde soms wat lang door ongrijpbare technische euvels, maar zodra deze uit de lucht waren, was het ook zo gedaan. Coby, het eerste wat ik me herinner is zo ongeveer het gooien van sneeuwballen bij een vrolijke lunchwandeling… en het volgende je hulp en kritische blik bij de 'iron removal from transferrin assay'. Zo waren er nog wat meer pilotexperimentjes en natuurlijk de hepcidinemetingen bij de muizen uit Boston, die terug te vinden zijn in dit proefschrift. Siem, jij stond ook steeds paraat, bij het vinden van ruimte in de vriezer en het last-minute printen van etiketten. Ook al is het (nog) geen begrip geworden op het lab, de betekenis van het woord 'strube' (stront-in-een-tube) zal ik niet snel vergeten. 'IJzercollega's' of 'IJzerstudenten' Joyce, Rieneke, Carla, Charlotte, Jos, Susanne, Lucas, Louise, Tessel, Nienke, Rachel, Sanne en Rens, we hebben niet veel samengewerkt, maar zoals voor de hele IJzergroep geldt: allemaal erg bedankt voor en/of al het werk, de goede samenwerking en de gezelligheid op het lab, bij congressen en bij uitstapjes!

Veel dank ook aan de enthousiaste studenten die ik heb mogen begeleiden. Anne-Claire, je hebt veel cellen en bacteriën gekweekt om te onderzoeken of de lipocalin-2 productie door darmcellen beïnvloed wordt door ijzer en bacteriën. Jouw werk was de basis en inspiratie voor een volgend project waarin we dit verder hebben uitgezocht. Je schreef ook een indrukwekkende scriptie en inmiddels ben je zelf met een promotieonderzoek bezig, veel succes daarmee! Michelle en Thijs, jullie maakten deel uit van dat volgende project. Een stoomcursus labvaardigheden, een stage in Boston en het eraan vastplakken van jullie wetenschappelijke stage op ons lab hebben uiteindelijk geleid tot hoofdstuk 4 in dit proefschrift en straks een mooie publicatie. Veel succes nog met jullie studie geneeskunde en wie weet welk nieuw onderzoek van jullie we nog gaan zien!

Vervolgens wil ik graag alle coauteurs bedanken, allereerst in het Engels. Next, I would like to thank all coauthors from abroad. Michael Zimmermann, Diego Moretti and especially Tanja Jaeggi from ETH Zürich, it was very nice working with you on the Kenyan infant gut microbiome project in which I learned a lot, and which lead to a very nice publication in 'Gut' and to chapter 2 in my thesis. Many thanks and I'm looking forward to work on the next gut microbiome project together! Bobby Cherayil from the Massachusetts General Hospital in Boston, thank you for all experiments performed in your lab and supervising Thijs and Michelle during their fruitful internships. The results of their projects make up a large part of chapter 4 in this thesis and that will hopefully be published soon. Albert Bolhuis from the University of Bath, of course you can also read Dutch, but the results of the 'bugs and worms' experiments also made a really nice contribution to the work in chapter 4, thank you. Manuela Raffatellu from the University of California at Irvine, thank you very much for your contribution to our literature review, which now has been published and which makes

up the introduction of this thesis. Nu terug naar het Nederlands. Annet Maathuis en Koen Venema van (destijds) TNO in Zeist, de experimenten met het indrukwekkende TIM-2 model waren erg waardevol en hebben veel resultaten en nieuwe inzichten opgeleverd die we terug zien in hoofdstuk 3 van dit proefschrift. Dank voor al het werk en de goede samenwerking, wellicht kunnen we in toekomst nog eens samen nieuwe TIM-2 experimenten doen! Udo Engelke en Leo Kluijtmans, jullie 1H-NMR-spectroscopie dan wel organisch zuren analyses waren erg waardevol bij het in kaart brengen van het bacteriële metaboloom in TIM-2, dat vinden we uitgebreid terug in hoofdstuk 3. Bas Dutilh, je metagenoomanalyses zijn een hele mooie toevoeging aan datzelfde hoofdstuk, bedankt voor het inzichtelijk maken van deze ingewikkelde data en je kritische blik op het manuscript. Harro Timmerman en Jos Boekhorst van NIZO Food Research BV in Ede, bedankt voor jullie erg nuttige input en bioinformatische analyses van het darmmicrobioom die belangrijk waren voor maar liefst de hoofdstukken 2 t/m 4. De pyrosequencingprojecten hebben veel inzicht gegeven in de effecten van ijzer op de samenstelling van het darmmicrobioom. Nu is het volgende interessante project alweer aanstaande. Sara Burt van de Universiteit Utrecht, onze posters hingen naast elkaar op de Gut day in Gent en hadden na jouw idee geleidelijk aan het ene na het andere experimentje tot gevolg. Zo ontstond hoofdstuk 6 en een leuke publicatie, bedankt voor deze samenwerking.

Door de promotiejaren heen heb ik op verschillende laboratoria en gastlaboratoria binnen het Radboudumc gewerkt. Die gastvrijheid en samenwerking waren onmisbaar voor dit proefschrift. Wilbert Peters en analisten Hennie en Rene van het Laboratorium Maag, Darm en Leverziekten, bedankt voor de mogelijkheid om bij jullie te celkweken en de ultracentrifuge te gebruiken. Peter Hermans en Marien de Jonge van lab Kindergeneeskunde en infectieziekten (LKI), bedankt voor de vele uren die ik bij jullie op het ML-II lab mocht werken. Daarnaast waren de tips, interacties en discussies op het lab en tijdens de werkbesprekingen erg nuttig en een meerwaarde voor dit proefschrift. Eigenlijk wil ik jullie allen bij naam noemen, maar dat zou een lange lijst worden. Daarom veel dank aan alle LKI-collega's en ook dank aan de collega's van het lab Kindergeneeskunde en oncologie (LKO)! Het eerste jaar van mijn promotietraject waren we onderdeel van het Laboratorium Klinische Chemie (LKC), een dank aan de collega's waarmee we toen het lab deelden. Daarna werd het Laboratorium Genetische Endocriene en Metabole ziekten mijn thuislab. Ook hier ontstonden waardevolle samenwerkingen en kwam er hulp vanuit de verschillende onderzoeksgroepen en diagnostiekgroepen op verscheidene vlakken, die allen bijdroegen aan dit proefschrift. Daarnaast, vooral de collega's aan de Q-kant, ook bedankt voor de gezelligheid op het lab, tijdens de pauzes en bij uitjes. In het bijzonder noem ik daarin mijn collega's van de Neurochemie Research, de verschillende activiteiten, o.a. de Efteling, pubquizzes, barbecue en diner rouler waren gezellig. En wat was het een eer om de hoofdrol te spelen in de speelfilm 'Toren Q'… Marcel, nu heeft de palindroom 'Nelli plaatst op ene parterretrap ene pot staalpillen' het toch nog tot in mijn proefschrift geschopt.

Het was vaak hard werken, maar er was gelukkig ook een fijn privéleven en ruimte voor vrije tijd. Als ik terugdenk aan mijn eerste tijd in Nijmegen, denk ik meteen aan QHarmony. Dit

leuke studentenorkest maakte mede dat ik me snel thuis voelde in Nijmegen en zorgde voor fijne vriendschappen tot op heden en voor in de toekomst. Beste vrienden, bedankt daarvoor en voor de ontspanning bij het fijne muziek maken samen, en daarbuiten bij bijvoorbeeld feestjes, uitstapjes en spelletjes spelen. Ik kan me eigenlijk geen leven zonder muziek maken voorstellen. In de afgelopen jaren was het ook een belangrijke vorm van ontspanning en vaak een goede reden om het werk even te laten liggen. Daarom een bedankt alle muziekvrienden en bekenden van QHarmony, het Symfonisch Blaasorkest Nijmegen, Philharmonie Gelre, de Sint Plechelmusharmonie en De Luttermuzikanten. Ode aan de muziek!
Tot slot, maar heel belangrijk, nog even terug naar mijn roots in De Lutte. Mijn broers Roel en Stef, ook heel erg bedankt. Sinds ik in Nijmegen woon zien we elkaar minder vaak dan vroeger, maar als we bij elkaar zijn is het zeker gezellig, met vaak de nodige ongein. Leuk en bedankt dat jullie nu mijn paranimfen willen zijn! Lieve Pap en Mam, wat zou een dankwoord zijn zonder jullie te noemen. Jullie hebben mede gemaakt dat ik hier sta en het is erg fijn dat ik altijd bij jullie terecht kan. Ontzettend bedankt voor jullie steun, vertrouwen, liefde en nog veel meer!

Curriculum Vitae

Guus Kortman was born in Oldenzaal on July 9, 1985. He followed pre-university education at 'Twents Carmel College De Thij' in Oldenzaal and graduated in 2003 with a profile in Nature and Health (including chemistry, biology, physics and mathematics). Afterwards he studied Biology & Medical Laboratory Research at the university of applied sciences in Enschede. During this bachelor study he followed two internships within the Radboud University Medical Center in Nijmegen, at the department of Cell Biology and at the department of Pathology. After obtaining his bachelor degree (Cum Laude) in 2006, Guus studied Biomedical Sciences at the Radboud University Medical Center. His master program consisted of the subjects Human Pathobiology and Nutrition & Health and involved two internships. In the first, at the Institute for Food Research in Norwich (UK), he studied the regulation of gene-specific DNA methylation by DNA methyltransferases. In the second, at the department of Biomolecular Chemistry of the Radboud University, he investigated the possible involvement of miRNAs in multiple sclerosis pathology.
Guus obtained his MSc degree in 2010 and was subsequently appointed as a PhD student at the department of Laboratory Medicine of the Radboud University Medical Center. His research focused on elucidating the effects of supplementary iron on the growth, pathogenicity, and metabolism of gut bacteria, in the context of the whole human gut microbiome. The results of this work are described in this thesis and in several publications in scientific literature. Since July 2014, Guus is working as a postdoctoral researcher at the department of Laboratory Medicine (Radboud University Medical Center) on several projects, including gut microbiota and iron as topics.

Guus Kortman werd geboren op 9 juli 1985 in Oldenzaal. In 2003 behaalde hij zijn VWO-diploma met het profiel Natuur en Gezondheid aan het Thijcollege in Oldenzaal. Vervolgens studeerde hij Biologie & Medisch Laboratoriumonderzoek aan de Saxion Hogeschool in Enschede. Tijdens deze studie liep hij twee stages binnen het Radboudumc, bij de afdeling Celbiologie en bij Pathologie. Na het behalen van deze bachelorstudie in 2006 (Cum Laude) is hij Biomedische Wetenschappen in Nijmegen gaan studeren. Zijn master bestond uit het hoofdvak Humane Pathobiologie en het bijvak Voeding en Gezondheid, waarvoor hij ook twee stages voltooide. In de eerste, bij het Institute for Food Research in Norwich (Verenigd Koninkrijk) bestudeerde hij genspecifieke DNA-methylering door DNA methyltransferases. In de tweede, bij de afdeling Biomoleculaire Chemie (Radboud Universiteit), onderzocht hij de mogelijke betrokkenheid van miRNAs bij multiple sclerose. Guus behaalde zijn masterdiploma in 2010 en is aansluitend begonnen met zijn promotieonderzoek bij de afdeling Laboratoriumgeneeskunde van het Radboudumc. Zijn onderzoek concentreerde zich op het ophelderen van de effecten van ijzersupplementatie op de groei, pathogeniciteit en metabolisme van darmbacteriën, in de context van het humane darmmicrobioom. De resultaten van dit werk zijn beschreven in dit proefschrift en in meerdere wetenschappelijke tijdschriften. Sinds juli 2014 werkt Guus als postdoconderzoeker bij de afdeling Laboratoriumgeneeskunde (Radboudumc) op verschillende projecten, waaronder de darmmicrobiota en ijzer als onderzoeksonderwerpen.

List of publications

1. **Kortman GAM**, Raffatellu M, Swinkels DW, Tjalsma H. Nutritional iron turned inside out: intestinal stress from a gut microbial perspective. *FEMS microbiology reviews. 2014 (Epub ahead of print).*
2. Jaeggi T, **Kortman GAM**, Moretti D, Chassard C, Holding P, Dostal A, Boekhorst J, Timmerman HM, Swinkels DW, Tjalsma H, Njenga J, Mwangi A, Kvalsvig J, Lacroix C, Zimmermann MB. Iron fortification adversely affects the gut microbiome, increases pathogen abundance and induces intestinal inflammation in Kenyan infants. *Gut. 2014 (Epub ahead of print).*
3. **Kortman GAM**, Roelofs RW, Swinkels DW, de Jonge MI, Burt SA, Tjalsma H. Iron-induced virulence of Salmonella enterica Serovar Typhimurium at the intestinal epithelial interface can be suppressed by carvacrol. *Antimicrobial agents and chemotherapy. 2014;58(3):1664-70.*
4. Jaeggi T, Moretti D, Kvalsvig J, Holding PA, Tjalsma H, **Kortman GAM**, Joosten I, Mwangi A, Zimmermann MB. Iron Status and Systemic Inflammation, but Not Gut Inflammation, Strongly Predict Gender-Specific Concentrations of Serum Hepcidin in Infants in Rural Kenya. *PloS one. 2013;8(2):e57513.*
5. Kato I, Boleij A, **Kortman GAM**, Roelofs R, Djuric Z, Severson RK, Tjalsma H. Partial associations of dietary iron, smoking and intestinal bacteria with colorectal cancer risk. *Nutrition and cancer. 2013;65(2):169-77.*
6. Schaap CC, Hendriks JC, **Kortman GAM**, Klaver SM, Kroot JJ, Laarakkers CM, Wiegerinck ET, Tjalsma, H, Janssen MC, Swinkels DW. Diurnal rhythm rather than dietary iron mediates daily hepcidin variations. *Clinical chemistry. 2013;59(3):527-35.*
7. **Kortman GAM**, Boleij A, Swinkels DW, Tjalsma H. Iron availability increases the pathogenic potential of Salmonella typhimurium and other enteric pathogens at the intestinal epithelial interface. *PloS one. 2012;7(1):e29968.*
8. Boleij A, Dutilh BE, **Kortman GAM**, Roelofs R, Laarakkers CM, Engelke UF, Tjalsma H. Bacterial responses to a simulated colon tumor microenvironment. *Molecular & cellular proteomics : MCP. 2012;11(10):851-62.*
9. Koopman M, **Kortman GAM**, Mekenkamp L, Ligtenberg MJ, Hoogerbrugge N, Antonini NF, Punt CJ, van Krieken JH. Deficient mismatch repair system in patients with sporadic advanced colorectal cancer. *British journal of cancer. 2009;100(2):266-73.*
10. **Kortman GAM**, Dutilh BE, Maathuis JH, Engelke UF, Boekhorst J, Keegan KP, Nielsen F, Betley J, Weir JC, Kingsbury Z, Kluijtmans LAJ, Swinkels DW, Venema K, Tjalsma H. Gut microbial metabolism shifts towards a more toxic profile with supplementary iron in a kinetic model of the human large intestine. *Submitted.*
11. **Kortman GAM**, Mulder MLM, Richters TJW, Shanmugam NKN, Trebicka E, Boekhorst J, Timmerman HM, Roelofs R, Wiegerinck ET, Laarakkers CM, Swinkels DW, Bolhuis A, Cherayil BJ, Tjalsma H. Low Dietary Iron Intake Restrains the Intestinal Inflammatory Response and Pathology of Enteric Infection by Food-borne Bacterial Pathogens. *Submitted.*