The battle for iron in enteric infections

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Summary

Iron is an essential element for almost all living organisms, but can be extremely toxic in high concentrations. All organisms must therefore employ homeostatic mechanisms to finely regulate iron uptake, usage and storage in the face of dynamic environmental conditions. The critical step in mammalian systemic iron homeostasis is the fine regulation of dietary iron absorption. However, as the gastrointestinal system is also home to >1014 bacteria, all of which engage in their own programmes of iron homeostasis, the gut represents an anatomical location where the inter-kingdom fight for iron is never-ending. Here, we explore the molecular mechanisms of, and interactions between, host and bacterial iron homeostasis in the gastrointestinal tract. We first detail how mammalian systemic and cellular iron homeostasis influences gastrointestinal iron availability. We then focus on two important human pathogens, Salmonella and Clostridia; despite their differences, they exemplify how a bacterial pathogen must navigate and exploit this web of iron homeostasis interactions to avoid host nutritional immunity and replicate successfully. We then reciprocally explore how iron availability interacts with the gastrointestinal microbiota, and the consequences of this on mammalian physiology and pathogen iron acquisition. Finally, we address how understanding the battle for iron in the gastrointestinal tract might inform clinical practice and inspire new treatments for important diseases.

Keywords: Clostridia; gut microbiota; iron; nutritional immunity; Salmonella.

Iron in biological systems

Iron plays a central role in the biochemistry of nearly all life forms, where it is found within haem and inorganic compounds.1,2 Inorganic iron may occur as ferrous (Fe2+) and ferric (Fe3+) iron, the reduced and oxidized states of the element, respectively.3 The ubiquity of iron as a cofactor in biomolecules is in part due to its versatile chemistry. Iron has a large number of accessible oxidation states, allowing it to shuttle electrons, catalyse reactions and form a wide range of co-ordination complexes. Furthermore, iron was highly bioavailable during the early stages of the evolution of life, potentially rendering iron as a metallic cofactor.4 In the human body, iron occurs in inorganic compounds (e.g. iron oxalate, iron citrate, iron phytate, iron sulphate) and in iron-containing proteins. These can be categorized as (i) haemoproteins, (ii) iron–sulphur (Fe–S) cluster proteins, and (iii) non-haem, non-Fe–S, iron-containing proteins. A multitude of crucial biological processes in eukaryotes and prokaryotes depend on iron-containing proteins: DNA synthesis; metabolic energy

Abbreviations: aTf, apo-transferrin; DCYTB, duodenal cytochrome b; DMT1 (or NRAMP2), divalent metal transporter protein 1; DtxR, diphtheria toxin repressor; Fe-S, iron-sulphur; FPN (or SLC40A1), ferroportin; Ft, ferritin; FHH, ferritin heavy subunits; Fl, ferritin light subunits; Fur, ferric uptake regulator; HAMP, hepcidin; HIF2α, hypoxia-inducible factor 2, α; hTF, holo-transferrin; IRP, iron regulatory proteins; LCN2, lipocalin 2; Lf, lactoferrin; NRAMP1 (or SLC11A1), natural resistance-associated macrophage protein 1; ROS, reactive oxygen species; Tf, transferrin; TfR1 (or CD71), transferrin receptor 1

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production; biosynthesis of organic compounds, such as hormones; regulation of gene expression by histone and DNA demethylases; oxygen transport; and production of and defence against free radicals. 2,5–8

### Ingestion, absorption and intracellular handling of iron in humans

In mammals, excretion of iron is not extensively regulated; instead, iron homeostasis is achieved mainly by regulating intestinal absorption in the duodenum, 9 and possibly in the colon to some extent. 10 On average, humans ingest 20–25 mg of iron/day, the majority of which is inorganic iron (85%–90%), followed by haem-bound iron (10%–15%). However, only ~ 1 mg iron is absorbed per day, because of both poor iron bioavailability and tightly regulated iron homeostasis. 9,11

Of the two oxidation states that inorganic iron may take, Fe₂⁺ is the most stable in physiological pH and oxygen conditions, and also the most commonly found in dietary products. 3 However, inorganic iron uptake from the gastrointestinal lumen by enterocytes depends upon the divalent metal transporter protein 1 (DMT1 or NRAMP2), which is not able to import Fe³⁺. 12–14 The low pH of the stomach lumen reduces a significant fraction of Fe³⁺ to Fe²⁺, thereby facilitating its solubility and absorption. However, increased pH in the small intestine results in the oxidation and precipitation of a large fraction of dietary iron, rendering it inaccessible. To overcome the low availability of Fe²⁺, duodenal cytochrome b (DCYTB) enzymes that are present within the apical membrane of enterocytes are able to reduce any soluble Fe³⁺ to Fe²⁺ before DMT1-mediated Fe²⁺ import. 14,15

Organic molecules such as dietary vitamin C and microbe-produced short-chain fatty acids can also reduce iron and increase its bioavailability. 11,16,17 Finally, the activity of the Na⁺/H⁺ exchanger NHE3, which exports H⁺ from the enterocyte and generates an electrochemical gradient of H⁺ into the cell, facilitates the symport of Fe²⁺ and H⁺ by DMT1. 18

Despite haem’s small contribution to the total amount of iron ingested, it still accounts for over 40% of the daily absorbed iron, mainly as a result of its significantly higher bioavailability when compared with inorganic iron. 11 The mechanism of haem uptake into enterocytes is unclear, but may involve the haem transporter HRG1, followed by intracellular degradation by the haem oxygenase HMOX1 to finally release Fe²⁺. 19,20

Enterocytes have a relatively short lifespan (~ 4 days in humans), and, once sloughed off, all of their intracellular content is lost into the gut lumen. Therefore, in order to be absorbed, iron must promptly cross the basolateral membrane of enterocytes to reach the circulation. Cellular iron export – and, therefore, gastrointestinal absorption of iron – is uniquely mediated by the iron transporter ferroportin (FPN or SLC40A1). 21–24 As discussed in the next section, regulation of ferroportin-mediated iron export is central to systemic iron homeostasis. Immediately after crossing the basolateral membrane, Fe²⁺ is oxidized to Fe³⁺ by hephaestin, a multicopper oxidase co-localized with FPN. 25,26 FPN is also found within the membranes of intracellular vesicles – including pathogen-containing phagosomes, 27 particularly in macrophages.

Upon entering the circulation, most Fe³⁺ is rapidly bound to transferrin (Tf), the main form of circulating iron. Uptake of iron-bound Tf – holo-transferrin (hTf) – complexes by transferrin receptor 1 (TfR1 or CD71) is also the dominant mechanism of cellular iron acquisition by many cells and tissues. 28 When bound together, TfR1–hTf complexes are endocytosed, releasing Fe³⁺ in weakly acidic endosomes. Within these vesicles, ferrieductases, such as six-transmembrane epithelial of the prostate 3, convert Fe³⁺ into Fe²⁺ once again. 29 TfR1 remains bound to the iron-free Tf – apo-transferrin (aTf) – and the complex returns to the cell surface. There, aTf is released back into the circulation, where it may capture iron again. 30

In endosomes, Fe²⁺ enters the cytosol via DMT1. Free iron in the cytosol might partake in dangerous biochemical reactions; however, the factors that control trafficking of the cellular labile iron pool are only beginning to be understood. The moonlighting RNA-binding proteins PCBP1 and PCBP2 have been proposed to play a role in chaperoning cytoplasmic iron. 31,32 Labile iron can then be: (i) placed into cytosolic iron-containing proteins; (ii) transported to the mitochondria and incorporated into haem and Fe–S clusters; 2,33 (iii) sequestered inside ferritin (Ft); or (iv) exported to the extracellular space by FPN. 30 Nanocages of Ft, formed from 24 light (Fl4) and heavy (FtH) subunits, provide a long-term storage site for iron, shielding the cell from potential redox activity of free iron. 34 During iron deficiency, iron can be liberated from the Ft cage by a specific form of autophagy – ferritinophagy – mediated by the adaptor protein NCOA4. 35,36

### Host systemic iron metabolism and regulation of iron absorption

Every day 200 billion erythrocytes are degraded by reticuloendothelial macrophages in the liver and spleen; replacing these red blood cells has been estimated to require 2 × 10¹⁵ iron atoms or 20 mg of iron. 30 Hence, recycling of erythrocyte-derived iron via reticuloendothelial macrophages is essential for organism survival. 30 However, every day around 1 mg of iron is lost from the body in an unregulated way – predominantly via the sloughing off of epithelial cells – and this must be compensated by finely regulated intestinal iron absorption to keep total body iron content in balance.
The key to systemic iron homeostasis lies with hepcidin (HAMP) regulation. HAMP is a 25-amino-acid hormone protein mainly produced by liver hepatocytes, and its production is induced by iron loading. HAMP-deficient mice and humans present with a severe iron loading phenotype, and injection of synthetic HAMP decreases serum iron concentrations. Similarly, haemochromatosis is a genetic disease characterized by mutations in genes involved in iron homeostasis (HFE, HJV and TFR2). The proteins encoded by these genes play essential roles in controlling HAMP synthesis induced by intracellular and circulating iron stores, via the bone morphogenetic protein/SMAD signalling pathway. In haemochromatosis, intestinal iron absorption is disconnected from systemic iron availability, resulting in potentially lethal iron loading and susceptibility to fatal siderophilic bacterial infections, which exemplify the importance of regulating intestinal iron absorption.

HAMP regulates systemic iron metabolism through its capacity to bind, occlude and degrade the degradation of FPN on the surface of reticuloendothelial macrophages and duodenal enterocytes. In conditions of iron accumulation, the production of HAMP is stimulated, and, as a result, iron is subsequently trapped within enterocytes and macrophages, respectively inhibiting iron export to circulation or preventing recycling of iron derived from red blood cells. This acutely decreases serum iron and reduces dietary absorption to prevent further iron loading. Conversely, in conditions of iron deficiency, HAMP expression is down-regulated and surface expression of FPN is maintained, allowing iron export into the circulation and thereby replenishing overall iron levels (Fig. 1).

In addition to systemic regulation of iron flux out of the duodenal epithelium by HAMP, the organismal response to iron deficiency is also critically dependent on local signal integration. Iron deficiency results in stabilization of duodenal Hypoxia-Inducible Factor 2x (HIF2x), which in turn transcriptionally up-regulates expression of DMT1, DCTYB and FPN in enterocytes. Mice deficient in enterocyte HIF2x tolerate iron deficiency poorly, rapidly becoming anaemic as they cannot maximize iron flux through the duodenal enterocyte. Control of FPN-mediated iron efflux by HAMP has been proposed to modulate enterocyte iron content and therefore HIF2x-regulated iron uptake, indicating how both systemic and local iron homeostasis are coupled (Fig. 1).

Intracellular iron homeostasis in mammals is also controlled by two iron regulatory proteins (IRP1 and IRP2), which sense iron and post-transcriptionally regulate the expression of a number of iron homeostatic genes. As mentioned, iron can be stored in Fe⁺⁺, instead of exported out of the cell via FPN. IRPs negatively regulate Ft transcription to prevent inappropriate iron sequestration in enterocytes. Hence, multiple regulatory mechanisms exist within and acting upon duodenal enterocytes to regulate flux into, through and out of the enterocyte into the circulation. It is important to note that any mechanisms that reduce intestinal iron uptake will in turn relatively increase availability of iron in the gastrointestinal tract.

Bacteria and iron: sensing and homeostasis, not just thievery

Iron is an essential element for the growth of most bacteria, which rely almost exclusively on Fe²⁺ uptake mechanisms. However, due to the low solubility of Fe²⁺ over the soluble Fe³⁺, iron is frequently a limiting nutrient. The capacity to acquire sufficient iron to support proliferation, in the presence of iron-limiting host defence mechanisms, is an important determinant of pathogenicity. Iron deprivation acts as a sensory cue in bacterial pathogens, triggering the coordinated regulation of iron acquisition and virulence genes. Bacterial responses to iron depletion are largely controlled by two families of highly conserved iron-responsive regulators: the ferric uptake regulator (Fur) superfamily – mostly expressed in Gram-negative bacteria – and the diphtheria toxin repressor family (DtxR) – occurring in many species of Gram-positive bacteria. Despite their dissimilarities, Fur and DtxR-like proteins have equivalent domain architectures. Moreover, both metalloproteins function similarly, allowing/repressing transcription of target genes and modulating the expression of virulence factors such as toxin secretion, production of adhesins, formation of biofilms and regulation of quorum sensing.

Fur and DtxR both bear a DNA-binding domain as well as a metal co-repressor binding site. In iron-rich conditions, these metalloproteins bind Fe²⁺, undergoing a conformational change that favours their binding to specific motifs within the promoter region of target genes. Binding of Fur–Fe²⁺ and DtxR–Fe²⁺ upstream of iron uptake and storage operons-genes inhibits their transcription under iron-replete conditions. Conversely, in iron deficiency, Fe³⁺ dissociates from Fur/DtxR, which unbind from DNA, allowing the transcription of essential iron harvesting genes. Indeed, most iron acquisition strategies employed by bacterial pathogens are regulated by members of the Fur or DtxR superfamilies.

In this review, we particularly focus on two instructive gastrointestinal pathogens in the context of host–pathogen iron interactions. *Salmonella enterica* serovar Typhimurium (S. Typhimurium) is a Gram-negative facultative intracellular pathogen, which commonly causes localized gastrointestinal disease. In turn, despite a complex and multifaceted host immune response, the typhoidal strains *Salmonella* Typhi (S. Typhi) and Paratyphi – as well as other specific non-typhoidal serovars – have the potential to breach the mucosa and cause severe systemic infection, through initial intracellular infection of...
macrophages.\textsuperscript{64–66} \textit{Clostridium difficile} is a Gram-positive extracellular pathogen, which remains localized to the gastrointestinal tract but can cause severe toxin-mediated gastrointestinal pathology, with a mortality rate of up to 6\%.\textsuperscript{67,68}

**Iron homeostasis in \textit{Salmonella}**

Approximately 7\% of the S. Typhimurium genome is directly or indirectly regulated by iron,\textsuperscript{69} which highlights the importance of iron to \textit{Salmonella}. Central to iron homeostasis in \textit{Salmonella} is Fur, which controls essential mechanisms of iron uptake and storage.\textsuperscript{61,62} Furthermore, indirect regulation by the Fur-inhibited repressor RyhB sRNA suppresses expression of non-essential iron-requiring proteins during iron depletion.\textsuperscript{62,70–72} Fur also attenuates iron overload by controlling the production of bacterial Ft, which stores iron and protects the cell against iron-catalysed reactive oxygen species (ROS).\textsuperscript{73,74} Fur-independent iron-driven changes in gene expression
Figure 1. Intestinal iron homeostasis. Fe$^{2+}$ is reduced to Fe$^{3+}$ by duodenal cytochrome b (DCYTB) on the apical membrane of duodenal enterocytes and dietary compounds. Fe$^{2+}$ is taken up by enterocytes through divalent metal transporter protein 1 (DMT1). Inside the cell, iron is partitioned into various compartments, in part through chaperoning by proteins such as PCBP1/2. Iron may be used for biosynthesis of Fe-S clusters or haem in the mitochondria, or stored as ferritin in the mitochondria and cytosol. Cytoplasmic Fe$^{2+}$ is exported out of the enterocyte into the blood through ferroportin (FPN), where it is oxidized to Fe$^{3+}$ by hephaestin and bound by transferrin. In conditions of iron deficiency, hypoxia-inducible factor 2α (HIF2α) is stabilized, promoting the transcription of FPN, DMT1 and DCYTB, facilitating further iron uptake. Enterocyte iron deficiency also results in increased RNA-binding activity of iron regulatory proteins 1 and 2 (IRP1/2), which represses the translation of ferritin, reducing iron sequestration inside the enterocyte. Local iron homeostasis is further modulated by the microbiota, in conditions of iron deficiency microbial species such as Bifidobacterium generate small organic molecules such as 1,3-diaminopropane (DAP) which repress HIF2α and reduce transcription of the iron transporters. The production of HIF2α inhibitors by the gut microbiota may be a mechanism by which the microbiota competes with the host for luminal iron. Iron homeostasis is systemically regulated by hepcidin (HAMP) production in the liver; when iron levels are systemically raised, HAMP is produced. HAMP binds to, occludes and stimulates the degradation of FPN preventing iron export from the enterocyte and therefore limiting further iron uptake from the diet. In the absence of iron export, enterocyte iron will be stored in ferritin. The iron concentration in the intestinal lumen is set by the iron requirements of the gastrointestinal microbiota, iron uptake into enterocytes via DMT1 and release of enterocyte iron stores back into the lumen by epithelial sloughing.

have also been observed, for example, extracellular iron concentration is one of the stimuli of the two-component sensing pathway PhoQ–PhoP.75

Besides controlling the expression of genes directly involved in iron homeostasis, Fur regulation extends to other fundamental pathways, such as nitrate/nitrite respiration,76 acid tolerance response,77 and virulence – including the Type 3 secretion system encoded by Salmonella pathogenicity island-1.78–80 Fur-mediated regulation of virulence genes probably underpins the observed enhancement of Salmonella virulence in iron-supplemented conditions.81 Fur can also itself be regulated by the peroxide sensor OxyR, with ROS-mediated induction of Fur probably preventing oxidation of biomolecules by reducing intracellular iron concentration and subsequent formation of hydroxyl radicals.82

Overall, iron sensing via Fur and RyhB sRNA is essential for S. Typhi83 and S. Typhimurium84 infection. This finding underpins the multifaceted role that Fur plays in the survival of Salmonella in the host, by integrating iron handling, ROS, virulence and other fundamental signalling pathways. Therefore, it may be inferred that Salmonella harnesses iron availability to extrapolate information on its wider external environment and guide its behaviour.

In addition to Fur-mediated iron sensing, haem biosynthesis is also exquisitely regulated, with haem negatively regulating the activity of the rate-limiting biosynthetic enzyme HemA.85,86 Haem compounds are crucial cofactors for many bacterial cytochromes and catalases. In S. Typhimurium, the haem biosynthetic pathway is essential both for oxidative respiration and for protection against toxic oxygen intermediates (such as hydrogen peroxide).87,88 It also branches out into two distinct pathways related to the synthesis of sirohaem (a substrate in cysteine biosynthesis) and of cobalamin (or vitamin B12, a cofactor to many different enzymes).87 It was previously shown that S. Typhimurium hemA mutants were avirulent in mice,89 and their exposure to hydrogen peroxide resulted in extensive iron-mediated DNA damage and cell death.87

**Response to iron deficiency in C. difficile**

Similarly to Salmonella, exposure to low iron evokes a stress response in C. difficile, with many of the differentially expressed genes exhibiting binding sites for Fur.90 This results in altered expression of iron transporters – thus increasing iron uptake – and a metabolic switch, by suppressing the activity of metabolic pathways requiring iron-containing proteins and favouring alternative mechanisms. Specifically, glucose metabolism by pyruvate formate-lyase, formate dehydrogenase and [FeFe]-hydrogenase, ferredoxin-dependent amino acid fermentation, and cell motility are all suppressed by iron deficiency. Clostridium difficile also significantly changes the composition of its cell wall in response to iron deficiency, presumably to protect itself from other microorganisms, antibiotics or host immune responses.91 Moreover, iron depletion revealed significant up-regulation of C. difficile genes associated with virulence, including polyamine and histidine biosynthesis and uptake, as well as several flagella-associated genes, which represent well-known factors involved in adherence.92 The diverse changes in gene expression mediated by Fur underpin how sensing and responding to iron availability is synonymous with sensing the diverse environments to which a pathogen is exposed as it moves through the host.

**The host modulates systemic iron metabolism to limit extracellular iron availability**

Withholding of nutrients, particularly iron, from invading microorganisms has long been understood to play a key role in innate immune responses to infection.93 IRP, Ft and the siderophore-binding protein lipocalin 2 (LCN2) play pivotal roles in preventing and regulating
intracellular infection, underscoring iron’s key role in determining the outcome of pathogen invasion.\textsuperscript{94,95}

Additionally, and besides controlling iron homeostasis, the iron regulatory hormone HAMP can also reprogramme systemic iron distribution to protect against infection.\textsuperscript{96} HAMP was first identified as a liver-produced relative of the defensin antimicrobial peptide family.\textsuperscript{37} During inflammation, HAMP is induced in hepatocytes alongside a battery of other acute-phase proteins, downstream of interleukin-6 and toll-like receptor signalling-associated pathways.\textsuperscript{98} This increased production of HAMP during inflammation, and the subsequent block on iron export into the serum, drives the commonly observed hypoferraemia of inflammation (Fig. 2). Serum iron deficiency in humans is protective against the growth of some bacteria,\textsuperscript{99} and HAMP plays a role in preventing uncontrolled systemic infection with extracellular bacteria – including \textit{Vibrio vulnificus}, \textit{Yersinia enterocolitica} and some pathogenic \textit{Escherichia coli} variants.\textsuperscript{100–103} However, many bacteria are unaffected by host responses elicited by endogenous (or therapeutic) HAMP, which may be partly explained by their capacity to obtain iron from diverse sources \textit{in vivo}, such as haem.\textsuperscript{100}

In contrast to its clear inhibitory activity against some extracellular pathogens, HAMP-mediated hypoferraemia may promote intracellular bacterial growth in macrophages.\textsuperscript{104,105} HAMP-mediated degradation of FPN in infected macrophages greatly reduces iron export from the cytosol. Recent work suggests that HAMP can induce the degradation of phagosomal membrane FPN as well as FPN in the plasma membrane.\textsuperscript{27} Reduced inflow of cytosolic iron decreases intra-vacuolar iron, so reducing iron-catalysed ROS production and originating a more amenable intra-phagosomal environment, which facilitates \textit{Salmonella} survival and replication.\textsuperscript{27}

Although hepatic HAMP is necessary to control systemic iron homeostasis,\textsuperscript{106} transcription of \textit{HAMP} has been observed in a range of other cell types. Recently, in an experimental murine dextran sodium sulphate-driven colitis, HAMP production by dendritic cells was proposed to drive local iron sequestration in colonic myeloid cells, limiting increases in free iron driven by loss of barrier integrity and tissue damage. Failure to sequester iron locally resulted in dysbiosis, increased microbial translocation across the mucosa, persistent gut inflammation and failure to recover weight\textsuperscript{107} (Fig. 2). Independent work proposed a role for the Spi-C transcription factor and FPN-expressing macrophages in protection against dextran sodium sulphate-driven colitis.\textsuperscript{108} HAMP produced locally by keratinocytes may play a role in skin infection, in part by modulating the local innate immune response and neutrophil recruitment.\textsuperscript{109} These results pave the way for future work exploring the role of HAMP as a local regulator of iron–immune cell–microorganism interactions.

Another crucial host defence strategy lies on the expression of the natural resistance-associated macrophage protein 1 (NRAMP1, or SLC11A1), a divalent metal ion transporter expressed on the membranes of phagolysosomes of phagocytic cells.\textsuperscript{110} NRAMP1 polymorphisms can modulate the sensitivity of humans and mice to intracellular bacterial infections.\textsuperscript{111} NRAMP1 exerts its antimicrobial effects by two distinct mechanisms. First, it can modulate the phagolysosomal metal composition and the access of pathogens to important micronutrients, such as Fe\textsuperscript{2+} and Mn\textsuperscript{2+}.\textsuperscript{112,113} Iron depletion inhibits bacterial growth and pathogenicity, and low manganese levels decrease the resistance of microbes to oxidative stress.\textsuperscript{114} Recent results suggest manganese deprivation is the major NRAMP1-mediated \textit{Salmonella} resistance mechanism. Second, NRAMP1 activates pro-inflammatory immune pathways and antimicrobial effector mechanisms, resulting in increased production of reactive oxygen intermediates and reactive nitrogen intermediates.\textsuperscript{115}

**Host iron-binding proteins and mechanisms of bacterial iron uptake**

It is instructive to view bacterial iron acquisition mechanisms through the lens of countering host nutritional immune defences. Broadly, the mechanisms for iron uptake adopted by pathogenic bacteria include: (i) uptake of free inorganic iron, facilitated by reductases and associated Fe\textsuperscript{2+} permeases; (ii) acquisition of Tf- and lactoferrin (Lf) -bound Fe\textsuperscript{3+}, through direct or siderophore-mediated uptake, and (iii) extraction and capture of haem-iron from host haemoproteins, through haemolysin and/or

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**Figure 2.** Intestinal iron homeostasis during inflammation. Systemic inflammation increases hepcidin (HAMP) production by the liver, reducing iron absorption by the enterocyte and preventing iron recycling out of reticuloendothelial macrophages. Serum iron levels drop, and iron is sequestered within enterocytes. Furthermore, locally within the lamina propria, HAMP produced by dendritic cells has been shown to suppress ferroportin (FPN) on gut macrophages and regulate local iron availability during inflammation. In the gut lumen, pathogenic bacteria such as \textit{Salmonella} can take up Fe\textsuperscript{2+} directly (via systems such as FeoABC) or produce siderophores (such as enterobactins and salmochelins) to facilitate uptake of Fe\textsuperscript{3+}. To sequester iron in the gut lumen, enterocytes and immune cells, particularly neutrophils, produce iron-sequestering proteins such as Fe\textsuperscript{2+}-binding lactoferrin (Lf) and enterobactin-targeting lipocalin 2 (LCN2). Salmochelin is not bound by LCN2 and allows \textit{Salmonella} to acquire iron even in inflammatory situations. Some commensals, such as \textit{Escherichia coli} strain Nissle, disrupt iron acquisition by \textit{Salmonella} by competing for iron through the production of salmochelin and expression IroN, its uptake receptor.
Blood/Lamina propria

Dendritic cell

Ferroportin

Limited local recycling

Lactoferrin

LCN2

Local hepcidin

Macrophage

Neutrophil

Transferrin

Low serum iron/systemic iron availability

Ferritin

DMT1 DCYTB

IroN

Nissle

Exogenous siderophores also provide iron to Salmonella

FepA FhuBCD

Free iron FeoABC

FeoABC

Fep

IroC

Ent locus

Salmochelin

Lactoferrin and LCN2 made by neutrophils contribute to LCN and lactoferrin in gut lumen

Salmochelin chelates iron Fe³⁺

LcN2 bound to enterobactin

E. coli

FoxA

Hydroxymate siderophate

IroN

IroC

IroB

Salmonella

Fe³⁺

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haemophore secretion and specific cell surface-associated receptors.116

Uptake of free inorganic iron

Salmonella captures free Fe$^{2+}$ by a number of transporters, primarily the FeoABC system.117 An S. Typhimurium mutant for FeoB was shown to be outcompeted by the wild-type during mixed colonization of the mouse intestine.118,119 Availability of Fe$^{3+}$ in vivo might be increased by host- and bacteria-encoded ferric reductases.120 However, recently, the host Mn$^{2+}$/Zn$^{2+}$-chelating protein calprotectin was proposed to limit bacterial iron acquisition through its capacity to bind Fe$^{2+}$.121 Clostridium difficile expresses many genes – most of which are Fur-regulated – which encode paralogous Feo transport systems. From these, the operon feoI was consistently shown to be highly induced under iron-depleted conditions.90–92 In addition to Feo systems, other Fur-regulated putative iron transporters might also import free Fe$^{2+}$ from the external milieu, such as proteins similar to P-type cation transporters, and the low-affinity zinc transporter ZupT.90,91

Direct and siderophore mediated uptake of Tf- and Lf-bound Fe$^{2+}$

Tf and Lf are the dominant host iron-binding proteins in blood/tissue fluid and exocrine secretions, respectively. Both target free Fe$^{3+}$,122 and can therefore reduce its availability. Unsurprisingly, many pathogens are capable of circumventing iron withholding by Tf and Lf by directly taking up and degrading these glycoproteins. Primate Tf shows evidence of positive evolutionary selection at amino acid residues bound by bacterial Tf receptors, indicating the evolutionary pressure on the host to deprive pathogens of iron and the importance of Tf for host defence.123

As an alternative to directly taking up Tf or Lf, some bacteria produce siderophores; these are small molecules with extremely high affinity for Fe$^{3+}$, frequently employed as a tool to hijack iron from host glycoproteins (such as Lf, Tf and Ft), and to scavenge residual free iron from the external milieu. Siderophores are categorized based on the ferric-binding moiety, and include carboxylates, catecholates, hydroxamates and phenolates.124 Siderophores are particularly important for the pathogenesis of Salmonella, which – like many Enterobacteriaceae – synthesizes and utilizes the siderophore enterobactin through the Fep operon. In addition to supplying iron for growth, enterobactin has been proposed to inhibit iron-dependent host defence proteins such as myeloperoxidase.125 However, enterobactin can be sequestered by human LCN2, limiting its efficacy. LCN2 restricts the growth of bacteria that rely solely on enterobactin in a number of in vivo and in vitro models.126–129

Demonstrating the strong selective pressure that LCN2 has placed on pathogenic bacteria, Salmonella also produces salmochelin, a glucosylated form of enterobactin that avoids capture by LCN2. Salmochelin is synthesized and mobilized by the Fur-regulated tandem iroN iroABCDE gene cluster.130,131 Salmonella Typhimurium and S. Typhi defective in enterobactin or salmochelin production or handling exhibit attenuated virulence, demonstrating the importance of both siderophore systems.118,119,132 In particular, salmochelin facilitates pathogen iron acquisition and proliferation in infectious situations where inflammation induces LCN2 expression.133 In addition to producing enterobactin and salmochelin, Salmonella also has the capacity to pirate exogenous siderophores produced by other bacteria and fungi, such as the fungal siderophore ferrichrome – a hydroxamate siderophore.124 However, the role of this mechanism during infection is still unclear.134

The genome of C. difficile encodes several ferritin-, ferric-, and siderophore-bound iron uptake systems, which are almost exclusively Fur-regulated and highly responsive to iron fluctuations.91,135 Previously, it was shown that the catecholate’s precursor spermidine biosynthetic- and transport-coding genes (speAHEB and potABCD, respectively), as well as the catecholate siderophore import system YclNOPQ, are Fur-regulated and highly induced in C. difficile grown under iron-limiting conditions.91,136 Moreover, iron deprivation also induced expression of putative ferric hydroxamate uptake and sulphonate ABC transporter systems, which might facilitate iron uptake during iron-poor conditions.91,92

Clostridium difficile has been hypothesized to import siderophores produced by other microorganisms, but not to produce them. However, a recent study conducted by Shaw et al.137 identified a large genetic island from the genome of clade 3-associated strains of C. difficile, which, among other peptides, is predicted to code for the biosynthesis of a Yersiniabactin-like siderophore.

Extraction and capture of haem

Due to the relative abundance of haem in vivo, and the insolubility of ferric iron, invading bacteria have evolved several mechanisms to access it from dietary sources, intracellular haemoproteins, or circulating haemoglobin. Haemoglobin – or spontaneously dissociated haem/haemin – may be captured directly by bacterial cell surface-associated haemoglobin/haem-binding transporters, or acquired indirectly via small soluble high-affinity haem-binding proteins called haemophores.138 While
pathogenic strains of *E. coli*, *Yersinia* and *Pseudomonas* all express haem acquisition systems that seem to play a role in virulence, the importance of haem as a nutrient source for *Salmonella* remains unclear. Nevertheless, both salmoxylin and the Typhi-specific haemoxylsin E are proposed as being required for systemic infection. In other bacterial infections, haemoxylsin-mediated lysis of red blood cells may facilitate iron acquisition and infection. In response, host scavenging of free haem and hae-moglobin by the acute-phase proteins haemopexin and haptoglobin, respectively, is another aspect of reactive nutritional immunity.

Clostridium difficile infection is characterized by high levels of luminal haemoglobin from lysed erythrocytes. Until now, haem uptake mechanisms remained elusive in *C. difficile*. Bioinformatic analyses of *C. difficile*’s genome unravelled an incomplete haem biosynthetic pathway, despite the presence of both sirohaem and cobalamine biosynthetic genes. Recently, Knippel et al. identified a haem-sensing membrane protein system (HsmRA), able to hijack and internalize haem from the host, using it as a defence mechanism against ROS generated by extracellular haemoglobin and innate immune cells. By sensing low haem concentrations, HsmR activates expression of the operon *hsmRA*; this prompts the binding of haem to the membrane-bound HsmA, which, due to haem’s reactivity, is able to shield the bacterium from redox-active species. Simultaneously, the haem activated transporter system complex patrols the intracellular environment for toxic levels of haem, by allowing its export when deemed necessary. It remains unclear to what extent *C. difficile*’s toxin-driven tissue destruction and haemolysis may facilitate iron uptake; however, cholera toxin was recently shown to produce a growth-enhancing effect on *Vibrio cholerae* growth in the gut lumen by facilitating iron acquisition.

**Gut microbiota**

The human gastrointestinal tract harbours a complex and dynamic collection of microorganisms, collectively entitled the gut microbiota. Microbial composition undergoes temporal and spatial variations as a result of nutrient availability caused by environmental factors such as age, health status, dietary changes, inflammation, pathogen and/or antibiotic exposure. The mucosal intestinal immune system is generally tolerogenic, allowing the survival and proliferation of commensal microbes in exchange for immune and metabolic homeostasis and protection against pathogens. Therefore, an unfavourable compositional or functional change within the microbiome (dysbiosis) can incite infection, acute inflammatory responses and even chronic diseases such as inflammatory bowel disease and colorectal cancer.

Iron availability modulates the microbiota

The specific hallmarks of iron homeostasis in the 2000–4000 bacterial strains that constitute the gut microbiota remain to be described. Iron supplementation trials in infants in low- to middle-income countries showed increased incidence of diarrhoeal disease. This has been linked to higher levels of luminal iron influencing the composition of the gut microbiota. Although complex, one consistent result from *in vivo* studies is an iron-induced skew away from commensals such as lactobacilli or *Bifidobacterium* species, which have low iron requirements, towards *Enterobacteriaceae* (the bacterial family which includes *Salmonella* sp.). This skew and associated gut inflammation were particularly strong in low- to middle-income country cohorts, probably because relatively poor sanitary conditions increase the risk of underlying colonization by pathogenic microorganisms, the proliferation of which may be facilitated by extra iron availability. Indeed, iron availability in the colon lumen influences the expression of pathogen virulent genes. FPN-mediated effluxes of iron were shown to favour the growth of *S. Typhimurium*, conferring on them an advantage in the invasion of epithelial cells. Also, a recent study demonstrated that gut inflammation – and associated stressors, like production of reactive oxygen intermediates and reactive nitrogen intermediates – stimulate *Salmonella*’s SOS response, which triggers bacterio- phage lytic induction and transfer, thereby boosting horizontal gene transfer among bacteria and reassortment of their virulence factors. Whether iron supplementation alters the evolution of virulent bacterial strains remains to be investigated.

Experiments in animal models and *in vitro* fermentation systems broadly support the claims that iron supplementation remodels the gut microbiota, potentially skewing towards a dysbiotic phenotype. However, some studies suggest that profound iron limitation may also disrupt the normal microbiota. Although the picture is in no way clear-cut, it seems that increasing iron availability in the gastrointestinal tract probably alters both the composition and metabolic activity of the gut microbiota, creating a niche through which pathogenic microorganisms can enter and cause disease.

Gut microbiota modulates iron uptake and systemic iron homeostasis

Comparisons of germ-free and microbiota-colonized mice indicate that microbial colonization reduces luminal iron in the cecum. Conversely, when placed on a low-iron diet, germ-free mice exhibited milder iron-deficiency-induced anaemia, perhaps suggesting more efficient iron uptake in the absence of competing microbiota. The gut microbiota, specifically
microcins, small anti-bacterial molecules that bind *E. coli* in vitro. 

Pathogenic *S.* have also been proposed to antagonize strains with high iron sequestration properties of *Enterobacteriaceae* the uptake of siderophore-bound iron by pathogenic *E. coli* in infancy.124 Multi-species interactions within the gut remains in its understood, our understanding of iron homeostasis in single-species situations is relatively well homeostasis in the microbiota as a whole. The non-pathogenic *E. coli* strain Nissle targets the uptake of siderophore-bound iron by pathogenic *Enterobacteriacea* – such as *Salmonella* – by secreting microcins, small anti-bacterial molecules that bind bacteria via their catecholate siderophore receptors.170 *Bifidobacteria* strains with high iron sequestration properties have also been proposed to antagonize *S. Typhi* and pathogenic *E. coli in vitro*.171 Although bacterial iron homeostasis in single-species situations is relatively well understood, our understanding of iron homeostasis in the multi-species interactions within the gut remains in its infancy.124

**Future perspectives: therapeutic opportunities**

**Targeting the pathogen**

Iron uptake in the face of host and commensal defences is a major requirement for pathogen virulence, with iron acquisition systems being ubiquitous features of pathogenicity islands within genomes of virulent bacteria.172 However, although pathogens seem to suffer from iron deprivation, the dependence of innate and adaptive cellular immunity on iron makes therapeutically targeting iron availability problematic.173

An alternative approach to targeting iron directly may be to exploit our growing knowledge of pathogen iron uptake mechanisms. Improving host nutritional immunity through immunization against enterobactins reduced the severity of experimental gastrointestinal *Salmonella* infection, systemic dissemination and infection-associated dysbiosis. Importantly, this approach supported, rather than undermined, the non-pathogenic role of commensal microbiota.174 Alternatively, some investigators have proposed mirroring the mechanism of action of bacterially produced microcins and sideromycins, and exploiting siderophores as vehicles to facilitate the delivery of conjugated antibiotics to pathogens, overcoming resistance mechanisms such as enhanced drug efflux activity.176,177 Finally, siderophore-based therapies extend to exploiting probiotic bacteria that can more effectively compete with *Salmonella* for iron.169 These concepts of manipulating commensal–pathogen competition for nutrients, such as iron, may also prove relevant to *C. difficile* infection, where faecal microbial transfer has had therapeutic success through normalization of the commensal microbiota.149,176

**Targeting the host**

Probiotics and prebiotics may be useful in the treatment of iron deficiency and iron overload, by altering bioavailability of iron in the gastrointestinal tract and iron uptake by the enterocyte.166,177–179 Furthermore, dysbiosis in gastrointestinal and systemic auto-inflammatory disorders may in turn influence systemic iron metabolism, contributing to the anaemia of inflammation and functional iron deficiency commonly observed in these conditions.151,167,180

On the flip side, the potential for dysbiosis driven by dietary iron supplementation, thus exacerbating disease, complicates the treatment of anaemia in inflammatory bowel disease patients.181 A similar consideration must be made regarding the use of dietary iron supplementation to treat anaemia in low- to moderate-income countries, where aggravating already high rates of infant diarrhoea is a clear risk; reducing inflammation and improving sanitary conditions will be instrumental if oral iron supplementation to treat anaemia is to prove fruitful.156,182 Impaired gut health is a common reason for poor compliance with dietary iron supplementation regimens, even in high-income countries.183 There is interest in developing dietary iron supplements that are bioavailable to the host but not the microbiota, hopefully avoiding dysbiosis.184 By changing iron dosing schedules to reflect the negative feedback mechanisms intrinsic to mammalian iron homeostasis, fractional iron uptake can be maximized.185,186 This may also limit the adverse effects of iron supplementation on the gut.
Finally, chronically high dietary iron and haem, often associated with diets rich in red meat, have been proposed to predispose towards inflammatory bowel disease and colorectal cancer,\(^1\) both via direct effects on the epithelium and promoting dysbiosis.\(^1\) Conversely, iron-deficient diets in experimental systems seem to favour the growth of protective microorganisms.\(^1\),\(^1\)

Conclusions

Contemplating the role of iron provides some new insights into host–pathogen relationships in enteric infections, such as those involving *Salmonella* and *C. difficile*. Mechanisms of mammalian iron homeostasis and bacterial iron acquisition are well studied, at least in defined experimental systems. However, the interaction of iron with complex and diverse commensal and pathogenic gut microbial communities in the context of human disease still remains relatively uncharacterized. Targeting bacteria-specific iron acquisition apparatus and promoting commensal iron uptake at the expense of pathogens, while optimizing iron availability to the host, may together foster improved therapeutic approaches to gastrointestinal infectious and inflammatory disorders.

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None of the authors have any conflicts of interest.

Data availability statement

Data sharing is not applicable to this article as no new data were created or analysed in this study.

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