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Review

The Effects of Iron Supplementation and Fortification on the Gut Microbiota: A Review

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Abstract: Iron supplementation and fortification are used to treat iron deficiency, which is often associated with gastrointestinal conditions, such as inflammatory bowel disease and colorectal cancer. Within the gut, commensal bacteria contribute to maintaining systemic iron homeostasis. Disturbances that lead to excess iron promote the replication and virulence of enteric pathogens. Consequently, research has been interested in better understanding the effects of iron supplementation and fortification on gut bacterial composition and overall gut health. While animal and human trials have shown seemingly conflicting results, these studies emphasize how numerous factors influence gut microbial composition. Understanding how different iron formulations and doses impact specific bacteria will improve the outcomes of iron supplementation and fortification in humans. Furthermore, discerning the nuances of iron supplementation and fortification will benefit subpopulations that currently do not respond well to treatment.

Keywords: iron supplementation; gut microbiome; iron metabolism; gastrointestinal homeostasis

1. Introduction

The majority of living organisms require iron for survival. Iron can exist in one of two oxidation states, and due to this redox potential, can function in several fundamental processes, such as respiration, DNA replication, energy production, and cellular proliferation [1]. Humans absorb iron from their diet in a dynamic, tightly regulated process within the intestine [2]. In addition to controlling the amount of iron absorbed, this process dictates iron availability for the complex community of bacteria living in the intestine, hereafter referred to as the gut microbiota. As such, many bacteria have developed sophisticated systems to obtain, store, and regulate iron. Iron deficiency and excess both impact gut microbial health and lead to diseases, such as iron deficiency anemia and iron overload, respectively. Iron deficiency is highly prevalent worldwide and is commonly treated with oral iron supplements and fortificants [3]. In this review, we cover the effects of oral iron supplementation and fortification on gut health and disease. We begin with an overview of how the body acquires and utilizes iron. Then, we discuss the complex relationship between iron homeostasis and the gut microbiome. Finally, we summarize the microbial changes that occur following iron supplementation and fortification in animal and human trials, and we identify areas in need of continued research. In this literature review, we used PubMed and MEDLINE databases to search for articles related to

"human iron metabolism", "bacterial iron metabolism", "iron and gut flora", and "the effects of iron on the gut microbiota/microbiome in animals and/or humans".

2. Overview of Iron Absorption

Humans lose approximately 0.5–2 mg of iron every day from skin cell desquamation, intestinal epithelial cell (IEC) sloughing, and urine and sweat production [4]. Additional iron may also be lost during specific physiological processes, such as menstruation and lactation [5]. To balance this loss, the human duodenum and proximal jejunum absorb approximately 2 mg of dietary iron daily, a small proportion of the total daily dietary intake [6,7]. Iron from the diet is found primarily as heme, derived from myoglobin and hemoglobin, or nonheme iron, derived from plants and iron-fortified foods [6]. Nonheme iron exists in two forms as reduced ferrous iron or oxidized ferric iron. IECs, known as enterocytes, can absorb only ferrous iron (Figure 1). As such, ferric iron is reduced to ferrous iron by the membrane-bound ferric reductase duodenal cytochrome B (Dcytb) that is expressed on the apical brush border membrane of IECs [8]. Once in the ferrous form, iron is transported across the apical membrane of enterocytes by the 12 transmembrane domain protein, divalent metal transporter 1 (DMT1, also known as Nramp2) [9]. Within enterocytes, iron is stored in ferritin, used in a variety of cellular processes, or transported into systemic circulation by crossing the basolateral membrane through the 12 transmembrane domain protein, ferroportin [10]. Ferroportin is also expressed on macrophages and hepatocytes [10]. On the basolateral membrane, hephaestin oxidizes ferrous iron to ferric iron, enabling the transportation of iron in the blood by transferrin [5]. In comparison to nonheme iron, heme absorption remains enigmatic [11]. There are two current hypotheses for intestinal heme absorption: Either heme is endocytosed from the apical membrane or transported through a specific receptor into the cytosol [12].

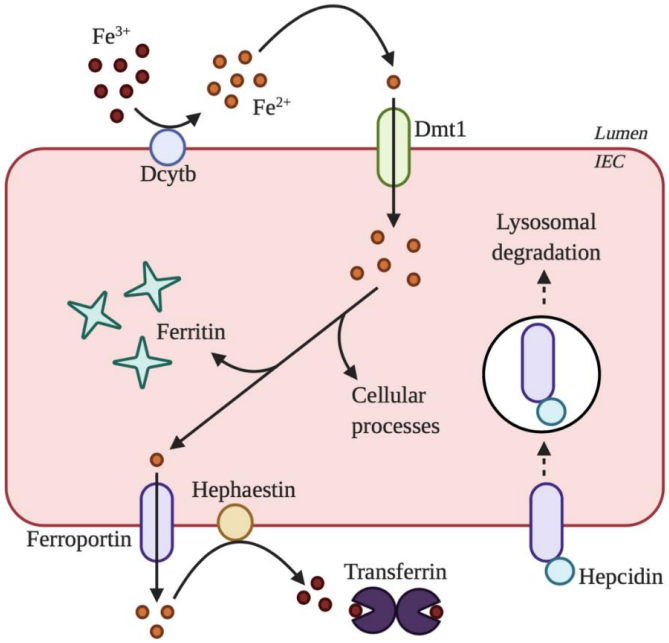

Figure 1. Absorption of nonheme iron by intestinal epithelial cells (IECs). Ferric iron is first reduced to ferrous iron by duodenal cytochrome B (Dcytb) on the apical membrane. Then, ferrous iron is transported across the apical membrane by divalent metal transporter 1 (DMT1). Once inside the cell, iron is stored in ferritin, transported across the basolateral membrane by ferroportin, or used in a variety of cellular processes. After transport across the basolateral membrane, ferrous iron is oxidized to ferric iron by hephaestin. Ferric iron is then transported by transferrin in circulation. Iron absorption is reduced when hepcidin binds to ferroportin because hepcidin causes the internalization and degradation of ferroportin. The figure created with www.BioRender.com.

3. Maintenance of Systemic Iron Homeostasis

Humans have no active iron excretory mechanism; therefore, systemic iron homeostasis is primarily regulated at the point of absorption. Hepcidin, a peptide hormone produced by the liver, is considered the master regulator of systemic iron homeostasis [13]. Hepcidin binds to and degrades ferroportin, which consequently impacts how iron is recycled by macrophages, absorbed by IECs, and stored by hepatocytes [14,15]. Hepcidin expression is upregulated when iron stores are adequate or high, or in response to inflammation, infection, or injury. Conversely, hepcidin expression is downregulated to improve iron absorption when iron stores are low, as in the case of iron deficiency or instances of certain genetic hemoglobinopathies, such as β-thalassemia [6,16,17]. As an aside, low hepcidin levels, as seen in some thalassemias and hereditary hemochromatosis, increase the risk of iron overload, due to increased intestinal iron absorption [18,19]. In hereditary hemochromatosis, a disease related to mutations in iron metabolism genes, excess iron is deposited throughout the body in the heart, pancreas, and liver, as well as the skin and joints [20]. Regardless of etiology, iron overload can result in several different diseases related to specific organ damage from oxidative stress. Iron overload also is known to increase the risk of infection.

Altered hepcidin levels are also associated with a variety of gastrointestinal conditions, including colorectal cancer (CRC) and inflammatory bowel disease (IBD). In CRC, hepcidin production is increased, which enables the tumor to retain more iron as tumoral ferroportin expression is decreased [21]. Intratumoral iron promotes oncogene activation, inflammation, and tumor growth [21]. In comparison, hepcidin levels in IBD do not follow a clear pattern, despite many patients with active disease experiencing reduced iron absorption [22–25]. Nevertheless, when examined using a dextran sulfate sodium (DSS) induced colitis mouse model, hepcidin levels were found to be reduced [26]. For both CRC and IBD, current research is focused on developing treatments and management strategies that reduce hepcidin levels. Vitamin D administration and anti-TNF-α monoclonal antibody therapy, for example, have shown promising results for the treatment and management of anemia in IBD [23,27].

4. Role of the Gut Microbiota in Maintaining Iron Homeostasis

Along the length of the intestine, there are physiological gradients (e.g., pH, oxygen, nutrient, etc.) that produce not only distinct bacterial habitats, but also influence the solubility and availability of iron [2]. The iron that is not absorbed by the duodenum passes into the colon, where it is thought to be metabolized by gut bacteria, as well as other microorganisms, such as parasites and fungi [1,28]. Iron is known to be a growth-limiting nutrient for both human cells and bacteria alike [29]. Accordingly, bacteria have developed two main strategies to obtain iron from their environment. The most prevalent mechanism involves the synthesis and secretion of siderophores, which are high-affinity ferric iron chelators [30]. *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Staphylococcus aureus*, and *Mycobacterium tuberculosis* are just some of the bacteria that use siderophores to acquire iron [31,32]. In addition to benefiting the bacterium, siderophores also appear to assist the host. As an example, Qi and Han (2018) observed in their study that enterobactin, an archetypical siderophore produced by many Gram-negative bacteria, promoted mitochondrial iron uptake and homeostasis by binding to the ATP synthase α subunit [33]. The results from this study emphasize that the battle for iron between microbes and host is far more complicated than currently understood, and opens the field for continued exploration of cooperative iron-mediated host-microbe interactions. The second strategy involves specific receptors that enable bacteria to acquire iron directly from host proteins, such as heme, transferrin, and lactoferrin [34]. Finally, some potentially beneficial gut bacteria, such as *Lactobacillus plantarum*, do not require iron at all but instead depend on manganese [35]. Therefore, in the presence of iron, iron-independent bacteria do not increase at a rate proportional to iron-dependent (and possibly pathogenic) bacteria [36].

Commensal gut bacteria play a vital role in maintaining iron homeostasis. In a recent study by Das et al. (2020), gut microbial metabolites were shown to suppress intestinal hypoxia-inducible factor 2-α activity (a master transcription factor of intestinal iron absorption) and upregulate ferritin

expression [37]. Additionally, Das et al. (2020) identified *Lactobacillus* species as the key sensors of intestinal iron [37]. Several studies have so far attempted to characterize the relationship between lactobacilli and iron. Using a mouse knockout model for iron regulatory protein 2, which increases fecal iron concentration, Buknik-Rosenblau et al. (2012) observed an increase in fecal *Lactobacillus* species abundance [38]. It remains to be seen whether the increase in lactobacilli was due to greater iron bioavailability or due to changes in the bacterial community that allowed lactobacilli to proliferate. Nevertheless, the work by Buknik-Rosenblau et al. (2012) demonstrates that deletions or mutations of iron metabolism genes affect the intestinal bacterial composition, which has clinically important implications. In human trials, a study by Balamurugan et al. (2010) found that young women in South India with iron deficiency had a low abundance of gut lactobacilli [39]. In comparison, the study by Kalipatnapu et al. (2017) observed an inverse relationship between fecal iron concentration and *Lactobacillus* species in rural children in India [40]. Regardless of the exact mechanism, several groups are testing whether specific *Lactobacillus* species improve iron absorption and status. In a meta-analysis of eight studies, Vonderheld et al. (2019) observed that *Lactobacillus plantarum 299v* significantly improved nonheme dietary iron absorption in humans [41]. Improvement in iron absorption may be due to an increase in Dcytb activity as Sandberg et al. (2018) observed activation of this axis following treatment with an *L. plantarum 299v* supplement in their human intestinal co-culture model of enterocytes and goblet cells [42]. In contrast, Rosen et al. found that *L. plantarum 299v* did not enhance iron absorption in iron-deficient pediatric patients treated with ferrous sulfate [43]. To improve the efficacy of *L. plantarum 229v* as a probiotic, future studies need to further assess the effects of dose, formulation, the timing of administration, and diet. In addition to *Lactobacillus*, other bacteria have been examined for their probiotic properties, and those studies are summarized in the recent review by Rusu et al. (2020) [44]. The use of prebiotics and synbiotics for the treatment of iron deficiency is also summarized in the same Rusu et al. (2020) review.

When the gut bacterial composition is altered or when gut bacteria are absent, iron homeostasis is disturbed. In experiments using germ-free mice [45] and rats [46], iron uptake and storage was reduced within IECs. In their study, Deschemin et al. (2016) also reported a decrease in ferroportin expression by IECs in germ-free mice, providing a mechanism for the observed reduction in iron absorption [45]. Similarly, iron absorption was reduced in rats [47] and rabbits [48] treated with antibiotics. These results, however, seem to conflict with a more recent study in mice that found iron absorption increased following antibiotic treatment [37]. These findings suggest that antibiotic administration may improve iron absorption in patients with iron deficiency.

While gut bacteria are important for maintaining systemic iron homeostasis, iron can also promote the replication and virulence of enteric pathogens, such as *Salmonella* spp., *Shigella* spp., and *Campylobacter* spp. [49,50]. When iron is abundant, bacteria proliferate, and form biofilms readily, which is hypothesized to be one of the reasons why individuals with iron overload are more susceptible to infection [7]. It has been shown that humans with iron overloading syndromes, including hemochromatosis and refractory anemias are more susceptible to bacterial infections, including *Yersinia* spp., *Listeria monocytogenes*, and *Vibrio vulnificus* [51–53]. Iron limitation, therefore, serves as an innate immune defence mechanism termed "nutritional immunity" [54]. Mediated by hepcidin, iron withholding strategies, such as hypoferraemia, denies iron to invading pathogens [55]. As an aside, iron retention within cells, such as macrophages, promotes the virulence of intracellular pathogens like *Salmonella enterica* [56]. Parmanand et al. (2019) observed in their in vitro colonic fermentation study that iron chelation resulted in a lower relative abundance of potentially pathogenic bacteria [57]. Similarly, Kortman et al. (2015) observed using a mouse model that dietary iron limitation reduced disease pathology upon oral challenge with *Citrobacter rodentium*, a well-established model for infectious gastroenteritis [58]. Finally, some of the pathogens that benefit from increased iron availability are procarcinogenic [59]. Specifically, *Streptococcus bovis*, *Bacteroides*, *Enterococcus faecalis*, and *Clostridia* are all implicated in carcinogenesis as these bacteria promote inflammation through the production of genotoxic metabolites [60]. These gut microbiota can contribute to the start and/or the

progression of colorectal cancer. With this knowledge in mind, there needs to be a reassessment as to whether providing oral iron therapy to colorectal cancer patients with iron deficiency is the best route of administration.

5. Effect of Iron Supplementation and Fortification on the Gut Microbiota

Iron supplementation and fortification are two different methods used to address iron deficiency. Iron supplementation is considered the more effective method, while iron fortification is often considered the safer method (as iron is delivered in smaller doses and is more amenable to physiological uptake when combined with food) [61]. While supplementation is a population-specific approach, iron-fortified foods, such as cereal products, milk, meal replacements, and infant foods, is a public health strategy to enhance the nutritional quality of diets in a population.

Iron salts are widely used in oral iron supplementation programs. As an inexpensive iron supplement, ferrous sulfate ($FeSO_4$) is one of the most commonly used iron salts. Unfortunately, ferrous sulfate is well known to irritate the stomach lining, causing gastrointestinal side effects, including stomach pain, nausea, diarrhea, and constipation, which makes supplementation adherence challenging [62,63]. Ferrous gluconate, another type of iron salt, appears to have fewer side-effects than ferrous sulfate [64]. Iron absorption from oral supplements is typically low, with less than 20% absorbed in the duodenum and the remainder passing unabsorbed into the colon [65]. Of the commonly used iron supplements, ferrous fumarate has the most iron per gram [64]. Emerging iron preparations, such as ferrous bisglycinate are marketed as having greater gastrointestinal tolerance, bioavailability, and protection against dietary iron inhibitors (e.g., phytates), as iron amino acid chelate does not form insoluble compounds with substances containing high content of iron absorption inhibitors, commonly found in cereal-based foods [66,67]. Nagpal and Choudhury (2004) previously conducted a comprehensive review of ferrous salts, ferric salts, iron amino acid chelates, iron polymaltose complex, and carbonyl iron; therefore, this review will not discuss the characteristics of these iron forms [68]. In comparison to iron supplementation, there are three primary forms of iron fortification: The fortificant can be added during food processing (e.g., flour) or during food preparation (e.g., multiple micronutrient powders—MNPs), or food can be genetically engineered to contain more iron (e.g., biofortified cereals) [17,69–71].

5.1. Animal Studies

In animal studies, microbial composition and metabolite production are altered by varying colonic iron availability. Mice weaned onto iron-deficient diets for eight weeks experienced a decrease in microbial richness compared to their baseline measurements and the control diet group [72]. Furthermore, iron repletion could not fully restore microbial richness [72]. In rats, iron deprivation increased *Lactobacillus* species while concomitantly reducing *Bacteroides* species and *Roseburia* species/*Eubacterium rectale* [73]. These compositional changes were associated with decreased levels of fecal propionate and butyrate (two types of short-chain fatty acids [SCFAs] used to fuel the gut) and were partially restored following ferrous iron repletion [73]. *Roseburia* species are found in a high abundance within the gut microbiota and are significant butyrate producers [74]. Similar findings were reported by Dostal et al. (2012) in their in vitro colonic fermentation models inoculated with immobilized fecal microbiota from a child [75]. Under very low iron conditions (0.9 mg Fe/L), *Roseburia* species/*E. rectale* decreased, as did butyrate levels, while *Lactobacillus* species increased [75]. The results from this experiment were subsequently confirmed in another Dostal et al. (2015) study that additionally showed that *Roseburia intestinalis* grown in low iron conditions preferentially produced lactate over butyrate [76]. Under high iron conditions, *R. intestinalis* increased expression of genes involved in butyrate production compared to results from the normal iron condition [76]. Another study in iron deprived rats also observed an increase in lactobacilli, in addition to total fecal anaerobes and *Enterococcus* species [77]. In comparison to iron deprivation, iron supplementation enabled bacterial taxa within the Clostridia class to proliferate in mice [78]. It should be noted that while iron

metabolism in mice is similar in many ways to humans, mice do not absorb iron, as well as humans; mice lose more iron relative to what they store, and thus, derive ~50% of their daily iron turnover from their diet, and mice have a more active iron excretory system that humans [78,79]. The fact that bacteria respond to iron bioavailability has led some researchers to pursue whether changes in fecal bacterial composition could provide unintrusive hints towards host iron status. Liu et al. (2020) showed in mice that five key bacterial taxa (*Porphyromonadaceae Parabacteroides*, *Clostridiales Peptostreptococcaceae*, *Akkermansia muciniphila*, *Clostridium perfringens*, and *Clostridia Clostridiales*) could be used as biomarkers to predict tissue iron levels in the small intestine and the liver (R^2 = 99.7% and 99.6%, respectively) [80]. Still, to be validated in humans, this method potentially offers a non-invasive way to diagnose iron-related diseases and monitor nutritional intervention. Not all studies, however, have observed changes to bacterial composition following changes to the availability of iron. Work by Alexeev et al. (2017) found that iron supplementation had no profound impact on individual rat pup bacterial operational taxonomic units [81].

Several studies have also examined the impact of iron supplementation on gut bacteria in animal models of IBD. Mahalhal et al. (2018) observed in their DSS-treated mouse model that any changes to dietary iron concentration (either an increase or decrease from standard) exacerbated the severity of colitis [82]. Interestingly, DSS-treated mice fed high iron diets did not lose as much weight as mice fed low iron diets, but had worse intestinal inflammation as measured by fecal calprotectin [82]. These same mice fed high iron diets experienced an increase in *Proteobacteria*, and a concomitant decrease in *Firmicutes* and *Bacteroidetes* [82]. Within the gut, *Firmicutes* are the most predominant producers of SCFAs, which have been shown to have anti-inflammatory properties [83]. In their DSS mouse model, Constante et al. (2017) observed worsened colitis in mice fed the iron preparation known as FEDTA (ferrous sulfate, ferrous bisglycinate, and ferric ethylenediaminetetraacetic acid) in comparison to the mice fed ferrous bisglycinate [84]. These mice had a reduced abundance of *Roseburia* species, which are known butyrate producers (a type of SCFA) [84]. Constante et al. (2017) also observed that iron supplementation with iron sulfate had a modest yet significant protective effect in mice treated with DSS by increasing survival [84]. These results are supported by research that shows that the severity of trinitrobenzene sulfonic acid-induced colitis can be reduced with oral ferric iron oral [85]. Still, these results conflict with research showing that ileitis in mice can be prevented by depleting luminal iron [86]. The discrepancy between studies may be a result of the different animal models as TNF-alpha ΔARE mice are susceptible to ileitis [86]; whereas, DSS- and trinitrobenzene sulfonic acid-treatment in mice results in colitis [84,85]. Werner et al. (2011) also observed that iron repletion by way of injection maintained the protective effect of the iron sulfate free diet, suggesting a role for luminal iron in the pathogenesis of chronic ileitis [86].

5.2. Human Studies

Iron supplementation and fortification have varying effects on human gut bacterial composition. Dostal et al. (2014) observed that South African children with iron deficiency who received high-dose iron supplements for 38 weeks (50 mg iron as $FeSO_4$ for four days/week) did not have significantly different concentrations of dominant gut bacterial groups or fecal SCFAs compared to children with no iron deficiency [87]. Similarly, Tang et al. (2016) observed in their study with American infants that iron supplementation did not create a more pathogenic microbial profile, rather, the abundance of *Escherichia* species decreased [88]. Likewise, Nitert et al. (2018) found no significant difference in the fecal microbiome at any taxonomic level between pregnant women receiving low-dose (0–10 mg Fe/day) or high-dose (>60 mg Fe/day) iron supplements [89]. Contrasting these studies, Jaeggi et al. (2015) and Tang et al. (2017) noted increased pathogen abundance in Kenyan infants receiving iron-containing micronutrient powder (12.5 mg/day) [90,91]. In healthy, non-anemic Swedish infants, consumption of high-iron formula (6.6 mg Fe/day) for 45 days did not increase the growth of pathogenic bacteria; however, the relative abundance of *Bifidobacterium* decreased [92]. In the same study, infants who received iron drops (6.6 mg Fe/day) had a lower relative abundance of *Lactobacillus* species than

infants who received high-iron formula. Despite the comparable doses, this study suggests that form of administration (i.e., as formula vs. drop) differentially influences gut microbial composition. Furthermore, as iron drops lead to a decrease in lactobacilli, which are important commensal bacteria, iron drops may increase susceptibility to infection. The varying microbial responses to iron reinforce the idea that multiple factors influence the gut bacterial composition. Future studies need to analyze how varying concentrations of iron influence fecal bacteria diversity and abundance. In addition, ethnicity and geography can influence gut microbial composition; therefore, future studies also need to investigate more disparate human populations [93]. These investigations will contribute to the developing field of microbiome-based personalized medicine [94]. Finally, another avenue for future research is examining the association between iron supplementation and exacerbation of infections such as malaria. In the infamous Pemba trial, iron supplementation in a malaria-endemic area was shown to increase the incidence of severe adverse events, including hospitalizations, due to malaria and other infections [95]. The potential mechanism for the worsening of malaria infection is thought to be excessive iron suppressing ferroportin, an iron exporter preventing iron excess in red blood cells, protecting against infection [96].

Human studies have also focused on characterizing the effects of iron supplementation and fortification on gut health as measured by inflammatory markers and incidence of diarrhea. Dostal et al. (2014) observed that the iron-deficient children on iron supplementation (study described in the previous paragraph) did not experience an increase in gut inflammation [87]. In contrast, Zimmermann et al. (2010) found that anemic children in Côte d'Ivoire who were iron-fortified biscuits (2 biscuits containing 20 mg Fe 4 times/week) presented with increased levels of fecal calprotectin, indicating increased levels of gastrointestinal inflammation. Several other studies in infants and toddlers from around the world also demonstrated increased intestinal inflammation following iron supplementation or fortification [88,90,97,98]. In their systematic review, Chanchi et al. (2019) examined 19 studies to evaluate the impact of oral iron supplementation and fortification on diarrhea incidence among children aged 4–59 months [99]. In 12 of the 19 studies, they found iron not to affect diarrheal incidence [90,100–110]. In the remaining studies, four recorded a significant increase in diarrheal incidence [111–114], and three recorded an increase within a specific subpopulation [115–117]. While most studies suggest iron supplementation and fortification do not induce diarrhea, there are two leading hypotheses to explain the sometimes-observed effect. Firstly, iron can produce reactive oxygen species within the intestine (through Haber-Weiss and Fenton reactions), which can cause intestinal damage and lead to inflammatory diarrhea [118]. This hypothesis is supported by in vitro experiments where intestinal epithelial cell lose their integrity following iron exposure [119,120]. Secondly (as previously discussed), iron can alter gut bacterial composition creating a more inflammatory environment [90,121]. In their review, Lönnerdal (2017) describes the other effects excess iron can have on children, such as impairing cognitive and motor development [29].

6. Conclusions

Iron supplementation and fortification studies have demonstrated that there are several factors, including diet, hygiene, inflammation status, disease burden, and genetics, that influence the complex interplay between iron and the gut. As the need for effective treatments for iron deficiency is indisputable, there are three main areas to explore related iron supplementation and fortification. Firstly, due to the previously mentioned complex interplay, future studies could benefit from a more thorough assessment and description of the study population to identify patterns and compare populations. Secondly, as newer iron preparations have been shown to have higher bioavailability and have been associated with fewer gastrointestinal side effects, there is a need to investigate the effects of varying doses, periodicity, and forms of iron supplementation on the gut microbiome. Similarly, as iron fortification within complex dietary matrices needs to overcome iron inhibitors, there is a need to examine the potential effects of iron-fortified foods on the gut microbiota. Finally, while this review has focused on oral iron supplementation and fortification, future reviews should assess how gut

microbial health is impacted by other iron therapies, including iron delivered intravenously and through biofortification.

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