Title: Deconstructing the role of iron and heme in the structure of healthy human gut microbial communities

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Summary

Responses of the human gut microbiome to disruptions are often difficult to discern without model systems that remove the complexity of the host environment. Fluctuations in iron availability provide a case in point: the responses of pathogenic bacteria to iron are much better understood than those of the indigenous human gut commensal microbiota because of an emphasis on *in vitro* studies of pathogens. In a study of iron supplementation in healthy humans, we identified gradual microbiota shifts in some participants correlated with bacterial iron internalization. To identify direct effects due to taxon-specific iron sensitivity, we used stool samples from participants as inocula to derive *in vitro* communities. Iron supplementation of these communities caused small shifts in structure, similar to those *in vivo*, whereas iron deprivation dramatically inhibited growth with irreversible and cumulative reduction in diversity and replacement of some dominant species. Sensitivity of individual species to iron deprivation during growth in axenic culture generally predicted iron dependency in a community context. Finally, exogenous heme acted as a source of inorganic iron to prevent depletion of some community members. Our results highlight the complementarity of *in vivo* and *in vitro* studies in understanding how environmental factors affect gut microbiome structure.

Introduction

The gut microbiota is often subjected to environmental perturbations with long-term effects, including dietary shifts[1], osmotic diarrhea[2], and antibiotics[3,4]. Typically, the complexities of a mammalian host pose challenges for disentangling possible direct effects of the perturbation on the microbiota versus indirect effects of an altered host environment. Recent studies have shown that *in vitro* microbial communities can be used to model the gut microbiota's response to antibiotics[5], other drugs[6], and colonization resistance[5,7]. However, it remains unclear to what extent *in vitro* communities can model other conditions, particularly ones that impact community composition based on taxon-specific metabolic needs, adaptation mechanisms, and sensitivities to the imposed condition.

A case in point is iron, which is essential for viability in all cells. Iron is incorporated directly into proteins involved in diverse processes such as DNA methylation and antibiotic biosynthesis[8], and is a component of essential cofactors such as iron-sulfur clusters and heme that are involved in important functions such as ATP production, protection against reactive oxygen species, and oxygen transport[8-10]. Despite its essentiality, iron can also be toxic when present at high levels. Hence, regulation of intracellular iron concentration is important, for both humans and the diverse set of microbes within the gastrointestinal tract[8,11].

Most existing knowledge about bacterial iron homeostasis stems from studies of pathogenic bacteria and in the context of infection[12,13]. In iron-limiting conditions, pathogenic bacteria produce high-affinity iron-binding molecules (siderophores) and/or proteins that aggressively scavenge iron[14]. Pathogens can also uptake heme from the environment and enzymatically degrade it to provide a source of inorganic iron[8,15,16]. When intracellular iron or heme concentrations become too high, iron- and heme-export machinery is deployed to avoid toxicity[17-19].

The extent to which these mechanisms extend to the diverse range of commensal bacteria, particularly those that typically live within communities such as in the human gut, has not been well characterized. During iron deprivation in isolation or in the context of infection, some commensals affiliated with the *Escherichia*[20,21], *Bifidobacterium*[22,23], *Enterococcus*[24], and *Bacteroides*[25,26] genera produce siderophores or use those produced by other species. Heme degradation as a source of iron has also been postulated for some commensal *Bacteroides* species[27,28], but unlike in pathogenic bacteria, the enzymes responsible for heme degradation have not been identified. Human studies on the effects of iron supplementation or iron deprivation have largely focused on populations involving confounding variables such as infection[29,30] or inflammation[31]; and studies in conventional mice[32] must contend with anatomic and resident differences with the human gut. Work based on *in vitro* fermentation models[33,34] has involved the cell-permeable iron chelator 2,2′-dipyridyl, which is not ideal due to its effects on both extracellular and intracellular iron concentrations. Thus, the relative sensitivities of gut commensal bacteria to excess or limited iron conditions, particularly in the context of a community and in the absence of confounding disease-associated variables, remain largely unexplored.

Here, we examined the effects of iron supplementation in healthy humans and then manipulated iron availability in communities derived *in vitro* using stool collected from these same humans to probe the iron requirements of commensal bacteria and the role of iron in shaping microbial community structure. In doing so, we sought to identify taxa that are resilient to changes in iron concentration and iron sources (i.e., inorganic iron versus heme-iron), in the absence of confounding host variables such as inflammation. We found that iron supplementation induces microbiota changes that are small, participant-specific, and positively correlated with increased bacterial intracellular iron concentrations during supplementation. We identified the Lachnospiraceae family as the most sensitive to iron supplementation *in vivo* and found that the specific genera and species affected within this family were participant-specific. Elevated iron availability in stable, diverse *in vitro* bacterial communities derived from our human participants recapitulated the small effects of iron supplementation *in vivo*, in contrast to iron deprivation, which induced irreversible changes to the community richness and composition that gradually accumulated. We found that resilience to iron deprivation varies across taxonomic families and that the variability of resilience to low iron within these families drives community shifts, as more resilient species occupy niches made available by less resilient ones. We discovered that the presence of heme buffers some of the effects of inorganic iron deprivation by rescuing specific species from depletion, and identify species that use heme as an alternate source of iron. Our results underscore the role of iron and heme availability in gut bacterial community structure, and highlight the general utility of *in vitro* communities for understanding the origins of human microbiota responses observed *in vivo*.

Results

Iron supplementation elicits small, participant-specific changes to gut microbiota composition in healthy humans

Inorganic iron supplements are available over-the-counter and are standard treatment for people diagnosed with iron deficiency (ID)[35]. ID is very common in children and women of LMIC, and occurs in ~40% of otherwise healthy pregnant women in the US[36] and up to 50% of healthy people who engage in endurance training[37-39]. To explore the response to iron supplementation of the gut microbiota in healthy humans, we recruited 20 participants to provide daily stool samples for one week before, the one week during, and for one week after a 7-day period of daily ingestion of a commercial iron supplement (65 mg iron/325 mg ferrous sulfate). One participant (#1) had been taking iron supplements for many years, and hence sampled daily for 21 days with their normal iron supplementation to determine baseline fluctuations. Of the sixteen participants that completed the study, all provided at least 18 samples, resulting in 301 samples total (Figure 1A).

We extracted DNA from each sample for 16S rRNA gene sequencing. Variation in community structure across all samples was analyzed by calculating alpha and beta diversities. Alpha diversity (Shannon index) did not exhibit significant changes throughout the study period for any of the participants (Figure S1), suggesting that iron supplementation did not have a large effect on the number of species that these communities harbored and that iron was not toxic at the ingested level. Beta diversity was visualized via Principal Coordinate Analysis (PCoA) of pairwise Bray-Curtis distances; other distance metrics led to similar conclusions (Figure S2). Samples largely clustered by participant (Figure 1B), indicating that the effect of iron supplementation on the gut microbiota at clinically relevant doses does not overcome inter-individual variation. Indeed, iron supplementation explained only 2.8% of the variability in microbiota structure throughout the study, while participant identity explained 87% (p<0.001, ANOVA). Nonetheless, when examining each participant of our current study individually, iron supplementation accounted for a much larger proportion of the variance: 20-33% for 9 participants with p<0.001 (ANOVA) and 16-21% for 4 of the remaining participants with p<0.05 (ANOVA) (Figure 1C). For two participants (#3 and #13), iron did not induce significant changes in microbiota structure; a similar analysis for our control participant #1 also did not show significant changes due to iron supplementation. For these three subjects, we attribute their measured variance to natural temporal fluctuations. Although we cannot disentangle variation due to iron supplementation from temporal variability, the highly significant changes (p<0.001) among the participants in our cohort are more likely to be iron-specific, with small but detectable individual-specific changes in the gut microbiota.

Given the high degree of participant specificity in the responses, we focused on subsets of the individuals to determine whether there were general structural changes to the gut microbiota. Among the 4 participants (#6, #7, #9, and #16) for which supplementation accounted for the largest proportions of variance, in participant #16, the gut community shifted during iron supplementation along one vector and continued to shift along that same vector after supplementation was halted (Figure 1C). Similar behavior was seen in participants #6, #7, and #9, suggesting that iron supplementation causes microbiota alterations that are either not reversible in the time frame of 1 week, or represent a delayed response to iron supplementation. In fact, iron-induced changes in community structure were of larger magnitude when comparing the “before” versus “after” periods (9 participants with $p<0.05$, 18-34% of total variance) than they were when comparing the “before” versus “during“ periods (4 participants with $p<0.05$, 14-26%), again suggesting that the effects of iron supplementation are gradual and delayed.

Next, we wondered whether the participant-specific responses to iron supplementation were associated with the timing of iron distribution or the local concentrations of iron throughout various parts of the gastrointestinal tract. Bowel movement frequency was uncorrelated with the percent variability attributable to iron across participants (Figure S3), suggesting that transit time of the iron through the gut was not a major determinant of participant-specific changes. We quantified extracellular and intracellular iron concentrations of each sample (Figure S4) by separating the supernatant from the bacterial pellets. The magnitude of change in extracellular concentration was uncorrelated with percent variability (R=0.001, p= 0.9, F-test), suggesting that while daily iron intake and/or iron absorption in the duodenum may vary across participants, the increase in the concentration of iron that ultimately reaches the colon is not the driver of microbiome structure. Conversely, intracellular iron concentration, was correlated with differences in percent variability (R=0.27, p< 0.05, F-test) (Figure 1D), suggesting that participant-specific responses might be explained by the capability of members of the community to internalize exogenous iron.

Participant-specific **Lachnospiraceae** *genera are the most responsive to iron supplementation*

To determine the taxonomic families most responsive to iron supplementation, we compared the relative abundance of several major families among the three study time periods (before, during, and after iron supplementation) in the four participants that were most responsive to iron supplementation (#6, #7, #9, and #16). The Akkermansiaceae, Enterococcaceae, and Enterobacteriaceae were present at very low abundances or not detected in most participants; hence, we focused on the Bacteroidaceae, Lachnospiracace, and Ruminococcaceae families. Although there were no consistent changes across the four participants at this taxonomic level (Figure S5), the relative abundance of certain genera or species affiliated with these families exhibited significant changes upon iron supplementation (p<0.05, HSD test), suggesting that iron-induced changes are most prominent at these lower taxonomic levels.

To identify structural changes that were specifically induced by iron supplementation, we calculated the fraction of genera/species that changed significantly within each family for each participant, and compared these fractions among the four participants that were most responsive to iron (26-33% variance explained, p<0.001) versus those that were least responsive to iron (#3,# 4, #13, and #5; 13-19% variance, p>0.05). The percent of varying genera in the Lachnospiraceae family was significantly greater in the four participants most responsive to iron supplementation (Figure 1E, p<0.05, HSD test), suggesting that genera/species within this family in particular responded to iron supplementation and merits deeper understanding of its response to iron levels.

In vitro ***passaging and study of an iron-responsive community recapitulates the observation that iron supplementation induces only small changes in community composition***

Given the inherent challenges in interpreting microbiota responses in the context of a complex host, we next sought to quantify the extent to which communities of gut commensals are sensitive to iron levels in a more controlled environment. Motivated by our previous study demonstrating that stool-derived *in vitro* communities can mimic the composition of the fecal microbiome and its sensitivity to ciprofloxacin observed *in vivo* [5], we used a pre-supplementation fecal sample from participant #16 (based on their relatively large response to iron) to derive *in vitro* communities via repeated passaging in BHI-S+0.05% mucin (Figure 2A). After five 48-h passage cycles, the community was stable and contained ~30 ASVs[40], which collectively represented 86% of the families detected in the fecal sample including members of the Bacteroidaeceae, Ruminococcaceae, Lachnospiraceae, Enterobacteriaceae, and Enterococcaceae families, all of which were predicted to respond to iron from our *in vivo* study (Figure 2B). Thus, this community provides a powerful starting point for interrogating the response to iron levels *in vitro*.

To study the effects of iron supplementation on this community, we passaged the stabilized community 12 times in 96-well plates in BHI-S+0.05% mucin medium (with the standard concentration of added heme (3.2 μM)), with and without supplemental 100 μM iron sulfate (Figure 2A). This concentration of iron is ~5-fold that of BHI-S medium (~16 μM) and was chosen based on the 2- to 3-fold increase in extracellular iron we observed in stool samples during *in vivo* iron supplementation (Figure S4).The maximum growth rate and carrying capacity of the community were unaffected by supplemental iron (Figure 2C), indicating that a surplus of iron does not enhance the growth of at least the fastest growing species. To determine whether supplemental iron induced changes in community structure, we performed 16S rRNA gene sequencing on passages 3, 6, 9, and 12. The number of observed species remained largely stable over time and the number was similar in the supplemented and non-supplemented communities (Figure 2D), suggesting that, as observed *in vivo*, the iron surplus did not have deleterious effects on most species. To determine the effects of iron supplementation on specific taxa, we examined the relative abundance of each major family over the 12 passages. Consistent with observations from our *in vivo* study, the supplemented and non-supplemented communities had very similar structure (Figure 2E, R=0.9, $p< 0.001$ *in vivo* and R=0.88, $p< 0.001$, F-test *in vitro*), indicating that the community was insensitive to a surplus of iron at the family and genus levels.

Although the relative abundances of most genera were similar between the supplemented and non-supplemented communities, of those that were detectable in both the *in vivo* and *in vitro* samples, a few displayed notable differences. Both *in vivo* and *in vitro*, the *Escherichia*/*Shigella* genus (*Enterobacteriaceae*) had a lower abundance during iron supplementation, while the *Coprococcus_3* (*Lachnospiraceae*) and *Bacteroides* (*Bacteroidaceae*) genera were at higher abundances (Figure 2F). The strong correlation between our *in vitro* and *in vivo* results (R=0.5, $p< 0.05$, F-test) provides further support that the changes observed *in vivo* are due to iron supplementation. Together, these results emphasize that supplemental iron is likely to have small but measurable effects on community structure and that our *in vitro* communities can be used more broadly to study the response to changes in iron levels.

Lack of iron inhibits the growth of complex communities* in vitro *and irreversibly reduces community richness

To further probe how changes in iron concentrations affect human gut communities and to identify taxa that are most sensitive to iron availability, we next sought to characterize the effects of iron deprivation on our *in vitro* community. We passaged the stabilized community multiple times in 96-well plates in BHI-S+0.05% mucin medium without added heme and supplemented with the extracellular iron chelator bathophenanthroline disulfonate (BPS) at increasing concentrations, thereby reducing iron levels in a graded fashion (Figure 2A). The maximum growth rate and carrying capacity of the community decreased in a chelator concentration-dependent fashion within a single 48-h passage (Figure 3A). Both growth parameters started to decrease substantially at 0.05 mM BPS, which is approximately the concentration necessary to chelate the 16 μM of iron in the medium given the 3:1 stoichiometry of BPS:Fe binding. The highest concentration of chelator (1.6 mM) reduced maximum growth rate and carrying capacity by 64% and 42%, respectively, suggesting severe inhibition of fastgrowing species and potential extinction of some species (Figure 3A). Maintaining low iron conditions with BPS for two additional 48-h passages further reduced maximum growth rate and carrying capacity, with a total decrease of up to 88% and 67%, respectively, relative to the untreated community (Figure 3B,C), indicating that growth defects due to iron deprivation accumulate over passages.

Reintroduction of the community to iron-sufficient concentrations (unaltered BHI-S) restored maximum growth rate by the third 48-h recovery passage, suggesting that growth of the fastest-growing species fully recovered (Figure 3D). However, the carrying capacity of communities previously treated with BPS remained up to ~30% lower than the untreated community, indicating either permanent loss of species whose niche remained unfilled or a change in metabolism that was irreversible within this time frame. Taken together, iron deficiency cumulatively destabilized human-derived *in vitro* communities over time and had long-lasting effects.

Motivated by the large, long-term changes in *in vitro* community growth due to iron deprivation, we performed 16S rRNA gene sequencing on all passages. The number of observed species in the community decreased in a BPS concentration-dependent fashion (Figure 3E), suggesting that the decrease in carrying capacity was at least partially due to severe depletion of some species. Reducing extracellular iron concentrations to nearly zero with 0.05 mM BPS decreased the number of observed species by ~20% over the first 48-h passage and by ~50% after a third 48-h passage relative to the untreated community (Figure 3F), suggesting that a large fraction of community members depend on iron to survive and/or grow and further emphasizing that the effects of iron deprivation are gradual and cumulative.

Virtually complete elimination of extracellular iron by adding excess chelator (BPS >0.1 mM) decreased the number of observed species to an even greater extent, by ~40% over one 48-h passage and ~80% by the third passage (Figure 3F), indicating variability in sensitivity and/or adaptation to low-iron conditions across species, whereby some could survive only at lower BPS concentrations. When the communities were reintroduced to iron-sufficient conditions, the number of observed species remained at approximately the same reduced level even after three 48-h recovery passages, suggesting irreversible loss of iron-dependent species (Figure 3F, passages 4-6).

Sensitivity to iron deprivation is heterogeneous across taxonomic groups

We next assessed changes in community structure to identify the taxa that were strongly affected by iron deprivation. The most striking changes were observed in the Lachnospiraceae and Ruminococcaceae families, whose relative abundances each decreased ~10-fold (from ~10% to ~1%) with 0.05 mM BPS and decreased below the limit of detection with >0.1 mM BPS (Figure 4A, passages 1-3). When iron levels were restored, both families re-equilibrated to only ~50-60% of their initial abundances (~5-6%) after treatment with 0.05 mM BPS and remained undetectable in communities treated with >0.1 mM BPS (Figure 4A, passages 4-6). These dramatic changes suggest that members of these families are highly dependent on exogenous iron and lack sufficiently effective mechanisms of adaptation to low-iron conditions. At BPS concentrations <0.5 mM, genera within these families exhibited slightly different sensitivities (Figure 4B), suggesting that adaptation mechanisms and/or overall sensitivity to low iron may be genus-specific.

To determine whether sensitivity to iron deprivation (extinction at >0.1 mM BPS) was intrinsic to these families rather than an emergent property dependent on community context, we acquired human isolates of several Lachnospiraceae and Ruminococcaceae species and grew them in iron-deprived conditions (0-0.4 mM BPS) for two 48-h passages (Figure S7A). Consistent with their behavior in the community, the isolates exhibited species-specific growth defects at 0.05 mM BPS, but all failed to grow after just one passage with ≥0.1 mM BPS (Figure 4C, S7B). These results suggest that overall sensitivity to iron deprivation is intrinsic to the Lachnospiraceae and Ruminococcaceae families, with subtle differences at the species level, and that isolate responses to iron deprivation can inform community-level changes.

The next most sensitive family to iron deprivation in the community was the Enterobacteriaceae family, which was represented by a single ASV in the *Escherichia*/*Shigella* genus. The relative abundance of this species decreased during iron deprivation in a BPS concentration-dependent manner and dropped below the limit of detection by the third 48-h passage with >0.1 mM BPS concentrations (Figure 4D, passages 1-3), suggesting that this commensal *Escherichia* species is highly dependent on exogenous iron. However, unlike the Lachnospiraceae and Ruminococcaceae families, when the community was reintroduced to iron-sufficient conditions, the *Escherichia* species fully recovered to its pre-treatment relative abundance by the third 48-h recovery passage in communities treated with ≤0.4 mM BPS (Figure 4D, passages 4-6), suggesting that this species possesses efficient low-iron adaptation mechanisms that allow it to endure long periods of iron restriction and then flourish once iron-sufficient conditions are restored. Notably, it did not recover in communities treated with >0.4 mM BPS, suggesting more complete BPS-mediated chelation of iron atoms at these higher concentrations and that BPS has a higher binding affinity for iron than the iron-binding molecules (e.g., siderophores) produced by the *Escherichia* species in this context. Taken together, these results suggest that commensal *Escherichia* species have a high dependency on iron and are equipped with efficient low-iron adaptation mechanisms that are likely missing in members of the Lachnospiraceae and Ruminococcaceae families.

Iron deprivation did not have detrimental effects on the Enterococcaceae and Burkholderiaceae families, which are low-abundance yet prevalent members of the human gut microbiome and are largely understudied. Most knowledge about the Enterococcaceae focuses on pathogenesis, and specifically on infection by the opportunistic pathogens *Enterococcus faecalis* and *Enterococcus faecium*[41-43]. Literature on iron responses of the Burkholderiaceae family is almost nonexistent[44]. When iron concentrations were reduced (0.05 mM BPS) or eliminated with excess chelator (>0.1 mM BPS), Enterococcaceae relative abundance gradually increased in each passage, by ~6-fold (from ~10% to ~60%) by the end of the third 48-h passage (Figure 4E, passages 1-3), suggesting that this family has little to no dependency on exogenous iron for survival and/or rapid, efficient mechanisms of adaptation to low iron. When iron-sufficient conditions were restored, Enterococcaceae relative abundance returned to near pre-treatment levels (Figure 4E, passages 4-6). Of the two *Enterococcus* species detected in the community, the initially less abundant *E. casseliflavus/faecium* (present at <1% in the untreated community) increased in relative abundance during iron deprivation, while the initially dominant *Enterococcus* species was at the same or lower relative abundance (Figure 4E), suggesting that Enterococcaceae members have different levels of resilience to low iron. Indeed, during growth in isolation, *E. faecium* was less sensitive to iron deprivation than *E. faecalis* and exhibited more growth during the second passage in iron-deprived media than in the first (Figure 4G and S7C). Both species immediately recovered when reintroduced to iron-sufficient medium regardless of the concentration of previous BPS treatment, suggesting that members of the Enterococcaceae family are highly resilient to iron deprivation.

The Burkholderiaceae family, which is represented by a *Sutterella wadsworthensis* ASV in the *in vitro* community, increased in relative abundance from ~5% in the untreated community to ~35-40% during the first passage with BPS (Figure 4F, passage 1). However, in subsequent passages with BPS, Burkholderiaceae relative abundance gradually declined (Figure 4F, passages 2 and 3), and upon reintroduction to iron-sufficient conditions its relative abundance returned to pre-treatment levels. This behavior suggests that *S. wadsworthensis* does not require high levels of iron for survival. When grown in isolation, *S. wadsworthensis* exhibited a BPS concentration-dependent decrease in growth rate and carrying capacity (Figure 4H, S7D). When reintroduced to iron-sufficient media, *S. wadsworthensis* recovered quickly, although not completely and in a BPS concentration-dependent manner (Figure 4H, S7D), suggesting a relatively high resilience to iron deprivation but lower than that of *Enterococcus* species.

Iron deprivation restructures the **Bacteroides** *genus*

Iron needs and acquisition strategies in commensal gut *Bacteroides* species have not been well characterized, but previous studies showed that some species such as *B. thetaiotaomicron* and *B. vulgatus* benefit from siderophores produced by other species, such as the commensal *E. coli* Nissle 1917, when deprived of iron *in vitro* or in the context of inflammation and the presence of a pathogenic *Salmonella* species in the mouse gut[25,26]. However, general features of the responses by commensal *Bacteroides* species to low-iron conditions in the context of a complex community remain unclear. In our community, the Bacteroidaceae family overall behaved differently from all other families. Bacteroidaceae relative abundance was ~30% during all three iron-deprivation passages at all BPS concentrations (Figure 5A, passages 1-3), suggesting that at least some family members have little dependency on iron or have efficient adaptation mechanisms that allow them to survive low-iron conditions. When the community was reintroduced to iron-sufficient conditions, Bacteroidaceae relative abundance increased in a BPS concentration-dependent manner (Figure 5A, passages 4-6).

Further examination revealed that *Bacteroides* species have distinct sensitivities to iron deprivation. *B. faecichincillae/faecis*, *B. vulgatus*, and *B. uniformis* decreased in relative abundance from ~5-10% to 0-1% during iron deprivation (Figure 5B), but recovered fully (*B. uniformis*) or partially (*B. faecichincillae/faecis* and *B. vulgatus*) when sufficient iron was restored (Figure 5B), suggesting that these species are iron-dependent but have adaptation mechanisms that allow them to survive. Importantly, this resilience disappeared for *B. uniformis* when communities were treated with >0.8 mM BPS or for *B. faecichincillae/faecis* and *B. vulgatus* when the communities were treated with >0.4 mM BPS, highlighting the variability in response to low iron within this family (Figure 5C). Since 0.4 mM BPS is the concentration at which the *Escherichia* species became undetectable, it is also possible that these *Bacteroides* species depend on the production of siderophores by *Escherichia* or another species with similar behavior to meet their iron needs and/or a cross-feeding metabolite [45]. *B. caccae, B. thetaiotamicron, B. nordii, B. salyersiae,* and *B. fragilis/ovatus*, which were all present at low (~1%) but consistently detectable levels in the untreated community, became undetectable when iron concentrations were reduced and did not rebound once iron was restored to sufficient levels (Figure 5B and 5C), suggesting that these species are strongly dependent on iron and have less efficient adaptation mechanisms to low iron than *B. faecichincillae/faecis*, *B. vulgatus*, and *B. uniformis*. Alternatively, it is also possible that *B. caccae, B. thetaiotamicron, B. nordii, B. salyersiae,* and *B. fragilis/ovatus* depend on a cross-feeding metabolite produced by an iron-sensitive species in the community, whose elimination means that these *Bacteroides* species can no longer survive.

Intriguingly, a *B. dorei/fragilis* ASV that was present at <1% in the untreated community increased in relative abundance during iron deprivation in a BPS concentration-dependent manner (Figure 5D, passages 1-3), comprising ~40% of the community by the third 48-h iron-deprivation passage at the highest BPS concentration and increasing to ~65% when sufficient iron levels were restored (Figure 5D, passages 4-6). The dominance of this *B. dorei/fragilis* ASV is presumably a combination of its lack of dependency on iron and/or efficient adaptation, with its ability to occupy the metabolic niches made available by species that did not survive the imposed low-iron conditions. Together, these results illustrate the dramatic extent to which differential iron sensitivities can substantially restructure the hierarchy within a single family.

A broad range of sensitivity to iron deprivation was also observed during isolated growth of six *Bacteroides* species in an initial passage in iron-deprived media (Figure 5E, S7E). During a second iron-deprivation passage, all *Bacteroides* species grew substantially worse than in the first passage or not at all (Figure 5E), again illustrating the gradual and cumulative effects of iron deprivation, as seen in the community (Figure 5B). However, the sensitivity of each species was more extreme in isolation than in the community, and growth defects were even observed in the absence of BPS, suggesting the absence of something other than iron from our BHI-S base medium, which lacks heme. The need for exogenous heme (heme auxotrophy) in some *Bacteroides* species (e.g., *B. fragilis* and *B. thetaiotamicron*), and the potential for its supply by the human host or other members of the gut microbiota is well known[10,25,45]. Thus, it is likely that the differences between growth in isolation versus in the community were at least partially due to the absence of heme. Because heme can be a source of iron, sensitivity to iron deprivation versus the requirement for heme as a cofactor are not easily disentangled in heme-deficient media. The substantial growth in the first passage without added heme and the gradual decline induced by iron deprivation suggest that *Bacteroides* species may harbor heme and iron stores that enabled their transient growth and survival. Together, these community and isolate findings demonstrate that sensitivity to low iron conditions can vary across species within a genus, illustrate the potential importance of the cross-feeding within a community , and suggest an explanation for why the inhibitory effects of iron deprivation accumulate over multiple passages.

Exogenous heme ameliorates the community destabilization induced by the lack of inorganic iron

Exogenous heme can be used as a cofactor and is directly incorporated into heme enzymes by heme auxotrophs. Moreover, heme is enzymatically broken down by heme degradases, providing a source of inorganic iron in pathogenic bacteria[46]. The enzymes responsible for heme degradation in commensal bacteria have not been identified, and the prevalence of heme degradation for meeting inorganic iron needs is not well known. To determine whether heme is used as an alternate source of inorganic iron by the commensal bacteria in our community during iron deprivation, we repeated the passaging experiment with the inclusion of the 3.2 μM of heme that is usually present in BHI-S medium (a concentration that is not expected to be toxic). Although maximum growth rate and carrying capacity still decreased in a BPS concentration-dependent manner in the first 48-h passage, the presence of heme reduced the extent of the decreases in maximum growth rate and carrying capacity to 59% and 40%, respectively, at the highest BPS concentration (Figure 6A), suggesting that the fastest-growing species were less growth-inhibited, fewer species were lost, and/or that heme sustained the growth of heme auxotrophs that could not survive in its absence and that are less sensitive to low inorganic iron levels.

When maintaining the community in low-iron conditions for two additional passages, heme prevented further decreases in maximum growth rate and carrying capacity. Instead, maximum growth rate was completely restored and carrying capacity was maintained by the third 48-h passage (Figure 6B and 6C), suggesting that heme was used as a source of iron by the fastest-growing species to restore their growth, and that the species that were sustained by heme in the first passage continued to thrive in low-iron conditions over an extended period. Reintroduction of iron to sufficient levels (unaltered BHI-S) in the presence of heme restored growth rate and carrying capacity almost completely by the third 48-h passage (Figure 6D), suggesting that the depletion of species that are highly dependent on exogenous iron can be prevented by heme.

To determine whether the beneficial effects of heme during iron deprivation were due to its use as an alternate iron source or to its ability to sustain the growth of heme auxotrophs, we compared the number of observed species and the relative abundance of species in our community grown with and without heme. In iron-sufficient conditions, the number of observed species (Figure 6E, 0 mM BPS) and their relative abundance (Figure 6F) were very similar between the added-heme and no-heme communities, indicating that exogenous heme did not alter community structure in these conditions. However, when iron concentrations were reduced (0.05 mM BPS) or virtually eliminated (>0.1 mM BPS), exogenous heme reduced the number of species that were lost from ~50% to ~30% and from ~80% to ~60%, respectively, indicating that exogenous heme rescued some species that would otherwise have dropped below the limit of detection (Figure 6E). Together these results suggest that heme buffers the effects of iron deprivation by acting as an alternate source of inorganic iron.

Heme rescues taxon depletion induced by iron deprivation in a family and species-specific manner

Given the buffering effects of heme during iron deprivation, we wanted to identify taxa that were rescued by heme and determine whether rescue was a family-level trait. Heme was most advantageous to the Enterobacteriaceae species in our community, almost completely preventing its drop in relative abundance induced by iron deprivation at ≤0.1 mM BPS and its apparent extinction up to the highest BPS concentration used (Figure 6G), suggesting that this commensal Enterobacteriaceae species uses heme as an alternate source of iron and has efficient mechanisms for demetallating this cofactor.

Heme also diminished the effects of iron deprivation and prevented the apparent extinction of all members of the Bacteroidaceae family except *B. nordii* and *B. salyersiae* (Figure 6H), suggesting that in addition to meeting the need of heme auxotrophs, heme can serve as an iron source for some but not all species in this family. Additionally, the relative abundance of *B. thetaiotaomicron*, *B. vulgatus*, and *B. fragilis/ovatus* actually increased when communities were iron-deprived in the presence of heme, suggesting that heme may provide an additional growth benefit to these species. In accordance with our hypothesis that the *B. dorei/fragilis* species has little dependency on iron and occupies niches that become available, the relative abundance of this species did not increase to the same extent (Figure 6H) as in the absence of heme (Figure 5B,C), consistent with the continued presence of members that became undetected in the nonheme community. Similar behavior was observed for the *Sutterella* (Burkholderiaceae) and Enterococcaceae species in this community (Figure 6I), again illustrating that heme diminishes the effects of iron deprivation within a community, enabling its composition to remain relatively stable when inorganic iron levels are depleted.

For the Lachnospiraceae and Ruminococcaceae families, heme enabled the survival at higher BPS concentrations of only one species in each family: *Flavonifractor plautii* (Ruminococcaceae) and *Coprococcus_3 comes* (Lachnospiraceae). However, heme did not prevent their eventual drop to undetectability (Figure 6J). These results suggest that heme can only act as an alternate source of iron for certain species within the Lachnospiraceae and Ruminococcaceae families, and their mechanisms for demetallating heme to obtain iron are not as efficient as those of the Bacteroidaceae and Enterobacteriaceae families.

Iron deprivation of isolates in the presence of heme largely resulted in similar behaviors as with communities. The growth rate and carrying capacity of all *Bacteroides* isolates decreased in a BPS concentration-dependent manner, and decreases were more pronounced in the second iron-deprivation passage, indicating the need for inorganic iron and not just heme as a cofactor (Figure 6K). However, heme ameliorated the negative effects of iron deprivation and enabled full or nearly full recovery when reintroduced to iron-sufficient conditions. Responses were species-specific: *B. thetaiotamicron*, *B. fragilis*, *B. vulgatus*, and *B. dorei* had the lowest growth defects and recovered fully when reintroduced to iron-sufficient conditions, while *B. uniformis* and *B. faecis* exhibited larger growth defects that lasted throughout the recovery passages. For Enterococcaceae species, heme reduced the effects of iron deprivation for *E. faecalis* but had no effect on the growth of *E. faecium* (Figure 6L).

In isolation, heme rescued Lachnospiraceae and Ruminococcaceae species from extinction due to iron deprivation at all BPS concentrations (Figure 6M). In addition, their growth was actually enhanced relative to growth in the absence of heme at all BPS concentrations, starting in the second iron-deprivation passage and lasting throughout the recovery passages (Figure 6M). Similar behavior was observed with *S. wadsworthensis* (Figure 6N). Although we currently cannot explain these positive effects on growth, it may be due to heme increasing expression of virulence genes and a shift from fermentation to more efficient anaerobic respiration, as has been reported for opportunistic pathogens[47,48].

Together, these findings show that heme serves as a source of inorganic iron for some commensal bacteria and plays an important role during iron deprivation in isolation and within a community.

BPS-induced effects are largely specific to deprivation of iron

A multitude of studies have used BPS to study the effects of iron deprivation on bacteria[26,49-51], fungi[52-54], and flies[55,56]. Although BPS binds with highest affinity to iron, it can form complexes with other divalent metals, specifically when these are in excess relative to iron[57,58]. To determine whether the effects of BPS on our *in vitro* community were specifically due to deprivation of iron, we treated the community with 0-200 μM BPS for 3 passages (with and without heme) as above, but also supplementing with one of the following divalent metals at a final concentration of 100 μM: Fe, Ca, Co, Cu, Mg, Mn, and Zn (Methods).

In the absence of BPS, metal supplementation did not have any notable effects on the growth rate or carrying capacity (Figure 7A), richness (Figure 7B), or composition of the community (Figure 7C,D), suggesting that these metals were not toxic at this concentration nor did they provide a differential fitness advantage to any species. At ≥50 μM BPS, supplementation with iron fully reversed the BPS-induced effects on growth rate, carrying capacity, community richness, and composition (Figure 7A-E), suggesting that the effects of BPS are due to the deprivation of iron. Supplementation with cobalt partially rescued the community relative to iron, reversing the BPS-induced decrease in growth rate and carrying capacity by the end of the first 48-h passage

(Figure 7A) and the reduction in number of observed species (Figure 7B). However, the time to reach maximum optical density was more than twice as long (~20-35 h versus ~10 h) and community composition was not restored as completely as with iron (Figure 7C-E). Supplementation with zinc was also able to partially rescue, but only at ≤100 μM BPS (Figure 7A-E). All other divalent metals tested did not rescue any BPS-induced effects on the community.

As expected, heme buffered the BPS-induced effects (Figure 7F, S8), but with some notable differences relative to supplementation with iron. Some taxa were rescued from dropping below the limit of detection by addition of iron, but not heme, suggesting that BPS-induced effects are largely due to iron deficiency and that species that did not recover in the presence of heme cannot use heme as an iron source (Figure S8E). To test this hypothesis, we assessed the growth of representative strains from families that recovered in the presence of heme (*Escherichia fergusonii*, Enterobacteriaceae) and those that did not (*Akkermansia muciniphila*, Akkermanciaceae) in 0 or 200 μM BPS supplemented with 100 μM iron, or 3.2 μM heme. Addition of 3.2 μM heme in 0 μM BPS did not perturb growth (Figure 7G), showing that heme is not toxic at this concentration. Both strains grew well in the absence of BPS and heme (Figure 7G), indicating that they are not heme auxotrophs. At 200 μM BPS, growth declined (Figure 7G), demonstrating that they are sensitive to iron deprivation. Supplementation with iron reversed this growth defect for both species (Figure 7G), confirming that BPS-effects are iron-specific. By contrast, addition of heme reversed the growth defect for *E. fergusonii* but not *A. muciniphila*, indicating that *E. fergusonii* can use heme as an iron source while *A. muciniphila* cannot.

Taken together, these results indicate that BPS-induced effects are generally a result of iron deficiency, that use of heme as a source of inorganic iron is species-specific, and that there is interplay between iron and other divalent metals, especially when they are present in excess of iron.

Discussion

A healthy cohort of individuals with variable levels of iron-deficiency (e.g., menstruating women and/or people with vegan or vegetarian diets), allowed us to study the effects of iron supplementation in the absence of disease-related confounding factors (e.g., inflammation during iron-deficiency anemia). Here, we showed that a typical clinical dose of supplemental iron can have gradual, long-lasting effects on the healthy human gut microbiota. However, the magnitude of the effects were participant-specific and small compared with antibiotic treatment[4], and some participants did not exhibit a significant response. The variation in responses across participants was partially due to differences in microbiota membership and the ability of these microbes to respond to increased iron via internalization (Figure 1D). Among participants that were most responsive to iron supplementation, the Lachnospiraceae family exhibited the largest shift in relative abundances (Figure 1E), with specific genera/species varying by participant. From these findings we conclude that iron supplementation can have varied effects on the human gut microbiome, making it difficult to predict which microbiome members will respond. *In vitro* communities derived from the stool of multiple participants in our study maintained most of the taxonomic diversity of the inocula (Figure 2B, S6), and iron supplementation of these *in vitro* communities yielded similar results as supplementation *in vivo* (Figure 2F), supporting the hypothesis that iron supplementation was a primary cause of the changes observed *in vivo* and emphasizing that effect sizes for healthy humans are expected to be small. Moreover, these findings further illustrate the ability of *in vitro* communities to model the effects of environmental perturbations on the gut microbiota *in vivo*.

Stool-derived *in vitro* communities provided an opportunity to interrogate the global effects of fluctuating iron levels on gut commensal communities, to gain a more nuanced understanding of the differences in iron sensitivities across taxa, and to identify commensals that benefit from heme as an alternate source of iron. Importantly, while we largely focused on a community derived from the participant that responded most strongly to iron supplementation, a community derived from another responder exhibited similar behaviors under changes to iron levels: Lachnospiraceae and Ruminococcaceae members became undetectable at ≥0.1 mM BPS, Enterobacteriaceae relative abundance decreased during iron deprivation but increased during recovery until their extinction at ≥0.4 mM BPS, and the Enterococcaceae family dominated at high BPS concentrations (Figure S6). By depriving *in vitro* communities of iron with the cell-impermeable chelator BPS, we showed that iron deprivation irreversibly reduces community richness, similar to previous studies in mice[32].

The environmental tunability of communities *in vitro* enabled us to discover that species depletion was BPS concentration-dependent and species-specific (Figure 3, 4, 5), indicating that commensal taxa have differential resilience to low-iron conditions, likely reflecting different iron needs and/or adaptation mechanisms. Members of the community covered the entire spectrum of sensitivity to resilience: Lachnospiraceae and Ruminococcaceae members were the most sensitive to low iron and rapidly became undetected (Figure 4A) , Enterobacteriaceae were sensitive but quickly recovered when iron-sufficient conditions were restored (Figure 4D), *Sutterella* (Burkholderiaceae) and *Enterococcus* species (Enterococcaceae) were resilient to low iron (Figures 4E and 4F), and members of the *Bacteroides* genus spanned the spectrum in a species-specific manner (Figure 5). Moreover, heme buffered the effects of iron deprivation, preventing the extinction of species (Figure 6, 7), and the ability to use heme as an alternate source of iron was species-specific (Figure 6, 7). Different responses to varying iron concentrations within a genus created the potential for certain resilient species to become dominant when other species with overlapping niches were inhibited (Figures 4E, 5D), indicating that the diverse range of responses has the potential to restructure a community under conditions of iron limitation. Thus, future predictions of the response of a given gut microbiota will benefit from further characterization of the behavior of individual species, in both isolated and community contexts, particularly the mechanisms of adaptation to changing iron conditions.

Treatment of an *in vitro* community with BPS and subsequent supplementation with iron, heme, or other divalent metals confirmed that the effects of BPS are specific to iron deprivation and that use of heme as a source of inorganic iron is species-specific (Figure 7). Interstingly, addition of cobalt (and zinc to a lesser extent) partially reversed the effects of BPS, suggesting that cobalt and zinc can compete with iron for complexation with BPS and/or that cobalt and zinc can functionally satisfy intracellular iron needs. At the end of a 48 h passage, BPS-containing media supplemented with cobalt exhibited less of the red hue and a lower absorbance at 535 nm that results from the BPS-iron complex compared to non-supplemented medium or media supplemented with any other metal besides iron (Figure S8A). Thus, we propose that when cobalt is present in excess of iron, it can gradually displace BPS-bound iron, thereby freeing up iron for bacterial use. The longer time required to reach saturation in a BPS-treated community supplemented with cobalt compared to supplementation with iron (Figure 7A) is consistent with slow release of BPS-bound iron by cobalt, and the similar composition after passaging with cobalt and iron supplementation indicates that the inhibition of growth did not affect substantially disrupt the niches of each organism. The slight rescue of BPS-induced effects by excess zinc may involve more complex metal interplay, such as upregulation of iron-store release and iron uptake to increase intracellular iron, as has been shown to occur in *E. coli*[59], or via functional replacement of iron through incorporation into iron metalloenzymes to provide enzymatic activity that is temporary and not highly efficient[60]. Regardless, our findings highlight the importance of bacterial metal homeostasis and the cross-talk between the pathways that control their intracellular levels.

In the future, mechanistic studies with bacterial isolates and mutant libraries[61,62] may provide greater understanding of these responses and adaptation mechanisms, as well as provide the opportunity to engineer communities through manipulation of iron levels and sensitivities. Utilization of heme as both an iron source and a cofactor may be intertwined in low-iron conditions for some species, as they appear to be for *Bacteroides* species (Figure 4). Moreover, in some cases the effects of iron or heme deprivation are not immediately apparent but instead require multiple passages in low-iron conditions (Figure 4E), indicating that these species can store iron/heme and then utilize these stores when iron/heme is lacking. This storage may explain the gradual changes observed during iron supplementation *in vivo* (Figure 1C) and deprivation *in vitro* (Figure 3B-D). In some cases, species such as members of the *Bacteroides* genus were more sensitive in isolation than in the community (Figure 5E), reflecting the interaction of iron availability with sharing of other resources such as heme.

This study provides a foundation for further interrogation of community restructuring due to environmental perturbations. Our study suggests that even in the case of chemicals such as iron that affect the host and microbiota alike, *in vitro* bacterial communities can provide key insight into the microbiota response *in vivo*. For iron specifically, the taxon specificity of most responses suggests that metagenomic sequencing may prove useful for revealing whether changes to iron concentration select for particular strains and substrains. Our findings motivate identification and biochemical characterization of the enzymes that anaerobically extract iron from heme in *Bacteroides* and other species. Currently only one anaerobic heme degradase (ChuW) has been identified, in *E. coli*[63]. ChuW-like proteins in *Bacteroides*, *Enterococcus*, and *Sutterella* species are annotated as putative oxygen-independent coproporphyrinogen III oxidadases (HemN) or as radical SAM family heme chaperones (HemW). Deciphering whether thse enzymes are cannonial HemW/HemN homologs or misannotated ChuW homologs remains to be determined. Use of transposon mutant libraries[61,64] when iron conditions are perturbed could shed light on genotype-phenotype relationships, and the structural underpinnings of enzyme function could be facilitated by recent advances in deep learning-based protein folding[65]. Our approach combining *in vivo* and *in vitro* interrogation can also be applied to future human studies of supplementation of anemic participants and of supplementation with heme instead of ferrous sulfate, both of which should inform potential treatment for anemic patients. Finally, clinical investigations of whether fecal microbiota transplants can reverse the effects of iron deprivation on gut communities should be facilitated by the relative ease and throughput of testing responses of stool-derived *in vitro* communities, which can be used to tune conditions before translating to mice and humans.

Significance

Iron metabolism in commensal bacteria remains poorly understood, especially in the context of a microbial community and in the absence of disease-confounding factors such as inflammation. Human-derived *in vitro* communities are powerful tools that enable the separation of host-mediated effects from microbe-specific effects. By conducting a study of iron supplementation in healthy humans and interrogating the effects of iron supplementation and iron deprivation on *in vitro* communities derived from these participants, we identified direct effects of changes to iron levels on healthy gut microbiotas. Iron supplementation caused shifts in community structure that were small but consistent between *in vivo* and *in vitro*, and iron deprivation induced large and irreversible changes to the structure of *in vitro* communities. We show that the sensitivity of individual species to iron deprivation can predict iron-dependent changes in a community and that heme can act as a source of inorganic iron, significantly diminishing the effects of iron deprivation. These findings provide a foundation for future studies of iron metabolism in human gut commensal bacteria and highlight the complementarity of *in vivo* and *in vitro* studies for deconstructing how environmental factors affect gut microbiome structure.

Author Contributions

A.I.C., D.R., and K.C.H. designed the research; A.I.C. performed the research; A.I.C. analyzed the data; and A.I.C., D.R., and K.C.H. wrote the paper. All authors reviewed the paper before submission.

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STAR Methods

Resource availability

Lead contact

Communication regarding this article should be directed to the lead contact, Kerwyn Casey Huang (kchuang@stanford.edu).

Materials availability

This study did not generate new unique reagents.

Data and code availability

Sequencing data and code have been deposited at the Stanford Data Repository and are publicly available as of the date of publication. DOIs are listed in the Key Resources Table. Any additional information required to reanalyze the data in this paper is available from the lead contact upon request.

Experimental model details

Human fecal samples or *in vitro* gut bacterial community cultures were used to conduct analyses in this study as specified in the text. The *in vitro* communities were derived from participant stool samples and cultured anaerobically in Brain Heart Infusion-S medium (BHI supplemented with 3.2 μM hemin (referred to as ‘heme’ in this study), 0.5 mg/mL L-cysteine, and 5 μg/mL Vitamin K; BHI, BD, Cat. #211069) along with 0.05% mucin, and incubated at 37 °C for 24-48 h.

Method details

Non-clinical iron supplementation study recruitment and sampling

Twenty healthy adults (39.8±12.6 years of age) who had not taken antibiotics or been diagnosed with iron deficiency within the previous year were recruited to provide daily stool samples for 7 days before, 7 days during, and 7 days after daily intake of a 65 mg iron supplement (325 mg ferrous sulfate, Nature Made®). Sample collection consisted of resuspending ~50 mg of stool in 500 μL of DNA/RNA shield (Zymo Research, R1100) and placing ~5 g in empty sterile plastic tubes, separately. One participant had been taking iron supplements for years, and hence was sampled daily for 21 days without additional supplementation but continued their regular supplementation to determine baseline fluctuations. Participants collected samples themselves and were instructed to immediately freeze the samples at home until the samples were brought to the laboratory for storage at -80 °C, which happened at the end of the sampling period. Sixteen of 20 participants completed the study, with 14 participants providing at least 18 samples, resulting in 301 samples total (Figure 1A). Samples stored in DNA/RNA shield were used for 16S rRNA sequencing analysis. Aliquots of the 5-g samples were used for iron quantification and for *in vitro* community inoculation. Informed consent was obtained from participants and the study protocol (#25268) was approved by the Administrative Panel for Medical Research on Human Subjects (Institutional Review Board) of Stanford University.

DNA extraction and 16S rRNA library preparation

For human stool, samples stored in DNA/RNA shield were thawed on ice and vigorously mixed. Two hundred fifty microliters of each sample were transferred into 96-well PowerBead Plates (Qiagen, Cat. #27500-4-EP-BP). Seven hundred fifty microliters of RLT buffer from an AllPrep DNA/RNA 96 Kit (Qiagen, Cat. #80311) were added to each well and subsequent steps of DNA extraction were followed using manufacturer's instructions. For *in vitro* communities, 50 µL of saturated bacterial cultures were extracted using a DNeasy UltraClean 96 Microbial Kit (Qiagen, Cat. #10196-4).

Three microliters of extracted DNA were used in 75-µL PCR reactions containing Earth Microbiome Project-recommended 515F/806R primer pairs (0.4 µM final concentration) and 5PRIME HotMasterMix (Quantabio, Cat. #2200410) to generate V4 region 16S rRNA amplicons. BSA at a final concentration of 100 ng/µL was also added to extracted DNA from stool samples. Thermocycler conditions were: 94 °C for 3 min, 35 cycles of [94 °C for 45 s, 50 °C for 60 s, and 72 °C for 90 s], then 72 °C for 10 min. PCR products were individually cleaned up and quantified using the DNeasy UltraClean 96 PCR Cleanup Kit (Qiagen, Cat. #12596-4) and the Quant-iT dsDNA High Sensitivity Assay kit (Invitrogen, Cat. #Q33120) before 200 ng of PCR product for each sample were manually pooled. Pooled libraries were sequenced with 250- or 300-bp paired-end reads on a MiSeq (Illumina).

16S rRNA data analysis

Samples were demultiplexed with QIIME2 v. 2021.2[66] and subsequent processing was performed using DADA2[40]. truncLeF and truncLenR parameters were set to 240 and 180, respectively, and the pooling option parameter was set to “pool=FALSE” unless otherwise indicated. All other parameters were set to the default. Taxonomies of the resulting ASVs were assigned using the assignTaxonomy function and the Silva reference database.

Iron quantification

A colorimetric ferrozine-based assay protocol previously used for quantification of iron in cultured astrocyte cells[67] was modified and used to measure extracellular and intracellular iron concentrations in stool samples. In brief, ~100 mg of stool were resuspended in 1 mL of TE buffer (10 mM Tris-HCl [pH 8], 1 mM EDTA) and vortexed vigorously. Resuspensions were centrifuged (6,000 rpm, 5 min) and 100 µL of the supernatant were transferred to a 2-mL 96-well plate for extracellular iron quantification. Pellets were washed twice with PBS, resuspended in 500 µL of lysis buffer (20 mM Tris-HC [pH 8], 2 mM EDTA, 1.2% TritonX-100, 20 mg/mL lysozyme), and incubated at 37 °C for 30 min. Cell debris was pelleted (13,000 rpm, 10 min) and 100 µL of cell lysate were transferred to a 2-mL 96-well plate for intracellular iron quantification. To release all protein-bound iron and cofactor-bound iron, 100 µL of 10 mM HCl and 100 µL of iron releasing agent (1:1 (v/v) 1.4 M HCl and 4.5% (w/v) $KMnO_4$) were added to each of the intra/extracellular samples and incubated at 60 °C for 2 h. After samples were cooled to room temperature, 30 µL of iron detection reagent (6.5 mM ferrozine, 6.5 mM neocuproine, 2.5 M ammonium acetate, 1 M ascorbic acid in dH_2O) were added and mixtures sat for 30 min at room temperature. One hundred fifty microliters of this solution were transferred to a 96-well polystyrene microplate and absorbance was measured at 550 nm using an Epoch2 plate reader (BioTek). Iron concentrations were calculated by comparing sample absorbances to those of a range of $FeCl_3$ concentrations of equal volume and prepared in a similar manner as the stool samples (mixtures of 100 µL of $FeCl_3$ standard in 10 mM HCl, 100 µL NaOH, 100 µL iron releasing agent). Iron concentrations were normalized against stool sample wet weight.

Human-derived in vitro community inoculation and passaging

A pre-supplementation stool sample from each of participants #16 and #7 was chosen to derive two *in vitro* communities, based on these participants exhibiting large responses to iron supplementation. Approximately 100 mg of stool were homogenized in 1 mL of PBS in an anaerobic chamber. After large particulates were allowed to settle (~2 min), the mixture was diluted 1:100 in PBS, used to inoculate 3 mL of BHI-S+0.05% mucin medium (1:100), and incubated at 37 °C for 48 h. This culture was subsequently passaged (1:200) in test tubes every 48 h for a total of 5 passages to obtain a stable community composition.

To study the effects of iron supplementation, 1 μL of the stable community was used to inoculate 199 μL of BHI-S+0.05% mucin medium containing no heme and 100 μM $FeSO_4$ (100 mM stock freshly prepared in 10 mM HCl to prevent oxidation). Optical density at 600 nm (OD_{600}) was measured in a 96-well polystyrene microplate using an Epoch2 plate reader (BioTek) with continuous shaking every 15 min. The community was passaged with 1:200 dilution in this iron-supplemented medium every 48 h for 12 passages.

To study the effects of iron deprivation, 1 μL of the stable community was used to inoculate 199 μL of BHI-S+0.05% mucin medium, with or without heme, containing 0-

1.6 mM bathophenanthroline disulfonic acid disodium salt hydrate (BPS, Sigma, Cat. #146617) (Figure 2A). OD_{600} was measured as above and the community was passaged in BPS-containing media 3 times (treatment passages 1-3). After 3 iron-deprivation passages, the community was passaged 3 more times in BHI-S+0.05% mucin medium with or without heme in the absence of chelator (recovery passages 4-6).

To study the effects of supplementation with divalent metals on BPS-treated communities, 1 μL of a stable community was used to inoculate 199 μL of BHI-S+0.05% mucin medium, with or without heme, containing 0-0.2 mM BPS and 100 μM of the following divalent metal salts, separately: $FeSO_4$, $CaCl_2{\cdot}2H_2O$, $CuSO_4{\cdot}5H_2O$, $CoCl_2{\cdot}6H_2O$, $MnSO_4{\cdot}H_2O$, or $ZnSO_4{\cdot}H_2O$. The control condition involved no metal supplementation. OD_{600} was measured as above and the community was passaged in BPS-containing media with the respective divalent metal 3 times.

Measurements of gut commensal isolate growth

Twelve human bacterial isolates from five taxonomic families were chosen for measurements of growth in axenic culture. Eight were from obtained from BEI Resources as part of their Human Microbiome Project collection: *Coprococcus* sp. HPP0048 (Lachnospiraceae), *Lachnospiraceae* sp. *1_4_56FAA* (Lachnospiraceae), *Ruminococcaceae* sp. *D16* (Ruminococcaceae), *Enterococcus faecium* TX1330 and

Enterococcus faecalis TX1322 (Enterococcaceae), *Sutterella wadsworthensis* HGA0223 (Burkholderiaceae), and *Bacteroides thetaiotaomicron* CL09T03C10 and *Bacteroides dorei* CL02T00C15 (Bacteroidaceae). *Bacteroides faecis*, *Bacteroides fragilis*, *Bacteroides uniformis*, and *Bacteroides vulgatus* (Bacteroidaceae) were isolated from a stool sample from one of our study participants.

All isolates were streaked on BHI-S+0.05% mucin agar plates and allowed to grow for 48-72 h at 37 °C in an anaerobic chamber. For each strain, 3 mL of BHI-S+0.05% mucin liquid medium were inoculated with a colony and grown for 48 h at 37°C. One microliter of saturated culture was used to inoculate 199 μL of BHI-S+0.05% mucin medium, with or without heme, containing 0-0.4 mM BPS (Figure S7A). OD_{600} was measured as above. At the end of the first 48 h passage, cultures were passaged in this BPS-containing media for another 48 h. After 24 h and 48 h of the second passage, 1 μL was used to inoculate 199 μL of BHI-S+0.05% mucin medium, with or without heme, without chelator and OD_{600} was measured thereafter to assess recovery at these two growth time points.

Quantification and statistical analysis

Data were analyzed in R studio and MATLAB (The MathWorks, Inc). Statistical significance was calculated using ANOVA and HSD-tests, as specified in the text and in the figure legends. PERMANOVA analyses on human data were performed using the `vegan` package and HSD-tests using the `agricolae` package. *r*-values are Pearson's correlation coefficients.

Main Figure Titles and Legends

Figure 1: Iron supplementation results in participant-specific shifts in gut microbiota composition that are correlated with intracellular iron concentration.

A) A total of 301 stool samples were collected from 16 participants on a daily basis for 7 days before, 7 days during, and 7 days after a period of daily iron supplementation. All participants provided ≥18 samples. Of the sixteen, one participant (participant #1), a long-time user of iron supplements, took iron supplements all 21 days and thus served as a control for determining baseline fluctuations.

B) The effects of iron supplementation on gut microbiota composition do not overcome inter-individual variation. A Principal Coordinate Analysis of pairwise Bray-Curtis distances clustered samples largely by participant as compared to time relative to iron supplementation.

C) Examination of each participant individually showed that iron supplementation accounted for 20-33% of the variability among samples in 9 participants ($p<0.001$, percent variability in green) and 16-21% in 4 participants ($p<0.05$, percent variability in yellow). Iron did not induce significant changes in the microbiota composition of participants #3 and #13 ($p>0.05$, percent variability in red).

D) Intracellular iron concentrations measured in stool samples were correlated with percent variability in community structure (*R*=0.27, $p<0.05$), while extracellular concentrations showed no correlation (*R*=0.001, $p=0.9$).

E) The percent of genera or species in the Lachnospiraceae family that exhibited significant changes during and after iron supplementation was significantly greater in the four participants that were most responsive to iron as compared with the participants that were least responsive to iron (*: $p<0.05$, HSD test; n.s.: not significant).

Figure 2: *In vitro* passaging of stool from an iron-responsive participant preserves microbial diversity and recapitulates the small changes in community composition due to iron supplementation observed *in vivo*.

A) After five 48-h passages, a community derived from a participant #16 pre-supplementation stool sample was passaged in BHI-S+0.05% mucin medium 12 times with 100 μM iron sulfate to study the effects of iron supplementation or 3 times supplemented with 0-1.6 mM BPS to study the effects of iron deprivation. Passaging was performed with and without heme to determine the degree to which heme can be used as an iron substitute. Iron-deprived communities were subsequently passaged 3 more times in the absence of BPS to assess recovery of the affected species.

B) A stool-derived community from participant #16 retained 86% of the families (representing 89% of the relative abundance) detected in the stool sample after five 48-h stabilization passage cycles.

C) Addition of supplemental 100 μM iron sulfate did not affect the growth rate or carrying capacity of the *in vitro* community. Growth curves were measured after twelve 48-h passages in BHI-S+0.05% mucin medium.

D) The number of observed species in the community supplemented with 100 μM iron sulfate was stable over passages and similar to that of the non-supplemented community.

E) The relative abundance of most bacterial genera was unchanged after 12 passages of iron supplementation *in vitro* (left), indicating that iron did not have large effects on community composition, consistent with observations *in vivo* (right).

F) Both *in vivo* and *in vitro* iron supplementation did not affect the relative abundance of most genera, except for a decrease in the relative abundance of *Escherichia/Shigella* (Enterobacteriaceae) and an increase in the *Coprococcus_3* (Lachnospiraceae) and *Bacteroides* (Bacteroidaceae) genera. The correlation between our *in vitro* and *in vivo* results provides further support that the changes observed *in vivo* are due to iron supplementation.

Figure 3: Iron deprivation decreases community yield and diversity.

A) Growth rate and carrying capacity of the stool-derived community decreased in a BPS concentration-dependent manner in a single passage.

B,C) The effects of iron deprivation were cumulative, as maintaining the communities in low-iron conditions further reduced growth rate and carrying capacity.

D) The effects of iron deprivation were long lasting, as reintroducing the community to iron-sufficient conditions restored maximum growth rate but not carrying capacity, suggesting permanent loss of species.

E) The number of observed species decreased by ~20-60% in a BPS concentration-dependent manner within a single 48-h passage.

F) Maintenance of the community in iron-deprived media for 2 additional passages (passages 2 and 3) further decreased the number of observed species, and the number of species did not recover after the community was reintroduced to iron-sufficient conditions (passages 4-6). The red dashed vertical line separates passages in iron-deprived media from recovery passages in iron-sufficient conditions.

Figure 4: Sensitivity to iron deprivation is heterogeneous across families and across genera within a family.

A) The Lachnospiraceae and Ruminococcaceae families were highly sensitive to iron deprivation. Both decreased below the limit of detection at >0.1 mM BPS during passages 1-3 and did not recover when iron-sufficient conditions were restored (passages 4-6). Red dashed vertical lines indicate the transition from iron-deprived to iron-sufficient conditions.

B) *Flavonifractor* and *Coprococcus* species (members of the Lachnospiraceae and Ruminococcaceae families, respectively) were more resilient to iron deprivation (0.05 mM on left, 0.1 mM on right) than *Faecalibacterium* (Ruminococcaceae), *UCG-002* (Ruminococcaceae), and *Dorea* (Lachnospiraceae) species.

C) A *Coprococcus* (Lachnospiraceae) species and other Lachnospiraceae and Ruminococcaceae isolates were highly sensitive to iron deprivation in isolated monocultures. Area under the curve (AUC) was calculated for each growth passage: iron-deprivation passage 1 and 2 (D1 and D2), recovery at 24 h of the second passage (R24), or recovery at 48 h of the second passage (R48) (Figure S7, Methods). Red dashed vertical lines indicate the transition from iron-deprived to iron-sufficient conditions.

D) The Enterobacteriaceae family was sensitive to iron deprivation, decreasing below the limit of detection with >0.1 mM BPS. Nonetheless, Enterobacteriaceae relative abundance recovered in communities treated with ≤0.4 mM BPS when iron-sufficient conditions were restored. Red dashed vertical lines indicate the transition from iron-deprived to iron-sufficient conditions.

E, F) Enterococcaceae and Burkholderiaceae species increased in relative abundance during iron deprivation. Red dashed vertical lines indicate the transition from iron-deprived to iron-sufficient conditions.

G, H) In isolated monoculture, *E. faecium* (Enterococcaceae) was less sensitive to iron deprivation than *E. faecalis* (Enterococcaceae) and grew better in the second iron-deprivation passage than in the first passage. An *S. wadsworthensis* (Burkholderiaceae) isolate exhibited BPS concentration-dependent growth defects, but recovered effectively once iron-sufficient conditions were restored. Red dashed vertical line indicates the transition from iron-deprived to iron-sufficient conditions.

Figure 5: Iron deprivation can restructure community composition by decreasing competition.

A) The Bacteroidaceae family maintained relatively constant relative abundance during iron deprivation, and increased in relative abundance during the recovery passages in a BPS concentration-dependent manner, suggesting that some member species that were less sensitive to iron expanded into niches left available by more sensitive species.

B,C) *Bacteroides* species exhibited distinct sensitivities to low iron (0.4 mM BPS in (B) and 1.6 mM in (C)): *B. fragilis, B. faecis, B. vulgatus*, and *B. dorei/fragilis* were less sensitive to iron deprivation than *B. caccae, B. nordii*, and *B. salyersiae*.

D) A *B. dorei/fragilis* ASV increased in relative abundance from <1% to ~40% during iron deprivation and further increased to ~65% during the subsequent iron recovery passages.

E) *Bacteroides* isolates exhibited species-specific, BPS-concentration-dependent growth defects during iron deprivation. All species grew substantially more poorly in the second iron-deprivation passage despite the absence of BPS, suggesting that, even in the presence of inorganic iron, the absence of heme from our base medium inhibits growth after depletion of internal heme stores.

Figure 6: Heme supplementation buffers species extinction during iron deprivation in a species-specific manner.

A-C) Supplementation with heme ameliorated the decreases in community growth rate and carrying capacity during iron deprivation, suggesting loss of fewer species and the use of heme as a source of iron.

D) In the presence of heme, growth rate and carrying capacity were almost completely restored during the recovery passages.

E, F) Addition of heme did not alter the number of observed species (E) or the relative abundance (F) of most species in the initial community (0 mM BPS) but prevented the extinction of some species during iron deprivation (E, 0.05-1.6 mM BPS).

G) Heme ameliorated the decrease in relative abundance of the Enterobacteriaceae family at all BPS concentrations and even rescued it from extinction at 1.6 mM BPS, suggesting that its members have the ability to use heme as a source of iron.

H) Heme completely prevented the extinction of all Bacteroidaceae species except *B. nordii* and *B. salyersiae* at 0.1 mM (middle) and 1.6 mM (right) BPS, indicating that the ability to use heme as an iron source is species-specific. Heme addition did not affect *Bacteroides* species relative abundances in the absence of BPS treatment (0 mM, left). The *B. dorei/fragilis* ASV did not increase in relative abundance in the heme condition.

I) The Enterococcaceae and Burkholderiaceae families did not increase in relative abundance in the presence of heme, suggesting that members of these families do not depend on heme-acquired iron for their survival.

J) Of the taxa in the Ruminococcaceae and Lachnospiraceae families, heme rescued only *Flavonifractor plautii* (Ruminococcaceae) and *Coprococcus_3 comes* (Lachnospiraceae) and only at ≤0.2 mM BPS (middle), suggesting that the ability to benefit from heme is species-specific and that species in these families do not use heme as efficiently as those in the Enterobacteriaceae (G) and Bacteroidaceae (H) families. Heme addition did not substantially affect the relative abundances of any genera in the absence of BPS treatment (left), and all species decreased below the limit of detection regardless of heme addition with 1.6 mM BPS (right).

K) Heme ameliorated the negative effects of iron deprivation for all *Bacteroides* species during growth as isolated monocultures in a species-specific manner. *B. faecis* and *B. uniformis* exhibited the greatest growth defects during the second passage with ≥0.1 mM BPS in the presence of heme, suggesting that these two species cannot extract iron from heme as efficiently as the others or that they have a greater iron need than the amount of supplemented heme can provide. The BPS concentration-dependent growth defects in the presence of heme suggest that these species need both inorganic iron and heme as a cofactor.

L) For Enterococcaceae isolates, heme reduced the effects of iron deprivation for *E. faecalis* but had no effect on the growth of *E. faecium*.

M,N) Heme rescued the iron deprivation-induced extinction of Lachnospiraceae, Ruminococcaceae, and Burkholderiaceae isolates and substantially enhanced their growth even in the absence of BPS, suggesting that heme may trigger alternative metabolic pathways in these species.

Figure 7: BPS-induced effects are specific to inorganic iron deprivation.

A) Fe supplementation completely reversed the effects of BPS on *in vitro* community growth as measured by area under the curve (AUC), Co or Zn supplementation partially reversed the effects at some BPS concentrations, and the rest of the metals did not rescue at all. In the absence of BPS (0 μM), metal supplementation did not have any notable effects on growth.

B) Fe, Co, and Zn (up to 100 μM BPS) supplementation reversed the BPS-induced reduction in number of observed species. In the absence of BPS (0 μM), metal supplementation did not have any notable effects on number of observed species.

C,D) Only Fe reversed BPS-induced changes in community compostion. Fe, Co, and Zn (up to 100 μM BPS) generally rescued BPS-induced changes in composition at the family level (C). However, only BPS-treated samples supplemented with Fe clustered with samples not treated with BPS in a Principal Coordinate Analysis based on Bray-Curtis distances (D). The composition of BPS-treated communities supplemented with any other metal, including Co and Zn, was perturbed along a trajectory similar to that of communities without metal supplementation, indicating that these metals are unable to fully reverse the effects of BPS on community composition. In the absence of BPS (0 μM), metal supplementation did not have any notable effects on community composition.

E) Fe-supplemented and Co-supplemented communities treated with BPS differ at the ASV level. Heatmap shows the log-fold change in relative abundance of each ASV in communities treated with 200 μM BPS versus 0 μM BPS. Communities supplemented with Ca, Cu, Mg, Mn, and Zn underwent similar community rearrangements as the non-supplemented community. Supplementation with Fe and Co resulted in compositions distinct from the non-supplemented community and from each other. Gray indicates a species that was not detected in both the 0 μM and the 200 μM community. White indicates a species that was detected in the 0 μM, but not detected in the 200 μM community.

F) Heme generally buffered the effects of BPS, but with some notable differences to iron. Only a subset of taxa were rescued from dropping below the limit of detection by heme, suggesting that species that did not recover cannot use heme as an iron source.

G) Use of heme as a source of inorganic iron is species-specific. Representative strains from families that recovered in the presence of heme (*Escherichia fergusonii*, Enterobacteriaceae) and those that did not (*Akkermansia muciniphila*, Akkermanciaceae) were cultured in media containing 0 or 200 μM BPS and supplemented with 100 μM iron or 3.2 μM heme. Addition of heme reversed the growth defect for *E. fergusonii* but not *A. muciniphila*, indicating that *E. fergusonii* can use heme as an iron source while *A. muciniphila* cannot.

References

1. Faith, J.J., McNulty, N.P., Rey, F.E., and Gordon, J.I. (2011). Predicting a human gut microbiota's response to diet in gnotobiotic mice. Science *333*, 101-104. 10.1126/science.1206025.
2. Tropini, C., Moss, E.L., Merrill, B.D., Ng, K.M., Higginbottom, S.K., Casavant, E.P., Gonzalez, C.G., Fremin, B., Bouley, D.M., Elias, J.E., et al. (2018). Transient Osmotic Perturbation Causes Long-Term Alteration to the Gut Microbiota. Cell *173*, 1742-1754 e1717. 10.1016/j.cell.2018.05.008.
3. Ng, K.M., Aranda-Diaz, A., Tropini, C., Frankel, M.R., Van Treuren, W., O'Loughlin, C.T., Merrill, B.D., Yu, F.B., Pruss, K.M., Oliveira, R.A., et al. (2019). Recovery of the Gut Microbiota after Antibiotics Depends on Host Diet, Community Context, and Environmental Reservoirs. Cell Host Microbe *26*, 650-665 e654. 10.1016/j.chom.2019.10.011.
4. Dethlefsen, L., and Relman, D.A. (2011). Incomplete recovery and individualized responses of the human distal gut microbiota to repeated antibiotic perturbation. Proc Natl Acad Sci U S A *108 Suppl 1*, 4554-4561. 10.1073/pnas.1000087107.
5. Aranda-Diaz, A., Ng, K.M., Thomsen, T., Real-Ramirez, I., Dahan, D., Dittmar, S., Gonzalez, C.G., Chavez, T., Vasquez, K.S., Nguyen, T.H., et al. (2022). Establishment and characterization of stable, diverse, fecal-derived in vitro microbial communities that model the intestinal microbiota. Cell Host Microbe *30*, 260-272 e265. 10.1016/j.chom.2021.12.008.
6. Javdan, B., Lopez, J.G., Chankhamjon, P., Lee, Y.J., Hull, R., Wu, Q., Wang, X., Chatterjee, S., and Donia, M.S. (2020). Personalized Mapping of Drug

Metabolism by the Human Gut Microbiome. Cell *181*, 1661-1679 e1622. 10.1016/j.cell.2020.05.001.

7. Hromada, S., Qian, Y., Jacobson, T.B., Clark, R.L., Watson, L., Safdar, N., Amador-Noguez, D., and Venturelli, O.S. (2021). Negative interactions determine *Clostridioides difficile* growth in synthetic human gut communities. Mol Syst Biol *17*, e10355. 10.15252/msb.202110355.
8. Andrews, S.C., Robinson, A.K., and Rodriguez-Quinones, F. (2003). Bacterial iron homeostasis. FEMS Microbiol Rev *27*, 215-237. 10.1016/S0168-6445(03)00055-X.
9. Andrews, S.C. (1998). Iron storage in bacteria. Adv Microb Physiol *40*, 281-351. 10.1016/s0065-2911(08)60134-4.
10. Gruss, A., Borezee-Durant, E., and Lechardeur, D. (2012). Environmental heme utilization by heme-auxotrophic bacteria. Adv Microb Physiol *61*, 69-124. 10.1016/B978-0-12-394423-8.00003-2.
11. Andrews, N.C. (2000). Iron metabolism: iron deficiency and iron overload. Annu Rev Genomics Hum Genet *1*, 75-98. 10.1146/annurev.genom.1.1.75.
12. Celis, A.I., and Relman, D.A. (2020). Competitors versus Collaborators: Micronutrient Processing by Pathogenic and Commensal Human-Associated Gut Bacteria. Mol Cell *78*, 570-576. 10.1016/j.molcel.2020.03.032.
13. Lopez, C.A., and Skaar, E.P. (2018). The Impact of Dietary Transition Metals on Host-Bacterial Interactions. Cell Host Microbe *23*, 737-748. 10.1016/j.chom.2018.05.008.
14. Khasheii, B., Mahmoodi, P., and Mohammadzadeh, A. (2021). Siderophores: Importance in bacterial pathogenesis and applications in medicine and industry. Microbiol Res *250*, 126790. 10.1016/j.micres.2021.126790.

15. Cescau, S., Cwerman, H., Letoffe, S., Delepelaire, P., Wandersman, C., and Biville, F. (2007). Heme acquisition by hemophores. Biometals *20*, 603-613. 10.1007/s10534-006-9050-y.

16. Lyles, K.V., and Eichenbaum, Z. (2018). From Host Heme To Iron: The Expanding Spectrum of Heme Degrading Enzymes Used by Pathogenic Bacteria. Front Cell Infect Microbiol *8*, 198. 10.3389/fcimb.2018.00198.

17. Anzaldi, L.L., and Skaar, E.P. (2010). Overcoming the heme paradox: heme toxicity and tolerance in bacterial pathogens. Infect Immun *78*, 4977-4989. 10.1128/IAI.00613-10.

18. Knippel, R.J., Zackular, J.P., Moore, J.L., Celis, A.I., Weiss, A., Washington, M.K., DuBois, J.L., Caprioli, R.M., and Skaar, E.P. (2018). Heme sensing and detoxification by HatRT contributes to pathogenesis during *Clostridium difficile* infection. PLoS Pathog *14*, e1007486. 10.1371/journal.ppat.1007486.

19. Stauff, D.L., and Skaar, E.P. (2009). The heme sensor system of *Staphylococcus aureus*. Contrib Microbiol *16*, 120-135. 10.1159/000219376.

20. Pi, H., Jones, S.A., Mercer, L.E., Meador, J.P., Caughron, J.E., Jordan, L., Newton, S.M., Conway, T., and Klebba, P.E. (2012). Role of catecholate siderophores in Gram-negative bacterial colonization of the mouse gut. PLoS One *7*, e50020. 10.1371/journal.pone.0050020.

21. Searle, L.J., Meric, G., Porcelli, I., Sheppard, S.K., and Lucchini, S. (2015). Variation in siderophore biosynthetic gene distribution and production across environmental and faecal populations of *Escherichia coli*. PLoS One *10*, e0117906. 10.1371/journal.pone.0117906.

22. Vazquez-Gutierrez, P., Lacroix, C., Jaeggi, T., Zeder, C., Zimmerman, M.B., and Chassard, C. (2015). Bifidobacteria strains isolated from stools of iron deficient

infants can efficiently sequester iron. BMC Microbiol *15*, 3. 10.1186/s12866-014-0334-z.

23. Vazquez-Gutierrez, P., Stevens, M.J., Gehrig, P., Barkow-Oesterreicher, S., Lacroix, C., and Chassard, C. (2017). The extracellular proteome of two *Bifidobacterium* species reveals different adaptation strategies to low iron conditions. BMC Genomics *18*, 41. 10.1186/s12864-016-3472-x.

24. Lisiecki, P., Wysocki, P., and Mikucki, J. (2000). Occurrence of siderophores in enterococci. Zentralbl Bakteriol *289*, 807-815. 10.1016/s0934-8840(00)80006-7.

25. Rocha, E.R., and Krykunivsky, A.S. (2017). Anaerobic utilization of Fe(III)-xenosiderophores among Bacteroides species and the distinct assimilation of Fe(III)-ferrichrome by *Bacteroides fragilis* within the genus. Microbiologyopen *6*. 10.1002/mbo3.479.

26. Zhu, W., Winter, M.G., Spiga, L., Hughes, E.R., Chanin, R., Mulgaonkar, A., Pennington, J., Maas, M., Behrendt, C.L., Kim, J., et al. (2020). Xenosiderophore Utilization Promotes *Bacteroides thetaiotaomicron* Resilience during Colitis. Cell Host Microbe *27*, 376-388 e378. 10.1016/j.chom.2020.01.010.

27. Parker, A.C., Bergonia, H.A., Seals, N.L., Baccanale, C.L., and Rocha, E.R. (2020). The uroS and yifB Genes Conserved among Tetrapyrrole Synthesizing-Deficient Bacteroidales Are Involved in *Bacteroides fragilis* Heme Assimilation and Survival in Experimental Intra-abdominal Infection and Intestinal Colonization. Infect Immun *88*. 10.1128/IAI.00103-20.

28. Rocha, E.R., Bergonia, H.A., Gerdes, S., and Jeffrey Smith, C. (2019). *Bacteroides fragilis* requires the ferrous-iron transporter FeoAB and the CobN-like proteins BtuS1 and BtuS2 for assimilation of iron released from heme. Microbiologyopen *8*, e00669. 10.1002/mbo3.669.

29. Jaeggi, T., Kortman, G.A., Moretti, D., Chassard, C., Holding, P., Dostal, A., Boekhorst, J., Timmerman, H.M., Swinkels, D.W., Tjalsma, H., et al. (2015). Iron fortification adversely affects the gut microbiome, increases pathogen abundance and induces intestinal inflammation in Kenyan infants. Gut *64*, 731-742. 10.1136/gutjnl-2014-307720.

30. Zimmermann, M.B., Chassard, C., Rohner, F., N'Goran E, K., Nindjin, C., Dostal, A., Utzinger, J., Ghattas, H., Lacroix, C., and Hurrell, R.F. (2010). The effects of iron fortification on the gut microbiota in African children: a randomized controlled trial in Cote d'Ivoire. Am J Clin Nutr *92*, 1406-1415. 10.3945/ajcn.110.004564.

31. Kumar, A., and Brookes, M.J. (2020). Iron Therapy in Inflammatory Bowel Disease. Nutrients *12*. 10.3390/nu12113478.

32. Coe, G.L., Pinkham, N.V., Celis, A.I., Johnson, C., DuBois, J.L., and Walk, S.T. (2021). Dynamic Gut Microbiome Changes in Response to Low-Iron Challenge. Appl Environ Microbiol *87*. 10.1128/AEM.02307-20.

33. Dostal, A., Fehlbaum, S., Chassard, C., Zimmermann, M.B., and 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. 10.1111/j.1574-6941.2012.01461.x.

34. Dostal, A., Lacroix, C., Bircher, L., Pham, V.T., Follador, R., Zimmermann, M.B., and Chassard, C. (2015). Iron Modulates Butyrate Production by a Child Gut Microbiota *In Vitro*. mBio *6*, e01453-01415. 10.1128/mBio.01453-15.

35. Short, M.W., and Domagalski, J.E. (2013). Iron deficiency anemia: evaluation and management. Am Fam Physician *87*, 98-104.

36. Zimmermann, M.B. (2020). Global look at nutritional and functional iron deficiency in infancy. Hematology Am Soc Hematol Educ Program *2020*, 471-477. 10.1182/hematology.2020000131.

37. Epstein, D., Borohovitz, A., Merdler, I., Furman, M., Atalli, E., Sorkin, A., Stainfeld, Y., Isenberg, Y., Mashiach, T., Shapira, S., et al. (2018). Prevalence of Iron Deficiency and Iron Deficiency Anemia in Strenuously Training Male Army Recruits. Acta Haematol *139*, 141-147. 10.1159/000485736.

38. Parks, R.B., Hetzel, S.J., and Brooks, M.A. (2017). Iron Deficiency and Anemia among Collegiate Athletes: A Retrospective Chart Review. Med Sci Sports Exerc *49*, 1711-1715. 10.1249/MSS.0000000000001259.

39. Sinclair, L.M., and Hinton, P.S. (2005). Prevalence of iron deficiency with and without anemia in recreationally active men and women. J Am Diet Assoc *105*, 975-978. 10.1016/j.jada.2005.03.005.

40. Callahan, B.J., McMurdie, P.J., Rosen, M.J., Han, A.W., Johnson, A.J., and Holmes, S.P. (2016). DADA2: High-resolution sample inference from Illumina amplicon data. Nat Methods *13*, 581-583. 10.1038/nmeth.3869.

41. Keogh, D., Tay, W.H., Ho, Y.Y., Dale, J.L., Chen, S., Umashankar, S., Williams, R.B.H., Chen, S.L., Dunny, G.M., and Kline, K.A. (2016). Enterococcal Metabolite Cues Facilitate Interspecies Niche Modulation and Polymicrobial Infection. Cell Host Microbe *20*, 493-503. 10.1016/j.chom.2016.09.004.

42. Krawczyk, B., Wityk, P., Galecka, M., and Michalik, M. (2021). The Many Faces of *Enterococcus* spp.-Commensal, Probiotic and Opportunistic Pathogen. Microorganisms *9*. 10.3390/microorganisms9091900.

43. Saillant, V., Lipuma, D., Ostyn, E., Joubert, L., Boussac, A., Guerin, H., Brandelet, G., Arnoux, P., and Lechardeur, D. (2021). A Novel *Enterococcus faecalis* Heme

Transport Regulator (FhtR) Senses Host Heme To Control Its Intracellular Homeostasis. mBio *12*. 10.1128/mBio.03392-20.

44. Hiippala, K., Kainulainen, V., Kalliomaki, M., Arkkila, P., and Satokari, R. (2016). Mucosal Prevalence and Interactions with the Epithelium Indicate Commensalism of *Sutterella* spp. Front Microbiol *7*, 1706. 10.3389/fmicb.2016.01706.

45. Huus, K.E., Hoang, T.T., Creus-Cuadros, A., Cirstea, M., Vogt, S.L., Knuff-Janzen, K., Sansonetti, P.J., Vonaesch, P., and Finlay, B.B. (2021). Cross-feeding between intestinal pathobionts promotes their overgrowth during undernutrition. Nat Commun *12*, 6860. 10.1038/s41467-021-27191-x.

46. Wilks, A., and Ikeda-Saito, M. (2014). Heme utilization by pathogenic bacteria: not all pathways lead to biliverdin. Acc Chem Res *47*, 2291-2298. 10.1021/ar500028n.

47. Palmer, L.D., and Skaar, E.P. (2016). Transition Metals and Virulence in Bacteria. Annu Rev Genet *50*, 67-91. 10.1146/annurev-genet-120215-035146.

48. Yamamoto, Y., Poyart, C., Trieu-Cuot, P., Lamberet, G., Gruss, A., and Gaudu, P. (2005). Respiration metabolism of Group B *Streptococcus* is activated by environmental haem and quinone and contributes to virulence. Mol Microbiol *56*, 525-534. 10.1111/j.1365-2958.2005.04555.x.

49. Pal, R., Hameed, S., and Fatima, Z. (2019). Altered drug efflux under iron deprivation unveils abrogated MmpL3 driven mycolic acid transport and fluidity in mycobacteria. Biometals *32*, 49-63. 10.1007/s10534-018-0157-8.

50. Javvadi, S., Pandey, S.S., Mishra, A., Pradhan, B.B., and Chatterjee, S. (2018). Bacterial cyclic beta-(1,2)-glucans sequester iron to protect against iron-induced toxicity. EMBO Rep *19*, 172-186. 10.15252/embr.201744650.

51. Arbon, D., Zeniskova, K., Mach, J., Grechnikova, M., Malych, R., Talacko, P., and Sutak, R. (2020). Adaptive iron utilization compensates for the lack of an inducible uptake system in *Naegleria fowleri* and represents a potential target for therapeutic intervention. PLoS Negl Trop Dis *14*, e0007759. 10.1371/journal.pntd.0007759.

52. Ror, S., and Panwar, S.L. (2019). Sef1-Regulated Iron Regulon Responds to Mitochondria-Dependent Iron-Sulfur Cluster Biosynthesis in *Candida albicans*. Front Microbiol *10*, 1528. 10.3389/fmicb.2019.01528.

53. Romero, A.M., Ramos-Alonso, L., Alepuz, P., Puig, S., and Martinez-Pastor, M.T. (2020). Global translational repression induced by iron deficiency in yeast depends on the Gcn2/eIF2alpha pathway. Sci Rep *10*, 233. 10.1038/s41598-019-57132-0.

54. Hameed, S., Prasad, T., Banerjee, D., Chandra, A., Mukhopadhyay, C.K., Goswami, S.K., Lattif, A.A., Chandra, J., Mukherjee, P.K., Ghannoum, M.A., and Prasad, R. (2008). Iron deprivation induces EFG1-mediated hyphal development in *Candida albicans* without affecting biofilm formation. FEMS Yeast Res *8*, 744-755. 10.1111/j.1567-1364.2008.00394.x.

55. Rudisill, S.S., Martin, B.R., Mankowski, K.M., and Tessier, C.R. (2019). Iron Deficiency Reduces Synapse Formation in the *Drosophila* Clock Circuit. Biol Trace Elem Res *189*, 241-250. 10.1007/s12011-018-1442-7.

56. Missirlis, F., Holmberg, S., Georgieva, T., Dunkov, B.C., Rouault, T.A., and Law, J.H. (2006). Characterization of mitochondrial ferritin in *Drosophila*. Proc Natl Acad Sci U S A *103*, 5893-5898. 10.1073/pnas.0601471103.

57. Hirayama, T., and Nagasawa, H. (2017). Chemical tools for detecting Fe ions. J Clin Biochem Nutr *60*, 39-48. 10.3164/jcbn.16-70.

58. Blair, D., and Diehl, H. (1961). Bathophenanthrolinedisulphonic acid and bathocuproinedisulphonic acid, water soluble reagents for iron and copper. Talanta *7*, 163-174.

59. Xu, Z., Wang, P., Wang, H., Yu, Z.H., Au-Yeung, H.Y., Hirayama, T., Sun, H., and Yan, A. (2019). Zinc excess increases cellular demand for iron and decreases tolerance to copper in *Escherichia coli*. J Biol Chem *294*, 16978-16991. 10.1074/jbc.RA119.010023.

60. Imlay, J.A. (2014). The mismetallation of enzymes during oxidative stress. J Biol Chem *289*, 28121-28128. 10.1074/jbc.R114.588814.

61. Liu, H., Shiver, A.L., Price, M.N., Carlson, H.K., Trotter, V.V., Chen, Y., Escalante, V., Ray, J., Hern, K.E., Petzold, C.J., et al. (2021). Functional genetics of human gut commensal *Bacteroides thetaiotaomicron* reveals metabolic requirements for growth across environments. Cell Rep *34*, 108789. 10.1016/j.celrep.2021.108789.

62. Price, M.N., Wetmore, K.M., Waters, R.J., Callaghan, M., Ray, J., Liu, H., Kuehl, J.V., Melnyk, R.A., Lamson, J.S., Suh, Y., et al. (2018). Mutant phenotypes for thousands of bacterial genes of unknown function. Nature *557*, 503-509. 10.1038/s41586-018-0124-0.

63. LaMattina, J.W., Delrossi, M., Uy, K.G., Keul, N.D., Nix, D.B., Neelam, A.R., and Lanzilotta, W.N. (2017). Anaerobic Heme Degradation: ChuY Is an Anaerobilin Reductase That Exhibits Kinetic Cooperativity. Biochemistry *56*, 845-855. 10.1021/acs.biochem.6b01099.

64. Wetmore, K.M., Price, M.N., Waters, R.J., Lamson, J.S., He, J., Hoover, C.A., Blow, M.J., Bristow, J., Butland, G., Arkin, A.P., and Deutschbauer, A. (2015).

Rapid quantification of mutant fitness in diverse bacteria by sequencing randomly bar-coded transposons. mBio *6*, e00306-00315. 10.1128/mBio.00306-15.

65. Jumper, J., Evans, R., Pritzel, A., Green, T., Figurnov, M., Ronneberger, O., Tunyasuvunakool, K., Bates, R., Zidek, A., Potapenko, A., et al. (2021). Highly accurate protein structure prediction with AlphaFold. Nature *596*, 583-589. 10.1038/s41586-021-03819-2.

66. Caporaso, J.G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F.D., Costello, E.K., Fierer, N., Pena, A.G., Goodrich, J.K., Gordon, J.I., et al. (2010). QIIME allows analysis of high-throughput community sequencing data. Nat Methods *7*, 335-336. 10.1038/nmeth.f.303.

67. Riemer, J., Hoepken, H.H., Czerwinska, H., Robinson, S.R., and Dringen, R. (2004). Colorimetric ferrozine-based assay for the quantitation of iron in cultured cells. Anal Biochem *331*, 370-375. 10.1016/j.ab.2004.03.049.