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## Dietary iron variably modulates assembly of the intestinal microbiota in colitis-resistant and colitis-susceptible mice

Melissa Ellermann<sup>a</sup>, Raad Z. Gharaibeh<sup>b,c</sup>, Nitsan Maharshak<sup>d</sup>, Ernesto Pérez-Chanona<sup>a</sup>, Christian Jobin<sup>e,f</sup>, Ian M. Carroll<sup>g,h</sup>, Janelle C. Arthur<sup>ib,a,h,i</sup>, Scott E Plevy<sup>j</sup>, Anthony A. Fodor<sup>c</sup>, Cory R. Brouwer<sup>ib,b,c</sup>, and R. Balfour Sartor<sup>a,g,h</sup>

<sup>a</sup>Department of Microbiology and Immunology, University of North Carolina, Chapel Hill, NC, USA; <sup>b</sup>Bioinformatics Services Division, Department of Bioinformatics and Genomics, University of North Carolina at Charlotte, Kannapolis, NC, USA; <sup>c</sup>Department of Bioinformatics and Genomics, University of North Carolina at Charlotte, Charlotte, NC, USA; <sup>d</sup>Department of Gastroenterology and Liver Diseases, Tel Aviv Medical Center, Affiliated to the Sackler Faculty of Medicine, Tel Aviv University, Tel Aviv, Israel, USA; <sup>e</sup>Department of Medicine, University of Florida, Gainesville, FL, USA; <sup>f</sup>Department of Infectious Diseases and Pathology, University of Florida, Gainesville, FL, USA; <sup>g</sup>Department of Medicine, University of North Carolina, Chapel Hill, NC, USA; <sup>h</sup>Center for Gastrointestinal Biology and Disease, University of North Carolina, Chapel Hill, NC, USA; <sup>i</sup>Lineberger Comprehensive Cancer Center, University of North Carolina, Chapel Hill, NC, USA; <sup>j</sup>Immunology Research and Development, Janssen Pharmaceuticals, Spring House, PA, USA

### ABSTRACT

Iron deficiency, a common comorbidity of gastrointestinal inflammatory disorders such as inflammatory bowel diseases (IBD), is often treated with oral iron supplementation. However, the safety of oral iron supplementation remains controversial because of its association with exacerbated disease activity in a subset of IBD patients. Because iron modulates bacterial growth and function, one possible mechanism by which iron may exacerbate inflammation in susceptible hosts is by modulating the intestinal microbiota. We, therefore, investigated the impact of dietary iron on the intestinal microbiota, utilizing the conventionalization of germ-free mice as a model of a microbial community in compositional flux to recapitulate the instability of the IBD-associated intestinal microbiota. Our findings demonstrate that altering intestinal iron availability during community assembly modulated the microbiota in non-inflamed wild type (WT) and colitis-susceptible interleukin-10-deficient (*Il10*<sup>-/-</sup>) mice. Depletion of luminal iron availability promoted luminal compositional changes associated with dysbiotic states irrespective of host genotype, including an expansion of Enterobacteriaceae such as *Escherichia coli*. Mechanistic *in vitro* growth competitions confirmed that high-affinity iron acquisition systems in *E. coli* enhance its abundance over other bacteria in iron-restricted conditions, thereby enabling pathobiont iron scavenging during dietary iron restriction. In contrast, distinct luminal community assembly was observed with dietary iron supplementation in WT versus *Il10*<sup>-/-</sup> mice, suggesting that the effects of increased iron on the microbiota differ with host inflammation status. Taken together, shifts in dietary iron intake during community assembly modulate the ecological structure of the intestinal microbiota and is dependent on host genotype and inflammation status.

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Dietary iron; IBD; gut microbiome; colitis; *Escherichia coli*; Animal models of GI-diseases with microbial components; Defining/profiling gut microbiome; Role of gut microbiome in GI disease

## Introduction

Iron deficiency anemia (IDA) is the most common micronutrient deficiency, affecting approximately 1 billion individuals worldwide<sup>1</sup>. Although oral iron supplementation can effectively treat IDA,<sup>2,3</sup> the safety and tolerance of oral iron supplementation within specific population subsets such as infants or patients with chronic inflammatory diseases remains controversial. Diarrhea, enhanced burdens of intestinal pathogens and increased fecal markers of inflammation have been reported in iron-deficient infants

receiving iron-supplemented foods.<sup>4–8</sup> Increased disease activity and functional gastrointestinal (GI) symptoms with oral iron supplementation have also been observed in patients with inflammatory bowel diseases (IBD).<sup>9,10</sup> The latter observations have been recapitulated with increased dietary iron administration in rodent models of experimental ileitis and colitis.<sup>10–12</sup> Diet-mediated increases in intestinal iron concentrations also enhance luminal production of reactive oxygen species,<sup>13</sup> a byproduct of the Fenton reaction, which in turn can increase colonic oxidative

**CONTACT** R. Balfour Sartor  [rbs@med.unc.edu](mailto:rbs@med.unc.edu)  Department of Microbiology and Immunology, University of North Carolina, Room 7309A Biomolecular Building, CB# 7032, Chapel Hill, NC 27599-7032, USA

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stress<sup>10</sup> and potentially exacerbate the development of intestinal inflammation in susceptible populations.

The GI tract is home to a complex community of microbes referred to as the intestinal microbiota. Dietary factors, including macronutrient and micronutrient constituents, can compositionally and functionally modulate the intestinal microbiota,<sup>11, 14–16</sup> which in turn can impact numerous host processes, including metabolism and immune function.<sup>17–22</sup> Iron is an essential cofactor for numerous bacterial enzymes and therefore serves as an important micronutrient for most members of the intestinal microbiota.<sup>23–26</sup> Iron requirements vary between bacterial taxa, as does the capacity to acquire iron when availability is limited, which includes secretion of small molecules with high affinity for iron known as siderophores. An estimated 5–15% of dietary non-heme iron is absorbed at the duodenum in iron-replete hosts, leaving the remaining unabsorbed iron to pass through the intestines for microbial use. Thus, dietary iron likely impacts the growth and function of resident intestinal bacteria, which in turn may contribute to the development of unfavorable side effects that can occur with oral iron supplementation.

Studies investigating the impact of dietary iron on the intestinal microbiota have primarily focused on rodent models with an established microbial community. However, population subsets that are most vulnerable to IDA, including the infant and IBD populations, frequently harbor an intestinal microbiota that is unstable in composition.<sup>27–29</sup> Therefore, we sought to determine how dietary iron restriction and supplementation influences the ecological structure of a microbiota in compositional flux, using the conventionalization of germ-free (GF) mice as a model system. Our findings demonstrate that dietary iron restriction initiated prior to conventionalization of GF mice promoted compositional changes frequently associated with a dysbiotic state, including decreased microbial richness and an increased abundance of Enterobacteriaceae family members such as *Escherichia coli*. Moreover, decreased intestinal iron availability corresponded with an increased capacity for bacterial iron scavenging. Similar shifts in community composition were observed with dietary iron restriction in colitis-susceptible interleukin-10-deficient (*Il10*<sup>-/-</sup>) mice. In contrast, dietary iron supplementation had distinct effects on the microbiotas of WT versus *Il10*<sup>-/-</sup> mice. Taken together, luminal

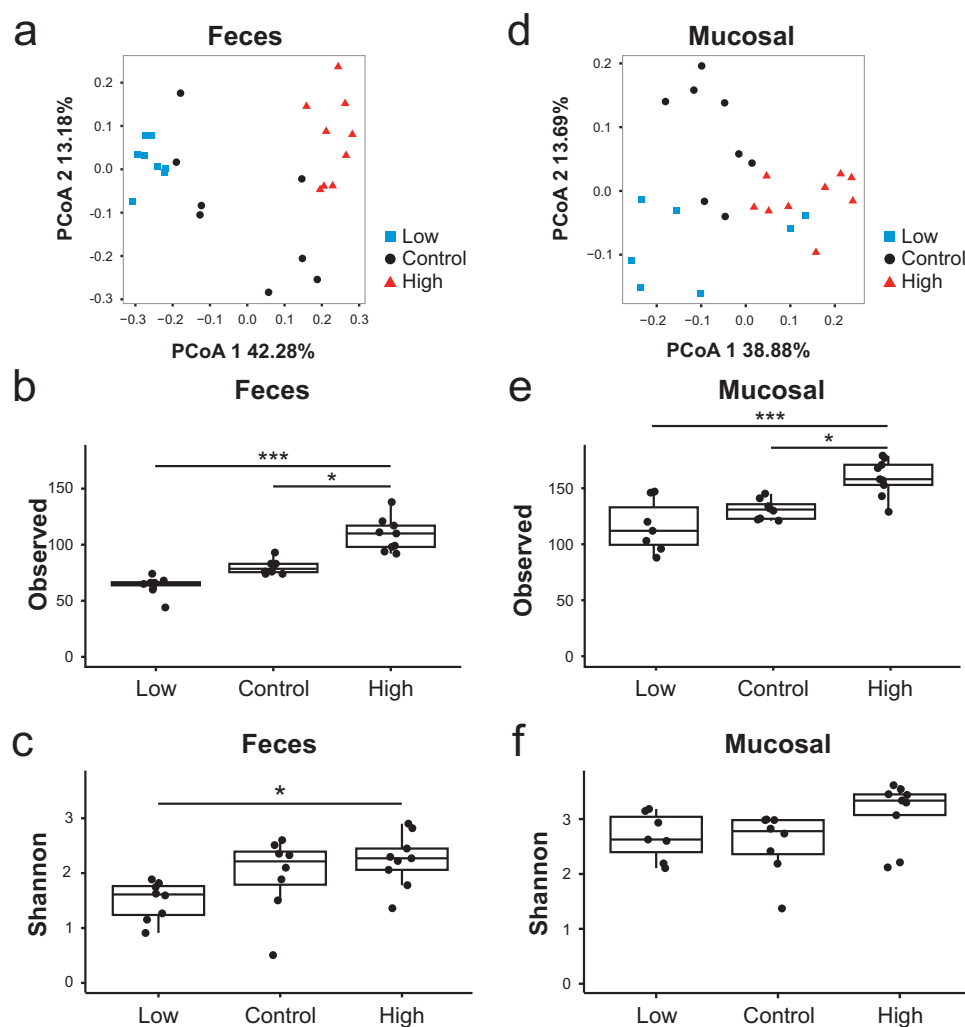
iron availability during community assembly influences the resulting structure of the intestinal microbiota and is dependent on host genotype and inflammation status.

## Results

### ***Intestinal iron availability during community assembly impacts the resulting structure of the intestinal microbiota***

Dietary iron interventions are frequently administered during infancy when the intestinal microbiota is undergoing ecological succession<sup>28,29</sup> or during disease states when the gut microbiota exhibits transient instability.<sup>27,30</sup> We, therefore, investigated the impact of dietary iron restriction or supplementation on the developing intestinal microbiota as a model of a bacterial community in compositional flux. GF WT mice were placed on an iron-deficient (“low”), control or iron-supplemented (“high”) diet, and then were colonized with a fecal microbiota from a WT donor. Fecal and cecal tissue samples were collected for 16S rRNA sequencing at 4 weeks post-colonization, when the microbiota exhibits initial compositional stability following conventionalization.<sup>31,32</sup> Because host systemic iron levels have been correlated with compositional changes to the intestinal microbiota<sup>7,33,34</sup> and iron deficient diets can induce systemic iron deficiency as early as 4 weeks,<sup>35,36</sup> mice on the low iron diet received weekly i.p. iron injections to prevent systemic iron depletion. Following the 4-week dietary intervention and i.p. injections, liver iron stores did not differ between dietary groups (Figure S1). Moreover, as described,<sup>13,37</sup> dietary iron restriction and supplementation significantly altered fecal iron concentrations (Figure S2), confirming that our diets modulated luminal iron concentrations.

Principal Coordinate Analysis (PCoA) using Bray–Curtis dissimilarity at the operational taxonomic unit (OTU) level revealed compositional differences in the fecal microbiota between WT mice administered the different iron diets at 4 weeks post-conventionalization (Figure 1(a)). Fecal communities in the high iron diet mice significantly differed from those in the low iron diet mice (PCoA axis 1; FDR-corrected  $p$ -value =  $4.4 \times 10^{-9}$ ). The composition of the fecal microbiota in mice fed the control diet exhibited the most



**Figure 1.** Dietary iron alters the structure of the fecal and mucosal microbiota. A + D) Principal coordinate analysis based on Bray–Curtis metrics for the (a) fecal and (d) mucosal microbiota in ex-GF WT mice maintained on an iron-deficient (low), control or iron supplemented (high) diet 4 weeks after fecal bacterial colonization. Microbial richness as measured by (b, e) observed OTUs and (c, f) Shannon diversity index for the (b, c) fecal and (e, f) mucosal microbiota. Each symbol represents an individual mouse,  $n = 7–9$  mice per group. Box and whisker plots show the minimum, first quartile, median, third quartile, and maximum values. Comparisons and FDR-corrected  $p$ -values were determined using a mixed linear model with the cage as a random effect. \*  $p < 0.05$ , \*\*\*  $p < 0.001$ .

interindividual variability as assessed by Bray Curtis distances between samples within each diet group (Figure S3), suggesting that dietary iron supplementation or depletion exerts strong selective pressure in shaping the fecal microbiota. Dietary iron restriction also significantly reduced microbial richness relative to iron supplementation (Figure 1(b–c)). Thus, manipulation of intestinal iron availability through dietary interventions impacts the ecological structure of the fecal microbiota in WT mice.

We next examined the impact of dietary iron supplementation and restriction on the structure of the mucosal microbiota. PCoA analysis revealed

partial clustering of the mucosal microbiota by dietary treatment, including significant differences between the low and high iron diet groups (PCoA axis 1; FDR-corrected  $p$ -value = 0.02) (Figure 1(d)) and increased microbial richness with dietary iron supplementation (Figure 1(e–f)). Collectively, these results suggest that dietary iron influences biodiversity and community assembly of the fecal and mucosal microbiotas.

### **Dietary iron supplementation alters fecal abundances of distinct bacterial taxa**

Because the compositional differences by iron diet were more pronounced in the feces, we next evaluated the impact of dietary iron supplementation and depletion on specific taxa within the fecal microbiota. A heat map was generated to highlight OTUs that significantly differed in relative abundance (Figure 2(a)) (Table S2). Statistical analysis using a mixed linear model revealed that the relative abundances of 59 OTUs significantly differed at an FDR-corrected  $p$ -value  $< 0.05$  between at least two dietary treatments. Iron supplementation resulted in the enrichment of 18 OTUs relative to the control diet and 37 OTUs relative to the low iron diet (Figure 2(b), Table S3). In contrast, dietary iron restriction decreased the relative abundances of 7 OTUs compared to the control diet and 8 OTUs compared to the high iron diet (Figure 2(c), Table S3). These data suggest that increased intestinal iron availability during community assembly corresponds with an alteration of specific bacterial taxa within the fecal microbiota with predominant enrichment.

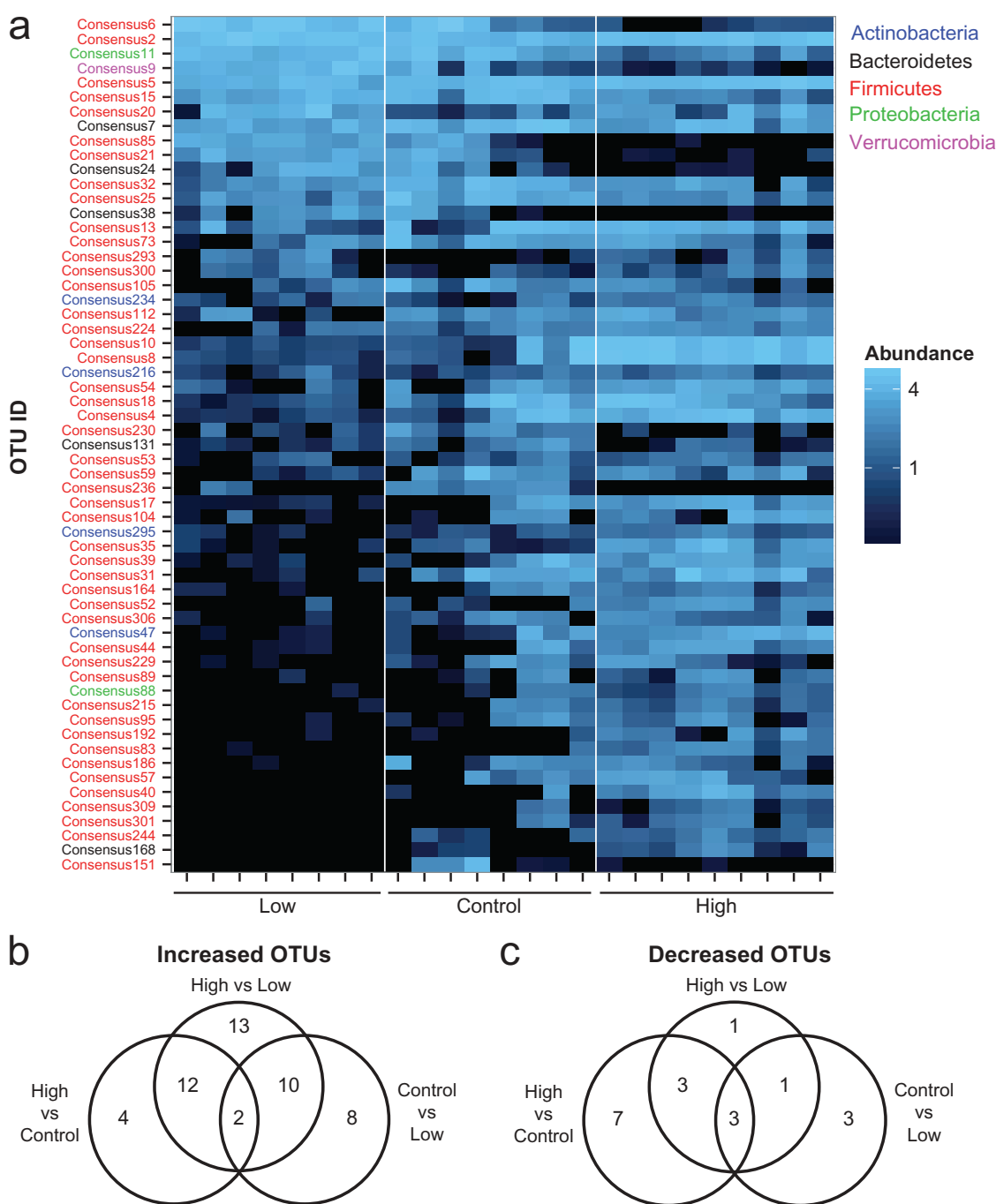
### **Dietary iron restriction promotes a bloom of Enterobacteriaceae and predicted bacterial iron uptake systems**

Given that increases in Enterobacteriaceae are associated with inflammation-induced dysbiosis,<sup>38,39</sup> we assessed the impact of dietary iron restriction or supplementation on Enterobacteriaceae abundance during community assembly using the Ribosomal Database Project (RDP) pipeline. In the control and low iron diet groups, the relative fecal abundances of Proteobacteria phylum and Enterobacteriaceae family members including *Escherichia coli* were significantly higher relative to the high iron diet group (Figure S4, 3(a–b)). This suggests that increased intestinal dietary iron concentrations as a result of iron supplementation may accelerate negative selection against Enterobacteriaceae during community assembly.

Because dietary iron restriction decreases fecal iron concentrations, we investigated whether low luminal iron promotes an increase presence of microbial genes involved in scavenging iron within the fecal microbiota. Using the metagenomic inference tool PICRUSt, we observed a significant

increase in the predicted presence of *tonB* (K03832), *exbB* (K03561) and *exbD* (K03559) in mice maintained on the low iron or control diets when compared to high iron diet mice (Figure 3(c), Table S4). These three genes are required for bacterial import of siderophore-bound iron.<sup>40</sup> Also increased was the relative abundance of additional genes involved in siderophore-mediated iron acquisition and utilization, including a predicted enterochelin esterase (K07214) that hydrolyzes ferric enterobactin<sup>41</sup> and a predicted iron complex outer membrane receptor (K02014). Transcript levels of these and other *E. coli* iron acquisition genes are increased *in vitro* under iron-limiting conditions,<sup>42,43</sup> including in a resident intestinal *E. coli* strain (Table S5). Moreover, these findings generated by PICRUSt are consistent with the increased abundance of Enterobacteriaceae observed using the RDP pipelines. Taken together, these data suggest that decreased intestinal iron concentrations observed with the low iron and control diets relative to the iron supplemented diet select for siderophilic bacteria including *E. coli* with a greater capacity to scavenge iron.

Because the predicted abundance of *tonB* (which encodes a protein known to be required for importing siderophore-bound iron<sup>44</sup>) was among the iron acquisition genes that were increased in the low iron and control diet groups, we next determined whether TonB-mediated iron acquisition provides resident intestinal *E. coli* a relative growth advantage when iron availability is restricted. To address this, we created an isogenic *tonB* deletion mutant in *E. coli* to simultaneously inactivate all siderophore-mediated iron import.<sup>45</sup> The growth of the parental strain exceeded that of the *tonB* mutant in the presence of the membrane impermeable iron chelator DTPA (Figure S5A). Addition of exogenous iron to the chelated cultures rescued growth of the *tonB* mutant (Figure S5A), demonstrating that siderophore-mediated iron scavenging limits the impact of extracellular iron chelation on *E. coli* growth. We then competed the parental or *tonB* mutant *E. coli* strain with a non-siderophilic bacterium, *Enterococcus faecalis*, that exhibits minimal iron requirements (Figure S5B).<sup>24</sup> When iron was chelated, the relative abundance of the parental *E. coli* strain – but not the *tonB* mutant – was significantly increased over co-cultured *E. faecalis* (Figure 4(a,c)),

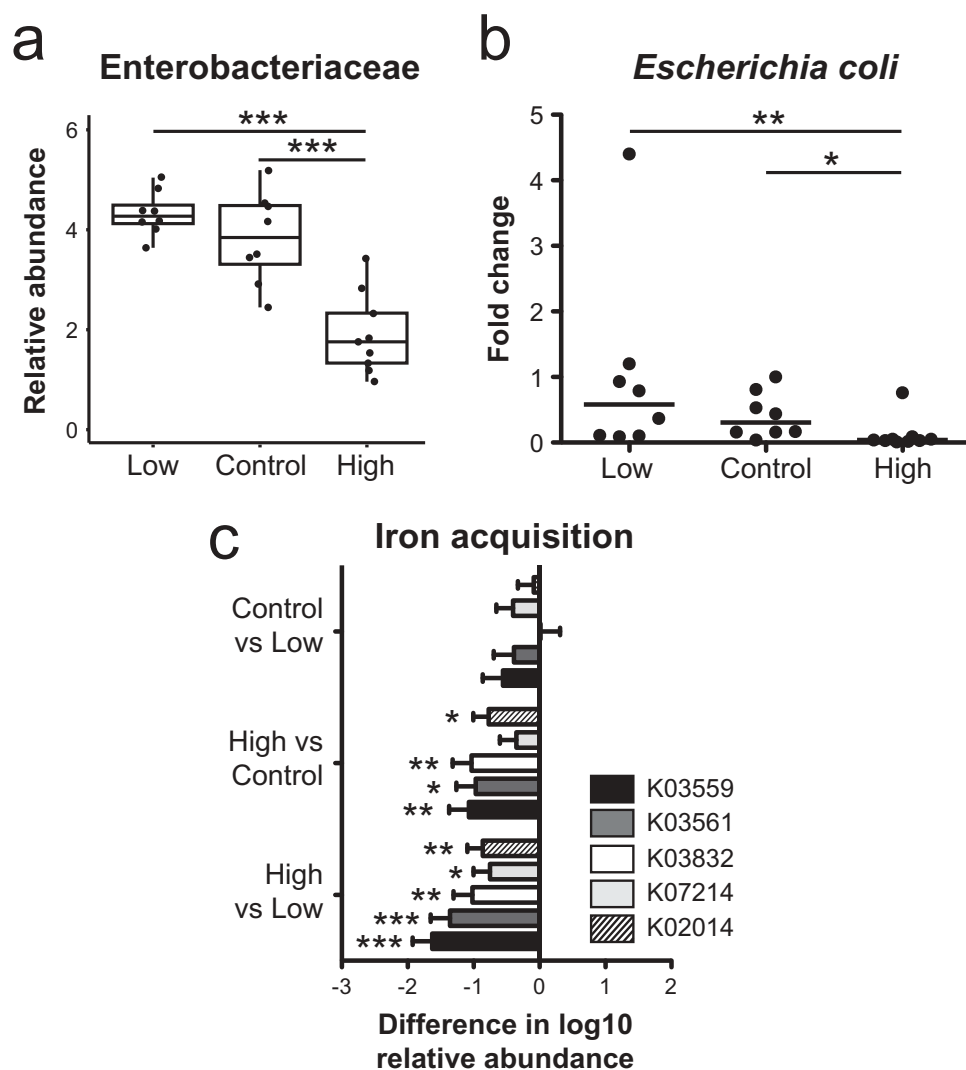


**Figure 2.** Dietary iron enhances the relative fecal abundances of numerous bacterial taxa. (a) Heat map of OTUs that are significantly different in abundance with variable iron diets (FDR-corrected  $p < 0.05$ ). Each column represents an individual WT mouse. Each row represents individual OTUs, color coded by phylum, that are significant between at least two diet groups as determined using a mixed linear model. The colors of the heat map represent the mean relative abundance (normalized and log transformed) of each OTU. B + C) Venn diagrams of OTUs that are significantly (b) increased or (c) decreased in relative abundance (FDR-corrected  $p < 0.05$ ) in the feces as determined using a mixed linear model.

which corresponded with an enrichment of *E. faecalis* in the presence of the *tonB* mutant (Figure 4(b,d)). When exogenous iron was added to the chelated cultures, the *tonB*-mediated enrichment of *E. coli* over *E. faecalis* reverted to baseline

values (Figure 4, Figure S5D-G). Consistent with these *in vitro* observations, our sequencing data revealed minimal changes in the relative fecal abundance of *Enterococcaceae* family members as intestinal iron concentrations were altered (Figure S5C).



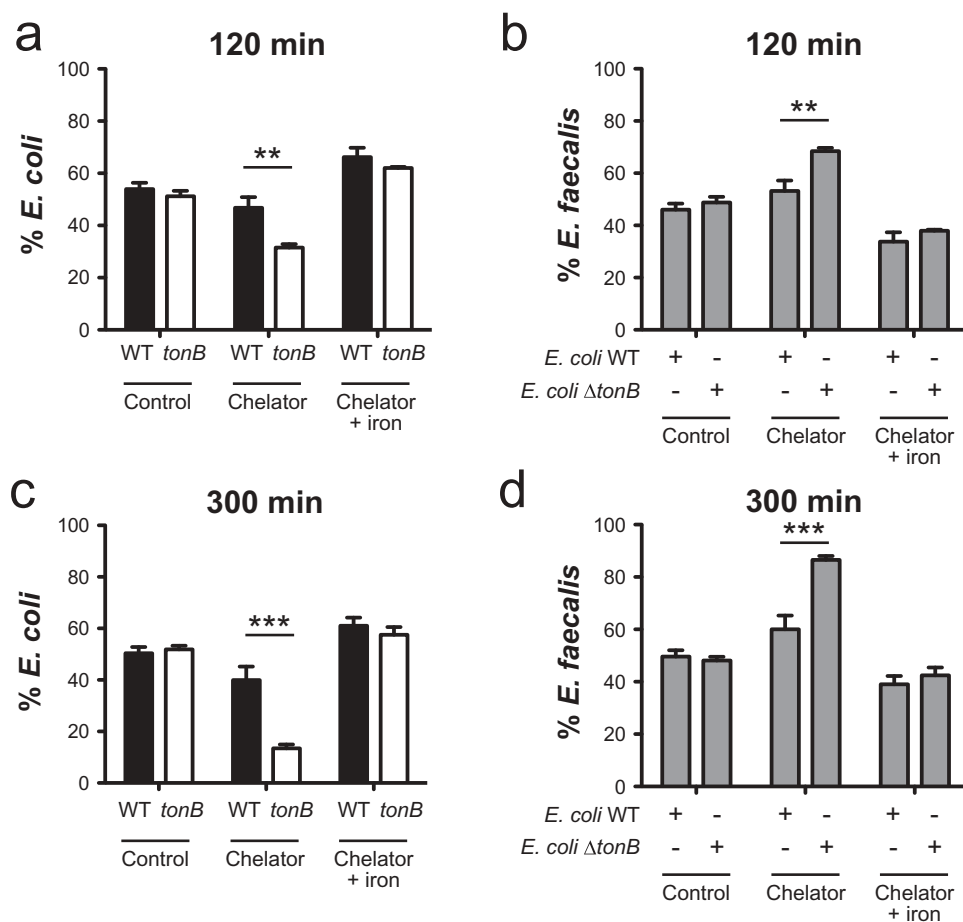


**Figure 3.** Dietary iron restriction promotes a bloom of Enterobacteriaceae and predicted bacterial iron uptake systems. (a) Relative fecal abundance of Enterobacteriaceae. (b) Abundance of fecal *E. coli* 16S sequences relative to total bacteria 16S sequences as determined by quantitative PCR. Data are presented as the fold change relative to the low iron diet group. Each point represents an individual mouse,  $n = 8-9$  mice per group. Lines are at the medians and  $p$  values were determined by pairwise comparisons by the Kruskal–Wallis test. (c) Differences in the least square mean between diet groups of log<sub>10</sub> normalized counts of predicted genes involved in iron acquisition as determined by PICRUSt. (a) Box and whisker plots show the minimum, first quartile, median, third quartile, and maximum relative abundance. (a, c) FDR-corrected  $p$ -values were determined using a mixed linear model. \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ .

Taken together, these data suggest that TonB-dependent iron acquisition may help promote the *in vivo* bloom of *E. coli* via efficient iron scavenging through siderophore-mediated iron acquisition when luminal intestinal iron availability is decreased.

### Dietary iron impacts microbiota structure in colitis-susceptible *Il10*<sup>-/-</sup> mice

We previously observed expanded Enterobacteriaceae during the onset of colitis in inflammation-susceptible, ex-GF *Il10*<sup>-/-</sup> mice following conventionalization.<sup>32</sup> Because we observed that dietary iron restriction also promotes a bloom of Enterobacteriaceae in uninflamed WT mice, we investigated the impact of iron restriction on the microbiota and colitis development in ex-GF *Il10*<sup>-/-</sup> mice.



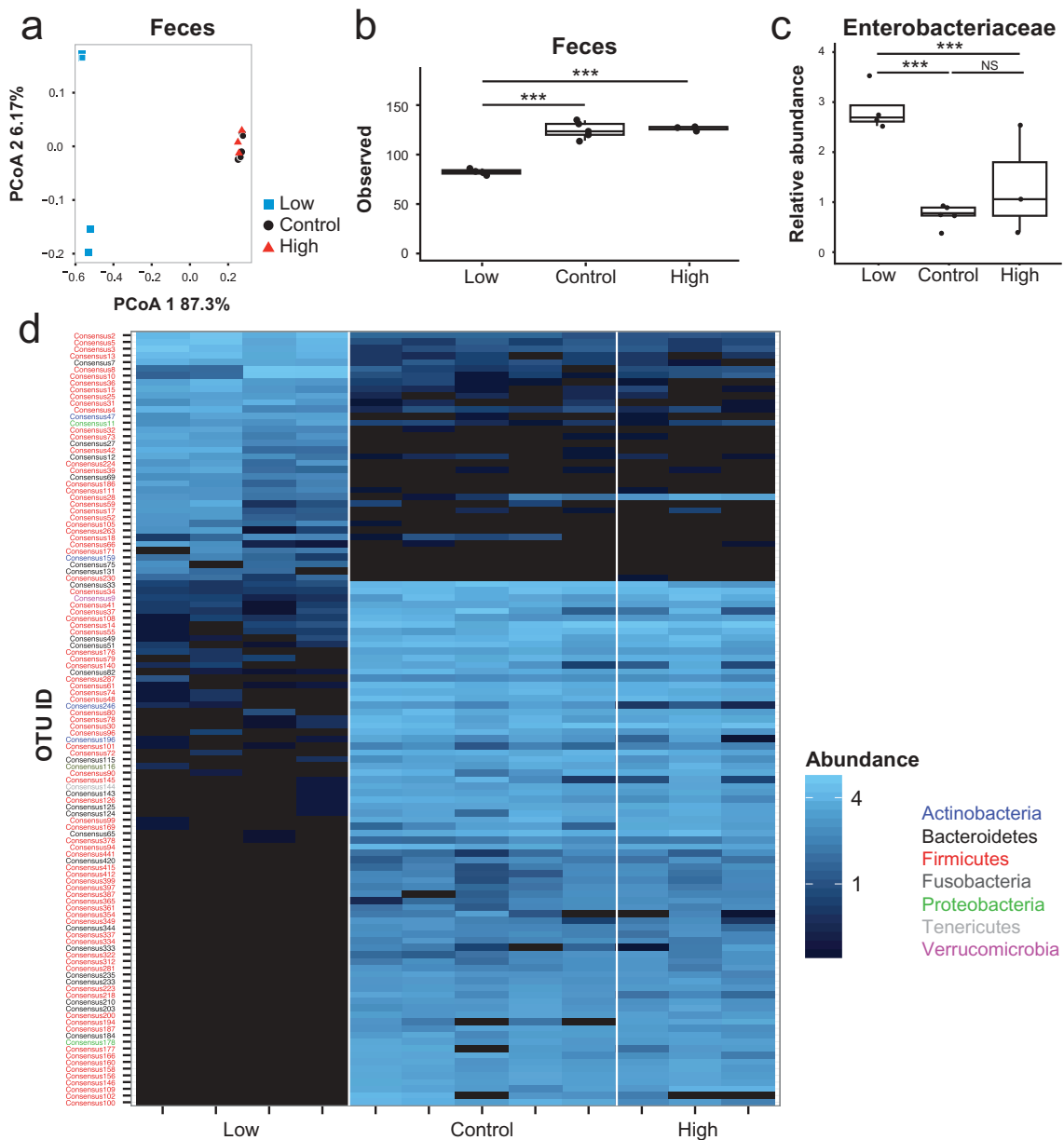
**Figure 4.** TonB-dependent iron acquisition enhances the relative abundance of *E. coli* when iron availability is restricted. *E. coli* WT or the *tonB*-deficient mutant were grown in the presence of *E. faecalis* in rich medium (control), in rich medium with the iron chelator DTPA (chelator) or in rich medium with DTPA and ferrous iron (chelator + iron). Abundance of bacteria was determined by quantitative selective plating. The % abundance of (a, c) *E. coli* WT or the *tonB* mutant in the presence of *E. faecalis* and (b, d) the % abundance of *E. faecalis* in the presence of *E. coli* WT or the *tonB* mutant after 120 or 300 min of growth. Data are represented as the mean  $\pm$  SEM of at least three independent experiments. *P*-values were determined by two-way ANOVA. \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ .

As was the case with the WT cohort, fecal communities assembled by iron availability in *Il10*<sup>-/-</sup> mice (Figure 5(a), PCoA axis 1; FDR-corrected  $p$ -value =  $2.7 \times 10^{-6}$ ). However, in contrast to WT mice, PCoA demonstrated that fecal communities from mice fed the control and high iron diets clustered together. Similar to WT mice, microbial richness was reduced (Figure 5(b)) and the relative abundance of Enterobacteriaceae was enhanced (Figure 5(c)) with dietary iron restriction. While 112 taxa were different when comparing the low iron and control groups and 106 taxa when comparing the low and high iron groups, few taxa (11 OTUs) were significantly different between the control and high diet groups (Figure 5(d), Table S6). PCoA analysis also revealed modest clustering of the mucosal microbiota by diet (Figure

S6A). This did not achieve statistical significance in contrast to WT mice (Figure 1(d)). However, as observed with WT mice, iron supplementation corresponded with increased microbial richness and abundances of numerous OTU in the mucosal microbiota relative to control diet (Figure S6B-C). Together, these data suggest that while dietary iron restriction alters the microbial community in *Il10*<sup>-/-</sup> mice, additional iron supplementation does not further alter the structure of the *Il10*<sup>-/-</sup> fecal microbiota relative to the control diet.

To determine the functional consequences of these changes to the *Il10*<sup>-/-</sup> microbiota, we characterized the development of colitis in *Il10*<sup>-/-</sup> mice subjected to iron supplementation or restriction. Four weeks post-conventionalization, high iron diet

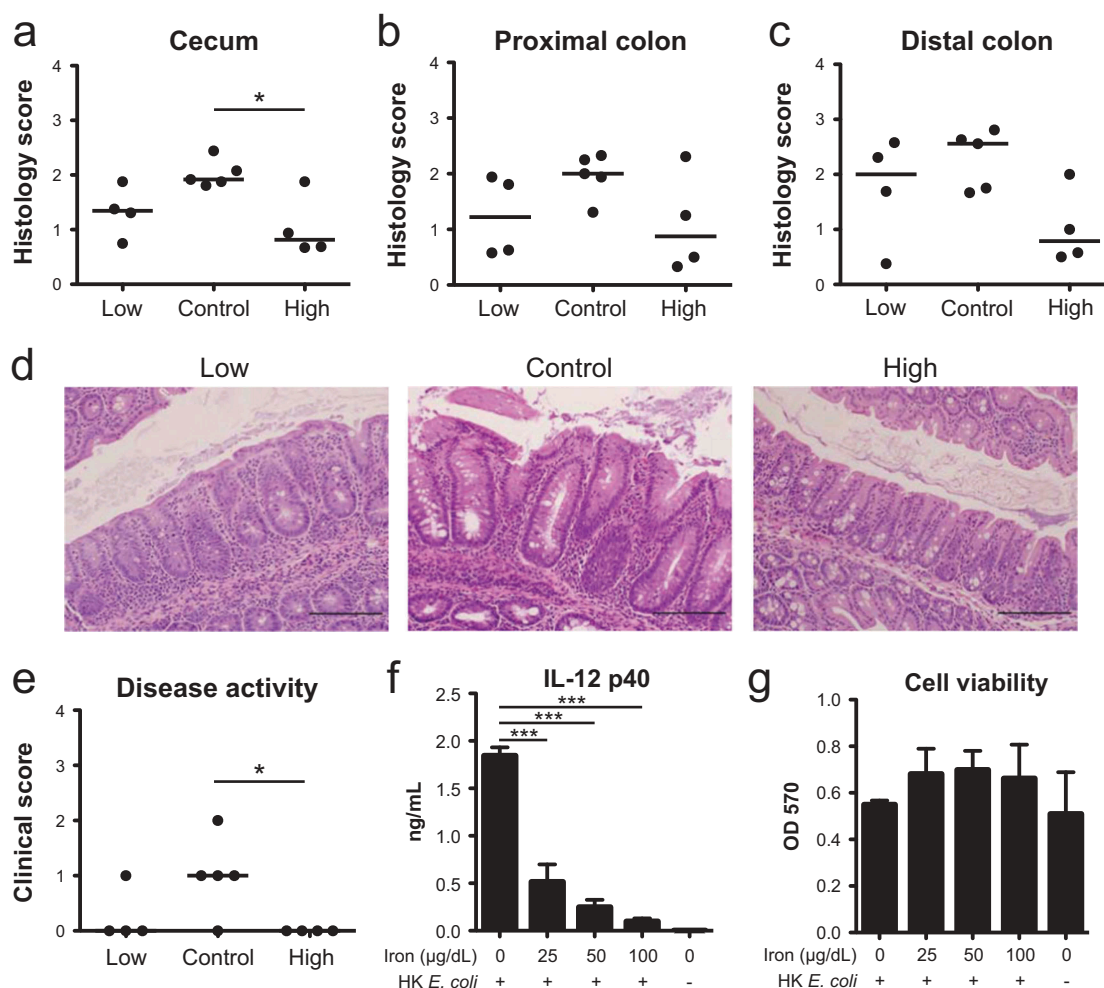




**Figure 5.:** Fecal community composition is insensitive to additional dietary iron supplementation in *Il10<sup>-/-</sup>* mice. (a) Principal coordinate analysis based on Bray–Curtis metrics for the fecal microbiota in ex-GF *Il10<sup>-/-</sup>* mice. (b) Microbial richness as measured by observed OTUs. (c) Relative fecal abundance of Enterobacteriaceae. (b, c) Box and whisker plots show the minimum, first quartile, median, third quartile, and maximum relative abundance. Each symbol represents an individual mouse,  $n = 3\text{--}5$  mice per group. FDR-corrected  $p$ -values were determined using a mixed linear model. (d) Heat map of OTUs that significantly differ in relative fecal abundance (FDR-corrected  $p < 0.05$ ) as determined using a mixed linear model. Each column represents an individual *Il10<sup>-/-</sup>* mouse. Each row represents individual OTUs, color-coded by phylum. The colors of the heat map represent the mean relative abundance (normalized and log transformed) of each OTU.

mice exhibited a modest but significant decrease in colitis severity compared to control mice as assessed by histological inflammation (Figure 6(a–d)) and clinical disease activity (Figure 6(e)). We also compared the spontaneous secretion of IL-12 p40 by colonic explant cultures and serum IL-12 p40 levels

between experimental groups as increased levels of this cytokine correspond with more severe colitis in this model.<sup>46</sup> A trend towards decreased IL-12 p40 in the colon (Figure S7A) and in the serum (Figure S7B) was observed in both the high iron and low iron versus the control group (Figure 6(a, b, c, e)).

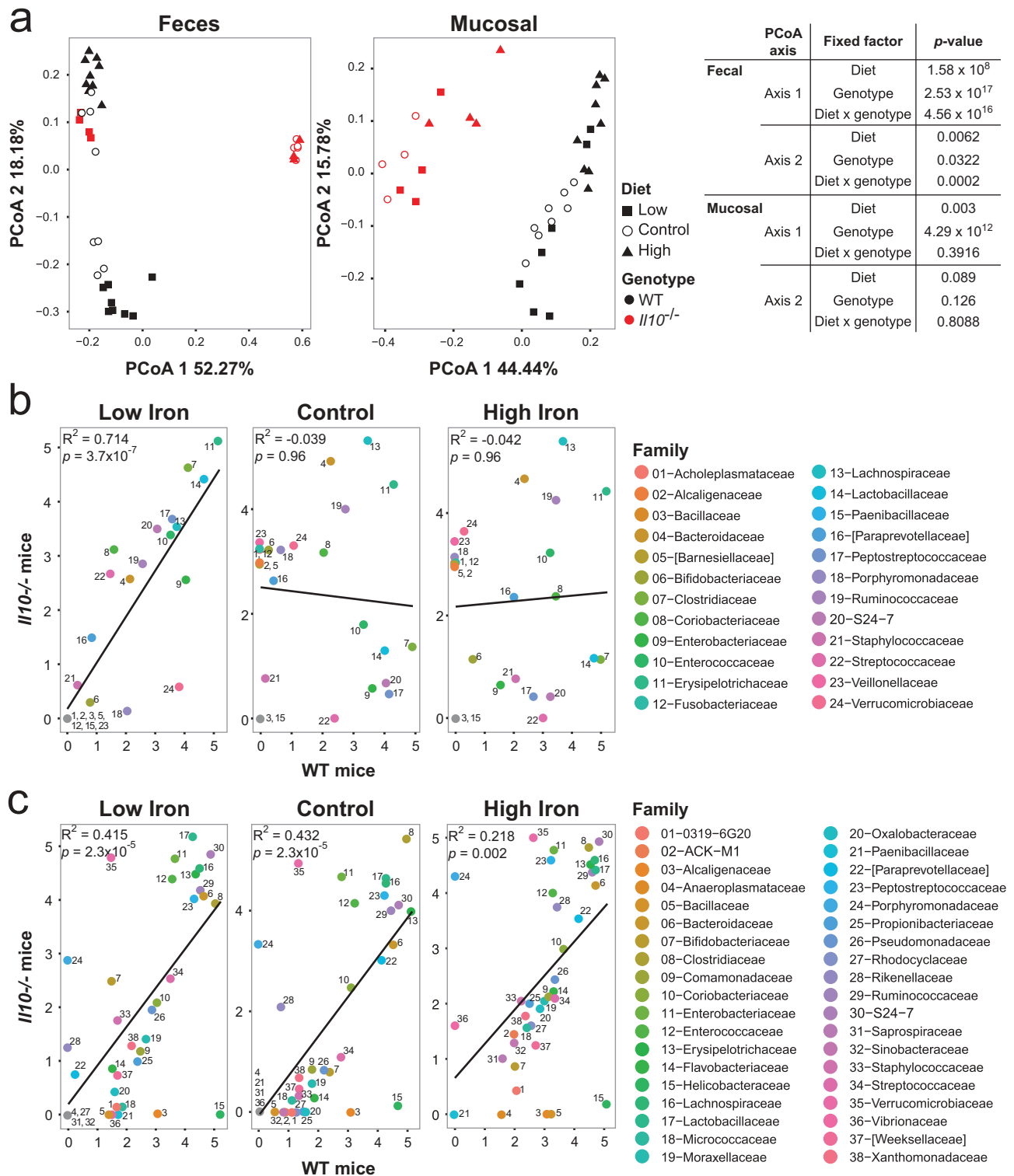


**Figure 6.:** Dietary iron impacts the development of colitis in *Il10*<sup>-/-</sup> mice. Ex-GF *Il10*<sup>-/-</sup> mice were maintained on an iron-deficient (low), control or iron supplemented (high) diet. (A–C) Histology scores (0–4) of the (A) cecum, (B) proximal colon and (C) distal colon. (D) Representative H&E histology at 200x of the ceca. Scale bar = 20 µm. (E) Clinical activity scores (0–4) after 4 weeks. (F + G) Macrophages were stimulated with heat-killed *E. coli* and cultured with iron for 8 h to assess: (F) IL-12 p40 secretion and (G) macrophage viability by MTT assay. Data are represented as the mean ± SEM of three independent experiments. *P*-values were determined by one-way ANOVA. \* *p* < 0.05, \*\* *p* < 0.01, \*\*\* *p* < 0.001.

Similarly, a trend towards reduced histopathology and disease activity was observed in low iron diet mice compared to the control group. Taken together, our results indicate that both iron restriction and supplementation modestly attenuate colitis development in the *Il10*<sup>-/-</sup> colitis model.

The lack of significant compositional changes between the fecal microbiotas of control *Il10*<sup>-/-</sup> mice with increased colitis versus *Il10*<sup>-/-</sup> mice fed a high iron diet suggests that host factors may also contribute to the impact of dietary iron supplementation on colitis development. Given that iron homeostasis and macrophage function are intimately linked,<sup>47</sup> we tested whether iron impacts *in vitro* macrophage production of IL-12 p40 as a marker

for proinflammatory macrophage activity. Consistent with our IL-12 p40 *in vivo* observations (Figure S7), iron reduced macrophage IL-12 p40 secretion (Figure 6(f)) without impacting viability (Figure 6(g)). Taken together, these results support previous findings that iron impacts innate immune function and may contribute to the protective effect of dietary iron supplementation on colitis development in the *Il10*<sup>-/-</sup> mouse model. Furthermore, these findings suggest that the protective effects of low iron on colitis development in this model likely occur through a different mechanism.



**Figure 7.** Dietary iron modulation differentially impacts the WT and *Il10*<sup>-/-</sup> fecal microbiotas. (a) Principal coordinate analysis based on Bray-Curtis metrics in WT and *Il10*<sup>-/-</sup> mice for the fecal and mucosal microbiota. Each symbol represents an individual mouse,  $n = 3-9$  mice per group. FDR corrected  $p$ -values were determined using a mixed linear model, evaluating the null hypothesis that the fixed factor indicated in each table does not impact the coordinate. (b, c) Correlations between the median log normalized counts of bacterial taxa at the family level of WT and *Il10*<sup>-/-</sup> mice in the (b) fecal and (c) mucosal microbiota.

### **Dietary iron modulation differentially impacts the WT and *Il10*<sup>-/-</sup> fecal microbiotas**

Given that dietary iron supplementation compositionally alters the microbiotas of WT and *Il10*<sup>-/-</sup> mice in distinct ways, we directly compared the microbiotas of both genotypes under conditions of dietary iron restriction and supplementation. PCoA and our mixed linear model revealed that genotype and genotype x diet interactions strongly associated with microbiota composition in the feces (Figure 7(a), S8). In the mucosa, however, only genotype strongly associated with community composition. We next determined whether bacterial taxa respond to iron availability in similar ways regardless of host genotype. In the fecal community, there was a significant positive correlation between bacterial abundance in WT and *Il10*<sup>-/-</sup> mice maintained on the low iron diet (Figure 7(b)), demonstrating a similarity in biological response of the luminal microbiota to iron restriction. In contrast, there was no correlation between bacterial abundance in WT and *Il10*<sup>-/-</sup> mice maintained on the control or high iron diets, suggesting that inflammation or other intrinsic factors associated with the *Il10*<sup>-/-</sup> genotype cause the fecal microbiota of these mice to respond differently to increased iron availability than in WT mice. By contrast, in the mucosa, bacterial abundance in WT and *Il10*<sup>-/-</sup> mice was positively correlated regardless of the diet group (Figure 7(c)). Consistent with our mixed linear results (Figure 7(a)), this suggests that niche selection pressure may be a stronger driver in modulating the abundance of specific bacterial taxa in the mucosa rather than genotype or dietary iron content. More broadly, diet and host genetics strongly impact community composition in the lumen, whereas only host genetics strongly influences the mucosal community.

### **Discussion**

Treatment of iron deficiency through oral iron supplements or iron-fortified diets can modulate luminal intestinal iron concentrations<sup>13,37</sup> and likely influences bacterial iron availability, consequent bacterial growth, and community structure. We, therefore, investigated the ecological impact of dietary iron restriction and supplementation on the intestinal microbiota. Our findings demonstrate that dietary iron restriction during community assembly resulted

in significant compositional changes frequently associated with a dysbiotic state. Other 16S rRNA community profiling studies report complementary findings in established intestinal microbial communities.<sup>36,48</sup> For example, dietary iron restriction initiated at weaning-induced compositional changes to the fecal microbiota, including decreased microbial richness relative to both the baseline community at weaning and the control diet.<sup>36</sup> Interestingly, subsequent dietary iron repletion did not fully restore microbial richness or community composition.<sup>36</sup> In contrast, in adult mice with an established microbiota, microbial richness was not affected by the dietary iron restriction.<sup>11</sup> Thus, the early assembly of the intestinal microbiota may be uniquely susceptible to iron restriction.

Our findings demonstrate that dietary iron restriction that is the result of administering a diet with trace iron levels is associated with the reduced luminal abundance of numerous taxa within the Firmicutes and an increase in endogenous Enterobacteriaceae. The abundance of resident Enterobacteriaceae did not differ upon iron restriction in other rodent studies harboring an established microbiota.<sup>11,36</sup> However, consistent with our results, dietary iron restriction initiated at weaning results in a bloom of endogenous Enterobacteriaceae that was reversed with subsequent dietary iron repletion.<sup>35</sup> Decreased Enterobacteriaceae was similarly observed in human infants with low initial intestinal pathogen burden receiving iron-fortified foods.<sup>49</sup> Taken together, these findings suggest that dietary iron restriction may perturb the assembly of the luminal intestinal bacterial community, promoting compositional changes, including increased resident Enterobacteriaceae, that have been linked to numerous microbially driven diseases. This may be mediated through the creation of an intestinal environment that favors the growth of early colonizers with multiple inducible iron acquisition systems such as *E. coli*. However, an important distinction between resident and pathogenic Enterobacteriaceae should be noted. In contrast to the effects of iron supplementation on endogenous *E. coli*, increased oral iron intake enhances the abundance of pathogenic enterobacteria including Enterotoxigenic *E. coli* (ETEC) and *Salmonella*.<sup>5,7,50</sup> Taken together, the initial composition of the intestinal microbiota is an important factor that affects how dietary iron will ecologically alter the microbiota, with important implications for

identifying IDA patients that may respond favorably to oral iron supplementation regimens.

In response to low iron availability, bacteria upregulate iron acquisition genes to scavenge iron from the environment. Bacterial taxa such as Enterobacteriaceae encode numerous siderophore-mediated iron transport systems that enhance their competitive advantage when iron availability is restricted. Indeed, controlled *in vitro* growth competitions between *E. coli* and non-siderophilic *E. faecalis* demonstrated that TonB-mediated iron acquisition enhanced the relative fitness of *E. coli* when the iron was sequestered by the extracellular chelator DTPA. While DTPA can sequester other metals, the addition of exogenous iron reversed the TonB-mediated fitness advantage of *E. coli* over *E. faecalis*, suggesting that these observations are likely the result of DTPA chelation of iron rather than other metals. However, it should be noted that these *in vitro* growth competitions were performed under aerobic conditions. Under steady state conditions, the gut lumen is an anaerobic environment, but becomes increasingly aerobic with inflammation, although not to the same extent as a shaking culture.<sup>51</sup> Nonetheless, consistent with our findings, iron chelation in colonic fermentators inoculated with a fecal microbiota and subjected to anaerobic growth results in a similar expansion of Enterobacteriaceae.<sup>52</sup> Thus, decreasing iron availability in the intestine likely provides a fitness advantage for resident bacteria that are more effective at scavenging iron. Supporting this model, metagenomic predictions using the PICRUSt algorithm suggest an increased capacity for siderophore-mediated iron acquisition in the microbiotas of mice administered an iron-deficient diet. Furthermore, the presence of genes involved in flagella biosynthesis and assembly, an iron-dependent process, as well as zinc, manganese and hemoglobin utilization is increased with decreased dietary iron consumption. However, while PICRUSt only predicts functional capacity using 16S profiling data, the PICRUSt algorithm has been shown to recapitulate metagenomic results with approximately 80% accuracy when applied to data obtained from the Human Microbiome Project.<sup>53</sup> Nonetheless, further studies assessing transcript levels of iron acquisition genes and *in vivo* growth competitions utilizing

siderophore deficient mutants are necessary to confirm this ecological model.

Intestinal inflammation is associated with compositional changes to the intestinal microbiota, including an expansion of Enterobacteriaceae.<sup>38,39,54–56</sup> Because dietary iron restriction induced similar perturbations to the fecal microbiota, we investigated the impact of dietary iron on the development of colitis in ex-GF *Il10*<sup>-/-</sup> mice. Interestingly, dietary iron supplementation modestly limited the development of inflammation. However, an important limitation of our study is that iron supplementation was provided to iron replete hosts rather than iron-deficient hosts, as often occurs in IBD patients. However, many IBD patients with anemia of chronic disease are encouraged to take supplemental iron despite normal iron stores, and IBD patients are routinely encouraged to take multi-vitamins that contain iron, regardless of their iron status. Nonetheless, in agreement with our observations, Dostal and colleagues reported higher baseline inflammation in the ilea and ceca of rats on a control diet compared to iron-deficient rats receiving iron supplementation.<sup>48</sup> Similarly, dietary iron restriction has also been reported to minimize intestinal inflammation.<sup>11,48</sup> In chemically induced models of colitis, increased dietary iron exacerbated colitis.<sup>10,12</sup> However, in many of these studies, dietary iron was administered at doses approximately 100 times higher than what is present in normal mouse chow and may therefore not reflect amounts consumed with iron fortification or oral iron supplements. Indeed, similar to our study, oral iron supplementation ranging from 2 to 10 fold higher than iron levels in the control diets protected against TNBS-induced and DSS-induced colitis.<sup>57,58</sup> Administration of an iron-fortified diet in infants with high initial pathogen burden also resulted in increased fecal markers of inflammation.<sup>7</sup> However, two other studies demonstrated no impact of dietary iron supplementation on inflammation markers in infants or children with low pathogen burden.<sup>49,59</sup> Interestingly, dietary iron restriction has been reported to minimize intestinal inflammation,<sup>11,48</sup> consistent with our statistically insignificant trends. Finally, it should also be noted that a recent study demonstrated that the formulation of iron also impacts the effect of oral iron supplementation on colitis severity, where ferrous iron and ferrous



bisglycinate both ameliorated DSS-induced colitis and ferric-EDTA had the opposite effect.<sup>58</sup> Together, these studies provide evidence that dietary iron influences colitis development, albeit in a complex manner that depends on the iron formulation as well as microbial and host factors that remain to be fully defined.

In *Il10*<sup>-/-</sup> mice, fecal communities clustered by diet, suggesting that intestinal iron concentrations had a stronger impact in shaping fecal community structure than colitis severity, while mucosally associated bacterial communities were influenced more by colonic inflammation. Interestingly, dietary iron restriction in *Il10*<sup>-/-</sup> mice did not exacerbate colitis relative to the control group despite compositional changes to the fecal microbiota frequently associated with inflammation.<sup>27,38,39,55,56</sup> Consistent with our findings, oral iron supplementation induced compositional changes to the fecal microbiota in UC and CD patients that were not associated with altered disease activity.<sup>60</sup> This suggests that in our model, the impact of iron supplementation on the development of colitis may not be mediated through compositional changes to the luminal microbiota, but rather through direct effects on the host. Indeed, oral iron supplementation increases the generation of free radicals in the intestines and serum markers of lipid peroxidation.<sup>11,61</sup> Cellular iron status is also intricately linked with epithelial and macrophage function including secretion of proinflammatory cytokines and susceptibility to infection.<sup>62-64</sup> Similarly, we also observed that iron modulates *E. coli*-stimulated macrophage production of the proinflammatory cytokine IL-12 p40, which correlates with severity of colitis in the *Il10*<sup>-/-</sup> mouse model.<sup>46</sup> It should be noted that the iron concentrations used for the macrophage stimulations are lower than total fecal iron concentrations. However, it is also unclear what fraction of total iron in the colonic lumen is bioavailable to both microbe and host. Finally, these findings do not exclude the possibility that dietary iron may impact the course of inflammation by altering the function of resident bacteria. Indeed, decreasing iron availability reduces the fermentative activity of the intestinal

microbiota,<sup>35,48,52</sup> a functional change that has been associated with dysbiosis. Oral iron supplementation has also been shown to alter the fecal metabolome in anemic and IBD patients,<sup>60</sup> which may reflect altered microbial function. Thus, longitudinal studies investigating the impact of dietary iron on the function of the intestinal microbiota prior to, during, and after the onset of inflammation are warranted to further elucidate the contribution of the intestinal microbiota on iron-mediated effects on colitis. Together our data support a model where iron-mediated modulation of the intestinal microbiome and host immune function both contribute to colitis development. Given the high prevalence of IDA in individuals with gastrointestinal inflammatory disorders such as IBD, the precise mechanisms underlying the effects of iron on colitis development should be explored in future studies.

## Materials and methods

### *Mice and experimental design*

*Il10*<sup>-/-</sup> and WT 129S6/SvEV mice were maintained in GF conditions at the National Gnotobiotic Rodent Resource Center at UNC-Chapel Hill. Three groups of age-matched, 8–14 week-old GF WT ( $n = 8-9$ , 3 cages/group) and *Il10*<sup>-/-</sup> mice ( $n = 4-5$ , 2–3 cages/group) were transferred to conventional housing and placed on iron-deficient (<10 ppm iron/kg diet,  $n = 5$  males, 3 females for WT mice;  $n = 3$  males, 1 female for *Il10*<sup>-/-</sup> mice), control (35 ppm iron/kg diet,  $n = 5$  males, 3 females for WT mice;  $n = 3$  males, 2 females for *Il10*<sup>-/-</sup> mice) or iron supplemented diets (200 ppm iron/kg diet,  $n = 5$  males, 4 females for WT mice;  $n = 1$  male, 3 females for *Il10*<sup>-/-</sup> mice). The following day, mice were colonized by oral/rectal swab with fecal slurry from a WT SPF 129S6/SvEV mouse housed in the same facility. At 2 and 4 weeks of dietary intervention, fecal samples were collected with fecal iron measurements. After 4 weeks of dietary intervention, mice were killed and fresh feces and cecal tissue were collected and processed for 16S rRNA sequencing as previously described.<sup>32,38</sup> One WT low iron and *Il10*<sup>-/-</sup> control mucosal sample was excluded from the analysis because of low DNA



yield or similar technical reasons. The WT and *Il10*<sup>-/-</sup> cohorts were run at different times and were therefore colonized with different donors; thus subsequent 16S rRNA sequencing analyses were run separately for both cohorts. Mice on iron-deficient diets received 5 µg/g body weight of dextran iron sulfate (D8517, Sigma) i.p. Mice on control and iron supplemented diets received PBS i.p. as a vehicle control. Fecal iron content was assessed in a separate cohort of WT and *Il10*<sup>-/-</sup> mice (Figure S2). Envigo produced all diets, based on the TD.99397 iron-deficient diet. Diets differed only in iron content, added as ferrous sulfate and ferric citrate at 1:1 ratio. All animal protocols were approved by the UNC-Chapel Hill Institutional Animal Care and Use Committee.

### Assessment of colitis and disease activity

At necropsy, colonic segments were fixed in 10% neutral buffered formalin. Histological inflammation scores (0–4) of cecal, proximal and distal colonic sections were blindly assessed as described.<sup>46</sup> Clinical disease activity (0–4) was assessed as described.<sup>65</sup> Unstimulated colonic explant cultures were prepared to assess spontaneous cytokine secretion as described.<sup>66</sup>

### Bacteria strains and growth conditions

Bacteria strains are listed in Table S1. Bacteria were grown overnight in brain heart infusion (BHI) medium at 37°C prior to inoculation into experimental conditions: BHI control, BHI with 250 µM of iron chelator diethylene triamine pentaacetic acid (DTPA; D6518, Sigma), or BHI with 250 µM DTPA and 250 µM ferrous sulfate (I146, Fisher Scientific). Bacterial growth was assessed by spectrophotometry (OD600) and quantitative plating. For dual cultures, selective media was utilized to distinguish *E. faecalis* (BHI agar with 50 µg/mL kanamycin) and *E. coli* (MacConkey agar). Deletion mutants in *E. coli* were created using the λ-red recombinase system as described.<sup>67</sup>

### Quantification of iron in fecal and liver samples

Iron contents in liver and feces were measured using atomic absorption spectrophotometry (AA) as described<sup>168</sup> with modifications: briefly, samples were

weighed, desiccated in a 90°C oven overnight and weighed again to obtain wet and dry sample weights. Samples were digested in 50% HNO<sub>3</sub> and acid was evaporated for 24 h at room temperature. Samples were diluted in 2% HNO<sub>3</sub> and analyzed by AA.

### Quantitative PCR

Quantitative PCR was performed on fecal DNA to quantify the abundance of 16S rRNA sequences from *E. coli* and all bacteria using previously reported primers.<sup>32</sup> Sensifast SYBR No-ROX Kit (Bioline) was utilized with the following PCR conditions: 95°C for 2 min, followed by 40 cycles at 95°C for 5 s, 60°C for 10 s and 72°C for 20 s.

### Bacterial RNA isolation and microarray hybridization

*E. coli* NC101 was grown in M9 medium with the indicated concentrations of ferrous sulfate. After 1 h, aliquots were collected for RNA isolation, which was isolated using the RNeasy isolation kit (Qiagen) following the manufacturer's instructions. Purified RNA was treated with on-column DNase (Qiagen) and DNA-free DNase (Ambion) following the manufacturer's instructions. RNA samples were prepared for microarray hybridization using Affymetrix *E. coli* Genome 2.0 arrays as previously described<sup>69</sup> at the UNC Functional Genomics Core. Statistical analysis was performed using GeneSpring 7.2 software.

### Bone marrow-derived macrophage (BMDM) cultures

Bone marrow cells were isolated as described.<sup>70</sup> Conditioned medium from murine fibroblast line L929 served as a source of M-CSF for macrophage differentiation.<sup>71</sup> BMDMs were seeded in 24-well plates and maintained in RPMI 1640 (Gibco) with 10% heat-inactivated fetal bovine serum (FBS, Gibco) and 1% penicillin/streptomycin/antimycotic (Gibco) at 37°C, 5% CO<sub>2</sub>. To assess the impact of iron on BMDM cytokine production, BMDMs were stimulated with heat-killed *E. coli* NC101 (MOI = 10) following the addition of the indicated amounts of ferrous sulfate to cultures. After 8 hrs, supernatants were collected and stored at -20°C to assess cytokine production. BMDM viability was assessed via the

reduction of tetrazolium dye, MTT, using spectrophotometry.

### Quantification of cytokine production

IL-12 p40 and interferon gamma were quantified by enzyme-linked immunosorbent assay (ELISA; BD Biosciences).

### Illumina 16S library construction, sequencing, and processing

Amplification of the hypervariable V6 region of the 16S rRNA gene via a two-step PCR strategy was performed as described.<sup>38</sup> DNA concentrations were quantified using a NanoDrop spectrophotometer and equal amounts of all samples were pooled together and subjected to paired-end Illumina Hi-Seq sequencing. A total of 97,286,872 raw reads were generated for a total of 74 samples. Raw reads were preprocessed as described.<sup>31,72</sup> Briefly, forward and reverse reads were merged if they satisfied having 70 bases overlap with 100% similarity. Successfully merged reads were quality trimmed at Q Score 20 followed by length check. Reads shorter than 50 bases were discarded. This resulted in 45,486,399 high quality reads that were incorporated into the subsequent analysis. These reads were then clustered into 1,301 operational taxonomic units (OTUs) using AbundantOTU+ v.0.93b (<http://omics.informatics.indiana.edu/AbundantOTU/>) (command option “-abundantonly” was used) at 97% similarity incorporating 99.84% of the input sequences. OTUs were checked for chimeric sequences using uchime v. 4.2.40 (<http://www.drive5.com/uchime/>)<sup>73</sup> utilizing the GOLD database. Uchime identified nine OTUs as chimeras, which were excluded from further analysis. Taxonomy was assigned through the Quantitative Insight into Microbial Ecology (QIIME) (v. 1.8.0)<sup>74</sup> using uclust consensus taxon assigner. Finally, we retained OTUs that have  $\geq 0.005\%$  of the total number of sequences according to Bokulich and colleagues<sup>75</sup> and excluded one sample due to technical reasons, resulting in a final dataset of 73 samples with an average of 611,168.6 reads/sample (median = 491,900; min = 299,100; max = 1,072,000 reads/sample).

### Functional prediction

We used Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PiCRUST)<sup>53</sup> to predict metagenomic functional content from our 16S rRNA survey. Briefly, the same reads fed to AbundantOTU+ were used to produce close reference OTUs using QIIME (v. 1.8.0) and GreenGenes dataset (gg\_13\_5). PiCRUST software was applied to the resulting biom file to generate KEGG orthologs and metagenomic predictions.

### Statistical analysis

OTU, phylum, family or KEGG ortholog raw counts were normalized and  $\log_{10}$  transformed according to the following formula:

$$\log_{10} \left\langle \left( \frac{\text{raw count}}{\text{Number of sequences in sample}} \times \text{Average number of sequences per sample} \right) + 1 \right\rangle$$

PCoA plots were produced from Bray–Curtis dissimilarity statistic on the normalized and  $\log_{10}$  transformed reads. Alpha diversity (Observed OTU estimate and Shannon diversity index) was calculated after rarefying the raw counts to a depth of the minimum count in all samples. We used a mixed linear model utilizing SAS (v. 9.3) software (SAS Institute Inc, Cary, NC) for analysis including accounting for possible effects from co-housing.<sup>31</sup>

The mixed linear model, in which dietary iron level (low, control, or high) and source (mucosal or fecal) are fixed effects and the cage is a random effect, is formulated as follows:

$$Y_{ijkl} = \mu + D_i + S_j + (SD)_{ij} + C_{k(i)} + \varepsilon_{ijkl},$$

where  $Y_{ijkl}$  represents either PCoA axis value,  $\log_{10}$  normalized OTU count,  $\log_{10}$  normalized phylum count,  $\log_{10}$  normalized family count,  $\log_{10}$  normalized KEGG ortholog count or richness value for dietary iron level  $i$ , source  $j$ , cage  $k$  and replicate  $l$ .  $D_i$  is the effect of the  $i^{\text{th}}$  dietary iron level.  $S_j$  is the effect from the  $j^{\text{th}}$  source.  $(SD)_{ij}$  is the interaction effect between dietary iron level  $i$  and source  $j$ .  $C_{k(i)}$  is the effect from the  $k^{\text{th}}$  cage that is nested within the  $i^{\text{th}}$  dietary iron level and

$\varepsilon_{ijkl}$  denotes the error associated with measuring  $Y_{ijkl}$ . For Figure 7(a), fixed terms  $S_j$  and  $(SD)_{ij}$  denote the  $j^{\text{th}}$  Genotype and interaction between dietary iron level  $i$  and Genotype  $j$ , respectively. Parallel analysis using QIIME (v. 1.8.0) close reference OTU picking approach with the GreenGenes dataset (gg\_13\_5) and employing UniFrac distance metric after OTU count normalization and  $\log_{10}$  transformation (as described above) or after rarefying the counts produced similar results to those obtained from AbundantOTU+ (Figure S7). We controlled for false discovery rate (FDR) by correcting  $P$ -values using Benjamini and Hochberg (BH) approach.<sup>76</sup>

For *in vitro* experiments,  $p$ -values were calculated using one-way ANOVA with Tukey's multiple comparison post-test for  $\geq 3$  experimental groups or two-way ANOVA with Bonferroni multiple comparison post-test when  $\geq 2$  variables were compared. For animal experiments,  $p$ -values were determined using a non-parametric Kruskal-Wallis test.

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## Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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## Additional Information

The sequences will be available upon acceptance/publication of the manuscript.

## ORCID

Janelle C. Arthur  <http://orcid.org/0000-0002-4796-0207>

Cory R. Brouwer  <http://orcid.org/0000-0003-4136-3249>

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