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RESEARCH ARTICLE

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Comparative analysis of dietary iron deprivation and supplementation in a murine model of colitis

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Abstract

Inflammatory bowel diseases are chronic inflammatory conditions with growing prevalence in western populations. Iron is an essential component of erythrocytes hemoglobin. Under the influence of elevated hepcidin production, iron is sequestered in cells during inflammation which, in turn, leads to iron restriction for red blood cell synthesis. As a consequence, iron deficiency and anemia of inflammation are the most prevalent extraintestinal complications in IBD patients. Iron deficiency is commonly treated with oral iron supplements, with limited efficacy as iron absorption is blunted during intestinal inflammation. Moreover, iron supplementation can cause intestinal complications, as previous studies have shown that it can worsen the inflammatory response. However, a comparative analysis of the effects of low, adequate, and high dietary iron content matching the iron supplementation given to patients has not been performed in mice. We therefore tested the impact of dietary iron deprivation and supplementation in a murine model of colitis induced by dextran sodium sulfate. We found that both dietary iron deprivation and supplementation were accompanied by a more severe inflammation with earlier signs of gastrointestinal bleeding compared to mice fed an iron-adequate diet. The manipulation of dietary iron led to a profound dysbiosis in the colon of control mice that differed depending on the dietary iron content. Analysis of this dysbiosis is in line with a pronounced susceptibility to colonic inflammation, thus questioning the benefit/risk balance of oral iron supplementation for IBD patients.

KEYWORDS

anemia, colitis, inflammation, iron, microbiota

1 | INTRODUCTION

Iron is a trace element essential for nearly all living species, but excess iron catalyzes the generation of reactive oxygen species. In humans, most of the plasma iron destined for erythropoiesis is provided by the recycling of iron from senescent red blood cells by splenic macrophages, and 25% of the body iron is stored in hepatocytes.[1]

Iron losses are minor and compensated by the absorption of dietary iron by duodenal enterocytes. Iron is released into the circulation by the exporter ferroportin present at the cell surface of macrophages, enterocytes, and hepatocytes. This process is orchestrated by the liver-produced hormone hepcidin,[2] which binds to ferroportin and induces its occlusion and degradation, leading to decreased iron uptake. In conditions associated with chronic inflammation, increased hepcidin production[3] prevents iron efflux into the bloodstream and causes a profound iron restriction that paves the way for the development of anemia, a frequent condition in hospitalized or chronically ill patients.

Ulcerative colitis and Crohn's disease, collectively termed inflammatory bowel diseases (IBD), are chronic forms of intestinal inflammation affecting the ileum and the large intestine of more than 300/100.000 individuals in Western populations.[4] Symptoms include abdominal pain, rectal bleeding, diarrhea, weight loss, and nutritional deficiencies. Since iron deficiency and anemia of inflammation are the most prevalent extraintestinal complications,[5] IBD patients are commonly treated with oral iron supplements that can cause abdominal pain and gastrointestinal complications[6–8] and increase ROS production and the risk of carcinogenesis.[9] Indeed, oral iron supplementation in IBD patients and increased dietary iron concentration in murine models of colitis are associated with exacerbated intestinal inflammation, blunted immune response,[9] and colonic dysbiosis.[10,11] In contrast, mice fed an iron-deficient diet seemed less susceptible to intestinal inflammation during experimental colitis.[12] In most studies, a standard rodent chow was used to study the effect of dietary iron, but a concomitant comparison of the outcomes of low and high dietary iron content mimicking oral iron supplementation during colitis has not been performed. Here, we sought to compare the impact of dietary iron deprivation and supplementation on intestinal inflammation in a mouse model of colitis induced by dextran sulfate sodium.

2 | METHODS

2.1 | Animals

Five-week-old C57Bl/6J male mice ($n=48$) were purchased at Janvier laboratories (Le Genest St-Isle, France) and housed in the CREFRE US006 animal care facility. Custom-made diets of identical composition (Ssniff) were supplemented (50 and 8000 μg/g) or not (10 μg/g) with carbonyl iron. At 6 weeks of age, mice were fed either an iron-deficient (ID, 10 μg/g Fe) or an iron adequate (IA; 50 μg/g Fe) diet for 2 weeks, with access to water ad libitum. A 2-week period was chosen to lower the body iron stores without the installation of iron-deficiency anemia. After 2 weeks, a group of mice fed an IA diet was given an iron-enriched diet (IE; 8000 μg/g Fe) on the day the colitis protocol was initiated. The iron content of the IE diet was chosen based on the physiological iron needs in humans and rodents. Every day, a healthy 70 kg adult human needs to absorb 1–2 mg of iron from the diet, which equals to 0.03 mg/kg. Mice require a daily intake of 10 μg iron, which, on the basis of a 20 g mouse, amounts to 1 mg/kg, approximately 33 times more than humans. Patients are usually supplemented with 100–200 mg of oral iron supplements daily, thus exceeding the iron needs by 100-fold, which would translate to an intake of 1000 μg in mice. We applied the correction factors of mice versus human needs (×33) and decided to supplement the diet to reach an intake of 32,000 μg Fe/day. As mice consume 4 g of food a day, our custom-made IE diet therefore contains 8000 μg/g of iron. All mice were caged in a specific pathogen-free animal facility with controlled temperature, humidity, and dark–light cycles of 12 h each. Samples were harvested, immediately frozen in liquid nitrogen, and stored at −80°C. Procedures were approved by the Animal Care and Ethics Committee of US006/CREFRE (CEEA-122; application number APAFIS 2016092912382223).

2.2 | Experimental colitis

Mice were administered 2% Dextran Sodium Sulfate (DSS, MP Biomedicals) in drinking water for 7 days and switched to regular drinking water. Animals were monitored daily for signs of illness and/or welfare impairment and were euthanized by cervical dislocation on day 9. Body weight and disease activity index (DAI) calculated using the scoring system shown in Table 1A were recorded every day.

2.3 | Histological assessment of colonic injury

Colonic tissue specimens were excised 2 cm proximal to the anus and immediately transferred into 4% formaldehyde before paraffin embedding. Five-micrometer colonic sections were stained with hematoxylin–eosin. Histological damage was evaluated based on inflammatory cell infiltration, epithelial/mucosal alteration (including vasculitis, goblet cell depletion, and crypt abscesses), mucosal architecture alteration (including ulceration and crypt loss), and submucosal edema (Table 1B). Colonic sections were

TABLE 1 Assessment of inflammatory phenotype. (A) Disease activity index (DAI) scoring chart. (B) Histological microscopic scores chart.

Score	0	1	2	3
(A)				
Weight loss (%)	0	0 to 10	10 to 20	>20
Stool consistency	Normal	Loose	Diarrhea	–
Blood in the feces	No	Yes	–	–
Mouse appearance	Normal	Prostrate	Prostrates with spiky hairs	Lethargic
Maximal score	9			
(B)				
Submucosal oedema	Absent	Mild	Moderate	Severe
Vasculitis	No	Yes	-	-
Mucosal architecture	Conserved	Mild	Moderate	Loss
Cell infiltration	No	Mild	Moderate	Severe
Muscle thickening	No	Yes	–	–
Crypt abscesses	No	Yes	–	–
Goblet cell depletion	No	Yes	–	–
Maximal score	13			

incubated in 1% periodic acid (Sigma, 375,810) for 15 min, in Schiff reagent (Sigma, 320,680) for 15 min, and counter-stained with Mayer's hematoxylin (RAL DIAGNOSTICS, 361075) to stain for polysaccharides, glycoproteins, and acidic mucopolysaccharides.

2.4 | Iron and hematological parameters

Serum iron concentration and unsaturated iron binding capacity were determined using iron direct and UIBC kits (Biolabo) according to the manufacturer's instructions. Liver iron content and iron deposition in the colon (Perl's stain) were determined as previously described.[13] A complete blood count was performed with a Cell-Dyn Emerald hematology analyzer (Abbott).

2.5 | Gene expression

Total RNA was isolated using Trizol-Chloroform. 10 µg of RNA was purified using the Dynabeads mRNA Direct kit to eliminate residual DSS. Complementary DNA was synthesized using MMLV reverse transcriptase (Promega). Real-time qPCR reactions were prepared with Takyon SYBR Mastermix (Eurogentec) and run on a LightCycler 480 System (Roche Diagnostics). The following forward (F) and reverse (R) primers were used:

Hprt: *F*-CTGGTTAAGCAGTACAGCCCCAA, R-CGACAGGTCCTTTTCACCAGC; *Hamp*: F-AAGCAGGGCAGACATTGCGAT, R-CAGGATGTGGCTCTAGGCTATGT; *Tnfα*: F-AATGGCCTCCCTCTCATCAG, R-GCTACGACGTGGGCTACAGG;*Infγ*:F-CAGCAACAGCAAGGCGAAA, R-AGCTCATTGAATGCTTGGCG; *Il6*: F-CTCTGCAAGAGACTTCCATCCAGT, R-CGTGGTTGTCACCAGCATCA; *Il1β*: F-ACCTTCCAGGATGAGGACATGAG, R-CATCCCATGAGTCACAGAGGATG; *Il10*: F-AGGCGCTGTCATCGATTTCTC, R-TGCTCCACTGCCTTGCTCTTA; *Il17a*: F-TCCAGAAGGCCCTCAGACTA, R-CAGGATCTCTTGCTGGATG; *Il22*: F-AGGTGGTGCCTTTCCTGACC, R-ACCGCTGATGTGACAGGAGC; *Cxcl9*: F-GGCAAATGTGAAGAAGCTGATG, R-TTTTTCCCCCTCTTTTGCTTTT; *Cxcl10*: F-TGTTGAGATCATTGCCACGA, R-CCAGTTAAGGAGCCCTTTTAGACC; *Reg3β*: F-TGGTTTGATGCAGAACTGGC, R-TGGAGGACAAGAATGAAGCCT; *Reg3γ*: F-CCTCCATGATCAAAAGCAGTGG, R-GGATTCGTCTCCCAGTTGATGT; *Ocln*: F-TGGATGACTACAGAGAGGAGAGT, R-TCCTCTTGATGTGCGATAATTTGC; *Tjp1*: F-CACAGCCTCCAGAGTTTGACAG, R-TCCACAGCTGAAGGACTCACAG; *Cldn2*: F-TCTCAGCCCTGTTTTCTTTGGT, R-GGGCCTGGTAGCCATCATAGTA; *Cldn8*: F-GGAGGAGCACTGTTCTGTTGTG, R-GTGGAAACTCCGTTGAGTGGT; *Muc2*: F-TGTCCCGACTTCAACCCAAG, R-TCTGGTTTTGAGGGATGCATGT; *Muc4*: F-AGAGGCAGAAGAGGAGTGGAGA, R-GGTGGTAGCCTTTGTAGCCATC; *Ptgs2*: F-GCCTCCCACTCCAGACTAGA, R-ACAGCTCAGTTGAACGCCTT; *Chac1*: F-AAGATGAGCACCTGGAAGCC, R-CTTGGCTCCTCAGGTCAGTG; *Ascl4*: F-TGGTCAGGGATATGGGCTGA, R-CCACCTTCCTGCCAGTCTTT; *Sod1*: F-AGGAGAGCATTCCATCATTGG, R-CCCAGCATTTCCAGTCTTTGT; *Sod2*: F-GGGCTGGCTTGGCTTCAATAA, R-TCCCACACGTCAATCCCCAG; *Lcn2*: F-TCTGTCCCCACCGACCAAT, R-CCAGTCAG

CCACACTCACCAC; *Hmox1*: F-CAGATGGCGTCACT TCGTCA, R-CTCTGCAGGGGCAGTATCTTG; *Nqo1*: F-AGCGGCTCCATGTACTCTCT, R-GCATCTGGTGGA GTGTGGC; *Atg5*: F-CAACCGGAAACTCATGGAAT, R-CGGAACAGCTTCTGGATGA; *Atg7*: F-GGTCTTAC CCTGCTCCATCA, R-TGTGGTTGCTTGCTTCAGAG; *Xbp1s*: F-GAACATCTTCCCATGGACTC, R-CCCAAAAG GATATCAGACTCAG.

The target gene expression was normalized to the Hypoxanthine Phosphoribosyl Transferase (*Hprt*) and quantified using the comparative −ΔCt (threshold cycle) method.

2.6 | Serum endotoxin measurement

Plasma Gram-negative bacterial endotoxin was quantified using the Thermo Scientific Pierce LAL Chromogenic Endotoxin Quantitation Kit according to the manufacturer's instructions.

2.7 | Microbiota analysis by 16S rDNA sequencing

DNA was extracted from fecal samples by using the QIAamp PowerFecalPro DNA kit (Qiagen, Hilden, MD) according to the manufacturer's recommendations. DNA extracts were quantified on a Qubit4 fluorometer using the dsDNA HS Assay Kit (Life Technologies; USA). For 16S rDNA gene sequencing, a two-step PCR library preparation was applied, according to the recommendations of the 16S Metagenomic Sequencing Library Preparation Guide (Illumina, San Dego, CA, USA). Briefly, for the first PCR reaction, the V4 hyper-variable region of the 16S rDNA was amplified using the primers 515-F: 5′-*TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG G*TG CCA GCM GCC GCG GTAA-3′ and 806-R: 5′-*GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA G*GG ACT ACH VGG GTW TCT AAT-3′ (italicized sequence is the Illumina adapter following by barcode, bold sequence is the conserved bacterial sequence). Amplicons were then purified using AMPure XP beads (Beckman Coulter, Indianapolis, IN). A second PCR reaction was performed to incorporate the sample-specific barcode, using the Nextera XT index kit (Illumina, USA). After amplicon purification, DNA concentration was controlled by qPCR using the KAPA Library Quantification kit (Roche). A master DNA pool was then generated in equimolar ratios. PhiX Control v3 (Illumina, USA) was added to check the quality of the run. The pooled products quantity was controlled on Qubit4 fluorometer, loaded into an Illumina MiSeq cartridge, and sequenced (paired-end reads, 2 × 300 bp) on the Illumina MiSeq sequencer. At the end of the run, FastQ files were generated.

Sequences were demultiplexed, reads quality control was done and paired-end amplicon reads were processed using the FROGS pipeline (Find Rapidly OTU with Galaxy Solution, Version 4.1.0) on the Galaxy Migale platform (https://galaxy.migale.inra.fr/).[14] Briefly, forward and reverse reads were trimmed for adaptor and PCR primers removal, merged, and chimeric sequences were removed. Reads were then clustered in Amplicon Sequence Variants (ASVs) with an aggregation distance of 1, and filtered with a minimum relative abundance threshold of 0.005% and blast coverage >0.95. Taxonomic assignment was performed against the 16S SILVA 138 pintail 100 database. Before further analysis, all samples were rarefied to the same depth, with a minimum reads number of 74,959. Bacterial composition and diversities were estimated using FROGSTAT phyloseq tools. Alpha-diversity within group was estimated by calculating richness (Observed) and Shannon diversity index. Beta-diversity between groups was evaluated by calculating Bray-Curtis distances between samples. Ordination using principal coordinates analysis (PcoA) was performed to represent biodiversity distribution at the ASV level between groups. To identify differentially abundant taxa, DESeq2[15] was used. The functional potential of the bacterial communities was predicted using PICRUSt2[16] and the R package ggpicrust2.[17]

2.8 | Statistical analysis

The statistical significances were assessed by Student's *t*-test, one-or two-way analysis of variance (ANOVA) specified in the figure legend using Prism 10 (GraphPad). For bacterial abundance and alpha diversity, significant differences were analyzed by ANOVA with the Bonferroni multiple comparison test. The significance of beta diversity metrics was assessed by the PERMANOVA test (Permutational Multivariate Analysis of Variance Using Distance Matrices).

3 | RESULTS

3.1 | Iron metabolism during DSS-induced colitis

Six weeks old C57BL/6 male mice were switched from a standard rodent chow to an IA or ID diet for 2 weeks (Figure 1A). A third group was fed an IA diet for 2 weeks and switched to an IE diet when the colitis was initiated in order to mimic the supplementation given to patients. Mice were then administered 2% DSS in drinking water for 7 days and returned to regular water until the peak inflammation was reached, which, according to our institutional

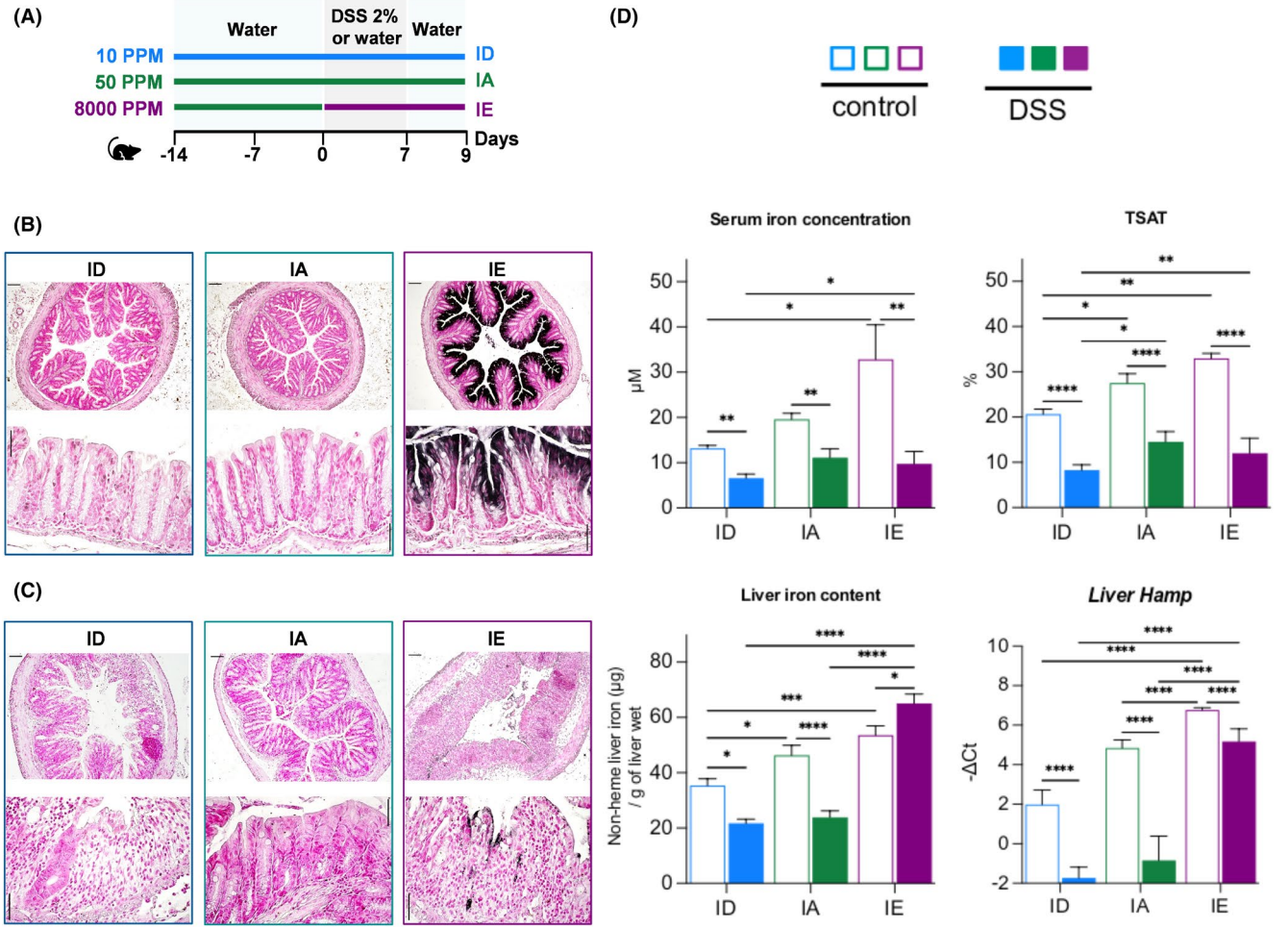

FIGURE 1 Iron metabolism during DSS-induced colitis. (A) Mice fed an ID, IA, or IE diet were given regular drinking water (control; empty bars) or 2% dextran sodium sulfate (DSS) for 7 days and regular drinking water for 2 days (DSS; plain bars). Iron deposition was assessed by Perls' staining on colon tissue section from control (B) or DSS-treated mice (C). Upper row scale bar = 100 µm; lower row scale bar = 50 µm. (D) Serum iron concentration, transferrin saturation, liver iron content and liver hepcidin (*Hamp*) mRNA expression in control and DSS-treated mice. Data shown are means ± s.e.m. and were compared between DSS-treated mice and control mice for each diet ($n = 8$) by Two-way ANOVA and corrected for multiple comparisons by Holm-Šidák method. ****$p < 0.0001$, ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$.

regulations, required euthanasia by day 9. Control mice for each diet were given water for 9 days. Histological assessment of non-heme iron distribution in the colon by Prussian blue staining showed that iron accumulated in the lumen and enterocytes of control mice fed an IE diet, whereas no iron was detectable in the colon of mice fed an ID or IA diet (Figure 1B). A milder accumulation of iron was observed in the colon of DSS-treated mice fed an IE diet (Figure 1C). Serum iron concentration and transferrin saturation decreased significantly in DSS-treated mice compared to control mice, regardless of the diet (Figure 1D). After DSS, mice fed an ID diet had slightly lower transferrin saturation compared to mice fed an IA diet, but plasma iron levels were similar between mice fed an IA or IE diet. While liver iron concentration decreased in DSS-treated mice fed an ID or IA diet compared to their respective controls, liver iron content was increased in mice fed an IE diet. Similarly, liver hepcidin expression correlated with the dietary iron content in control mice, but hepcidin levels were significantly reduced in DSS-treated mice fed an ID or IA diet, presumably as a result of iron deficiency and blood loss. However, hepcidin levels in DSS-treated mice fed an IE diet were similar to those of control mice fed an IA diet (Figure 1D). These results indicate that iron supplementation was effective at preserving the iron stores and mitigating the iron deficiency caused by colonic inflammation.

3.2 | Iron deprivation and supplementation worsen the inflammatory phenotype

Control mice did not show any significant difference in body weight (Figure 2A) or disease activity index

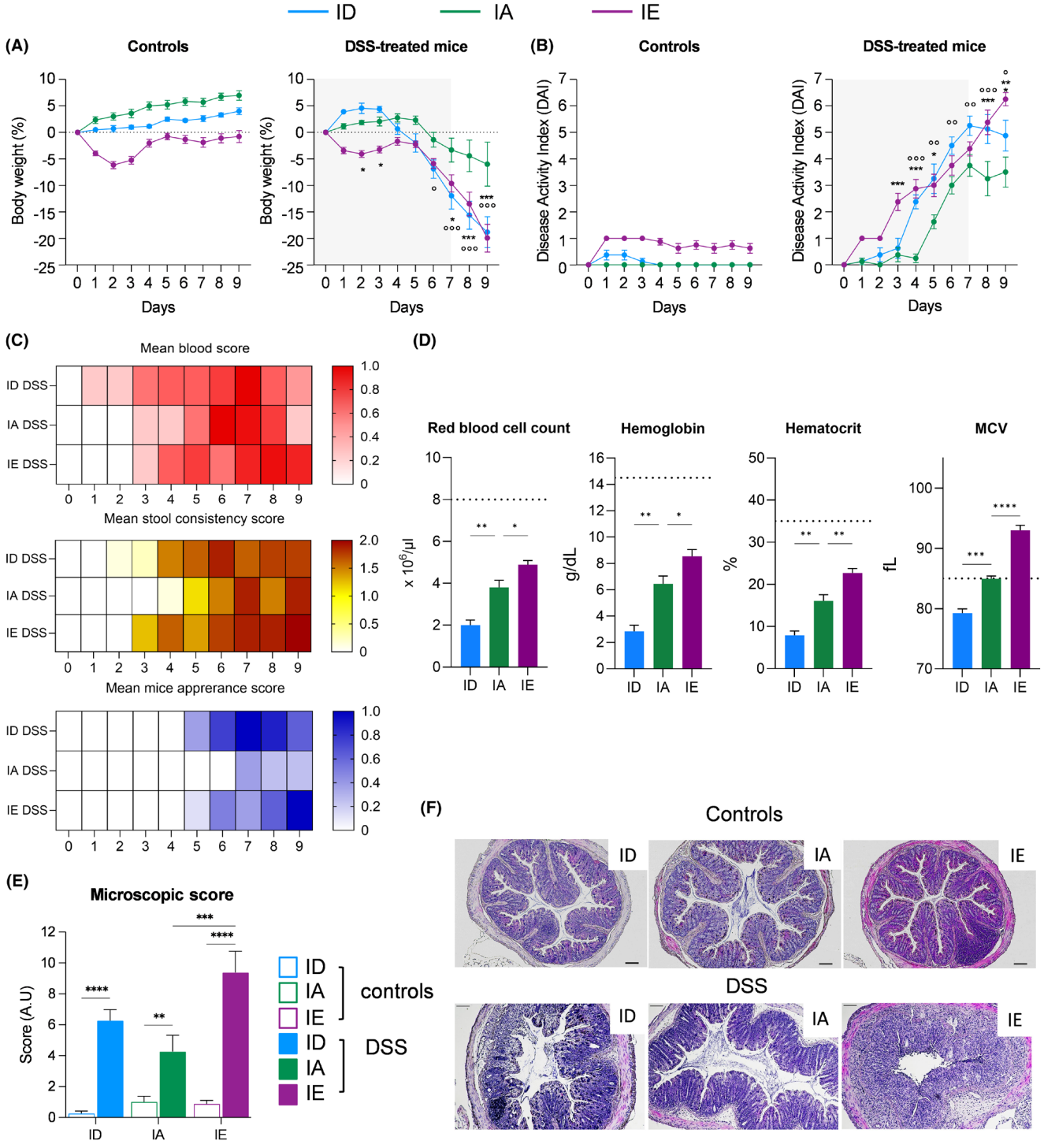

FIGURE 2 Iron deprivation and supplementation worsen the inflammatory phenotype during DSS-induced colitis. (A) Body weight (%), (B) disease activity index (DAI), (C) mean blood, stool consistency and appearance score, (D) hematological parameters (red blood cell count, hemoglobin, hematocrit and mean corpuscular volume – MCV). Dotted lines represent for each parameter the mean value in control mice fed an IA diet. (E) histological microscopic score in control and DSS-treated mice. (F) hematoxylin–eosin staining of colon sections from control and DSS-treated mice. Scale bar = 50 µm. Data shown are means ± s.e.m. and were compared between DSS-treated mice and control mice for each diet ($n = 8$) by One or Two-way ANOVA and corrected for multiple comparisons by Holm-Šidák method. (A, B) IE versus IA: $^{***}p < 0.001$, $^{**}p < 0.01$, $^{*}p < 0.05$; ID versus IA: $^{°°°}p < 0.001$, $^{°°}p < 0.01$, $^{°}p < 0.05$. (D) ID or IE versus IA: $^{****}p < 0.0001$, $^{***}p < 0.001$, $^{**}p < 0.01$, $^{*}p < 0.05$. (F) $^{****}p < 0.0001$, $^{***}p < 0.001$, $^{**}p < 0.01$, $^{*}p < 0.05$.

(Figure 2B). Only a trend toward a decrease in body weight that was rapidly compensated was observed in mice fed an IE diet, presumably as a result of the change in diet. However, mice fed an ID and IE diet exhibited a more pronounced weight loss than mice fed an IA diet 6 to 9 days after DSS administration (Figure 2A) with an average body weight loss of 20%. The weight loss was accompanied by a more severe disease activity index in mice fed an ID or IE diet compared to mice fed an IA diet, with a significant increase as early as 3–4 days after DSS administration (Figure 2B). The presence of blood was detected in the cage of mice fed an ID diet 1 day after DSS administration, and the decrease in stool consistency was observed after 2–3 days in mice fed an ID or IE diet, 2 days before the first signs in mice fed an IA diet (Figure 2C). The change in overall mouse appearance was also more pronounced in mice fed an ID and IE diet compared to mice fed an IA diet. Consistent with the development of anemia, red blood cell count, hemoglobin, hematocrit, and mean corpuscular volume were decreased in DSS-treated mice compared to control mice (Figure 2D). Compared to mice fed an IA diet, mice fed an ID diet showed a more severe anemia, whereas iron supplementation was effective at mitigating the extent of anemia in mice fed an IE diet. While the microscopic scores increased in all the mice subjected to DSS compared to their respective controls, mice fed an IE diet showed more severe microscopic inflammatory scores (Table 1B) compared to mice fed an IA diet (Figure 2E,F). Altogether, these results indicate that mice fed an ID or IE diet exhibit a more severe colonic inflammation compared to mice fed an IA diet.

3.3 | Transcriptional response to dietary iron challenge during colonic inflammation

Consistent with an alteration of epithelial barrier integrity during DSS-induced colitis,[18] tight junction proteins Occludin 1 (*Ocln*) and tight junction protein 1 (*Tjp1*) mRNA expression was significantly reduced 9 days after DSS administration in the colon of mice fed an ID diet (Figure 3A). A comparable decrease in *Ocln* expression in mice fed an IE diet was accompanied by a decrease in claudin 2 and 8 (*Cldn2* and *Cldn8*) expression. The dietary iron content did not induce any change in *Ocln*, *Tjp1*, *Cldn2*, *Cldn8* expression in DSS-treated mice (Figure 3B). Expression of mucin 2 (*Muc2*) and 4 (*Muc4*) was increased in mice fed an IE diet compared to mice fed an IA diet (Figure 3A,B). However, plasma endotoxin levels were very low and unchanged between control and DSS-treated mice regardless of the diet (Figure 3C). Histological assessment of colon sections by periodic acid–Schiff (PAS) staining indicated that the number of goblet cells per crypt was reduced upon DSS treatment and to a greater extent in mice fed an ID or IE diet compared to mice fed an IA diet (Figure 3D). As epithelial damage is usually associated with mucosal inflammation, we next assessed whether dietary iron influenced the expression level of inflammatory cytokines (*Tnfα*, *Ifnγ*, *Il17a*, *Il1β*, *Il6*, *Il10*, and *Il22*), chemokines (*Cxcl9* and *Cxcl10*), and antimicrobial peptides (*Reg3β*, *Reg3γ*) known to contribute to the inflammatory response in the colon (Figure 4A,B). Expression of *Tnfα*, *Il17a*, *Il1β*, and *Il6* was increased in DSS-treated mice fed an ID or IE diet compared to their respective controls, whereas expression of chemokines *Cxcl9* and *Cxcl10* and antimicrobial peptides *Reg3β*, *Reg3γ* was markedly increased only in mice fed an IE diet and subjected to DSS-induced colitis (Figure 4A). A significant increase in *Tnfα*, *Il17a*, and *Cxcl9* expression was observed in DSS-treated mice fed an IE diet compared to mice fed an IA diet (Figure 4B) suggesting that iron supplementation fueled the immune response. Previous studies have shown that ferroptosis, oxidative stress, autophagy, and ER stress contribute to colonic inflammation during IBD.[19] We therefore assessed whether iron deprivation or supplementation altered the expression level of mediators of ferroptosis (*Ptgs2*, *Acsl4*, *Chac1*), oxidative stress (*Sod1*, *Sod2*, *Lcn2*, *Hmox*, *Nqo1*), autophagy (*Atg5*, *Atg7*), and ER stress (*Xbp1s*) in DSS-treated and control mice. DSS treatment only led to an increase in lipocalin 2 (*Lcn2*) expression regardless of the dietary iron content, which is consistent with its proposed function as an antimicrobial protein,[20] and expression of the other markers was unchanged by inflammation (Figure 5A). However, after DSS treatment, expression of *Acsl4*, *Chac1*, *Sod1*, *Sod2*, and *Nqo1* was significantly increased in the colon of mice fed an ID or IE diet compared to mice fed an IA diet, suggesting that ferroptosis and oxidative stress may be triggered upon iron deprivation and supplementation (Figure 5B).

3.4 | Dietary iron impact on fecal microbiota diversity

We next investigated the impact of the dietary iron content on bacterial abundance and composition by 16S rDNA sequencing from stool samples. Alpha-diversity was analyzed among the different subgroups of control mice. Richness (Observed feature) was significantly decreased in mice fed an ID or IE diet compared to mice fed an IA diet (means: 236.6 vs. 281.3, p-value = 2e-05 and 246.6 vs. 281.3, p = 4e-04, respectively, Figure 6A).

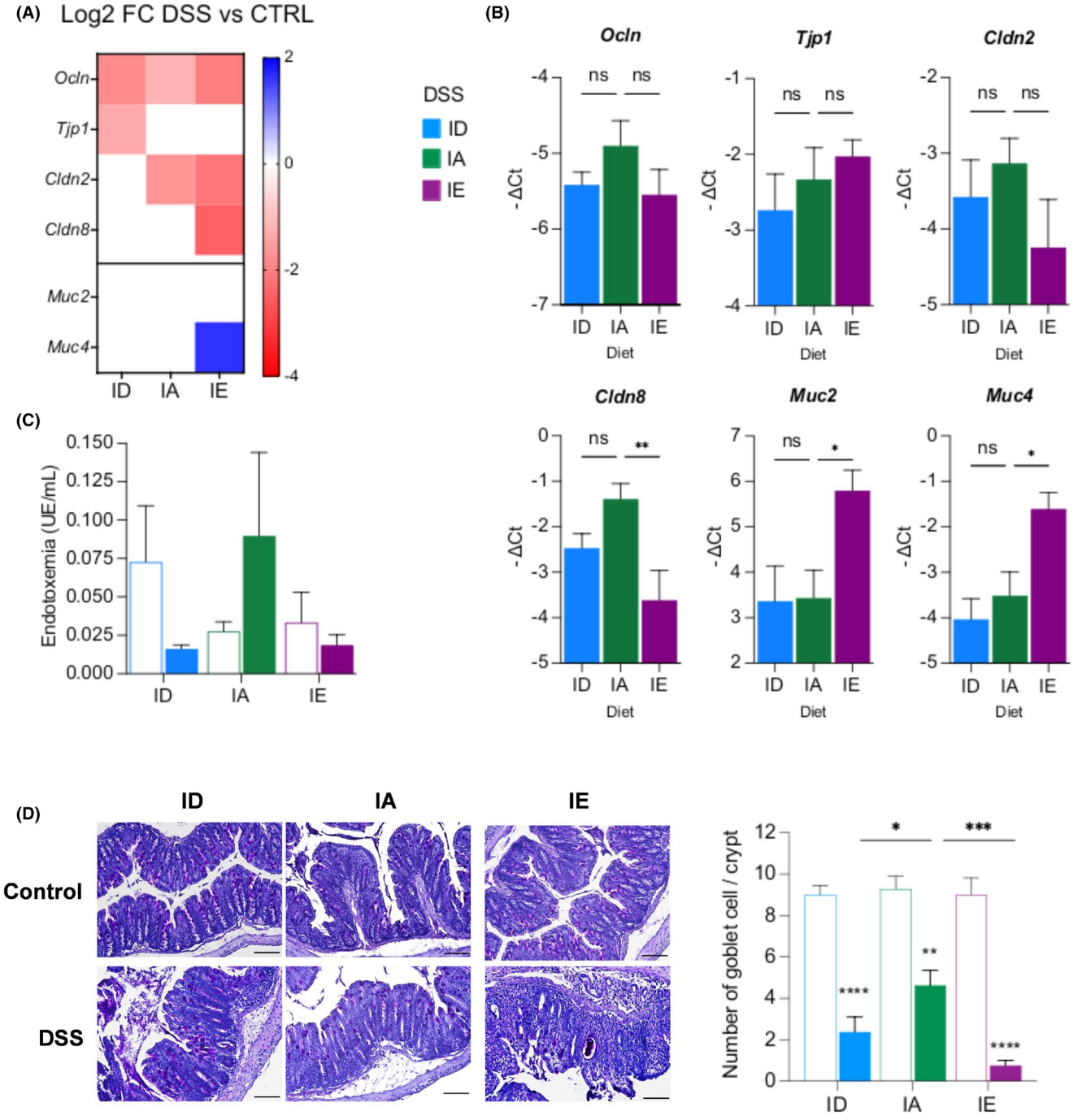

FIGURE 3 Intestinal barrier during colitis. (A) Representative heatmap of tight junction proteins Occludin 1 (*Ocln*), tight junction protein 1/Zona occludens 1 (*Tjp1*), claudin 2 and 8 (*Cldn2*, *Cldn8*) and mucin 2 (*Muc2*) and 4 (*Muc 4*) mRNA expression in the colon of DSS-treated mice compared to their respective controls. Only differences reaching statistical significance ($p < 0.05$) are represented. (B) mRNA expression in the colon of DSS-treated mice fed an ID or IE diet compared to mice fed an IA diet. (C) Endotoxemia and (D) Periodic acid Schiff staining of colon section and the observed number of goblet cells per crypt. Scale bar = 50 µm. Data shown are means ± s.e.m. and were compared between DSS-treated mice and control mice for each diet ($n = 8$) by Two-way ANOVA and corrected for multiple comparisons by Holm-Šidák method. ****$p < 0.0001$, ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$.

However, diversity differences (Shannon index) between mice subgroups were not significant, except for a trend toward a decrease in mice fed an ID diet compared to mice fed an IE diet. Beta-diversity was evaluated by calculating distances between samples using Jaccard and Bray-Curtis methods. Principal coordinates analysis (PcoA) was performed to represent the distribution of biodiversity at the OTU level between groups (Figure 6B). Subgroups of mice fed an ID, IA or IE diet were clustered and significantly different ($p < 0.0001$).

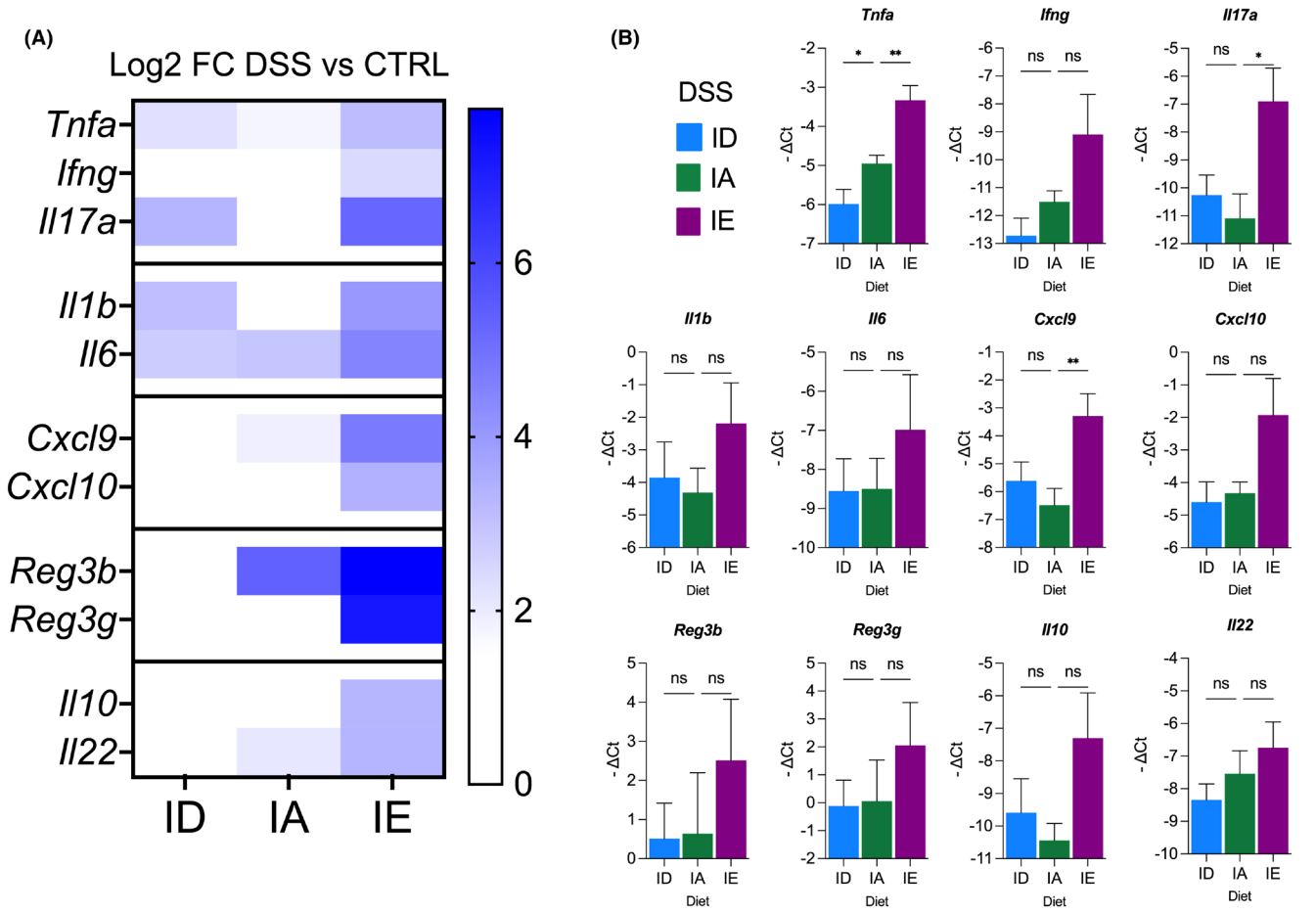

FIGURE 4 Expression of inflammatory and immune mediators during colitis. mRNA expression of inflammatory cytokines (*Tnfα*, *Ifnγ*, *Il17a*, *Il1β*, *Il6*, *Il10*, and *Il22*), chemokines (*Cxcl9* and *Cxcl10*), and antimicrobial peptides (*Reg3β*, *Reg3γ*) in the colon of control and DSS-terated mice. (A) Representative heatmap of expression level in DSS-treated mice compared their respective controls. Only differences reaching statistical significance ($p < 0.05$) are represented. (B) Expression level in DSS-treated mice fed an ID or IE diet compared to mice fed an IA diet. Data shown are means ± s.e.m. and were compared between DSS-treated mice and control mice for each diet ($n = 8$) by Two-way ANOVA and corrected for multiple comparisons by Holm-Šidák method. $^{**}p < 0.01$, $^{*}p < 0.05$.

We then examined the bacterial communities impacted by the dietary iron deprivation or supplementation. Relative abundance was analyzed at the phylum level (Figure 6C). A decreased proportion of *Bacillota* (previously known as *Firmicutes*) was observed in mice fed an ID (19%) or IE (10%) diet compared to mice fed an IA diet whereas an increase in the *Bacteroidota* (ID: 16%; IE: 4%) and *Pseudomonadota* (previously known as *Proteobacteria*) (ID: 4%; IE: 2%) was found. At the family level (Figure 6D), *Erysipelotrichaceae* (Bacillota phylum) was the most abundant family in mice fed an IA or IE diet, whereas *Tannerellaceae* (Bacteroidota phylum) was the predominant family in mice fed an ID diet. *Bacteroidaceae*, *Desulfovinrionaceae*, *Oscillospiraceae, Marinifilaceae*, and *Tannerellaceae* families were more abundant in mice fed an ID or IE diet when compared to mice fed an IA diet. In contrast, *Akkermansiaceae*, *Erysipelotrichaceae*, and *Rikenellaceae* families were less represented in the microbiota of dietary challenged mice. However, an increase in *Muribaculaceae* family was observed only in mice fed an IE diet. Finally, we assessed the significant changes in microbiota composition at the genus level depending on the dietary iron content using DESeq2. We observed a significant decrease or increase of 47 to 76 OTUs in mice fed an IE or ID diet compared to mice fed an IA diet (Figure 6E and supplemental data). In comparison to mice fed an IA diet, mice fed an ID or IE diet exhibited a significantly decreased abundance in *Akkermansia*, *Candidatus Saccharimonas*, *Faecalibaculum*, and *Rikenellaceae RC9* and an increase in *Escherichia Shigella* with a more pronounced alteration in mice fed an ID diet (Figure 6F). *Parabacteroides* was the most abundant genus during iron deprivation along with a significant increase in *Bacteroides* and a reduction in *Alloprevotella* whereas mice fed an IE diet showed an increased abundance in *Alistipes* and *Blautia*. We further studied the predicted functional alterations caused by the modifications in fecal microbiota using

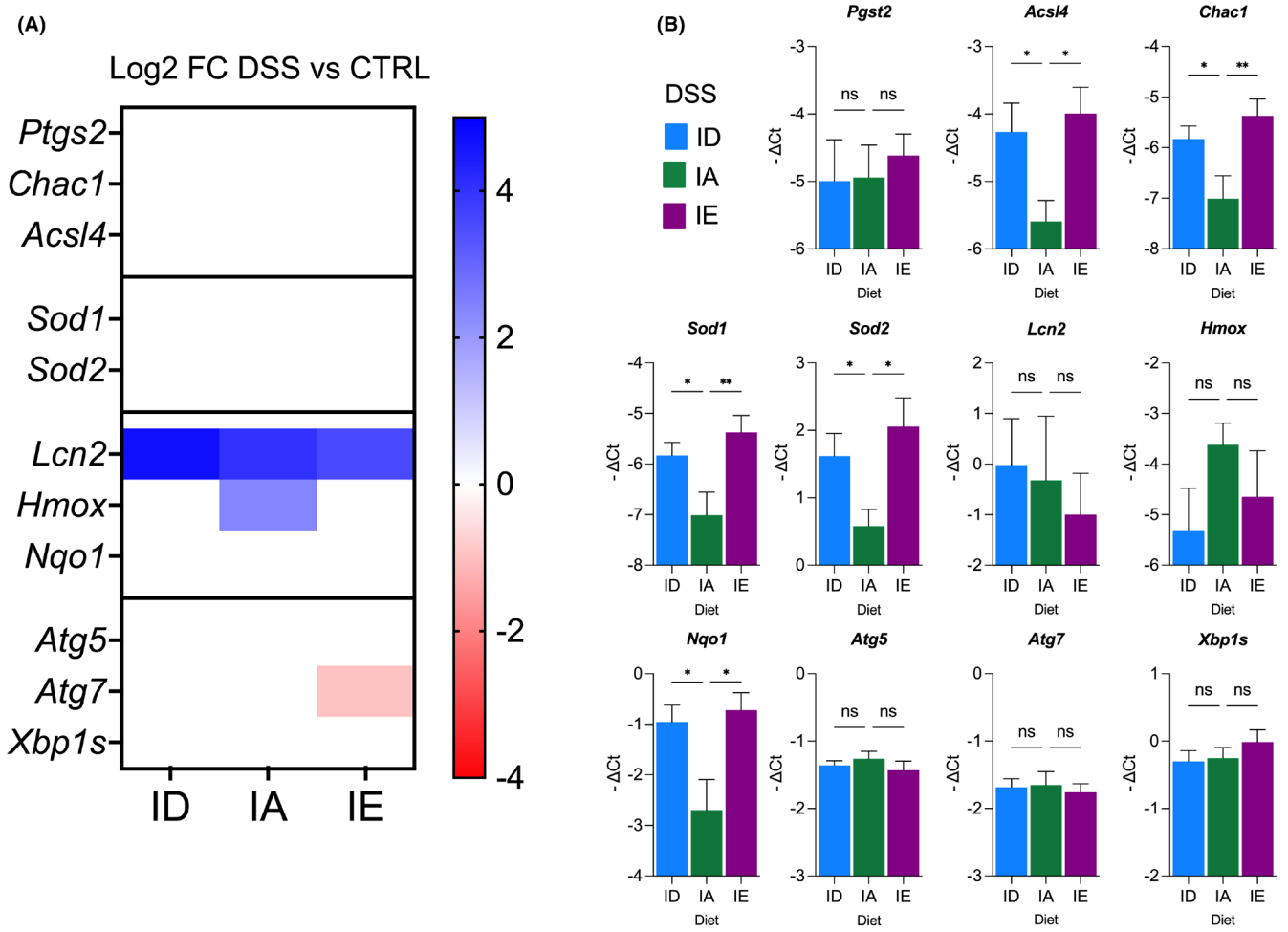

FIGURE 5 Dietary iron deprivation and supplementation induce oxidative stress and ferroptosis in DSS-treated mice. mRNA expression level of mediators of ferroptosis (*Ptgs2*, *Acsl4*, *Chac1*), oxidative stress (*Sod1*, *Sod2*, *Lcn2*, *Hmox*, *Nqo1*), autophagy (*Atg5*, *Atg7*), ER stress (*Xbp1s*) in the colon of control and DSS-treated mice. (A) Representative heatmap of expression level in DSS-treated mice compared to their respective controls. Only differences reaching statistical significance ($p < 0.05$) are represented. (B) Expression level in DSS-treated mice fed an ID or IE diet compared to mice fed an IA diet. Data shown are means ± s.e.m. and were compared between DSS-treated mice and control mice for each diet ($n = 8$) by Two-way ANOVA and corrected for multiple comparisons by Holm-Šidák method. $^{**}p < 0.01$, $^{*}p < 0.05$.

PICRUSt2[16,17] (Figure 7). The dysbiosis observed in mice fed an ID or IE diet resulted in significant differences in metabolic (MetaCyc) and KEGG (Kyoto Encyclopedia of Genes and Genomes) pathways (Figure 7A,B). In mice fed an ID or IE diet, the metabolic pathways primarily altered were the sulfur oxidation and the anhydromuropeptide recycling, respectively (Appendix S1). The KEGG pathway abundance analysis showed that iron deficiency altered more pathways than iron supplementation (Figure 7C,D). Among those, steroid hormone biosynthesis, glycosaminoglucan degradation, biotin and sphingolipid metabolism, TCA cycle (citrate cycle) were increased. Only the biotin metabolism and glycoaminoglycan degradation were reduced in mice fed an IE diet compared to mice fed an IA diet. The phosphotransferase system (PTS) activity and biosynthesis of ansamycins were decreased upon both dietary iron supplementation and depletion.

4 | DISCUSSION

Iron deficiency anemia is a common extra-intestinal complication affecting patients with IBD. The outcome of iron deficiency or iron supplementation in IBD has been extensively documented in mouse models and patients.[21,22] However, this is the first study that compares simultaneously the colitis and microbiota response to dietary iron restriction or supplementation in comparison to an iron-adequate diet. Most animal studies have been performed using dietary iron restriction or supplementation that were not necessarily translatable to human studies. While common standard rodent chows contain 150–300 μg/g of iron, 20–25 μg/g of iron in the diet is sufficient to meet the daily iron needs in mice. In some studies, the iron-deficient diet exceeded these daily requirements[9,23–25] whereas a standard rodent chow was considered an iron enrichment. A 10-fold increase in enteral iron was reported to be protective, whereas a 100-fold

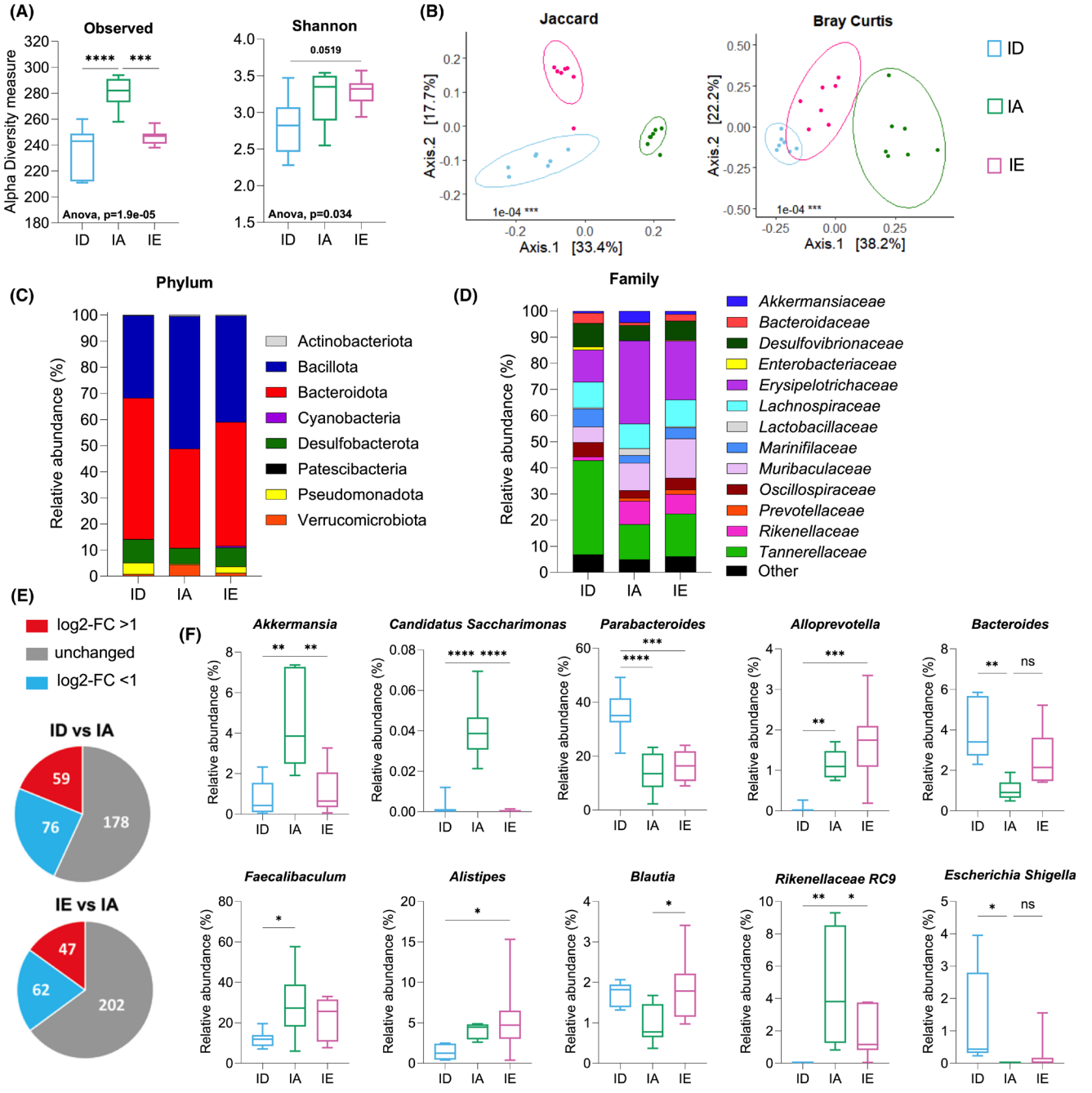

FIGURE 6 Dietary iron impact on fecal microbiota diversity. Assessment of the microbiome composition by 16S DNA sequencing from stool samples of control mice. (A) Amplicon sequence variants (ASV) distribution (alpha diversity): The observed number of species in each sample (richness) and Shannon (richness and evenness). (B) Principal component analysis of the similarities in microbial communities between groups (beta diversity) based on the Jaccard algorithm and Bray Curtis dissimilarity. (C) Phylum and (D) bacteria family. (E) Comparison of OTU levels depending on the dietary iron content. (F) Relative abundance of bacterial genus. Data shown are means ± s.e.m. and were compared between DSS-treated mice and control mice for each diet ($n = 8$) by One-way ANOVA and corrected for multiple comparisons by Bonferroni method. ****$p < 0.0001$, ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$.

increase exacerbated the colitis in mice.[9,11,26,27] In the present study, mice were fed an ID diet containing 10 µg/g Fe, the minimum achievable considering the amount of iron found in cereals and other components of the diet. The impact of the dietary iron was compared to the same diet enriched with carbonyl-iron at a concentration of 50 µg/g (IA) or 8000 µg/g (IE) to match the oral iron supplementation administered to patients with iron deficiency anemia. The dietary iron challenge allowed the accumulation of iron in the lumen of mice fed the IE diet, but mice fed an ID or IE diet did not develop an iron deficiency or an iron overload in comparison to mice fed an IA diet.

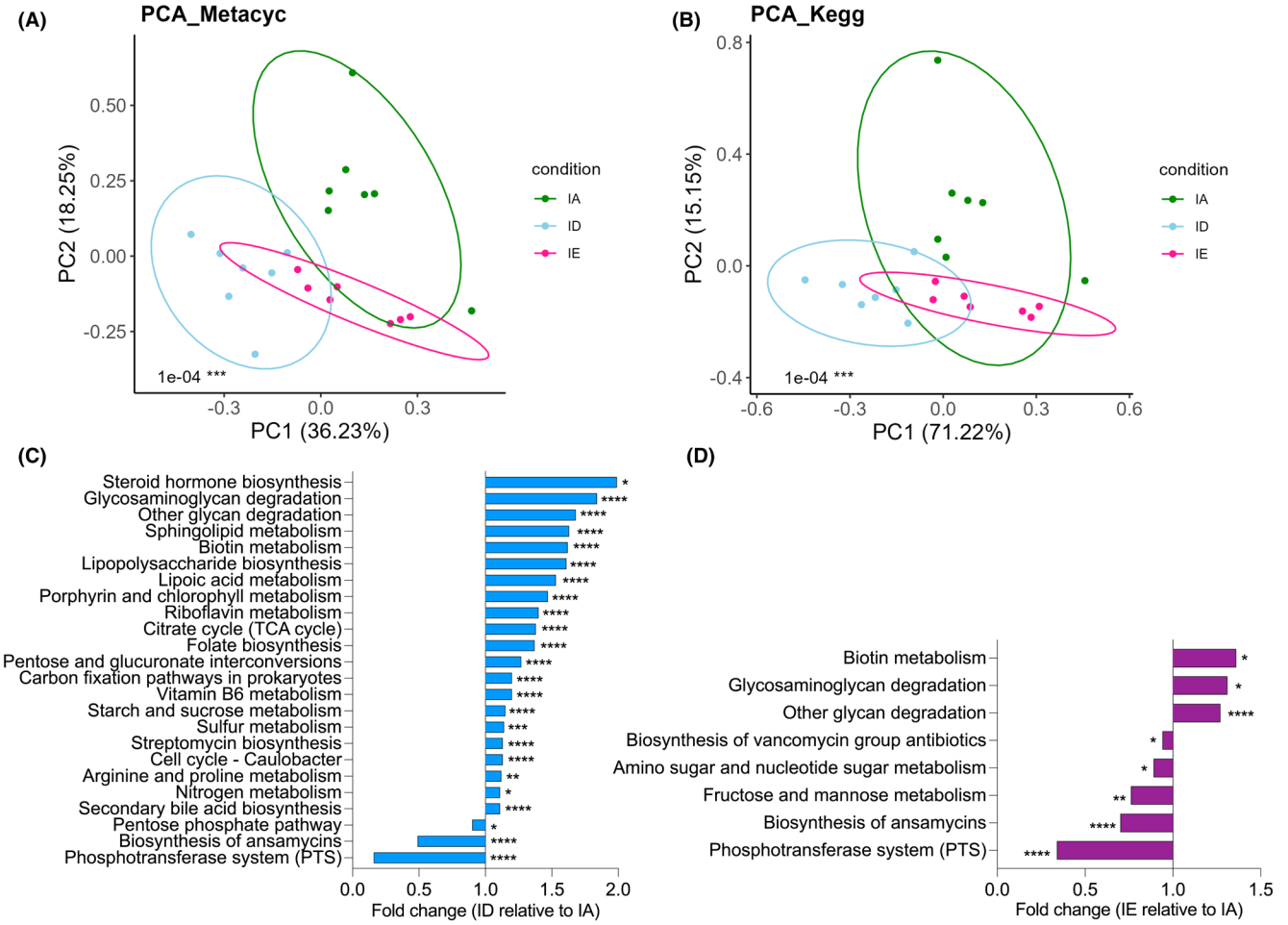

FIGURE 7 PICRUSt2 prediction of functions from 16S marker sequences. Principal component analysis of MetaCyc and KEGG pathways in mice fed an ID, IA, or IE diet (A, B). MetaCyc and KEGG pathways abundances are significantly altered in mice fed an ID or IE diet compared to mice fed an IA diet (C, D). Data are shown as a mean fold change and were compared between mice fed an ID or IE diet and mice fed an IA diet by two-way ANOVA followed by Dunnett's multiple comparisons. ****$p < 0.0001$, ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$.

To study the impact of dietary iron during colitis, mice were subjected to a murine model of chronic intestinal inflammation mimicking the flare-ups and recovery phases observed in patients, which is based on two repeated cycles of 2% DSS or normal drinking water for 7 days each.[28] However, after 9 days, mice fed an ID or IE diet had reached the maximal 20% body weight loss authorized by our ethical guidelines, which prompted us to compare the impact of dietary iron on the severity of colitis after 9 days (7 days 2% DSS, 2 days water). The impact of dietary iron deprivation or supplementation in a chronic DSS model could therefore not be tested. In this model, mice fed an IE diet also exhibited a less severe anemia compared to their counterparts fed an ID or IA diet. Of note, mice fed an ID diet exhibited earlier signs of gastrointestinal bleeding compared to mice fed an IE or IA diet, suggesting an increased susceptibility to epithelial and vascular damage. Indeed, iron is necessary for the proliferation of stem cells during the regeneration that follows a tissue injury induced by DSS.[29]

We did not detect major differences in mRNA expression of tight junction proteins, inflammatory mediators, and markers of ferroptosis and oxidative stress between mice fed an ID and IE diet, except for a higher expression of *Tnfa* and *Il17a* in the colon of DSS-treated mice fed an IE diet. These results suggest that the presence of iron in the lumen does not directly affect the expression of tight junction proteins. A potential explanation for the increase in *Tnfa* expression is that iron induces the polarization of proinflammatory macrophages (M1) through the mitogen-activated protein kinase (MAPK) pathway.[30] Mice fed an ID or IE diet exhibited a mild increase in markers of ferroptosis and oxidative stress mRNA compared to mice fed an IA diet, but lipid peroxidation and ROS levels were not assessed. Both groups of mice showed early signs of bleeding, and while heme iron is not detectable by Perls' staining, heme iron may accumulate in the colonic lumen, leading to oxidative stress, ferroptosis, and epithelial damage,[31] independently of the dietary iron content.

The amount of iron in the diet is known to alter the microbial composition in the colon by exerting a selective pressure or by promoting the proliferation of specific bacteria.[32] While a different dysbiosis has been reported depending on the iron formulation used to supplement the diet,[11] our results show that alterations in dietary content led to a profound remodeling of bacterial composition and an increased susceptibility to colitis. Indeed, the alpha- and beta-diversity analyses revealed a dysbiotic state of the fecal microbiota of mice fed an IE or ID diet compared to mice fed an IA diet. In addition to its essential roles in the host and microorganisms, iron disturbs the equilibrium of the microbial composition by promoting the growth and pathogenesis of siderophilic bacteria.[32] Interestingly, dietary iron deprivation and supplementation resulted in similar changes in the most abundant gut microbial phyla represented by a decreased proportion of Bacillota and an increase in Bacteroidetes and Pseudomonadota. The phylum Verrucomicrobiota was less abundant in mice fed an ID diet in comparison to mice fed an IA diet. Studies in IBD patients also reported a reduced level of short-chain fatty acid producers Bacillota and a pronounced abundance of Pseudomonadota.[33,34] Among the Bacillota, the abundance of the Erysipelotrichaceae family was reduced in mice fed an IE or ID diet similar to CD patients.[35] This family includes *Faecalibaculum*, a bacterium known to influence regulatory T cells and reduce colonic inflammation.[36] Similarly, manipulating dietary iron resulted in a decreased abundance of *Akkermansia*, a promising probiotic against colitis that is also reduced in IBD patients.[37] The most notable change in the microbial family observed only in mice fed an ID diet was the increased abundance of *Tannerellaceae* and specifically *Parabacteroides*, which have been shown to reduce intestinal and systemic inflammation and obesity.[38] The Pseudomonadota phylum includes pathogenic microorganisms such as the *Enterobacteriaceae* family and the well-known *Escherichia*. In the present study, we found a greater genus variation in *Escherichia shigella* between mice fed an ID or IE diet compared to mice fed an adequate chow. These siderophilic gram-negative species are responsible for enteric infections worldwide, as a result of the invasion and destruction of the colonic mucosa that leads to acute diarrhea, hemorrhage, and ulceration.[39] However, the physiological relevance of *E. shigella* in murine models is still debated.[40]

Changes in the microbiome composition are accompanied by changes in bacterial metabolism. The functional potential of the bacterial communities that could explain the inflammatory phenotype based on gene sequencing profiles was assessed using PICRUSt2. We observed that dietary iron deprivation had more profound yet different effects than iron supplementation. MetaCyc pathway abundances analysis indicated that dietary iron deprivation may affect mainly sulfur oxidation, an essential driver of energy production sustaining bacterial growth and resilience[41] whereas anhydromuropeptide recycling, involved in microbial-associated molecular patterns (MAMPs) activating inflammation[42] was altered in mice fed an IE diet. In addition, the KEGG pathway analysis highlighted cellular processes for which iron is an essential cofactor of numerous enzymes[43] involved in energy production, the TCA cycle, and carbon fixation pathways in prokaryotes. Iron also supports the phosphotransferase system (PTS) activity by participating in reduction–oxidation reactions that regulate sugar transport. Intriguingly, PTS activity was reduced upon iron deprivation and supplementation. Iron-dependent enzymes are also involved in streptomycin, ansamycin, folate, riboflavin, biotin, and lipoic acid metabolism, all of which are essential for bacterial growth and antibiotic production, and in the pentose phosphate pathway responsible for nucleotide synthesis during the response to oxidative stress. Furthermore, iron is implicated in the biosynthesis of lipopolysaccharides that compose the bacterial cell wall and in the metabolism of sphingolipid essential for eukaryotic membrane integrity. In host–microbe interactions, iron influences secondary bile acid and steroid hormone biosynthesis that affect gut microbiota and systemic metabolism. Overall, the disturbance of the host and bacterial metabolism correlates with the severity of the inflammation. A fecal microbiota transfer (FMT) experiment would be necessary to ascertain whether or not these changes caused by the manipulation of dietary iron could directly fuel the inflammation during colitis. Interestingly, the metabolites that were increased by iron changes in our study were also found to be detrimental to the remission of patients with ulcerative colitis (UC) after FMT. Similarly, the biosynthesis of ansamycins was shown to be beneficial, whereas it was reduced with the manipulation of dietary iron.[44] The detection of genes encoding bacterial siderophores (i.e., iron-chelating molecules) in the fecal microbiota is also predictive of the success of the FMT.[45] While siderophore-producing bacteria may influence gut microbiota composition and inflammatory responses, current data remain insufficient to confirm a causal relationship.

While the DSS-induced model of colitis is widely used to study the contribution of the intestinal microbiota and the innate immune system to the development of intestinal inflammation, its features resemble human ulcerative colitis rather than Crohn's disease. Whether the iron-mediated alterations in intestinal microbiota would have a similar outcome depending on the IBD subtype (Crohn's disease or ulcerative colitis) remains to be determined. The rapid change (9 days) in microbiota composition in mice fed an ID diet may also not be found in humans eating an omnivore diet with sufficient iron intake. Indeed, only 10% of the iron present in the food is absorbed in the duodenum while 90% transits through the intestinal tract. A systemic

iron deficiency caused by hepcidin-mediated iron restriction would not lower the amount of iron found in the lumen unless the patients consume only iron-poor products. However, as suggested in previous studies, this work confirms that oral iron supplementation can fuel the inflammation during IBD, thus questioning the benefit/risk balance of oral iron supplementation for IBD patients. As iron absorption is impaired in patients with IBD iron deficiency can be corrected with intravenous (IV) iron formulations to bypass the gastrointestinal tract absorption. The formulation, inconvenience of administration, and range of side effects of the IV iron preparations have greatly improved over the past decade.[46] Recent studies indicate that IV iron showed a superior response and tolerance in IBD patients compared to oral iron and thus should be the first line of treatment in patients with clinically active IBD.[47,48]

AUTHOR CONTRIBUTIONS

TM and AD performed experiments, analyzed data, and wrote the paper. US, PP, KC, and MR performed experiments; LK designed and supervised the study, analysed data, and wrote the manuscript. All authors edited the manuscript.

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CONFLICT OF INTEREST STATEMENT

The authors have no conflicts of interest in connection with this article.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available in the Materials and Methods, Results, and Supplemental Material of this article.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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