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***Long-term iron deficiency and iron supplementation
exacerbate acute DSS-induced colitis and are associated
with significant dysbiosis***

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30 **"Authors' contributions."**

31

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46 experiments, edited and revised manuscript, approved the final version of manuscript].

47

48 **Abstract:**

49 Patients taking oral iron supplementation often suffer from gastrointestinal side effects. We
50 have previously shown that acute alterations in oral iron exacerbate dextran sodium sulphate
51 (DSS) induced colitis and are associated with dysbiosis. As patients take iron supplementation
52 for long periods, we asked whether this too would influence colitis and the microbiome. We
53 assessed the impact of long-term changes in dietary iron, by feeding chow containing 100ppm,
54 200ppm and 400ppm (reflecting a deficient, normal or supplemented diet, respectively) for up
55 to 9 weeks to female wild-type C57BL/6 (WT) mice in presence or absence of chronic colitis,
56 or acute colitis induced after 8 weeks, induced by DSS. Assessment was made based on (i)
57 clinical and histological severity of colitis, and (ii) faecal microbial diversity, as assessed by
58 sequencing the V4 region of 16S rRNA. In mice with long term changes to their dietary iron,
59 reduced iron intake (100ppm iron diet) was associated with increased weight loss and
60 histology scoring in the acute colitis model. Chronic colitis was not influenced by altering
61 dietary iron however there was a clear change in the faecal microbiome in the 100 and 400ppm
62 iron DSS-treated groups and in controls consuming the 400ppm iron diet. Proteobacteria
63 levels increased significantly at day-63 compared to baseline and Bacteroidetes levels
64 decreased in the 400ppm iron DSS group at day-63 compared to baseline; mirroring our
65 previously published work in acute colitis. Long term dietary iron alterations clearly affects gut
66 microbiota signatures but do not appear to exacerbate chronic colitis. However, acute colitis
67 is exacerbated by changes in dietary iron. More work is needed to understand the impact of
68 iron supplementation of the pathogenesis of IBD and rise that possibility that the change in
69 the microbiome, in patients with colitis, is a consequence of the increase in luminal iron and
70 not simply the presence of colitis.

72 Introduction

73 Inflammatory bowel disease (IBD) is a debilitating, relapsing-remitting long-term condition of
74 the gastrointestinal tract that affects around 240,000 people in the UK (1) (2, 3). Approximately
75 one-third of patients develop iron deficiency anaemia because of intestinal bleeding and/or
76 malabsorption (4-7). Iron deficiency (ID) may be treated effectively by intravenous or oral iron
77 replacement (8). These therapeutic options have different side effect profiles (9), and may
78 have other off target effects e.g. iron is a growth factor for some bacteria (10). Unabsorbed
79 oral iron supplements and gastrointestinal bleeding result in an increase in luminal iron which
80 may exacerbate IBD and lead to increased proliferation and virulence of some bacteria (11-
81 13). Intestinal bacterial dysbioses have been associated with relapse of IBD (14, 15). It is not
82 clear whether relapsing inflammation leads to dysbiosis by modulating luminal iron (16).

83

84 Chronic inflammation of the intestinal tract is the main feature of IBD. Intestinal epithelial cells
85 (IECs) provide a single superficial layer on the intestinal mucosa and act as the first defensive
86 barrier against the luminal content of the gut and protector of the underlying tissues. IECs
87 have important roles, secreting antimicrobial substances [defensins] and communicating with
88 intestinal immune cells using soluble mediators, chemokines and cytokines (17, 18). There is
89 mounting evidence that alterations in immune regulatory pathways, including inflammasome
90 activation pathways drive changes in gut microbial diversity (19). The mucosal barrier not
91 only defends against luminal pathogens, but also actively shapes the peri-mucosal niche,
92 thereby regulating the composition of the mucosa-associated microbiota (20).

93

94 Based on this evidence, we hypothesised that iron supplementation (and or bleeding) in IBD
95 patients could change the composition of the gut microbiota and potentially influence the
96 natural history of IBD. To investigate this, we assessed the long-term effects of altering dietary
97 iron consumption on intestinal microbiota in murine models of colitis to eliminate any
98 confounding factors based on background genetics that would be inevitable in a human
99 population.

100 **Materials and Methods**

101 **Animals**

102 Wild type C57BL/6 female mice, aged 8-9 weeks old, were purchased from Charles River
103 Laboratories (Margate, UK). Six groups of 8 mice were studied: three control groups and three
104 DSS-treated groups, all of which were maintained for 63-days. Mice received standard chow
105 and water *ad libitum*, during an acclimatisation period of at least one week. Animals were then
106 individually caged in a room with controlled temperature, humidity and a pre-set dark-light
107 cycle (12 h: 12 h) in a specific pathogen-free animal facility. For each group of experiments,
108 mice were matched for age and body weight. The care of, and experimentation on, mice was
109 carried out in accordance with UK Home Office regulations (project licence no: 70/8457) and
110 the project was reviewed by the University of Liverpool Animal Welfare and Ethical Review
111 Body (AWERB).

112

113 **Diets**

114 When eating a normal (standard) iron diet, mice were fed Rat and Mouse Breeder and Grower
115 Pelleted CRM (P) chow (Special Diets Services (SDS), Witham, Essex, UK) which contained
116 200 part per million (ppm) iron in 10mm compression pellets. Two modifications of this
117 standard iron diet were also used: the first was CRM (P) 100ppm iron (Fe) diet where the CRM
118 (P) formulation was used with reduced iron content (0.01% Fe), this was called the half
119 standard iron diet (100ppm iron). The second modification was the CRM (P) 400ppm iron diet:
120 again the CRM (P) formulation was used, but the iron content was increased (0.04% Fe), this
121 was called the double standard iron diet (400ppm iron).

122

123 **Controls**

124 Mice in the three control groups received the standard, half standard or double standard iron
125 diets respectively for 63 days. After 53-days, each group was divided into two, half carried on
126 as controls and half were treated with 2% DSS as described below (Supplementary Figure 1).
127

128 **Induction of chronic colitis using dextran sodium sulphate** 129 **(DSS)**

130 Three groups of 8 mice (taking standard, half standard and double standard iron diets
131 respectively) were given a 1.25% solution of dextran sulfate sodium (M.W. 36,000 – 50,000Da;
132 Catalogue number: 160110; Lot number: 6683K; MP Biomedicals, LLC, UK) in their drinking
133 water for 5-days to induce colitis (Supplementary Figure 1). Mice were allowed to recover for
134 16 days and then the DSS-treatment was repeated for a total of three cycles (21).
135

136 **Induction of acute colitis using DSS**

137 Three groups of 4 mice which had been on the diets for 53 days (control groups) received 2%
138 DSS for 5-days in drinking water, followed by 5-days of plain drinking water, to induce acute
139 colitis. All mice were euthanised on day-63.
140

141 **Histopathological scoring of colonic inflammation**

142 The distal colon was removed, fixed in 4% neutral buffered formalin, dehydrated, wax-
143 embedded and then cut into 4µm sections. The sections were stained with haematoxylin and
144 eosin (H&E). Inflammation was reported using the inflammatory scoring system described by
145 Bauer *et al.* (22). Fibrosis was assessed using Masson's trichrome staining (NovaUltra™
146 Masson's Trichrome Stain Kit (Fisher Scientific UK Ltd)) (23). A researcher blinded to the
147 treatment group assessed all slides.
148

149 **Assessing the degree of gut inflammation by measuring** 150 **faecal calprotectin concentrations**

151 Faecal calprotectin concentration was measured using the S100A8/S100A9 ELISA kit
152 (Immundiagnostik AG, Stubenwald-Allee 8a, Bensheim, Germany) from faecal samples
153 collected from each mouse, on day-1, 21, 42 and 63 in the chronic colitis study, and on day-1
154 and day-10 of the acute colitis study in control mice.

155

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157

158 **Faecal iron concentration**

159 The faecal iron (Fe²⁺ and Fe³⁺) concentration was measured using an iron immunoassay kit
160 (MAK025, Sigma-Aldrich) from the same faecal pellets that were collected for calprotectin
161 assessment.

162

163 **Faecal bacterial DNA extraction and sequencing**

164 2g of faeces was used for bacterial DNA extraction using Stratec Kit (PSP® Spin Stool DNA
165 Plus Kit, STRATEC Molecular GmbH, D-13125 Berlin) following the supplier's protocol. The
166 extracted DNA was sent to the Centre for Genomic Research at the University of Liverpool to
167 undertake the rest of amplicon library protocol 16S [Metagenomic Sequencing Library].
168 Primers described by Caporaso *et al.* (24) were used to amplify the V4 region of 16S rDNA F:
169 5'ACACTCTTTCCCTACACGACGCTCTTCCGATCTNNNNNGTGCCAGCMGCCGCGGTAA
170 3' and R: 5'GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTGG
171 ACTACHVGGGTWTCTAAT3'.

172

173 Approximately 5 µg of extracted DNA was used for first round PCR with conditions of 20 sec
174 at 95°C, 15 secs at 65°C, 30 sec at 70°C for 10 cycles then a 5 min extension at 72°C. Samples
175 were purified using Axygen SPRI Beads. The second-round PCR was performed to

176 incorporate Illumina sequencing adapter sequences: 15 cycles of PCR were performed using
177 the same conditions. Samples were re-purified then quantified using Qubit and assessed using
178 the Fragment Analyser. Successfully-generated amplicon libraries were sequenced (25).

179

180 The final libraries were pooled in equimolar amounts using the Qubit and Fragment Analyser
181 data and 350-550 bp size-selected on the Pippin Prep. The quantity and quality of each pool
182 were assessed by Bioanalyzer and subsequently by qPCR using the Illumina Library
183 Quantification Kit from Kapa on a Roche Light Cycler LC480II according to manufacturer's
184 instructions. The pool of libraries was sequenced on one lane of the MiSeq at 2x250 bp paired-
185 end sequencing. To help balance the complexity of the amplicon library 15%, PhiX was spiked
186 in (25).

187

188 **Bioinformatics analysis**

189 Initial processing and quality assessment of the sequence data was performed using an in-
190 house pipeline. Base-calling and de-multiplexing of indexed reads were conducted by
191 CASAVA version 1.8.2 (Illumina). The raw fastq files were trimmed to remove Illumina adapter
192 sequences where any reads that matched the adapter sequence over at least three bp was
193 trimmed off. The reads were further trimmed to remove low-quality bases (reads <10 bp were
194 removed). Read pairs were aligned to produce a single sequence for each read pair that would
195 entirely span the amplicon. Sequences with lengths outside the expected range were excluded
196 (25). The sequences passing the above filters for each sample were pooled into a single file.

197 A metadata file was created to describe each sample. These two files were analysed using
198 Qiime, version 1.8.0 (Caporaso *et al.*, 2010) (26). Similar sequences were clustered into
199 groups, to define OTUs of 97% similarity. OTU-picking was performed using USEARCH7
200 (Edgar *et al.*, 2010) (27). The Greengenes database version 12.8 (McDonald *et al.*, 2012) (28),
201 was used for reference-based chimera detection (25). OTU tables were repeatedly
202 sub-sampled (rarefied). For each rarefied OTU table, three measures of alpha diversity were
203 estimated: chao1, the observed number of species, and the phylogenetic distance. For

204 inter-sample comparisons (beta-diversity), all datasets were rarefied, and tables were used to
205 calculate weighted and unweighted pair-wise UniFrac matrices using Qiime. UniFrac matrices
206 were then used to generate UPGMA (Unweighted Pair-Group Method with Arithmetic mean)
207 trees and 2D principal coordinates plots (PCoA).(25)

208

209 **Statistics**

210 Normally distributed physiological and biochemical data were assessed by analysis of
211 variance followed by multiple comparisons Dunn's test and non-normally distributed data
212 have been evaluated by Kruskal-Wallis test followed by multiple comparisons Dunn's test
213 (Stats Direct version 3.0.171). For the bioinformatic analysis of microbiota data, Kruskal-
214 Wallis H-test was used with the false discovery rate (FDR) Storey's (multiple correction tests).
215 The q-value is the adjusted p-value based on FDR calculation, where statistical significance
216 was declared at $p < 0.05$.

217 **Results**

218 **Chronic DSS-induced colitis induces C57BL/6 weight loss**

219 Colitis was reproducibly induced by 1.25% DSS. All mice lost body weight from day-6 and
220 maximal weight loss occurred at day-8 of each cycle. Mice receiving the 100ppm iron diet
221 appeared to lose more weight than other groups, but this difference was not significant (Fig.
222 1-a). All control mice, irrespective of the iron dosing, showed a steady increase in body weight.
223 However, mice fed 400ppm iron diet showed a significant weight gain during the whole 63
224 days this reflect the nutritional factor effect (Supplementary Figure 2).

225

226 **Figure 1-a: Percentage of weight change in mice (100ppm iron (blue), 200ppm iron (red)**
227 **and 400ppm iron (green)) during three cycles of 1.25% dextran sulphate sodium-**
228 **induced colitis during the 63-day period. Data are presented as a mean \pm standard error**
229 **of the mean. Statistical differences were assessed by the Kruskal–Wallis test followed**
230 **by Dunn’s multiple comparison tests. (n=8 female mice per group).**

231

232 **Acute DSS-colitis induced weight loss is more severe in** 233 **mice fed 100ppm iron diets**

234 Acute DSS colitis was induced after 53 days of dietary manipulation in a subset of mice that
235 had consumed different amounts of dietary iron during this time: all developed colitis.
236 Weight loss began earlier (day-3) in the 100ppm iron group than in the 200 and 400ppm
237 iron DSS-treated groups (Fig. 1-b). During this acute DSS cycle, mice fed 100ppm iron lost
238 significantly ($P < 0.001$) more weight than the other treated groups.

239

240 **Figure 1-b: Percentage of weight change in mice (100ppm iron (blue), 200ppm iron (red)**
241 **and 400ppm iron (green)) during 2% dextran sulphate sodium-induced colitis. Data are**
242 **presented as a mean \pm standard error of the mean. Statistical differences were assessed**

243 **by the Kruskal–Wallis test followed by Dunn’s multiple comparison tests, compared**
244 **with standard chow group. (n=4 female mice per group). * P<0.05, ** P<0.01, *** P<0.001.**

245

246 **Histopathological changes caused by acute and chronic** 247 **DSS treatment**

248 At autopsy all mice that had been treated with repeated cycles of 1.25% DSS showed
249 histological evidence of mild chronic colitis (Fig. 2-a; I, II and III), and those receiving acute
250 DSS treatment had moderately severe acute colitis (Fig. 2-a; VII, VIII and IX). By contrast the
251 colons of control untreated mice appeared histologically normal (Fig. 2-a; IV, V and VI). The
252 colitis scores were significantly greater (P<0.01) in the mice that had been treated with 2%
253 DSS after consuming either 100 or 400ppm iron, compared with the mice that had received
254 200ppm iron and all the mice that received cycles of 1.25% DSS (Fig. 2-b).

255

256 **Figure 2-a: Illustrative H&E-stained segments of distal colon from untreated (n=4),**
257 **1.25% (n=8) and 2% DSS-treated mice (n=4). Mice received either water (control) (IV, V,**
258 **VI), 1.25% DSS for 5 days and full recovery period 16 days on normal water (I, II, III) or**
259 **2% DSS for 5 days and followed by another 5 days on plain drinking water before they**
260 **were euthanised (VII, VIII, IX). Arrowheads highlight submucosal oedema; arrows**
261 **highlight almost complete loss of colonic epithelium. Scale Bar: 100 µm.**

262 **Figure 2-b: Inflammation (colitis) scores for all groups’ DSS-treated (n=8 (63-days) and**
263 **n=4 (10-days) mice per group) and untreated (controls) mice on different iron diets n=4**
264 **per group (63-days). Horizontal lines at the median. Differences tested by One-way**
265 **ANOVA followed by multiple comparisons Dunn’s test. **P<0.01.**

266

267

268 **Analysis of intestinal fibrosis in chronic colitis in mice**
269 **treated with repeated cycles of dextran sulphate sodium**

270 Masson's trichrome staining was used to assess the degree of fibrosis following chronic DSS
271 treatment (Supplementary Figure 3). Mice in the 100ppm iron DSS-treated group had
272 significantly more fibrosis ($P<0.05$) than the DSS-treated mice receiving 200ppm iron and
273 400ppm iron diets (Supplementary Figure 4).

274

275 **Faecal calprotectin concentration in chronic and acute DSS-**
276 **treated mice**

277 Faecal calprotectin concentrations appeared to increase after each cycle of 1.25% DSS
278 treatment in mice consuming the 400ppm iron diet; the differences were statistically significant
279 ($P<0.01$) between day-21 and day-63 (Supplementary Figure 5): this was not seen in other
280 mice. Thus, mice with double standard iron diet appeared to develop more inflammation at
281 molecular level by assessment of faecal calprotectin concentration.

282

283 For the acute DSS experiment, faecal calprotectin concentration increased significantly in
284 each DSS-treated group. The change in faecal calprotectin was greater in the 100ppm iron
285 diet DSS-treated mice than in the other groups (Supplementary Figure 6). Thus, mice
286 consuming half-standard iron diets also appeared to develop more molecular inflammation
287 after acute colitis induced.

288

289 **Faecal iron concentrations**

290 In the chronic colitis experiment, DSS-treated mice consuming 400ppm iron showed a
291 difference in faecal iron concentration between day-1 and day-63 only. Mice in the 100 and
292 200ppm treated groups that received DSS both showed significant differences at day-1 vs
293 day-21, 42 and 63 (Fig. 3-a) consistent with the presence of luminal iron from bleeding

294 resulting from colitis. Faecal iron concentration increased significantly in control mice (63 days
295 on diet alone) taking 200 and 400ppm diets, but did not change with time in those mice
296 consuming 100ppm iron (Fig. 3-a).

297

298 In the acute DSS experiment, faecal iron concentration increased significantly in all DSS-
299 treated mice. This was more pronounced in the 400ppm iron group (Fig. 3-b).

300

301 **Figure 3-a: Faecal iron concentration at four different time points day-1, 21, 42 and 63**
302 **separately. (I) Faecal iron in 100ppm iron DSS-treated and untreated groups (II) faecal**
303 **iron in 200ppm iron DSS-treated and untreated groups (III) faecal iron in 400ppm iron**
304 **DSS-treated and untreated groups. Data are presented as a mean \pm standard error of**
305 **the mean. Differences were tested by Kruskal– Wallis test followed by multiple**
306 **comparison Dunn’s test. ** P<0.01.**

307 **Figure 3-b: Faecal iron concentration at two different time points day-1 and 10**
308 **separately. (I) Faecal iron in 100ppm iron DSS-treated and untreated groups (II) faecal**
309 **iron in 200ppm iron DSS-treated and untreated groups (III) faecal iron in 400ppm iron**
310 **DSS-treated and untreated groups. Data are presented as a mean \pm standard error of**
311 **the mean. Differences were tested by Kruskal– Wallis test followed by multiple**
312 **comparison Dunn’s test.**

313

314

315 **Bacterial diversity data analysis at phylum and family level** 316 **for chronic experiments**

317 Tables of rarefied OTU data were prepared, and three measures of alpha diversity were
318 estimated: chao1, the observed number of species, and the phylogenetic distance. These
319 estimates were plotted as rarefaction curves using Qiime (Supplementary Figure 7). Similarly,
320 for beta-diversity, weighted and unweighted pair-wise UniFrac matrices UPGMA trees were
321 prepared (Supplementary Figure 8).

322

323 Principal component analysis (PCA) was used to identify linear combinations of gut microbial
324 taxa associated with the duration on a diet (Fig. 4). Our data showed an overlap in the samples
325 of 100 and 200ppm iron DSS-untreated and 200ppm iron DSS-treated mice (Figure 4-a, c and
326 d). There was clustering with little separation of samples pre- and post-DSS treatment for 100
327 and the 400ppm iron DSS-treated groups as well as with control mice fed a 400ppm iron diet
328 (Figure 4-b, e and f). The double standard (400ppm) iron diet disturbed the microbial
329 community significantly in both DSS-treated and untreated mice.

330

331 **Figure 4: In chronic DSS, PCA plots of the unweighted UniFrac distances of pre-and**
332 **post-DSS-intervention stool samples from chronic (3 cycles) DSS-treated mice (b, d,**
333 **and f) and (a, c and e) untreated mice at Phylum-level, phylogenetic classification of**
334 **16S rRNA gene sequences. Symbols represent data from individual mice, colour-**
335 **coded by the indicated metadata. Statistical differences were assessed by Kruskal-**
336 **Wallis H-test followed by Storey's FDR multiple test correction.**

337

338

339 Post-hoc tests revealed a significant difference in the amount of *Proteobacteria* in 100ppm
340 iron chronic DSS-treated mice when day-1 and 63 were compared ($P < 0.017$) (Fig. 5-a). In
341 400ppm iron DSS-untreated mice there was a significant increase in two phyla (*Proteobacteria*
342 and *Actinobacteria*) comparing day-1, 21, 42 and 63 samples ($p < 0.011$ for both) (Fig. 5-b).
343 The analysis of faecal samples from mice in the 400ppm iron DSS-treated group showed
344 differences in *Bacteroidetes* and *Proteobacteria* comparing day-1, 21, 42 and 63:
345 *Proteobacteria* increased significantly ($P < 0.016$), and *Bacteroidetes* decreased ($P < 0.028$)
346 (Figure 5-c). Together these data suggest that *Proteobacteria* are dependent on luminal iron,
347 but *Bacteroidetes* are suppressed by inflammation and/or luminal iron.

348

349 **Figure 5-a: In chronic DSS, box plot showing the distribution in the proportion of**
 350 ***Proteobacteria* assigned to samples at day-1, 21, 42 and 63 from 100ppm iron DSS-**
 351 **treated mice.**

352 **Figure 5-b: In chronic DSS, box plot showing the distribution in the proportion of two**
 353 **phyla (*Actinobacteria* (I) and *Proteobacteria* (II)) assigned to samples from 400ppm**
 354 **iron untreated mice.**

355 **Figure 5-c: In chronic DSS, box plot showing the distribution in the proportion of two**
 356 **phyla (*Proteobacteria* (I) and *Bacteroidetes* (II)) assigned to samples from 400ppm iron**
 357 **DSS-treated mice.**

358

359 Further bioinformatics analysis identified 4 phyla and 15 taxa (genera) of interest. Of the four
 360 phyla (*Firmicutes*, *Bacteroidetes*, *Proteobacteria*, and *Actinobacteria*), one (*Firmicutes*) was
 361 highly abundant among all groups while the lowest abundance phylum was *Actinobacteria*.
 362 However, 100ppm iron and 400ppm iron chronic DSS groups showed seven different genera
 363 apart from the three genera (*Bacteroides*, *Lactobacillus* and *Bilophila*) that they shared.
 364 STAMP encourages the use of effect sizes and confidence intervals (29). The results of the
 365 relative abundances of various phyla and identified genera are summarised in Table 1: a-c.

366

367 **Table 1-a: Genus-level taxonomic composition of faecal samples from 100ppm iron DSS-**
 368 **treated mice (Day-1 vs 21, 42 and 63 samples)**

100ppm iron DSS-treated group			
Taxon	p-values	p-values (corrected)	Effect size
p_ <i>Bacteroidetes</i>; g_ <i>Bacteroides</i>	0.003	0.047	0.496
p_ <i>Bacteroidetes</i>; g_ <i>Odoribacter</i>	0.002	0.04	0.620
p_ <i>Bacteroidetes</i>; g_ <i>Prevotella</i>	0.0002	0.008	0.669
p_ <i>Firmicutes</i>; g_ <i>Clostridium</i>	0.002	0.04	0.431
p_ <i>Firmicutes</i>; g_ <i>Dorea</i>	0.003	0.047	0.138
p_ <i>Firmicutes</i>; g_ <i>Lactobacillus</i>	0.00002	0.002	0.880
p_ <i>Proteobacteria</i>; g_ <i>Bilophila</i>	0.0002	0.008	0.766

369 **Table 1-b:** Genus-level taxonomic composition of faecal samples from 400ppm iron DSS-
370 treated mice (Day-1 vs 21, 42 and 63 samples)

400ppm iron DSS-treated group			
Taxon	p-values	p-values (corrected)	Effect size
p_Firmicutes; g_Lactobacillus	0.0001	0.01	0.74

371
372

373 **Table 1-c:** Genus-level taxonomic composition of faecal samples from 400ppm iron untreated
374 mice (Day-1 vs 21, 42 and 63 samples)

400ppm iron untreated group (Controls)			
Taxon	p-values	p-values (corrected)	Effect size
p_Actinobacteria; g_Adlercreutzia	0.002	0.04	0.49
p_Bacteroidetes; g_Bacteroides	0.0005	0.02	0.68
p_Firmicutes; g_Candidatus Arthromitus	0.003	0.04	0.54
p_Firmicutes; g_Lactobacillus	0.0002	0.02	0.77
p_Firmicutes; g_Oscillospira	0.001	0.03	0.61
p_Firmicutes; g_Ruminococcus	0.002	0.04	0.46
p_Proteobacteria; g_Bilophila	0.001	0.03	0.55

375

376

377

378 Discussion

379 DSS-induced colitis in mice is a popular model for the study of human ulcerative colitis: its
380 mechanism of action is unclear but may be toxic to the colonic epithelium, activate
381 macrophages and/or alter the gut microbiota (30) (31). Most research has used the acute
382 colitis model, however Okayasu *et al* described a chronic colitis model in mice, which may be
383 more appropriate for research of chronic IBD in humans (31) (32) (33). Most studies of the role
384 of iron in relapse of IBD have focussed on the effect of supplementation, however we have
385 recently reported the effect of half standard and double standard dietary iron on acute DSS
386 induced colitis: both changes were associated with more severe colitis than the standard diet
387 (25). Here, we report the effects of the same dietary modification on a model of (1) chronic
388 colitis and (2) acute colitis, in the setting of chronic prior modification of the diet.

389
390 When acute colitis was induced after 7 weeks of dietary modification, mice consuming the
391 100ppm or 400ppm diet developed more severe colitis than mice taking the 200ppm iron diet:
392 clinical and histological data were concordant for 100ppm iron group. In contrast, mice in which
393 chronic colitis was induced while consuming 100ppm, 200ppm or 400ppm dietary iron showed
394 only modest, non-significant weight loss and histological colitis.

395
396 In this study, increasing dietary iron led to an increase in faecal iron in the 200 and 400ppm
397 treated mice. After induction of chronic colitis, faecal iron increased in all mice. In the acute
398 DSS experiment, the 400ppm iron group showed the most significant difference ($P < 0.0001$) in
399 faecal iron concentration. There is an obvious paradox: reducing dietary iron was associated
400 with an increase in loss of iron in faeces. The mechanism appears to be by exacerbating DSS-
401 colitis. We speculate that the low iron diet led to more severe colitis, which secondarily led to
402 an increase in bleeding and hence faecal iron.

403

404 Changing dietary iron concentration led to a significant difference in the microbiome in both
405 the 100 and 400ppm iron chronic DSS-treated and 400ppm iron untreated groups of animals.
406 Previous research has established a reduction in the biodiversity of commensal bacteria in
407 IBD (34). In mouse experiments, changes in bacterial composition resulted from colonic
408 inflammation and infection (35). In particular, intestinal pathogens (some types of
409 *Proteobacteria*) appeared to take advantage of this. This observation is in agreement with the
410 'food hypothesis' and 'differential killing' hypothesis. These two mechanisms are likely to
411 contribute to the loss of colonisation resistance in the inflamed gut (36). Nonetheless, the post-
412 hoc analysis of our data revealed that one bacterial phylum (*Proteobacteria*) was increased
413 significantly ($P<0.01$) in the 100ppm iron and 400ppm iron DSS-treated and 400ppm iron
414 untreated groups. *Bacteroidetes* decreased significantly ($P<0.028$) in the 400ppm iron DSS-
415 treated group.

416

417 Haller et al. (37) investigated the effects of dietary iron upon the microbiome. Eight bacterial
418 families and nine bacterial genera were significantly ($P<0.01$) affected by luminal iron (ferrous
419 sulphate) deficiency. The genera *Bifidobacterium* ($P<0.0018$), *Succinivibrio* ($P<0.0027$),
420 *Turicibacter* ($P<0.0020$) and *Clostridium* ($P<0.0017$) were significantly increased in mice fed
421 an iron depleted diet, whereas the genera *Desulfovibrio* ($P<0.0001$), *Dorea* ($P<0.01$) and
422 *Bacteroides* related were greatly reduced. The authors concluded that all significant
423 differences in bacterial abundance in wild-type mice appeared as a result of the interaction
424 between treatment and host-mediated inflammation (37, 38). There are several key
425 differences between that paper and our own: they investigated caecal contents, not faeces;
426 they induced ileitis, not colitis and they did not measure faecal iron concentration. Thus, their
427 paper and our data cannot be directly compared.

428

429 Our data analysis showed that seven genera were significantly different. In the half standard
430 iron diets (100ppm) DSS-treated group, we found reductions in *Lactobacillus* ($P<0.002$),
431 *Dorea*, *Clostridium*, *Bacteroides* and *Odoribacter* ($P<0.04$), *Bilophila* ($P<0.008$), and an

432 increase in (*Prevotella* $P < 0.008$), all belonging to three phyla [*Firmicutes*, *Bacteroidetes* and
433 *Proteobacteria*]. In the 400ppm iron DSS group, a significant reduction was shown in
434 *Lactobacillus* ($P < 0.01$). The only control group in which significant differences were found was
435 the 400ppm iron group, where four phyla [*Firmicutes*, *Bacteroidetes*, *Proteobacteria*, and
436 *Actinobacteria*] with seven genera showed statistically significant differences. Increases were
437 shown in *Lactobacillus* ($P < 0.02$), *Oscillospira* ($P < 0.03$), *Adlercreutzia* and *Candidatus*
438 *Arthromitus* ($P < 0.04$), whereas reductions occurred in *Bacteroides* ($P < 0.02$), *Bilophila*
439 ($P < 0.03$) and *Ruminococcus* ($P < 0.04$) (Table 1-c).

440

441 Dietary iron plays a role in modulating the susceptibility to DSS-induced colitis. Lower (half
442 standard) iron content in the diet significantly worsened acute colitis leading to an increase in
443 faecal iron. Double standard iron diets caused a dysbiosis. These observations demonstrated
444 the importance of luminal iron and inflammation. Manipulations in dietary iron administration
445 for a longer period significantly exacerbated susceptibility towards developing DSS-induced
446 intestinal inflammation suggesting that the time of iron supplementation may be crucial in
447 aggravating colitis. We cannot explain why the reduced iron diet exacerbates colitis. Further
448 studies will be necessary to investigate the relevance of our findings in humans.

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458 **Supplementary:**

459 All data files uploaded in supporting information file.

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461

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561

Figure 1-a:

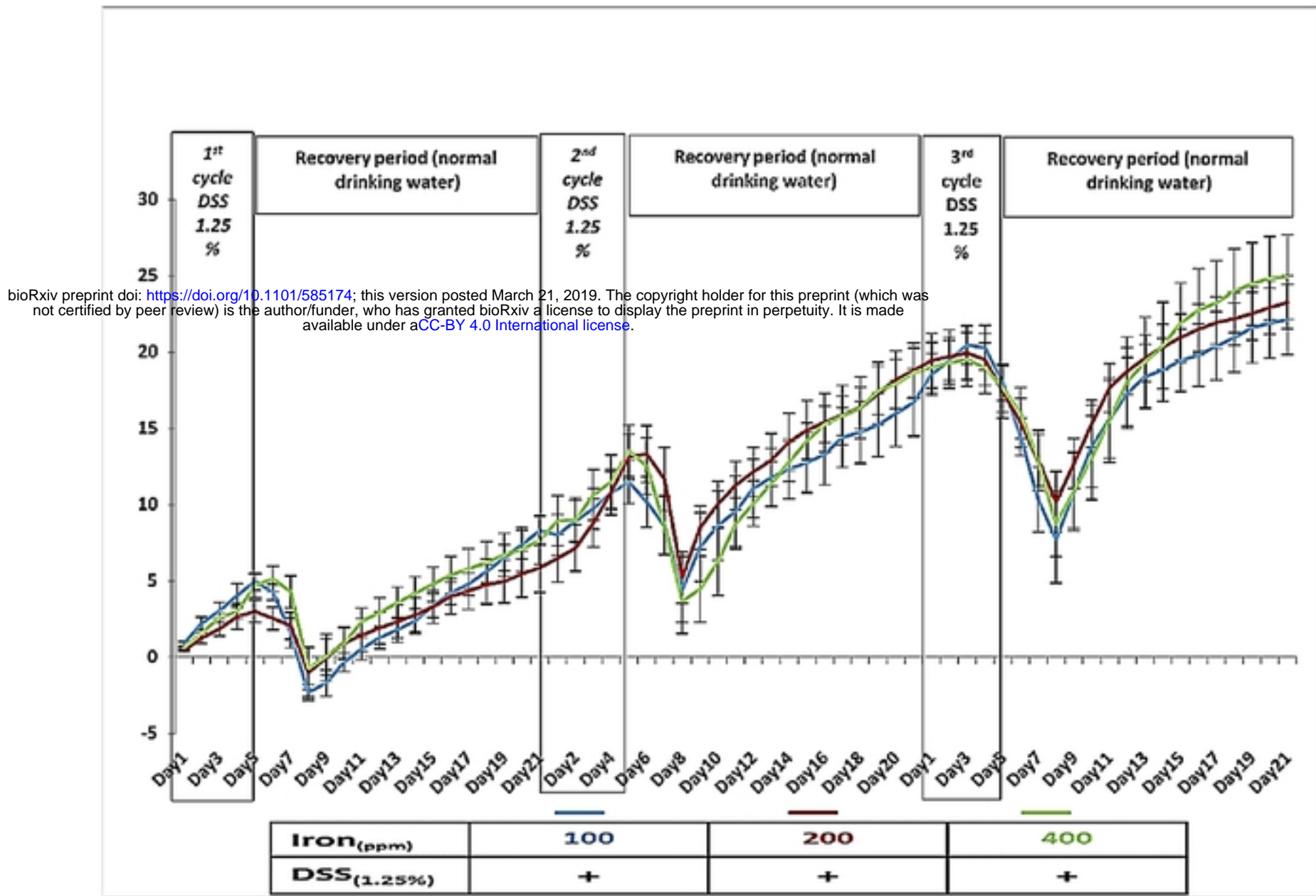


Figure 1-b:

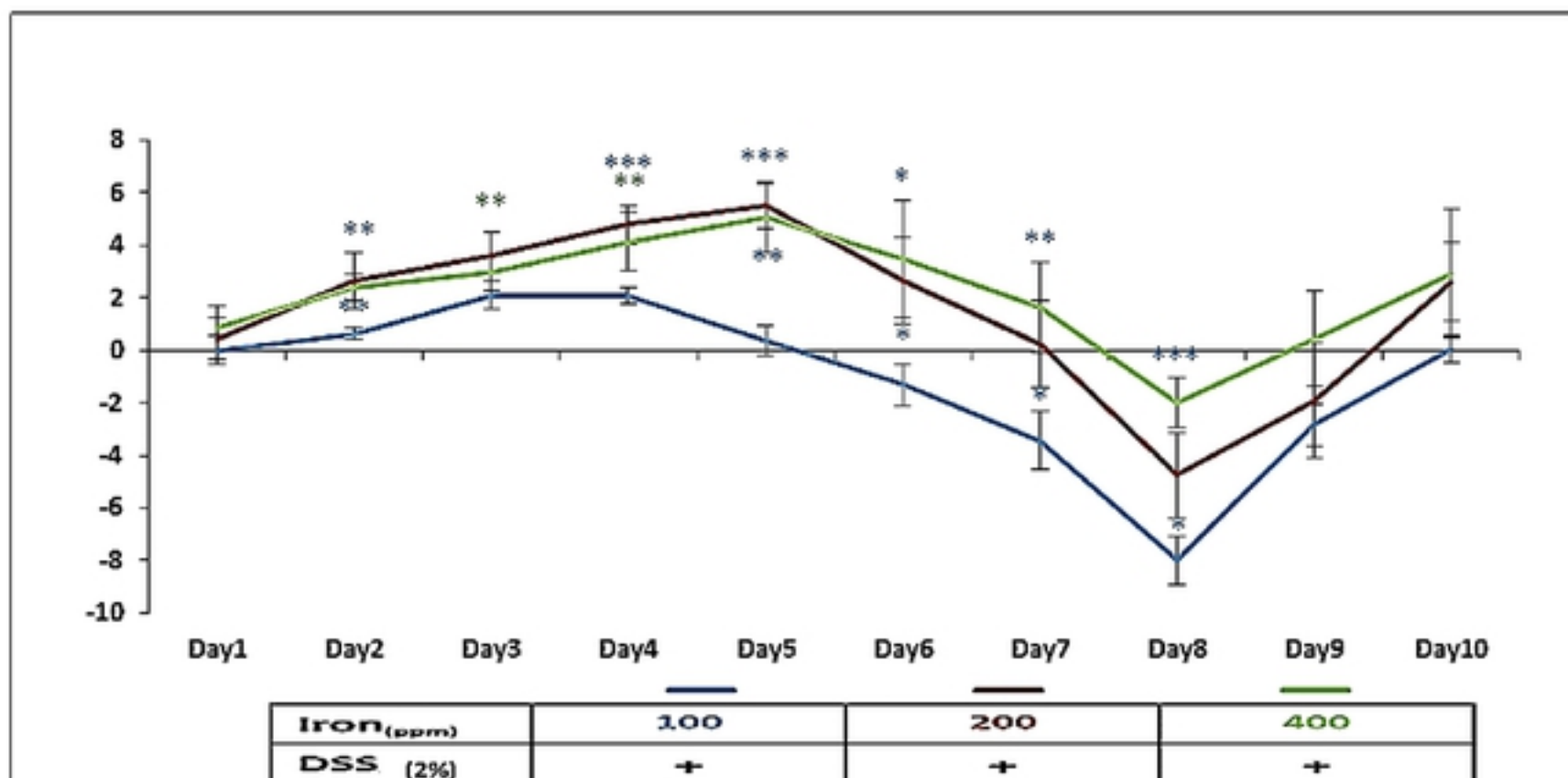


Figure 2-a:

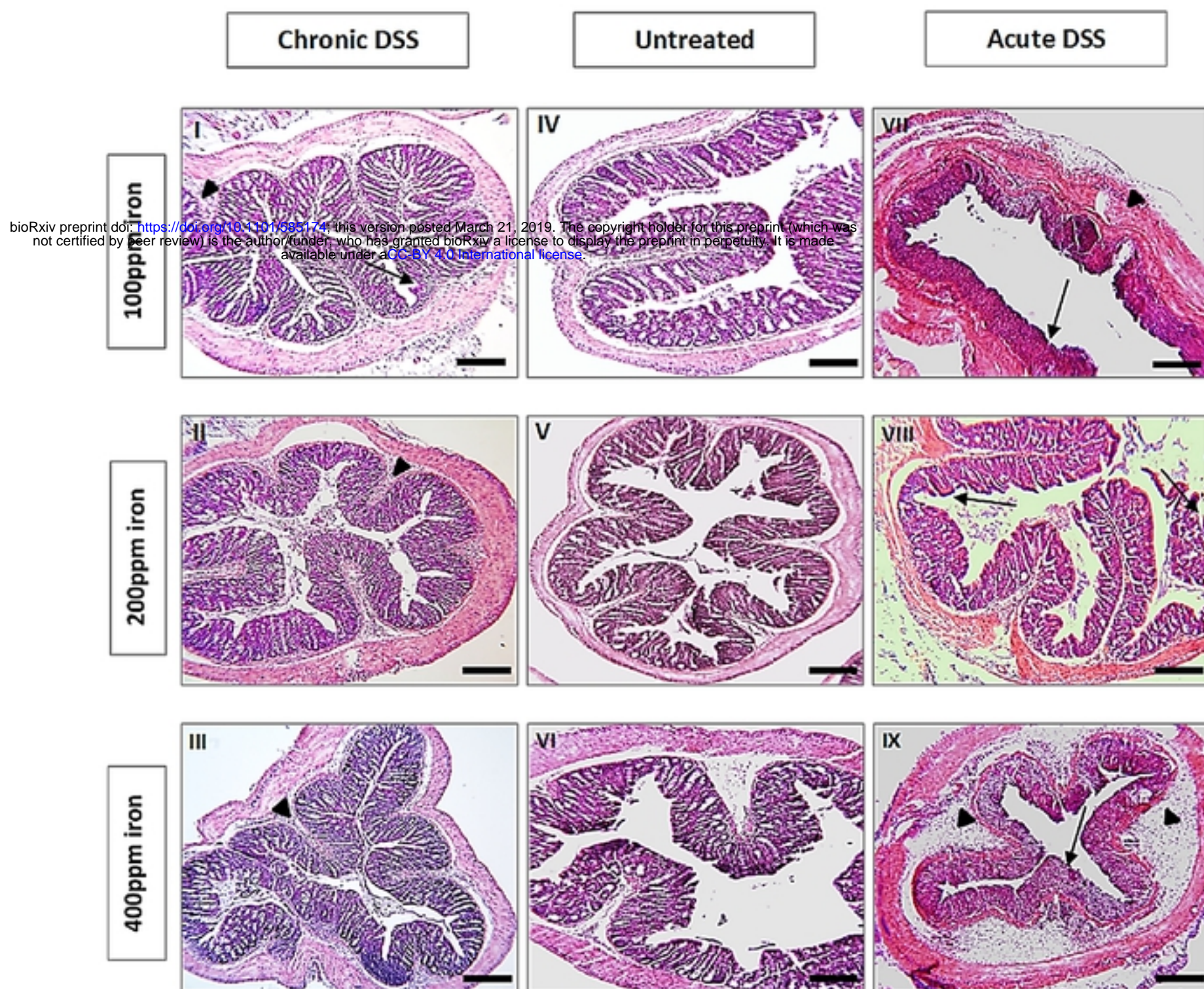
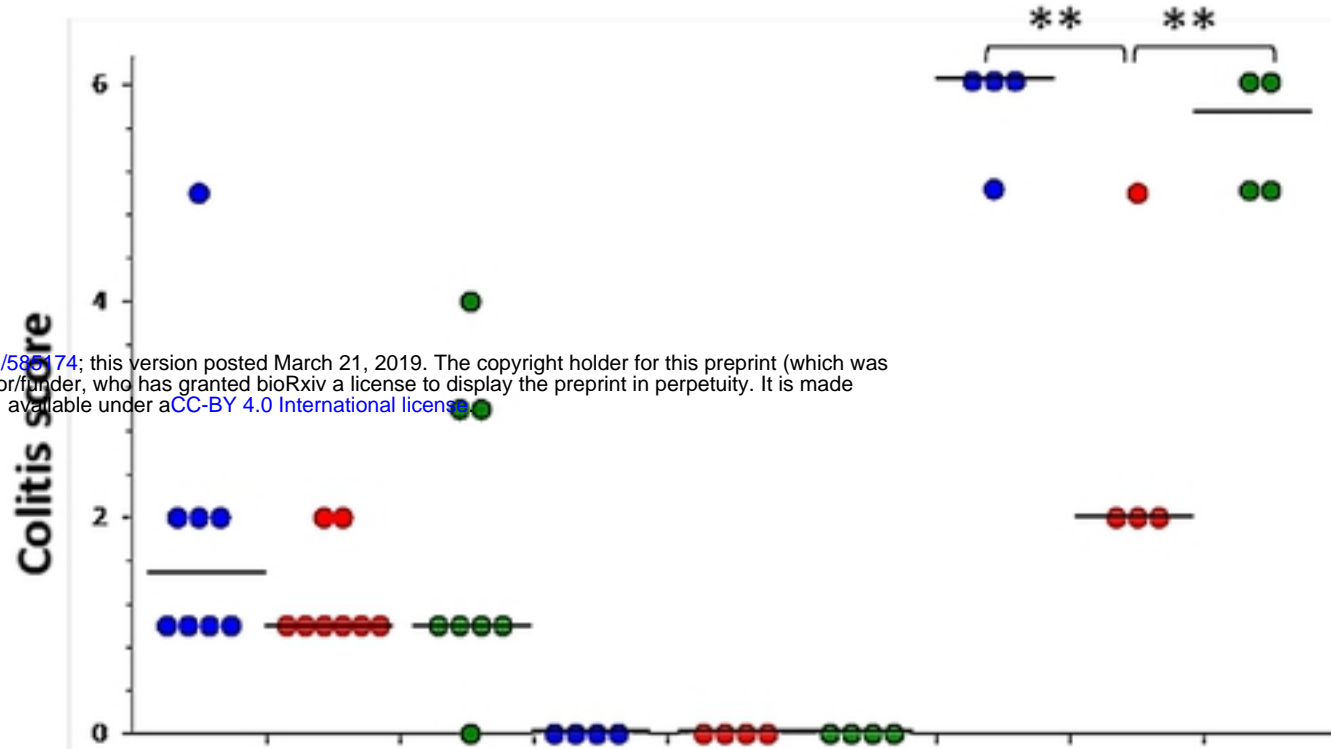


Figure 2-b:

Spread plot Chronic and Acute DSS groups



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100 PPM iron	+	-	-	+	-	-	+	-	-
200 PPM iron	-	+		-	+	-	-	+	-
400 PPM iron	-	-	+	-	-	+	-	-	+
1.25 % DSS x3	+	+	+	-	-	-	-	-	-
2 % DSS x1	-	-	-	-	-	-	+	+	+

Figure 3-a:

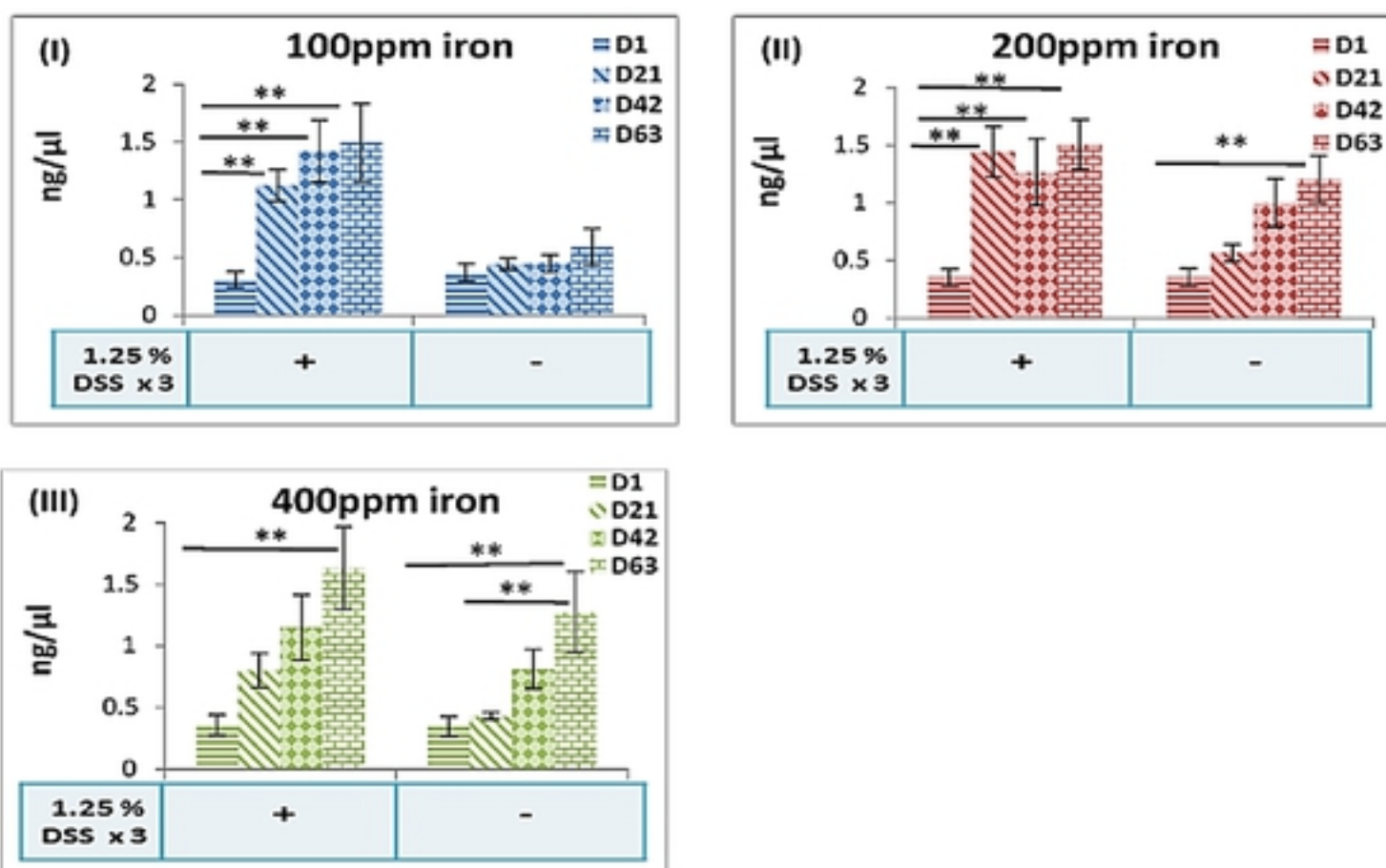


Figure 3-b:

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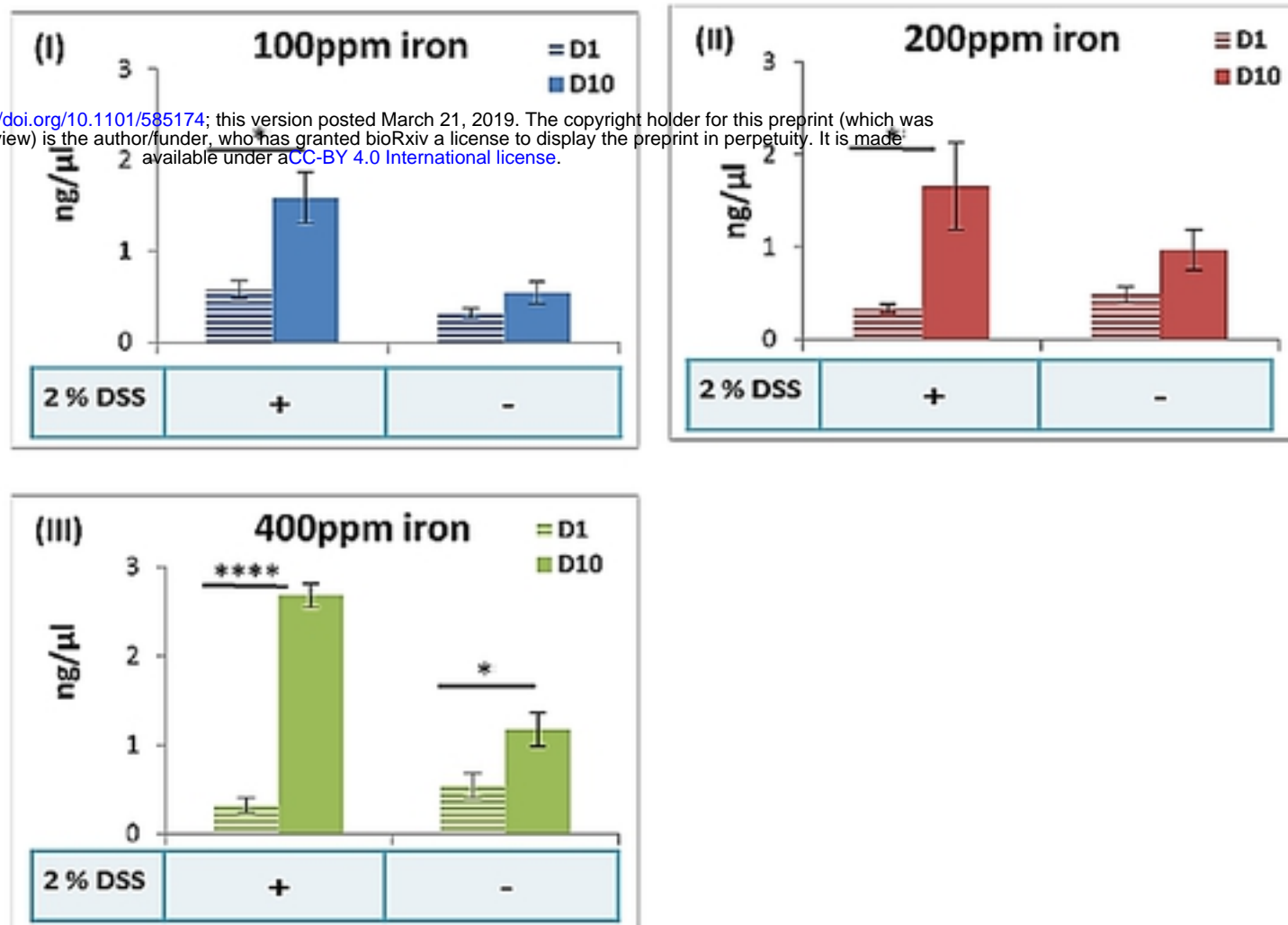
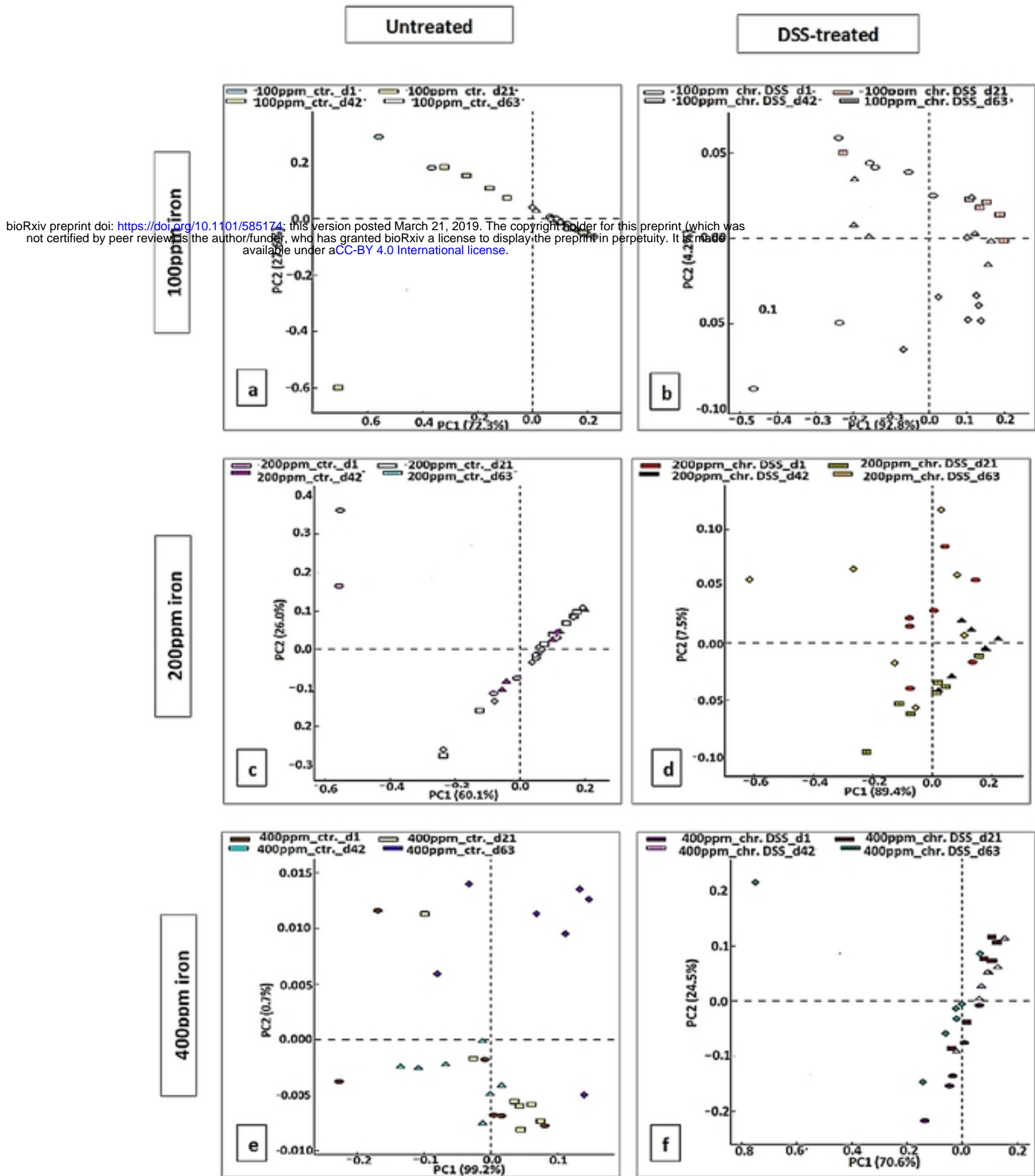
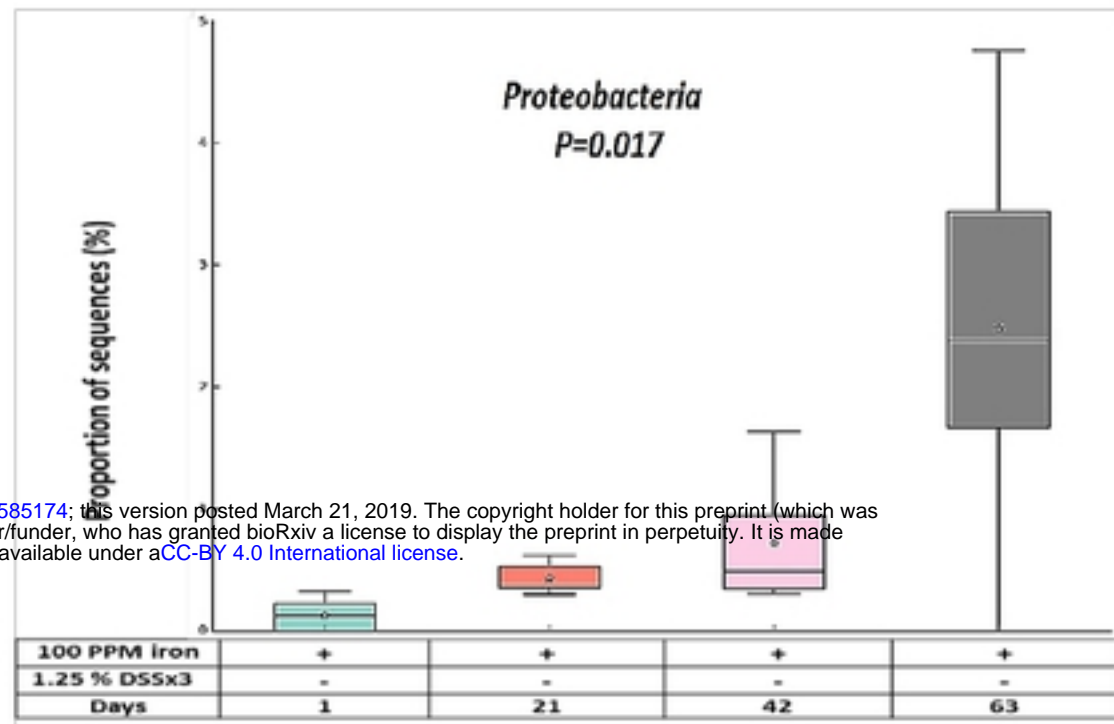


Figure 4:



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Figure 5-a:



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Figure 5-b:

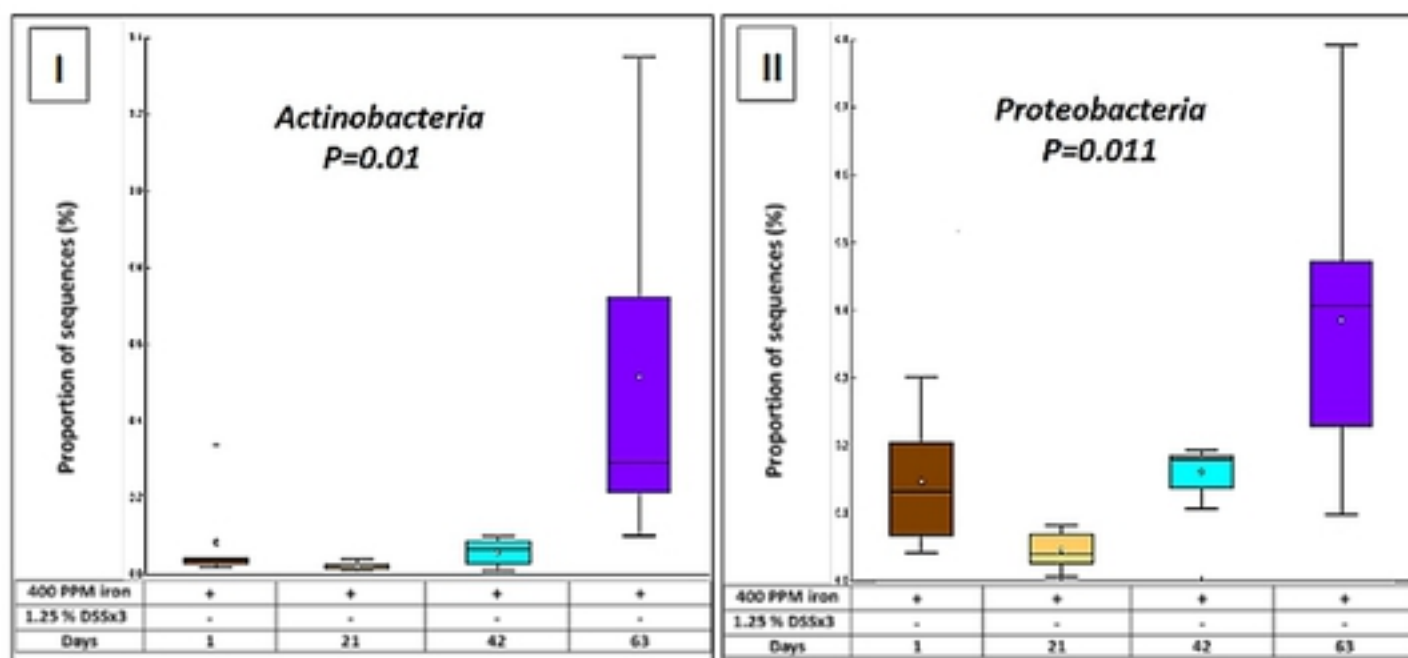


Figure 5-c:

