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***Oral iron exacerbates colitis and influences the intestinal
microbiome***

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27 **"Authors' contributions."**

28

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30 interpreted results of experiments, prepared figures, drafted manuscript, edited and revised
31 manuscript and approved the final version of manuscript]

32 **Jonathan M. Williams** [interpreted results of experiments, drafted manuscript, approved the
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48 experiments, edited and revised manuscript, approved the final version of manuscript].

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55 **Abstract:**

56 Inflammatory bowel disease (IBD) is associated with anaemia and oral iron replacement to
57 correct this can be problematic, intensifying inflammation and tissue damage. The intestinal
58 microbiota also plays a key role in the pathogenesis of IBD, and iron supplementation likely
59 influences gut bacterial diversity in patients with IBD. Here, we assessed the impact of dietary
60 iron, using chow diets containing either 100, 200 or 400 ppm, fed *ad libitum* to adult female
61 C57BL/6 mice in the presence or absence of colitis induced using dextran sulfate sodium
62 (DSS), on (i) clinical and histological severity of acute DSS-induced colitis, and (ii) faecal
63 microbial diversity, as assessed by sequencing the V4 region of 16S rRNA. Increasing or
64 decreasing dietary iron concentration from the standard 200 ppm exacerbated both clinical
65 and histological severity of DSS-induced colitis. DSS-treated mice provided only half the
66 standard levels of iron *ad libitum* (i.e. chow containing 100 ppm iron) lost more body weight
67 than those receiving double the amount of standard iron (i.e. 400 ppm); $p < 0.01$. Faecal
68 calprotectin levels were significantly increased in the presence of colitis in those consuming
69 100 ppm iron at day 8 (5.94-fold) versus day-10 group (4.14-fold) ($p < 0.05$), and for the 400
70 ppm day-8 group (8.17-fold) versus day-10 group (4.44-fold) ($p < 0.001$). In the presence of
71 colitis, dietary iron at 400 ppm resulted in a significant reduction in faecal abundance of
72 Firmicutes and Bacteroidetes, and increase of Proteobacteria, changes which were not
73 observed with lower dietary intake of iron at 100 ppm. Overall, altering dietary iron intake
74 exacerbated DSS-induced colitis; increasing the iron content of the diet also led to changes in
75 intestinal bacteria diversity and composition after colitis was induced with DSS.

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81 Introduction

82 Inflammatory bowel disease (IBD) is characterised by chronic inflammation of the
83 gastrointestinal tract. Inflammation is associated with intestinal ulceration in both ulcerative
84 colitis (UC) and Crohn's disease (CD). Bleeding and malabsorption may also occur in IBD ^{1,2},
85 and iron deficiency anaemia occurs in one-third of patients ^{1,3}. The best way to administer iron
86 replacement to patients with IBD is a subject of debate, with both oral iron and intravenous
87 (IV) iron supplements being considered effective ^{4, 5}. However, ferrous forms of oral iron
88 replacement appear to be poorly absorbed, and the resultant free luminal iron likely results in
89 enhanced catalytic activity and production of reactive oxygen species within the intestine ^{6, 7}.
90 High dose oral iron consumption appears to be associated with more side effects than half of
91 the standard dose of iron ⁸, perhaps as a result of unabsorbed iron reaching the colon.
92 Intravenous (IV) iron therapy offers effective alternative management of iron deficiency
93 anaemia. While the route of administration is not thought to influence disease activity; oral iron
94 supplements have been shown to disturb the microbiota, with disturbances in bacterial
95 phylotypes and associated aberrations in faecal metabolites compared with IV treatment ^{9, 10}.

96
97 The gut microbiota typically comprises greater than 10^{11} microorganisms per gram of intestinal
98 content ¹¹, playing an important role in the maintenance of gut health, including protection
99 against pathogens (colonisation resistance) and the synthesis of beneficial short-chain fatty
100 acids (SCFA) generated through fermentation of dietary fibre ^{12, 13}. IBD is associated with a
101 perturbation of gut microbiota ('dysbiosis'), with the observed reduction in microbial diversity,
102 including a decline in beneficial bacteria from the phyla Bacteroidetes and Firmicutes, although
103 within these classifications a much more complicated picture exists, alongside enhancement
104 of some potentially harmful Proteobacteria, particularly within the family *Enterobacteriaceae*
105 ^{14, 15}. The key mechanisms responsible for the development of this dysbiosis and its
106 contribution to IBD are to date poorly defined.

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108 Iron is an essential metal that is required by most organisms ¹⁶. It is a growth-limiting nutrient
109 for many gut bacteria, which compete for unabsorbed dietary iron in the colon ¹⁷. Lactobacilli,
110 considered to be beneficial intestinal barrier-maintaining bacteria, playing a significant role in
111 the inhibition of mucosal colonisation by enteric pathogens, do not require iron ¹⁸. For other
112 bacteria, acquisition of nutrient iron is an essential step for expression of key virulence factors,
113 including Gram-negative enteric pathogens within the family *Enterobacteriaceae*, such as
114 *Salmonella* spp., *Shigella* spp. and *Escherichia coli* pathovars ¹⁹. Consequently, an increase
115 in unabsorbed dietary iron could favour the growth of opportunistic pathogens over mucosal
116 barrier-maintaining species and alter the composition of the intestinal microbiota ²⁰. In the
117 context of IBD, excess colonic iron concentrations can occur as a result of ulceration and
118 bleeding, as well as excess unabsorbed iron from oral iron replacement therapy ²¹.

119

120 Iron supplementation, either oral or delivered intravenously, has been shown to have an
121 impact on the intestinal microbiota and metabolome of patients with IBD ¹⁰. Comparison of
122 oral versus intravenous routes of iron supplementation demonstrated no significant effects on
123 the human bacterial diversity, although specific species changes were noted. Four Operational
124 Taxonomic Units (OTUs) were observed to be less abundant after oral iron therapy, including
125 *Faecalibacterium prausnitzii*, low abundance of which, has been linked to relapse in Crohn's
126 disease ¹⁰. An OTU of the *Bifidobacterium* genus was noted to be increased with oral iron
127 therapy but, the effect of prebiotics and probiotics was a confounder in 4/6 patients studied.
128 Overall, this study suggested that oral iron therapy might have an adverse effect on the
129 microbiome; however, the contemporaneous effect of IBD was not studied.

130

131 Murine models of IBD offer the opportunity to investigate the gut microbiota and microbial
132 diversity changes that occur during IBD pathogenesis ^{22, 23}. We hypothesised that changing
133 the amount of dietary iron would influence IBD development in a murine model of colitis.
134 Hence, in this study, we modified the standard chow diet of C57BL/6 mice (at 200 parts per

135 million-ppm iron) to half of the standard levels (100 ppm), or to double that of standard (400
136 ppm) of iron and subsequently induced colitis using dextran sulfate sodium (DSS).
137 Clinicopathological outcomes were analyzed in parallel with the characterisation of the
138 composition of the gut microbial community by sequencing the 16S prokaryotic ribosomal
139 subunit.

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163 **Materials and Methods**

164 **Animals**

165 Female C57BL/6 mice (n=130), aged 8-9 weeks old, were purchased from Charles River
166 Laboratories (Margate, UK). Mice were fed a standard rodent chow pellet diet, during an initial
167 acclimatisation period of at least one week, with access to water *ad libitum*. All mice were
168 individually-caged in a specific pathogen-free animal facility with controlled temperature,
169 humidity and a pre-set dark-light cycle (12 h: 12 h). Eight groups were studied initially; two
170 control groups and six DSS-treated groups were maintained either for 8 days or up to 10 days.
171 Three additional control groups of mice (to compare the effects of diets alone) received
172 drinking water without DSS, but with varying amounts of dietary iron for a total of 10 days,
173 under conditions as described above (see Table 1). For each set of experiments, mice were
174 matched for age and body weight. The work described was conducted in accordance with UK
175 Home Office regulations under the Animals (Scientific Procedures) Act 1986 (ASPA). The
176 University of Liverpool Ethical Review Body also approved protocols. Study animals were
177 observed for signs of illness and/or welfare impairment and were euthanised by cervical
178 dislocation.

179

180 **Table 1:** Experimental animal groups' classification

181

Iron (ppm)	100ppm	200ppm	400ppm	100ppm	200ppm	400ppm
DSS (2% w/v)	-	-	-	+	+	+
Number of mice (day-10)	6	20	6	14	14	14
Number of mice (day-8)	-	8	-	16	16	16

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185 **Diets**

186 The standard 10 mm compression pellet chow diet utilised contained 200 ppm iron (Rat and
187 Mouse Breeder and Grower Pelleted CRM (P) - Special Diets Services, Witham, Essex, UK).
188 Two modifications of this diet were used. The first diet was formulated to contain half the
189 amount of iron found in standard chow, ie. 100 ppm, a dietary level selected to reduce luminal
190 bacterial exposure to iron without being harmful to the mice. The second formulation contained
191 double the amount of iron found in standard chow, i.e. 400 ppm, a diet to increase bacterial
192 exposure to iron without being overtly toxic to mice.

193

194 **Induction of acute colitis using dextran sulfate sodium** 195 **(DSS)**

196 Mice were given 2% w/v dextran sodium sulfate (M.W. 36,000 – 50,000Da; Catalogue number:
197 160110; Lot number: 6683K; MP Biomedicals, UK) in their drinking water for 5 days to induce
198 colitis (~150 mL/mouse over 5 days), followed by another 5 days of DSS-free drinking water.
199 Mice were euthanised on day-8 or day-10.

200

201 **Histopathological scoring of colonic inflammation**

202 The distal colon was removed, fixed in 4% neutral buffered formalin, dehydrated, paraffin wax-
203 embedded and then 4 µm sections were cut by microtomy. The sections were stained with
204 hematoxylin and eosin (H&E), and inflammation scored, using the system described by Bauer
205 *et al.* ²⁴.

206

207 **Measurement of faecal calprotectin as a marker of the** 208 **degree of intestinal inflammation**

209 Faecal pellets were collected from each cage (1 mice per cage), in all groups, on day 1, 8 and
210 10. Faecal calprotectin levels were measured using an S100A8/S100A9 ELISA kit
211 (Immundiagnostik AG, Bensheim; Germany) as per the manufacturer instructions.

212

213 **Assessment of faecal iron**

214 The faecal iron (Fe^{2+} and Fe^{3+}) concentration was measured using an iron immunoassay kit
215 [MAK025, Sigma-Aldrich]. This was performed using faecal pellets taken at the same time as
216 those for the faecal calprotectin ELISA.

217

218 **High-throughput sequence analysis of bacterial** 219 **communities from faecal samples**

220 Faeces (2 g) was sampled from each animal and bacterial DNA extracted using the Stratec
221 PSP® Spin Stool DNA Plus Kit (STRATEC Molecular GmbH, Berlin; Germany) following the
222 manufacturer recommended protocol. Isolated DNA was sent to the Centre for Genomic
223 Research at the University of Liverpool to generate the 16S Metagenomic Sequencing Library.
224 Primers described by Caporaso *et al.* ²⁵ were used to amplify the V4 region of 16S rRNA; F:
225 5'ACACTCTTTCCCTACACGACGCTCTTCCGATCTNNNNNGTGCCAGCMGCCGCGGT
226 AA3' and R: 5'GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTGGACTACHVGGGT
227 WTCTAAT3'.

228

229 Approximately 5 μL of extracted DNA was used for first round PCR with conditions of 20 sec
230 at 95°C, 15 secs at 65°C, 30 sec at 70°C for 10 cycles then a 5 min final extension at 72°C.
231 Amplicons were purified with Axygen SPRI Beads before a second-round PCR was performed

232 to incorporate Illumina sequencing adapter sequences containing indexes (i5 and i7) for
233 sample identification as described in ²⁵. Fifteen cycles of DNA amplification by PCR were
234 performed using the same conditions as above, i.e., 25 cycles overall. Again, samples were
235 purified using Axygen SPRI Beads before being quantified using Qubit and assessed using
236 the Fragment Analyser. Successfully generated amplicon libraries were used for sequencing.

237

238 The final libraries were pooled in equimolar amounts using the Qubit and Fragment Analyser,
239 data and size-selected on the Pippin Prep using a size range of 350-550 base pairs (bp). The
240 quantity and quality of each pool were assessed by Bioanalyzer, and subsequently by qPCR
241 using the Illumina Library Quantification Kit from Kapa on a Roche Light Cycler LC480II
242 system according to the manufacturer instructions. The pool of libraries was sequenced on
243 one lane of the MiSeq at 2 x 250 bp paired-end sequencing ²⁶. To help balance the complexity
244 of the amplicon library, 15% PhiX was spiked as described by Altschul *et al.* ²⁷.

245 **Bioinformatics analysis**

246 Initial processing and quality assessment of the sequence data was performed using an in-
247 house pipeline. Base calling and de-multiplexing of indexed reads were conducted by
248 CASAVA version 1.8.2 (Illumina) as described by Schubert *et al.* ²⁸. The raw fastq files were
249 trimmed to remove Illumina adapter sequences with any reads matching the adapter sequence
250 over at least 3 bp being trimmed off. The reads were further trimmed to remove low-quality
251 bases (reads <10 bp were removed). Read pairs were aligned to produce a single sequence
252 for each read pair that would entirely span the amplicon. Sequences with lengths outside of
253 the expected range (which are likely to represent errors) were also excluded. Sequences
254 passing the above filters for each sample were pooled into a single file. A metadata file was
255 created to describe each sample. These two files were used for metagenomics analysis using
256 Qiime, version 1.8.0 as described by Caporaso *et al.* ²⁹. Similar sequences were clustered into
257 groups, to define OTUs of 97% similarity. OTU-picking was performed using USEARCH7 as
258 described by Edgar *et al.* ³⁰ to cluster sequences, remove chimeras, and define OTU

259 abundance. The Greengenes database of ribosomal RNA sequences, version 12.8 as
260 described by McDonald *et al.*³¹, was used as a reference for reference-based chimera
261 detection. To reduce the effect of sample size and to estimate species richness within each
262 sample (alpha diversity), OTU tables were repeatedly sub-sampled (rarefied). For each
263 rarefied OTU table, three measures of alpha diversity were estimated: Chao1, the observed
264 number of species, and the phylogenetic distance. To allow inter-sample comparisons
265 (beta-diversity), all datasets were sub-sampled (rarefied). Rarefied OTU tables were used to
266 calculate weighted and unweighted pair-wise UniFrac matrices. UniFrac matrices were then
267 used to generate UPGMA (Unweighted Pair-Group Method with Arithmetic mean) trees and
268 2D principal component analysis (PCA) plots.

269

270 **Statistics**

271 Normally distributed physiological and biochemical data (as determined by Shapiro-Wilks
272 test) were assessed by one-way analysis of variance followed by multiple pairwise
273 comparisons of treatment means using Dunnett's test. Non-normally distributed data were
274 assessed by Kruskal-Wallis non-parametric-test followed by multiple pairwise comparisons
275 (Conover-Inman) test (Stats Direct version 3.0.171; Altrincham, UK).

276

277 For the bioinformatic analysis of microbiota data, Welch's t-test was used with the false
278 discovery rate (FDR) Storey's multiple correction tests. The q-value is the adjusted p-value
279 based on FDR calculation, where statistical significance was declared at $p < 0.05$.

280 **Results**

281 **Reduced dietary iron intake is associated with increased** 282 **weight loss and more severe colitis following the induction** 283 **of DSS colitis**

284 All mice treated with 2% w/v DSS lost body weight from day-5, with maximal weight loss
285 occurring on day-8. Mice ingesting a diet containing 100 ppm iron lost significantly more weight
286 ($13 \pm 1.53\%$) than seen with the other DSS treatment groups (i.e. $8.3 \pm 1.09\%$ for mice
287 ingesting 200 ppm iron, and $8.6 \pm 1.33\%$ for mice on 400 ppm iron); see Fig 1. Control mice,
288 ingesting dietary iron at 100 ppm, 200 ppm and 400 ppm, receiving no DSS treatment, showed
289 expected steady increases in body weight over the 10-day study period. No evidence of colitis
290 was observed in all untreated (controls) mice. In contrast, all mice treated with 2% w/v DSS
291 developed bloody diarrhoea within the last 5 days of the 10-day study. Histopathological
292 examination established the presence of DSS-induced colitis, which was localised mainly to
293 the distal part of the colon. Histological features of colitis observed included areas of mucosal
294 loss, inflammatory cell infiltration and oedema (Fig 2). Histological colonic inflammation
295 severity scores were significantly greater in mice consuming 100 ppm dietary iron and treated
296 with 2% w/v DSS, compared with those mice receiving 2% w/v DSS and ingesting a diet
297 containing 200 ppm or 400 ppm iron, at both day-8 and day-10 (Fig 3).

298
299 **Fig 1. This is the Fig 1 Title: Daily weight changes. This is the Fig 1 legend: Percentage**
300 **weight change in mice consuming diets containing iron [100 ppm (blue), 200 ppm (red)**
301 **and 400 ppm (green)] during dextran sulfate sodium (DSS)-induced colitis, and mice**
302 **consuming a diet containing 200 ppm iron without DSS treatment (orange) during the**
303 **10-day study period. Data are presented as a mean \pm standard error of the mean (SEM).**
304 **Statistical differences were assessed by Kruskal–Wallis test followed by multiple**

305 **comparisons (Conover-Inman) tests (* p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001).**
306 **(n=30 female mice per DSS-treated; n=22 mice per untreated group).**

307

308 **Fig 2. This is the Fig 2 Title: H & E histology. This is the Fig 2 legend: Representative**
309 **Haematoxylin- and eosin-stained sections of the distal colon from untreated and 2%**
310 **w/v DSS-treated mice. Mice received either water (control, I) or 2% w/v DSS for 5 days**
311 **followed by another 3 days on plain drinking water (II, III and IV) or 5 days on plain**
312 **drinking water (V, VI, and VII). Arrowheads highlight submucosal oedema; arrows**
313 **highlight almost complete loss of colonic epithelium.**

314

315 **Fig 3. This is the Fig 3 Title: Inflammation score. This is the Fig 3 legend: Inflammation**
316 **(colitis) scores for all groups of DSS-treated mice (n=16 [8-days] and n=14 [10-days]**
317 **mice per group) and untreated control mice (n=24) on diets containing different levels**
318 **of iron (100, 200 and 400 ppm). Horizontal lines represent medians. Significant**
319 **differences were assessed using one-way ANOVA followed by multiple comparisons**
320 **against untreated control by Dunnett's test; * p<0.05, ** p<0.01, **** p<0.0001.**

321

322

323 **Faecal calprotectin concentration in DSS-treated mice** 324 **during the 10-day course**

325 Faecal calprotectin concentrations were measured in faecal pellets collected from the cage of
326 each mouse in all groups at day-1, day-8, and day-10. Analysis of day-1 samples showed
327 similar levels (10.3 'mean') in all groups indicating no treatment effects were yet apparent.
328 Faecal calprotectin concentration data were normalised to the values found in control samples
329 with higher levels seen in the mice on modified (half and double of the standard content) iron
330 diet compared to those fed the standard 200 ppm iron diet (Fig 4). This finding was seen at
331 both day-8 and day-10 although the most striking levels were recorded at day-8 ($60 \pm 1.11\%$,

332 40 ± 1.12% and 80 ± 1.08% increase for the half of the standard, standard and the double of
333 the standard iron diets, respectively). The maximal faecal calprotectin levels were seen at day-
334 8 and correlated with the highest histological scores in DSS-treated mice that received 200
335 ppm and 400 ppm iron containing diets compared to their corresponding day-10 non-DSS
336 controls (p<0.001).

337

338 **Fig 4. This is the Fig 4 Title: Faecal calprotectin concentrations. This is the Fig 4 legend:**
339 **Faecal calprotectin concentrations at three different time points (day-1, 8 and 10) for**
340 **four groups, three DSS-treated groups (consuming diets containing 100, 200 and 400**
341 **ppm iron) and one untreated control group (consuming a standard 200-ppm iron**
342 **containing chow diet). Data are presented as a mean ± SEM. Significant differences**
343 **were identified using the Kruskal–Wallis test followed by multiple comparisons**
344 **(Conover-Inman) test; * p<0.05, *** p<0.001. (30 samples for all DSS-treated groups and**
345 **22 samples for untreated mice at each time point).**

346

347 **Faecal iron concentrations in DSS-treated mice**

348 Faecal iron concentrations were measured to investigate the net impact of dietary iron and
349 bleeding resulting from inflammation. Faecal pellets, from each mouse, were assessed for
350 faecal iron concentration (ferric and ferrous) from any (dietary and bleeding) source at the
351 different time points (day-1, 8 and 10). Data points from experimental groups were normalised
352 to the values found in the control samples (Fig 5). Faecal iron concentrations were increased,
353 at day-10, for all mice with DSS-induced colitis, compared to control mice, with the greatest
354 level of change being observed in mice receiving half of standard chow dietary iron levels, i.e.
355 100 ppm (Fig 5). DSS-treated mice receiving the standard levels of iron (200-ppm diet) had
356 significance (P<0.05) faecal iron concentrations at day-8 vs day-8 within the control group of
357 mice. Observed differences in faecal iron concentrations between mice on half of standard
358 chow dietary iron levels (100 ppm) and double the standard iron diet levels (400 ppm) were

359 not statistically significant. This suggests that colitis and bleeding likely had more pronounced
360 effects on the faecal iron concentration than the amount of iron consumed in the diet alone.

361

362 **Fig 5. This is the Fig 5 Title: Faecal iron concentrations. This is the Fig 5 legend: Faecal**
363 **iron concentration at three different time points (day-1, 8 and 10) for four groups, three**
364 **DSS-treated groups (consuming diets containing 100, 200 and 400 ppm iron) and one**
365 **untreated control group (consuming a standard 200-ppm iron containing chow diet).**
366 **Data are presented as a mean \pm SEM. Significant differences were identified using the**
367 **Kruskal–Wallis test followed by multiple comparisons (Conover-Inman) test; * $p < 0.05$.**

368

369 **Effect of iron on the microbiota composition in the colon** 370 **after DSS-induced colitis**

371 To determine the effect of DSS and oral iron on the gut microbiota, fresh faecal samples were
372 compared at baseline and the end of each experiment (after 10 days from the start). After
373 sequence processing and filtering, a total of 11,811,301 chimera-checked 16S rRNA
374 sequences ($166,356 \pm 59,353$ per sample) spanning a total of 204,331 OTUs were obtained.

375

376 Analysis of alpha-diversity (statistical significance of Shannon) indicated that there was a
377 significant reduction in species richness in faecal samples taken from the 400 ppm iron fed,
378 the DSS-treated group between day-1 and day-10 ($P < 0.0066$; Shannon diversity index) (Fig
379 6-a). To assess whether the differences in species richness were attributable to alterations in
380 the relative abundance of specific bacterial groups, we compared the proportions of various
381 taxonomic groups at the phylum level. Bacteroidetes was the most abundant phyla present,
382 followed by Firmicutes, Cyanobacteria and Proteobacteria (Table 2). Phyla changes were
383 seen in all DSS-treated groups when day-10 samples were compared to day-1. However,
384 these changes were only observed to be statistically significant for the mice consuming 400
385 ppm iron, with increases observed in the numbers of Proteobacteria (increased 1.40 ± 0.1 -

386 fold) and Actinobacteria (1.30 ± 0.1-fold increase) and concomitant reductions in Firmicutes
 387 (0.6 ± 0.1-fold) and Bacteroidetes (0.8 ± 0.04-fold); these changes have been explicitly
 388 accredited to Proteobacteria, Actinobacteria, Firmicutes and Bacteroidetes, which occurred in
 389 the presence of inflamed versus non-inflamed tissues even within the same group. Therefore,
 390 the double of the standard iron diet group had the highest relative abundance among inflamed
 391 (colitis) groups on the subject of reduction or increase changes (day-1 vs day-10) (Table 2
 392 and Fig 6-b).

393

394 **Table 2:** Comparison between all groups regarding proportions of bacteria at the phylum level at
 395 day-1 vs day-10

396

Taxonomy	Control Day-1	Control Day-10	100ppm DSS Day-1	100ppm DSS Day-10	200ppm DSS Day-1	200ppm DSS Day-10	400ppm DSS Day-1	400ppm DSS Day-10
Actinobacteria	6.63%	7.25%	6.77%	8.48%	7%	9.28%	7.35%	9.82%
Bacteroidetes	27.03%	24.98%	30.38%	30.73%	32%	31.33%	34.62%	27.27%
Cyanobacteria	19.13%	22.02%	19.80%	25.67%	20%	27.87%	20.70%	30.23%
Firmicutes	26.98%	24.15%	21.33%	6.20%	20%	10.2%	15.57%	6.22%
Proteobacteria	13.22%	15.17%	13.85%	17.55%	15%	19.65%	14.82%	20.58%
TM7	1.63%	1.63%	1.65%	2.02%	2%	2.27%	1.80%	2.48%
Tenericutes	2.42%	1.90%	3.55%	0.45%	1%	0.15%	2.53%	0.07%

397

398

399 **Fig 6. This is the Fig 6 Title: Relative abundance of bacteria. This is the Fig 6 legend:**
 400 **Effect of iron on the microbiota composition in the colon after DSS-induced colitis. (a)**
 401 **Shannon effective diversity boxplots display decreased numbers of dominant**
 402 **molecular species in all groups, day-1 versus day-10 of the study. (b) The Phylum-level**
 403 **taxonomic composition of all samples (average relative abundance). Ctr. = untreated**

404 **controls on a standard chow diet containing 200 ppm iron; DSS = 2% w/v dextran sulfate**
405 **sodium (DSS) treated mice on diets containing low iron (100 ppm), standard iron (200**
406 **ppm) and high iron (400 ppm) levels.**

407

408 We searched for differences between day-1 and day-10 samples by considering delta-values
409 calculated as differences in sequence abundances (before and after treatment). No,
410 statistically significant changes were observed in mice receiving diets containing half of the
411 standard iron levels where DSS was administered, despite showing similar trends to those
412 mice on double the standard diet iron levels (Fig 7).

413

414 **Fig 7. This is the Fig 7 Title: Proportions of sequences. This is the Fig 7 legend: Box**
415 **plot is showing the distribution in the proportion of four key phyla (Firmicutes,**
416 **Bacteroidetes, Actinobacteria and Proteobacteria) assigned to samples from all groups**
417 **at day-1 and day-10. Boxes indicate the interquartile ranges (75th to 25th IQR) of the data.**
418 **The median values are shown as lines within the box, and the mean values are indicated**
419 **by stars. Whiskers extend to the most extreme value within 1.5*IQR. Outliers are shown**
420 **as crosses. Statistical differences were assessed by Welch's t-test followed by Storey's**
421 **FDR multiple test correction.**

422

423 Principal Component Analysis (PCA) was used to identify linear combinations of gut microbial
424 taxa that were associated with specific diets. There was a clear separation of samples from
425 the mice consuming a chow diet containing 400 ppm before (day-1) and after (day-10) DSS-
426 treatment which was not seen in the other treatment groups (Fig 8). This suggests that DSS-
427 induced colitis, in the presence of double the standard level of dietary iron intake, affected the
428 bacterial community significantly more than that observed in all other diet groups ($P < 0.0066$;
429 Shannon diversity index) (Fig 6-a).

430

431 **Fig 8. This is the Fig 8 Title: Principal Coordinate/Component Analysis. This is the Fig**
432 **8 legend: Analysis of faecal microbiota shifts assessed by Principal**
433 **Coordinate/Component Analysis (PCA-PCoA) plots of the unweighted UniFrac**
434 **distances of pre-and post-DSS-intervention stool samples (I) PCoA ; all groups (II) PCA;**
435 **DSS-treated mice on diets containing low iron (100 ppm), standard iron (200 ppm) and**
436 **high iron (400 ppm) (b, c, and d respectively) and untreated control mice on a diet**
437 **containing standard 200 ppm iron (a) at phylum-level, phylogenetic classification of 16S**
438 **rRNA gene sequences. Symbols represent data from individual mice, colour-coded by**
439 **the indicated metadata. Statistical differences were assessed by Welch's t-test followed**
440 **by Storey's FDR multiple test correction.**

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457 Discussion

458 In this study, we used a murine model of IBD in which 2% w/v DSS was administered to mice
459 and investigated the impact of changing dietary iron intake on the degree of inflammation and
460 the bacterial components of the intestinal microbiome.

461

462 Alteration of iron content from standard chow diet levels (200 ppm) significantly influenced the
463 severity of colitis induced by DSS in mice. Clinically, for mice treated with DSS, those fed half
464 the standard iron levels developed more severe colitis (compared to those consuming chow
465 diets with iron levels at 200 ppm, or at higher levels of 400 ppm. DSS-treated mice that
466 received 100 ppm dietary iron significantly also lost more body weight than observed in the
467 other treatment groups. However, at molecular level increasing dietary iron 2-fold above
468 standard levels, to 400 ppm, led to worse inflammation and greater faecal calprotectin
469 concentrations at day-8, than was found in mice consuming a 100 ppm iron diet. Our
470 observation agrees with the findings of an earlier study performed by Carrier and colleagues
471 ³² in DSS-treated rats which emphasised the role of nutrient iron in modulating inflammation.
472 Specifically, the severity of colitis appeared to positively associate with the amount of iron
473 consumed. However, they did not investigate the effects of consumption of lower than normal
474 amounts of iron in their work ³². A study by Erichsen *et al.* ³³ reported that the addition of low-
475 dose oral ferrous fumarate (0.60 mg Fe/kg/d) to Wistar rats to levels present in standard chow
476 130 mg/kg (ferrous carbonate, 40 mg/kg; the remainder representing organic iron), also
477 increased the severity of DSS-induced colitis. In the same study, oral supplementation with
478 higher doses of ferrous fumarate caused a further increase in histological intestinal
479 inflammation ³³. Our study shows that a diet depleted in iron (100 ppm) can also exacerbate
480 colitis severity. The mice that consumed a diet containing 100 ppm iron, and treated with 2%
481 w/v DSS, showed greater increased intestinal inflammation than mice ingesting a standard
482 chow diet containing 200ppm iron, and treatment with 2% w/v DSS.

483 It has previously been suggested that iron formulations can be beneficial (ferrous bisglycinate)
484 or highly damaging (ferric ethylenediaminetetraacetic acid (FEDTA)) during DSS-induced
485 colitis experiments ⁹. Iron supplementation at different doses also induced shifts in the gut
486 microbial community and inferred metabolic pathways ⁹. Our findings indicate that any
487 significant alteration in standard dietary iron (above or below the standard chow levels of 200
488 ppm) may have a negative impact on the severity of DSS-induced colitis in mice.

489

490 For humans, faecal calprotectin measurement is commonly used as an assessment tool for
491 disease activity in IBD ^{34, 35}. We, therefore, used this additional approach and measured
492 murine faecal calprotectin levels to examine whether dietary iron levels affected inflammation.
493 The degree of colonic inflammation was found to be significantly higher for DSS-treated mice
494 receiving 400 ppm iron in their chow as assessed by faecal calprotectin concentration. The
495 histopathological changes observed were consistent with the faecal calprotectin levels
496 measured, which were higher at day-8 than at day-10, particularly in the high- and low-iron
497 fed, DSS-treated groups. A previous study in African infants by Jaeggi and colleagues ³⁶ also
498 noted that oral iron supplementation was associated with increased concentrations of faecal
499 calprotectin and with an increased rate of diarrhoea ³⁶. In contrast, a mouse study by Kortman
500 *et al.*³⁷ showed that faecal calprotectin concentrations were not influenced by dietary iron
501 intervention alone, but only following an enteric infection (*Citrobacter rodentium*), with faecal
502 calprotectin concentrations being significantly lower in mice consuming an iron-deficient diet.
503 Kortman *et al.* ³⁷ also found that Gram-positive *Enterorhabdus* appeared only after enteric
504 infection and its relative abundance, and faecal calprotectin concentrations observed, were
505 highest in a standard (45 mg/kg) dietary iron group ³⁷.

506

507 In the present study, all DSS-treated mice showed an increase in faecal calprotectin levels at
508 day-8; this was most prominent in the mice consuming 400 ppm dietary iron. However, all
509 DSS-treated groups showed greater levels of calprotectin in their stool at day-8 vs day-10.

510 This further supports the view that altering the standard levels of dietary iron may exacerbate
511 the severity of murine DSS-induced colitis.

512

513 One key source of iron accessible to the intestinal microbiota is unabsorbed, excess dietary
514 iron and any significant changes in luminal iron concentrations may have a potential impact
515 on structure, function and diversity of the intestinal microbiome^{36, 38}. Iron replacement therapy
516 is a common treatment in patients with anaemia and IBD, such as in Crohn's disease, although
517 such supplements may also influence intestinal inflammation as well as intestinal microbial
518 community structure and function^{32, 39}.

519

520 Measuring faecal iron concentrations would help to assess the severity of bleeding during
521 colitis. However, it is difficult to distinguish between the iron that comes from the diet and that
522 which has been released from red blood cells because of luminal bleeding during colitis.
523 Following a collection of faecal pellets at different time points from each mouse and calculating
524 the iron content (ferric and ferrous) (dietary and bleeding source) and comparing results
525 observed between groups at day-10, the absolute amount of faecal iron appeared to be
526 different for DSS-treated groups (3.3 -fold increase in half of the standard iron group, and 2.3-
527 fold increase in the double the standard iron group compared with the control group at day-
528 10). There was a significant increase in faecal iron at day-8, in standard iron diet group, but
529 not in the other groups. As there was as if an to increased (no significance) in faecal iron in
530 mice fed 100 ppm iron compared with those mice fed the standard chow diet level of 200 ppm,
531 this suggests that luminal bleeding may be a contributing factor to faecal iron quantitation in
532 the DSS-induced colitis model.

533

534 Overall in this study, changes (increase or decrease) in the iron content of the diet from
535 standard chow levels (200 ppm) appeared to significantly enhance colonic inflammation in a
536 DSS-induced mouse model of IBD. There appeared to be synergy between dietary iron levels
537 and DSS treatment of colonic inflammation and faecal calprotectin levels. Faecal iron

538 concentrations are known to be increased by inflammation, as well as oral iron intake³⁶. This
539 may explain the paradox in the half standard dietary iron fed group where luminal bleeding
540 during colitis caused an increase in the faecal iron concentration despite lower levels of iron
541 being consumed in the diet.

542

543 Changes in the microbiota are thought to be a major contributory factor in many human
544 diseases, including IBD^{40,41}. The most distinct phylum level alterations in IBD are a reduction
545 in the abundance of Bacteroidetes and Firmicutes and increased proportions of
546 Proteobacteria, in particular, increased numbers of bacteria from the family
547 *Enterobacteriaceae*^{14,40,42,43}. Murine models of IBD provide a means to investigate bacteria
548 in IBD²², and dysbiosis of the intestinal microbiota has been shown to induce murine colitis²³,
549⁴⁴. Here, we analysed inter- and intra-group differences and similarities between the intestinal
550 microbiota composition of 24 laboratory C57BL/6 mice (6 mice/group). Qualitative and
551 quantitative-based analysis of the faecal gut microbiota at two different time points (day-1 and
552 10) for DSS-treated groups (100, 200 and 400 ppm dietary iron) and untreated mice (controls)
553 was undertaken. Principal component analysis indicated an overlap of all microbial profiles,
554 except for the double standard dietary iron (400 ppm) fed DSS-treated mice. Based on the
555 PCA, ingestion of double the standard level of dietary iron was found to be the most important
556 factor responsible for clustering.

557

558 Some studies have shown that subsets of CD and UC intestinal tissue and faecal samples
559 have an abnormal gut microbiota, characterised by depletion of commensal bacteria, in
560 particular members of the phyla Firmicutes and Bacteroidetes, and an increase in
561 Proteobacteria^{14,43}. Doubling the standard level of iron in the chow diet (i.e. to 400ppm) here
562 led to significant alterations in microbiota composition in 2% w/v DSS-treated mice, with our
563 study showing a similar pattern of change to those observed in human IBD, including
564 increases in Proteobacteria and concomitant decreases in Firmicutes and Bacteroidetes.
565 Similar trends were found in the other DSS-treated groups of mice, but these changes in

566 microbiota composition did not reach statistical significance. An increase in the iron content of
567 the diet changed the microbiota after colitis was induced with DSS, which was not observed
568 in the standard or lower dietary iron groups. A shifting balance within the intestinal microbiota
569 could alter host immune response and open niches for the establishment of key
570 environmental-shaping bacteria in the intestine, for example, the significant decrease in
571 numbers of beneficial Firmicutes could create an opportunity for, and encourage the growth
572 of potential gut pathogens ⁴⁵. Bacteria species within the Firmicutes phylum are predominant
573 in the generation of short-chain fatty acids, particularly butyrate, from dietary metabolism of
574 insoluble fibre, resistant starches and fermentable soluble fibres (non-starch polysaccharides),
575 ⁴⁶, thereby providing a key anti-inflammatory effectors to ameliorate animal models of colitis
576 ⁴⁷.

577

578 This is the first study to use models of colitis to contemporaneously assess the influence of
579 dietary iron content on both disease activity and the microbiome. It emphasises the detrimental
580 effects of both halving and doubling the amount of iron in the diet on a murine model of IBD.
581 The diet with double the standard level of iron (400 ppm) led to key changes in the microbiome
582 and this would imply that these changes observed were not simply driven by the severity of
583 inflammation, but rather that luminal free iron can also contribute to the complex interaction
584 of factors that lead to the development of a dysbiotic state as has frequently been observed
585 in IBD. There is more to understand how all sources of luminal iron influence IBD.
586 Furthermore, work is needed to outline the physiological impact on the gut microbiota resultant
587 from increased availability of luminal iron and how this may affect bacterial phyla and diversity.
588 Future intervention studies in humans will be invaluable to further define the complex effects
589 of different doses of therapeutic oral iron on the human gut microbiota, particularly to
590 understand the metabolic consequences of observed phyla changes.

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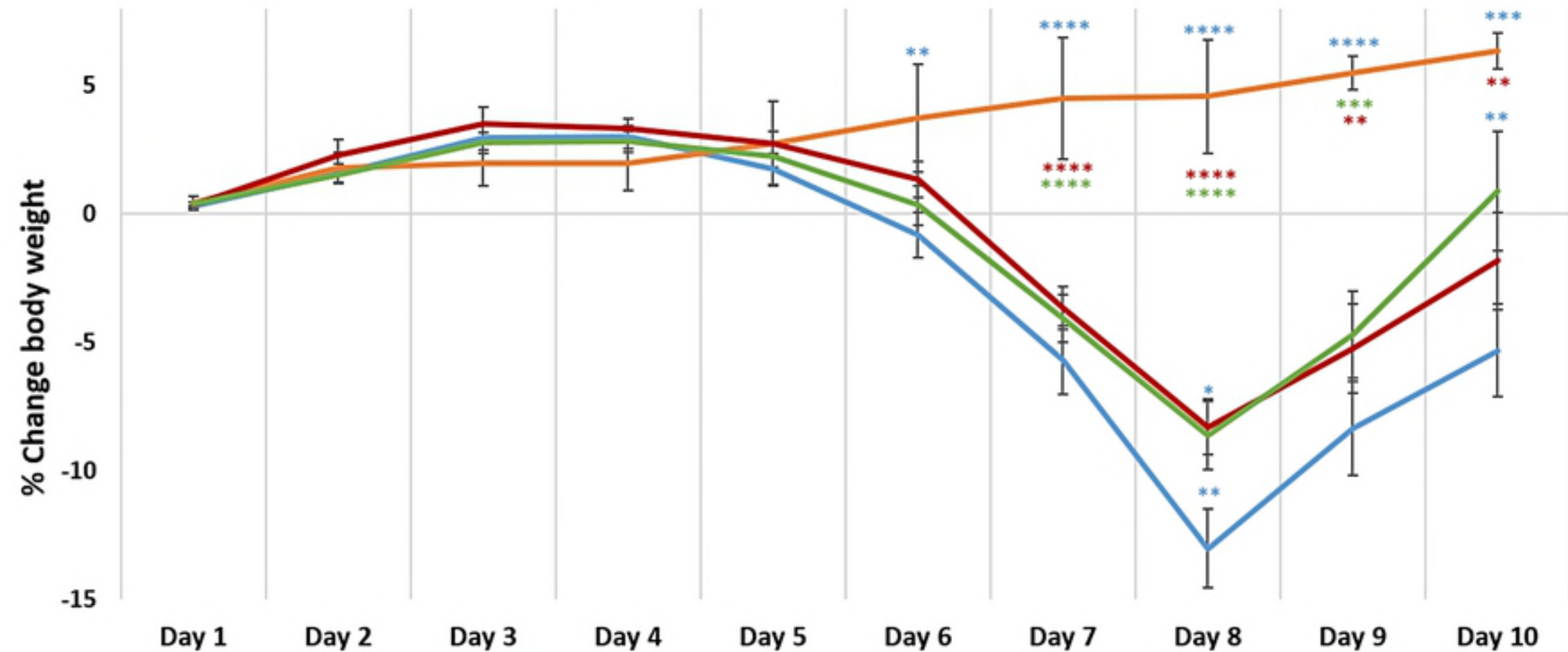
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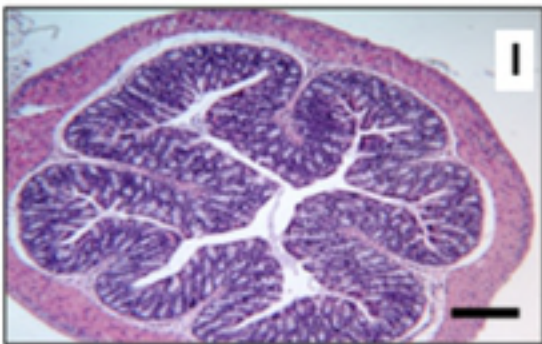
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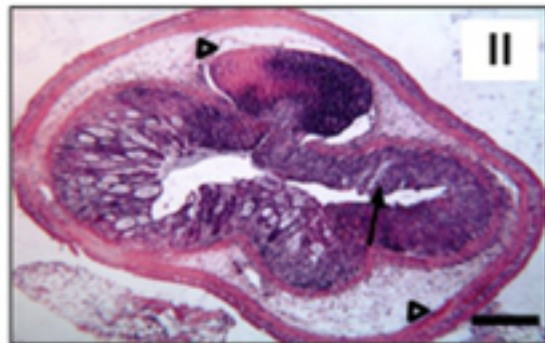
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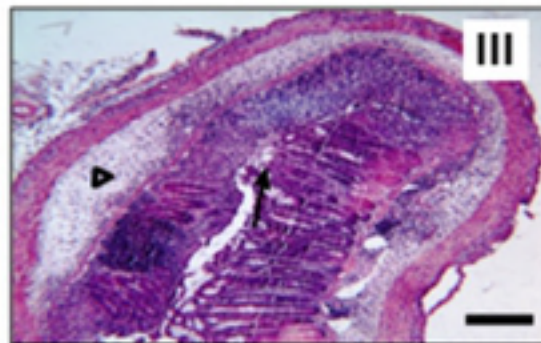
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DSS (2%)	+	-	+	+



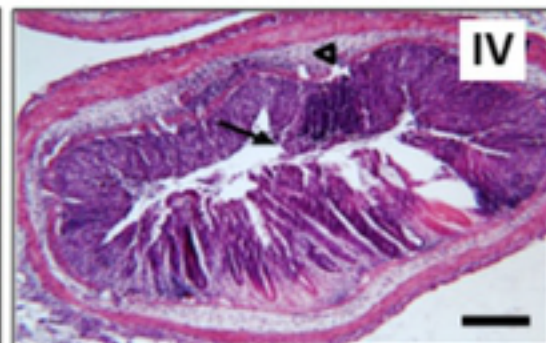
100ppm iron + 2% DSS



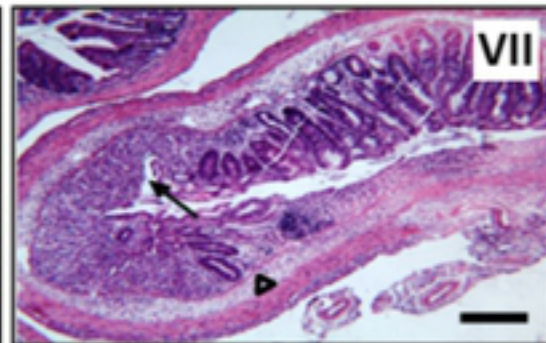
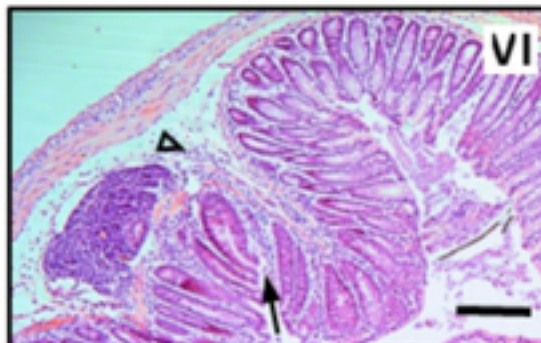
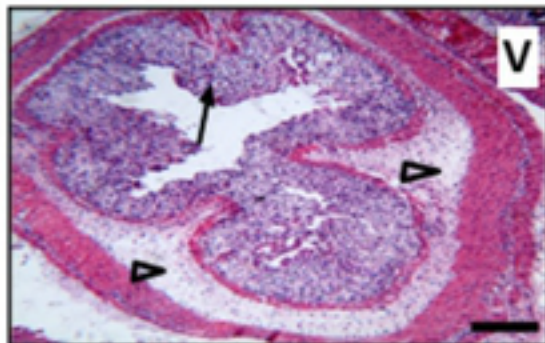
200ppm iron + 2% DSS



400ppm iron + 2% DSS

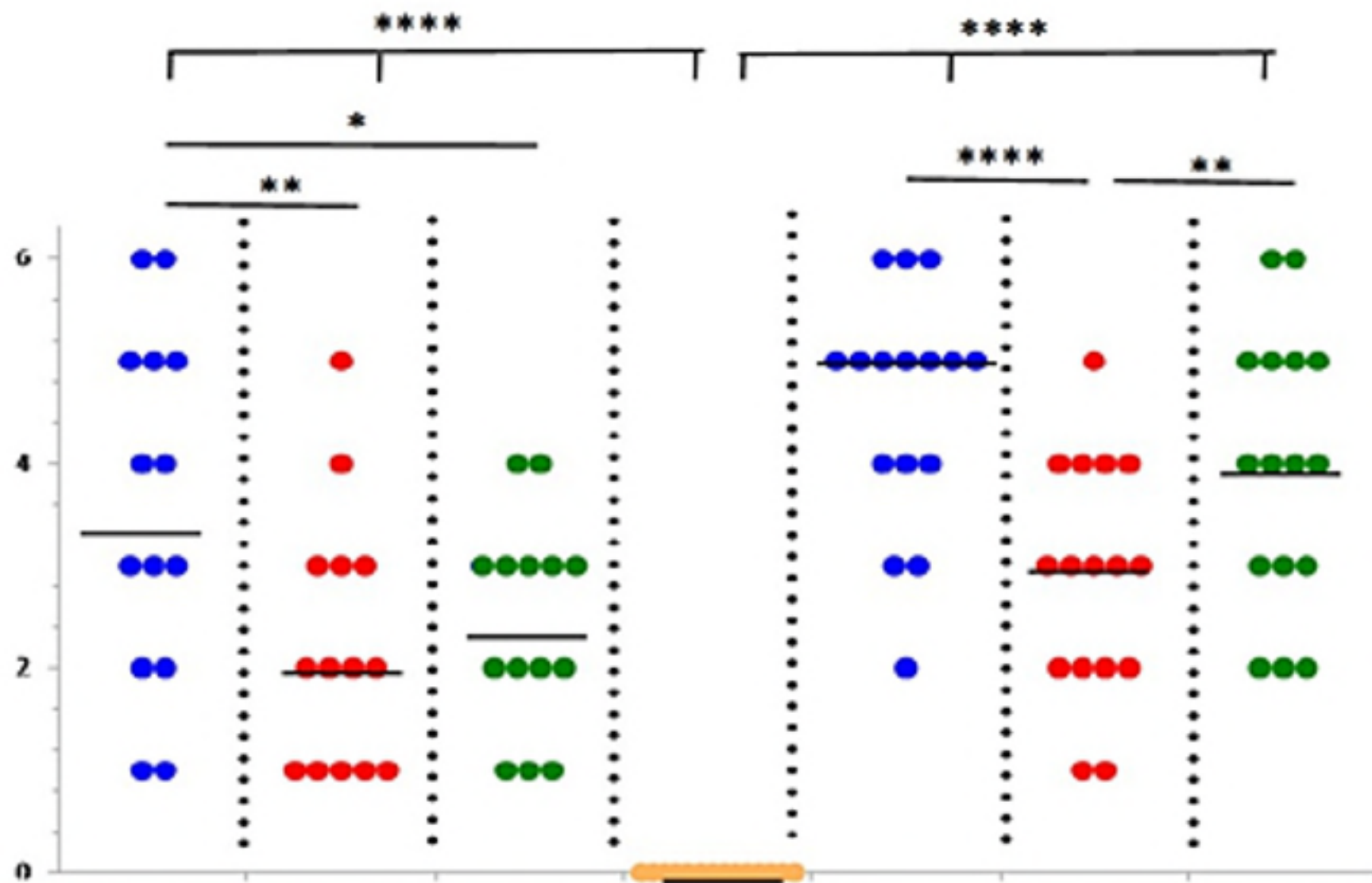


DAY 8



DAY 10

Inflammation score



Iron (ppm)

100

200

400

200

100

200

400

DSS (2%)

+

+

+

-

+

+

+

Time (days)

10

10

10

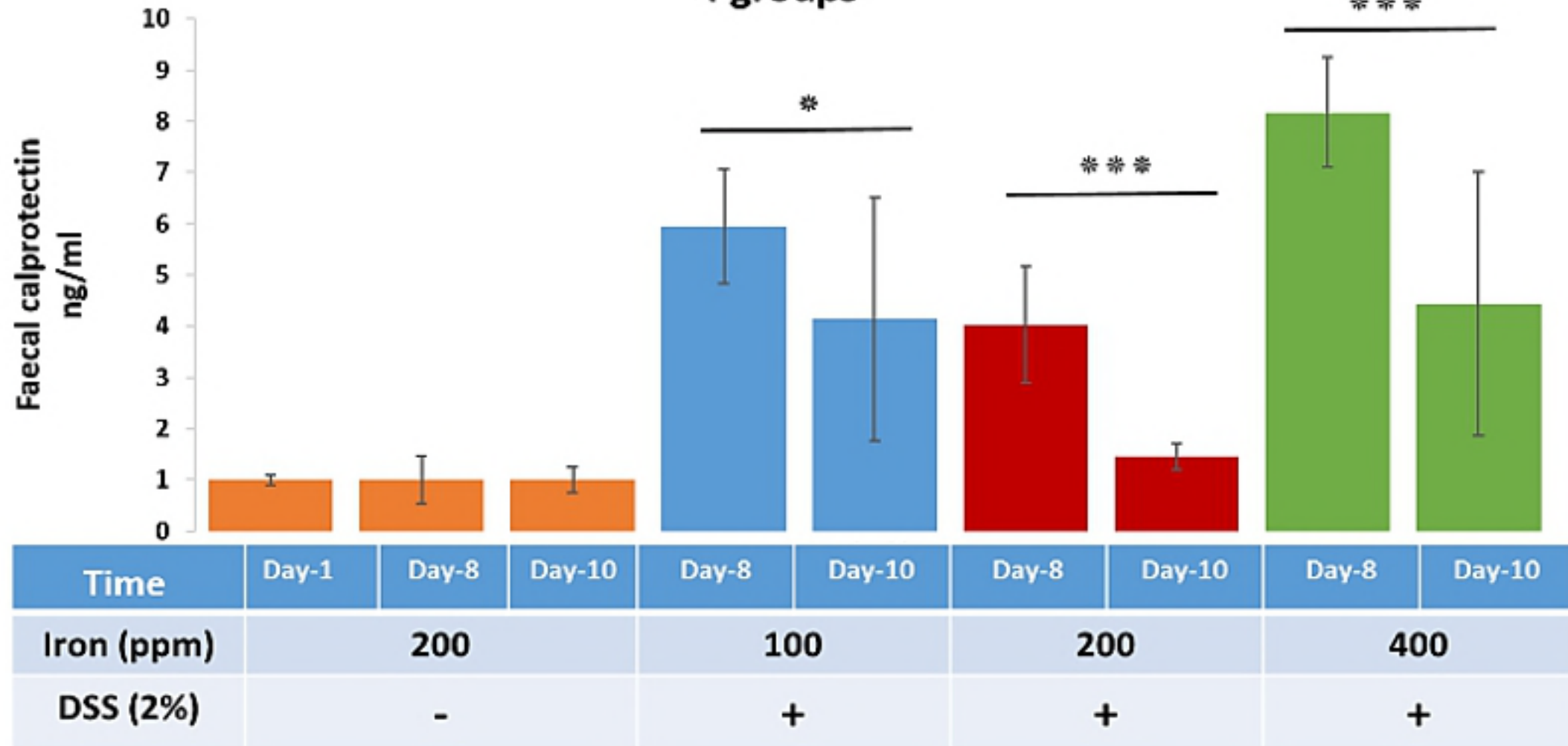
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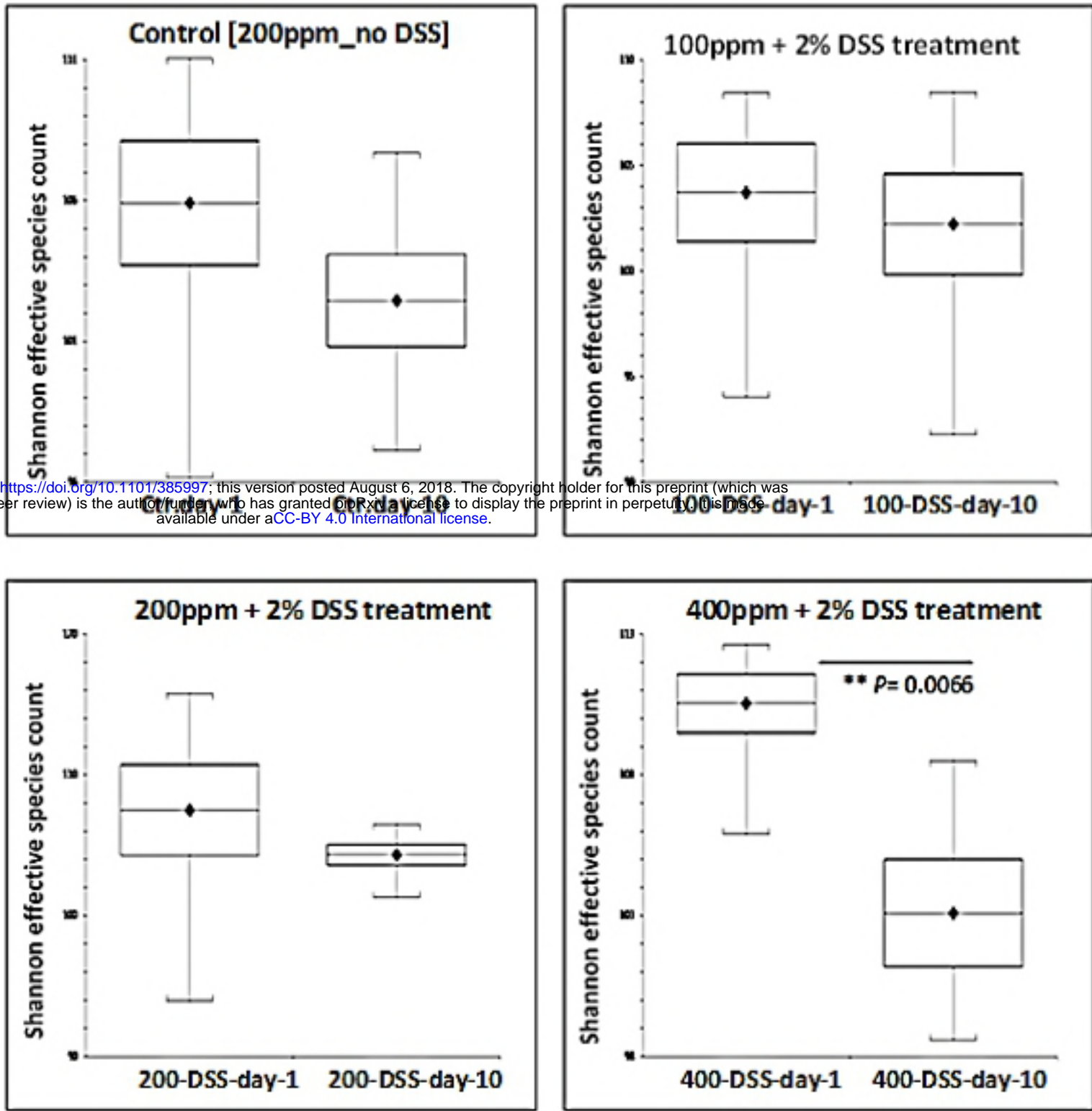
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Faecal calprotectin concentrations 4 groups



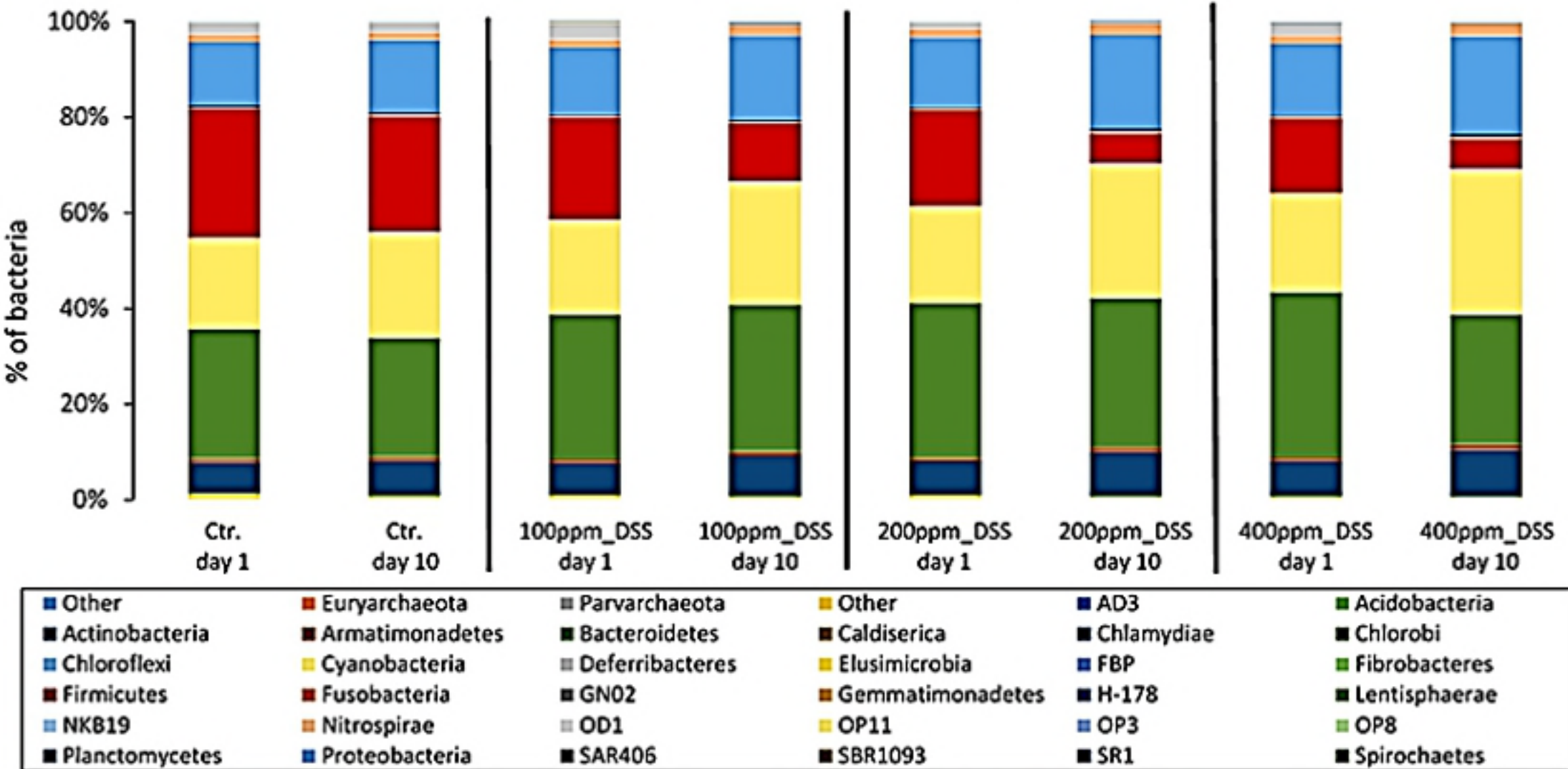
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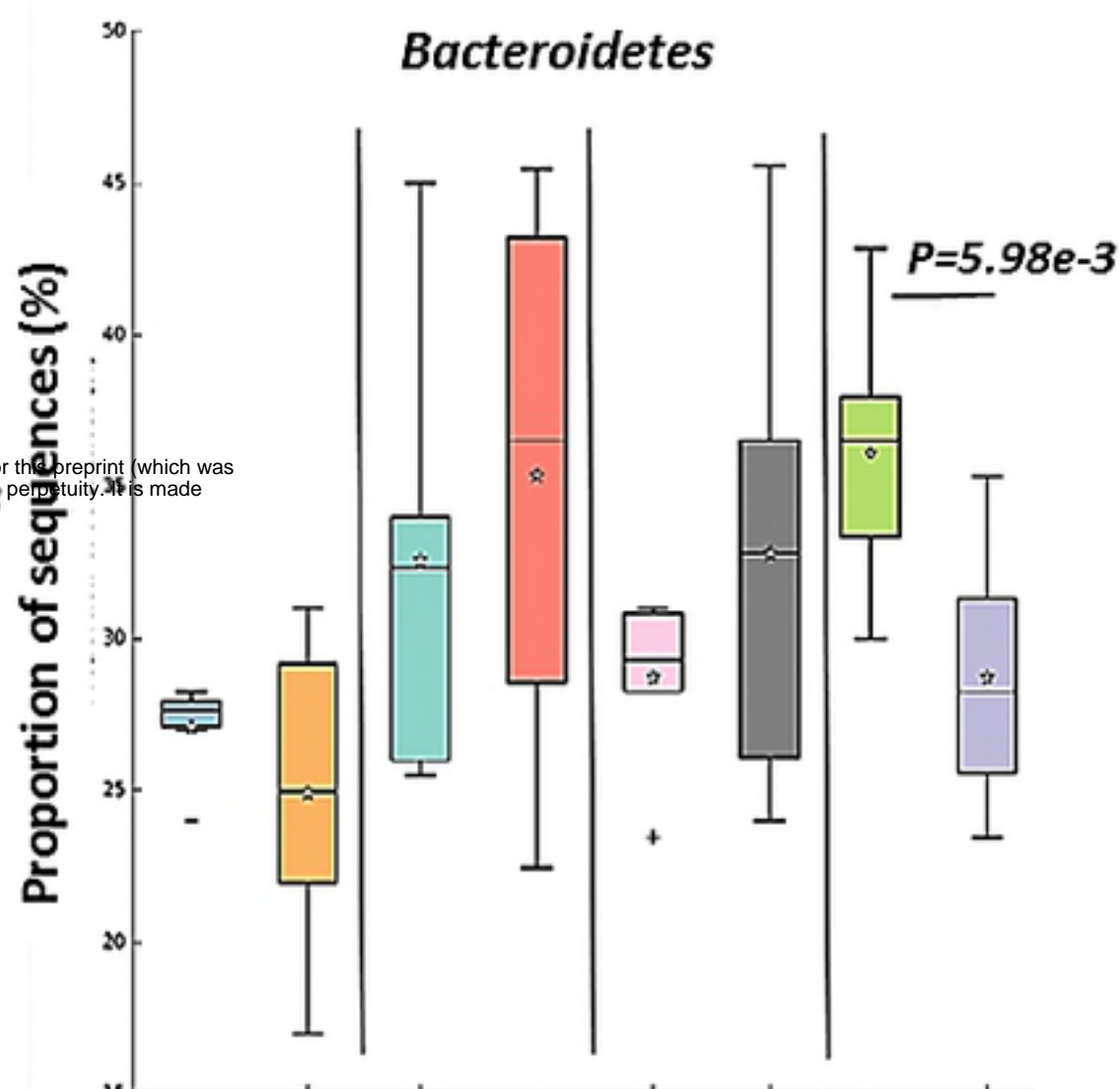
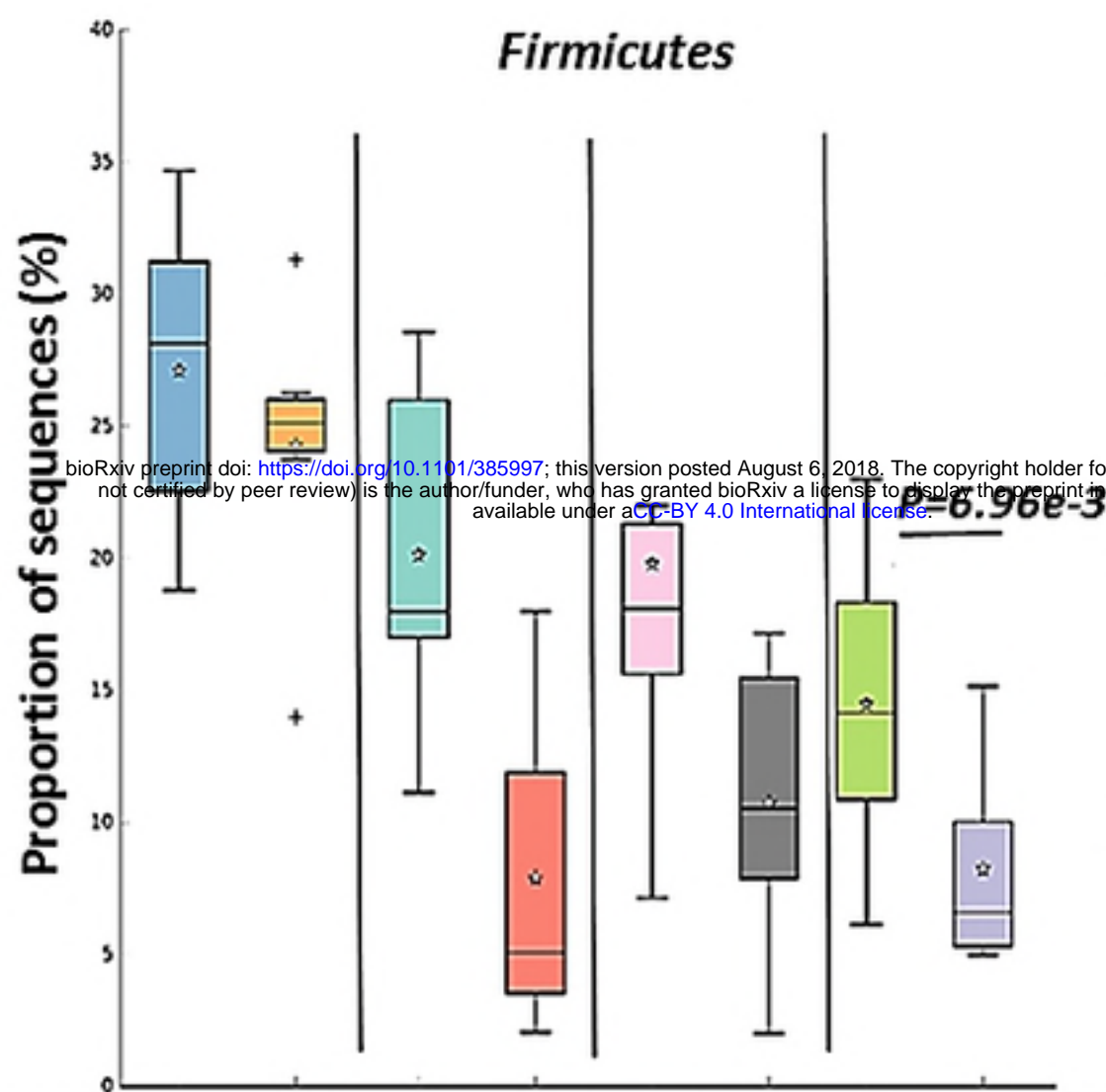


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(b)

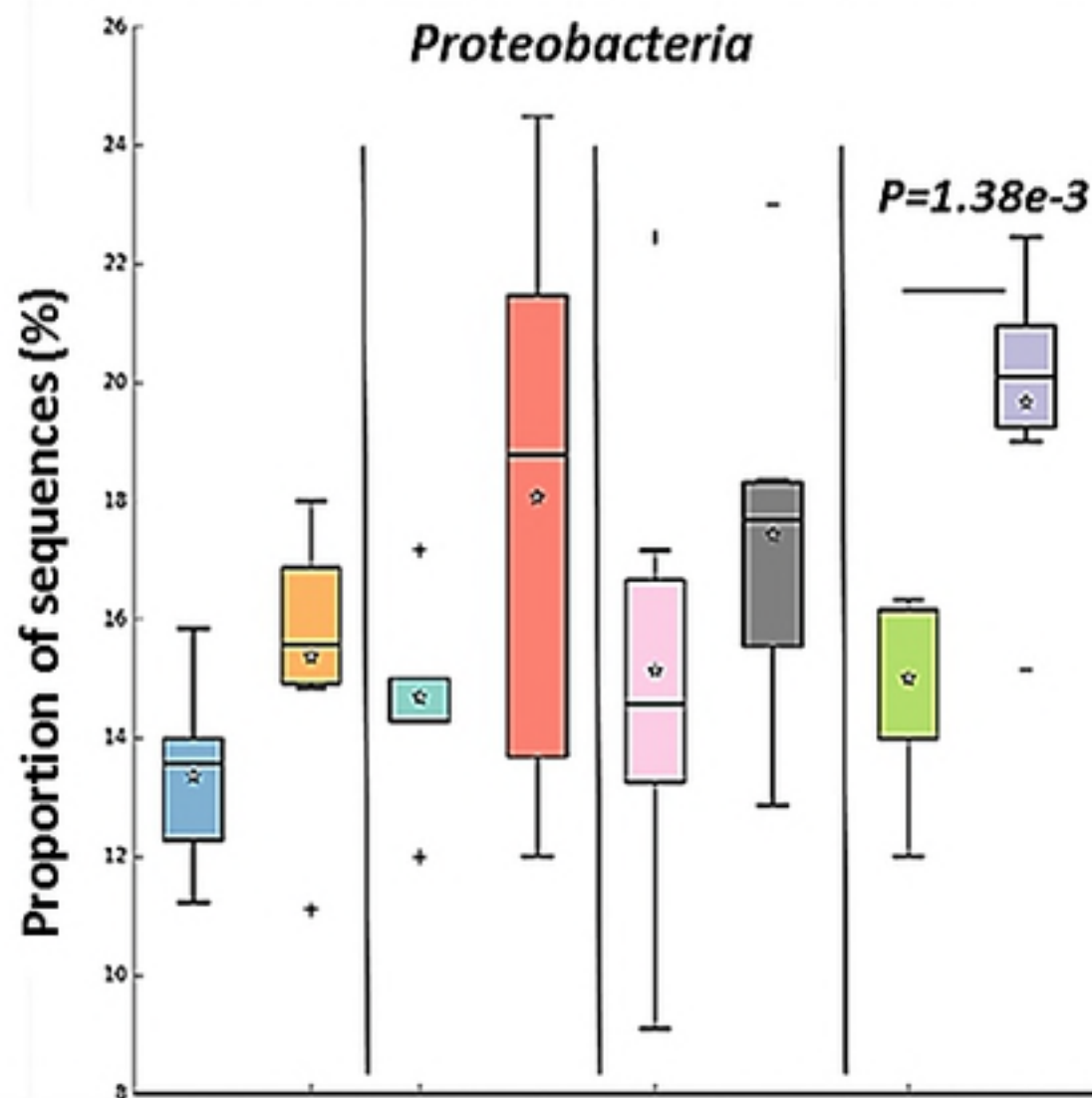
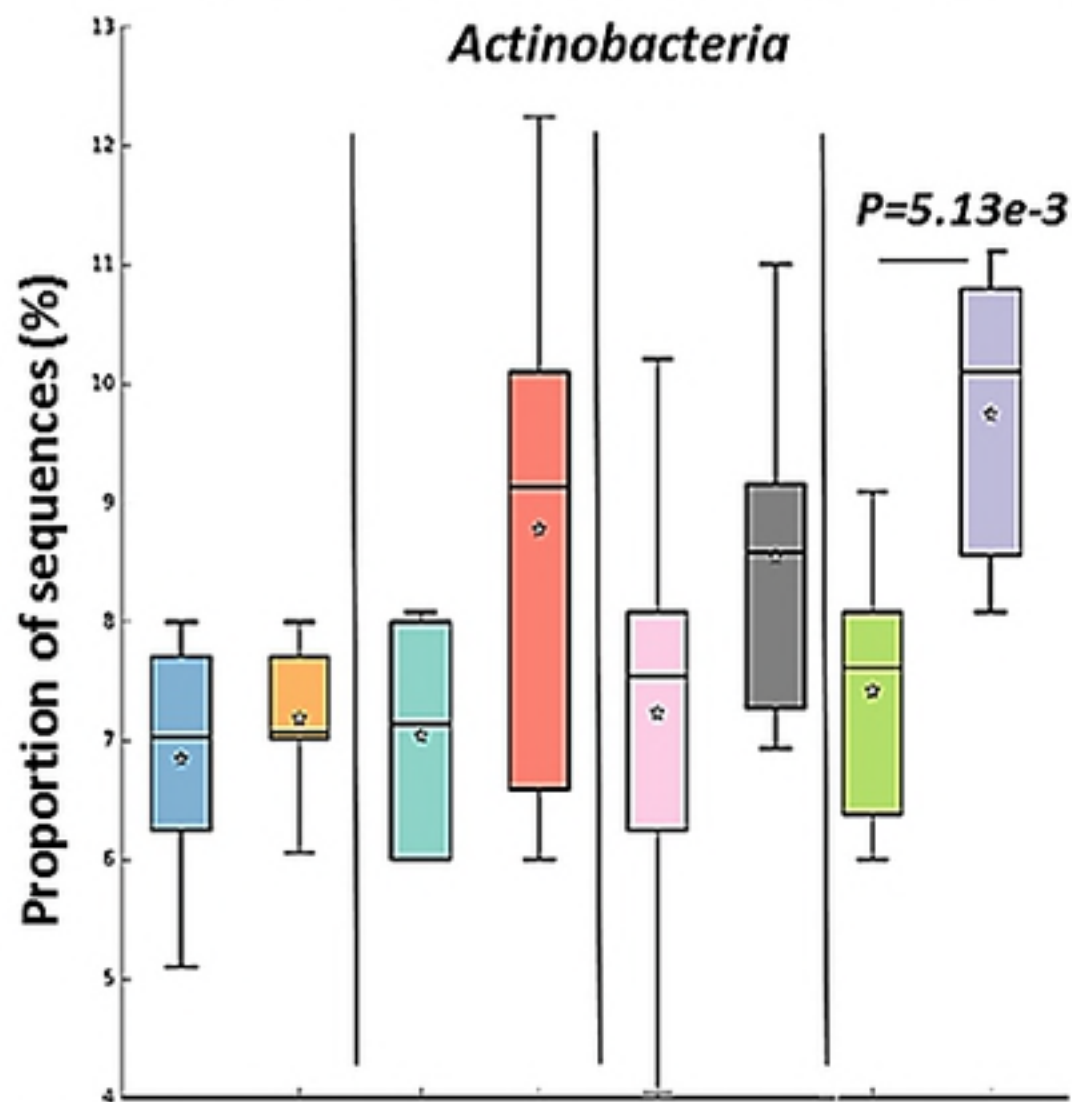
Phylum level taxonomic composition of all samples (relative abundance)





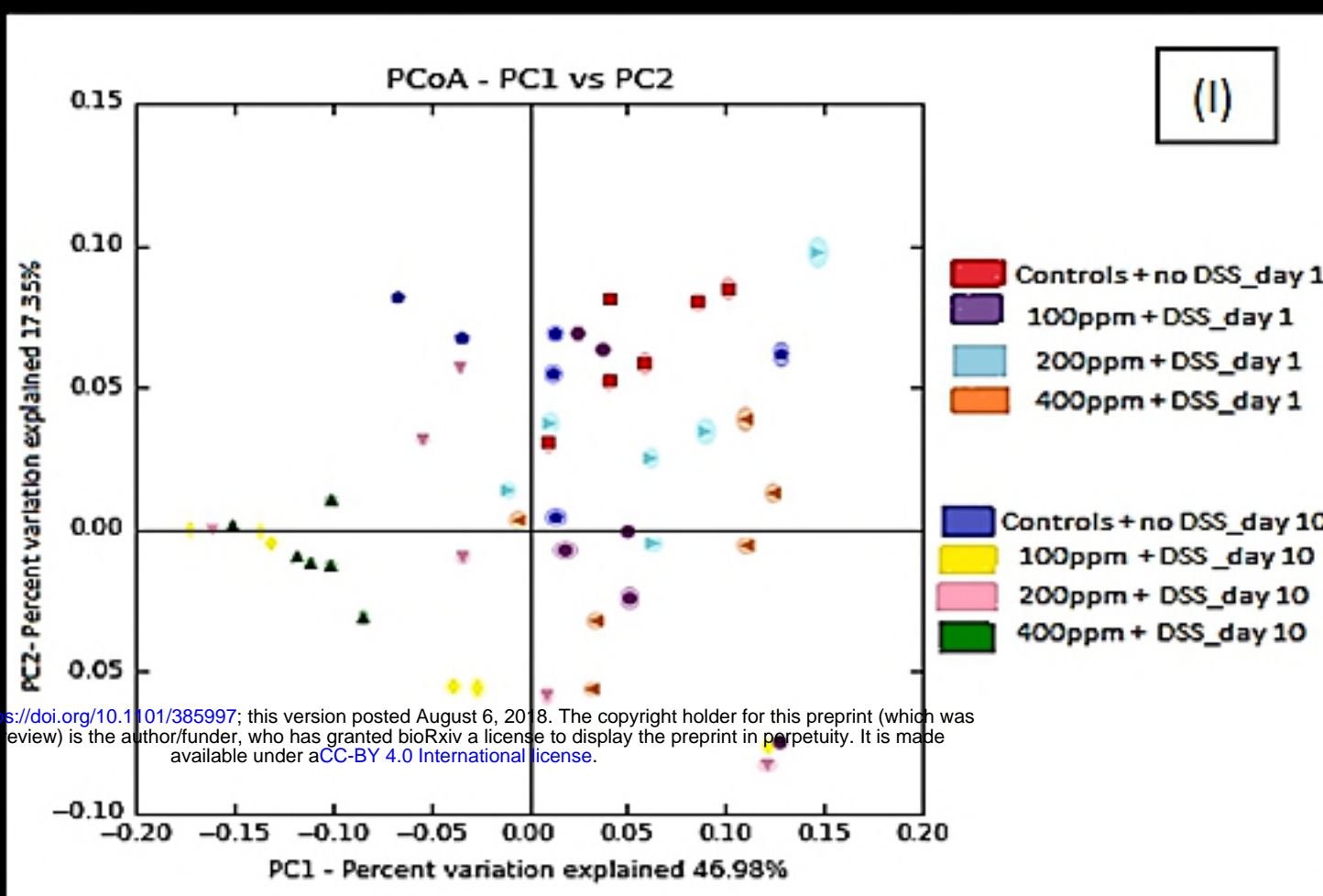
Time _{day}	1	10	1	10	1	10	1	10
Iron _{ppm}	200	200	100	100	200	200	400	400
DSS _{2%}	-	-	+	+	+	+	+	+

Time _{day}	1	10	1	10	1	10	1	10
Iron _{ppm}	200	200	100	100	200	200	400	400
DSS _{2%}	-	-	+	+	+	+	+	+



Time _{day}	1	10	1	10	1	10	1	10
Iron _{ppm}	200	200	100	100	200	200	400	400
DSS _{2%}	-	-	+	+	+	+	+	+

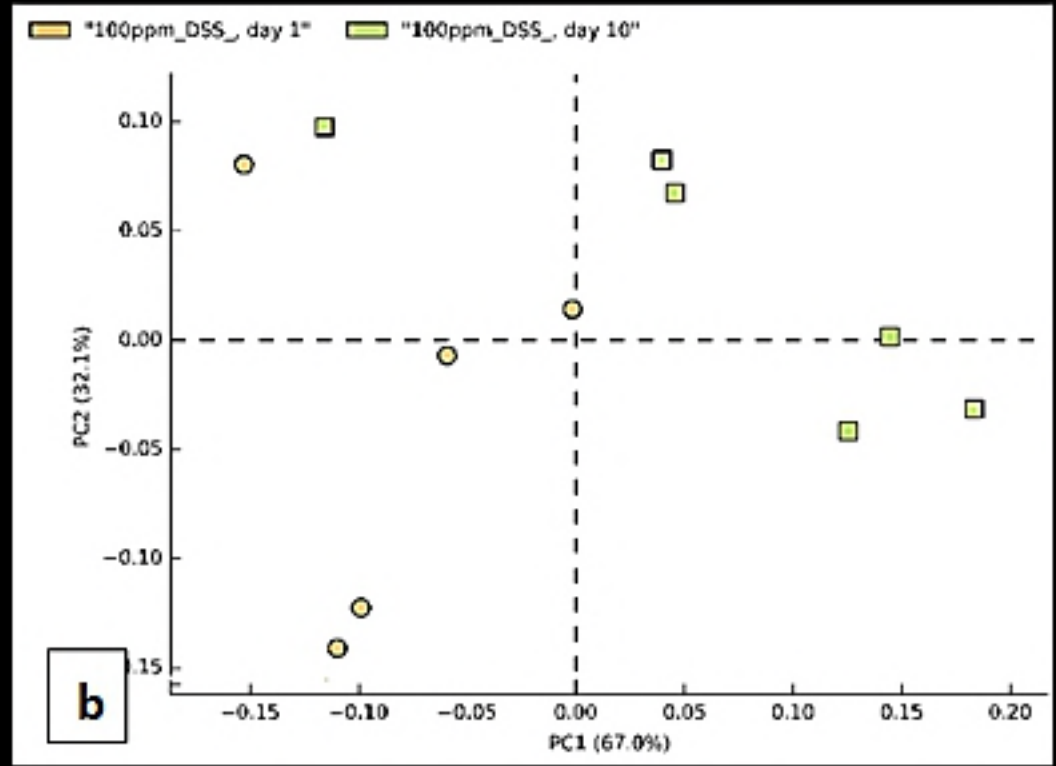
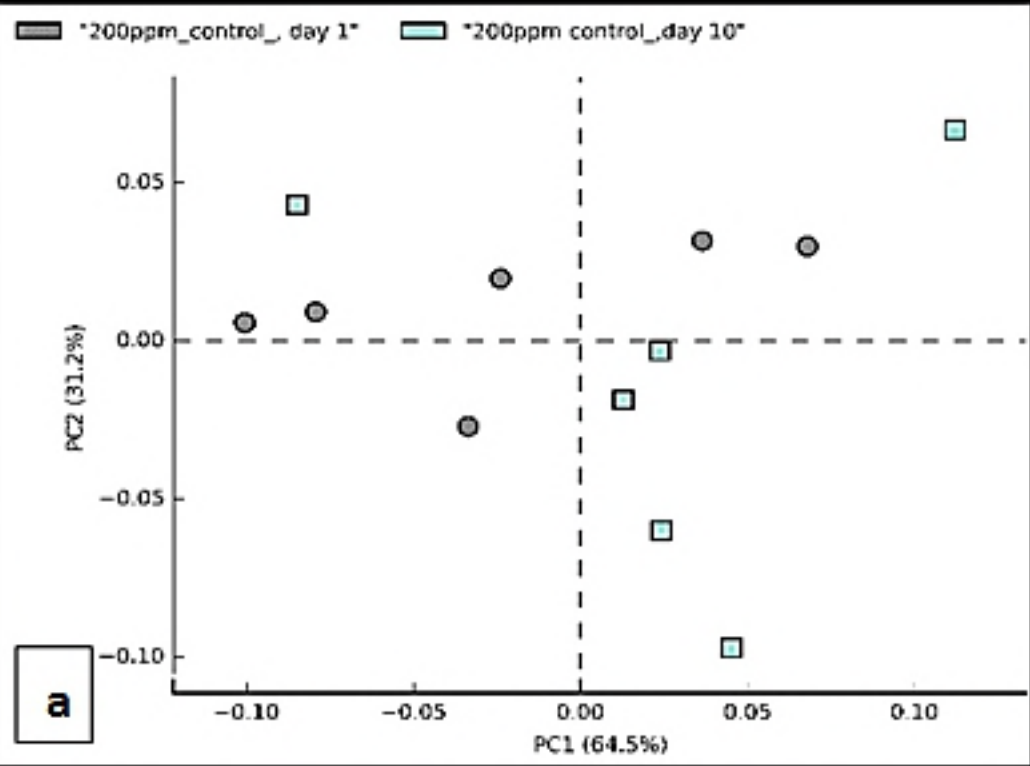
Time _{day}	1	10	1	10	1	10	1	10
Iron _{ppm}	200	200	100	100	200	200	400	400
DSS _{2%}	-	-	+	+	+	+	+	+



(II)

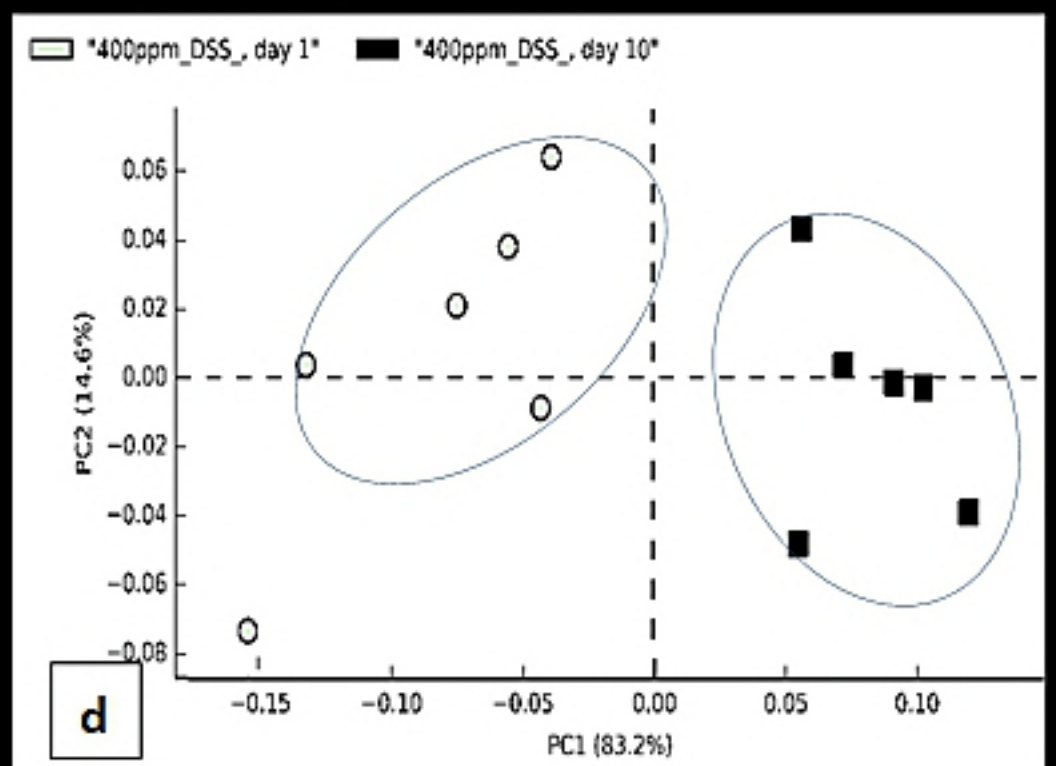
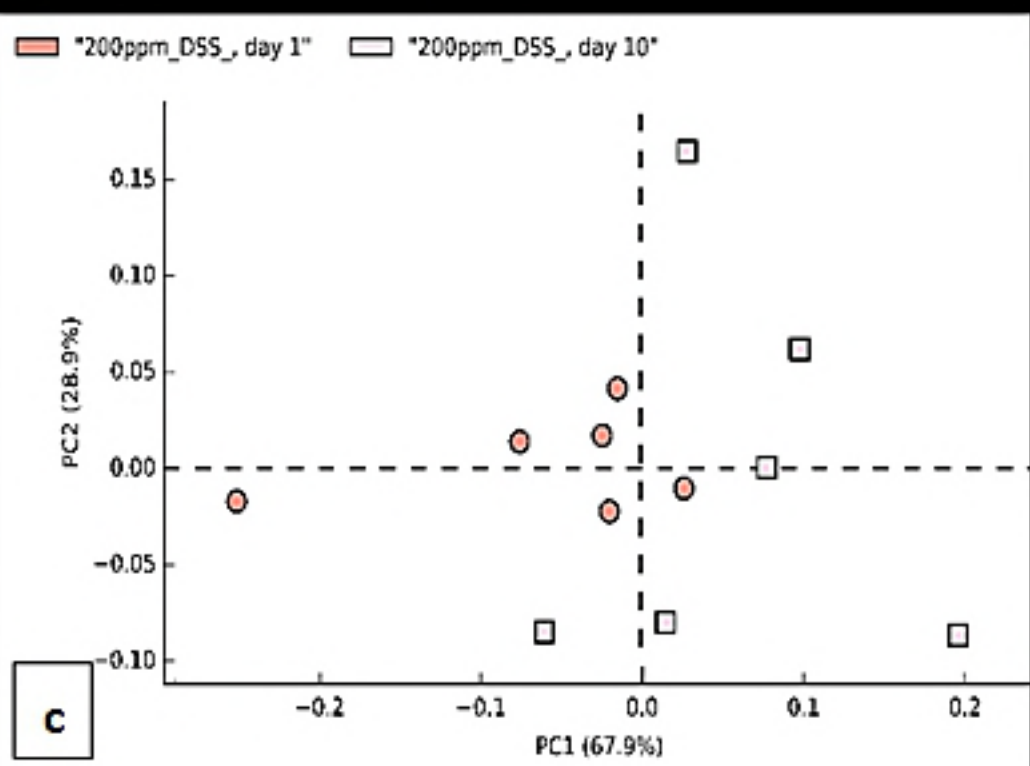
200ppm iron, Untreated

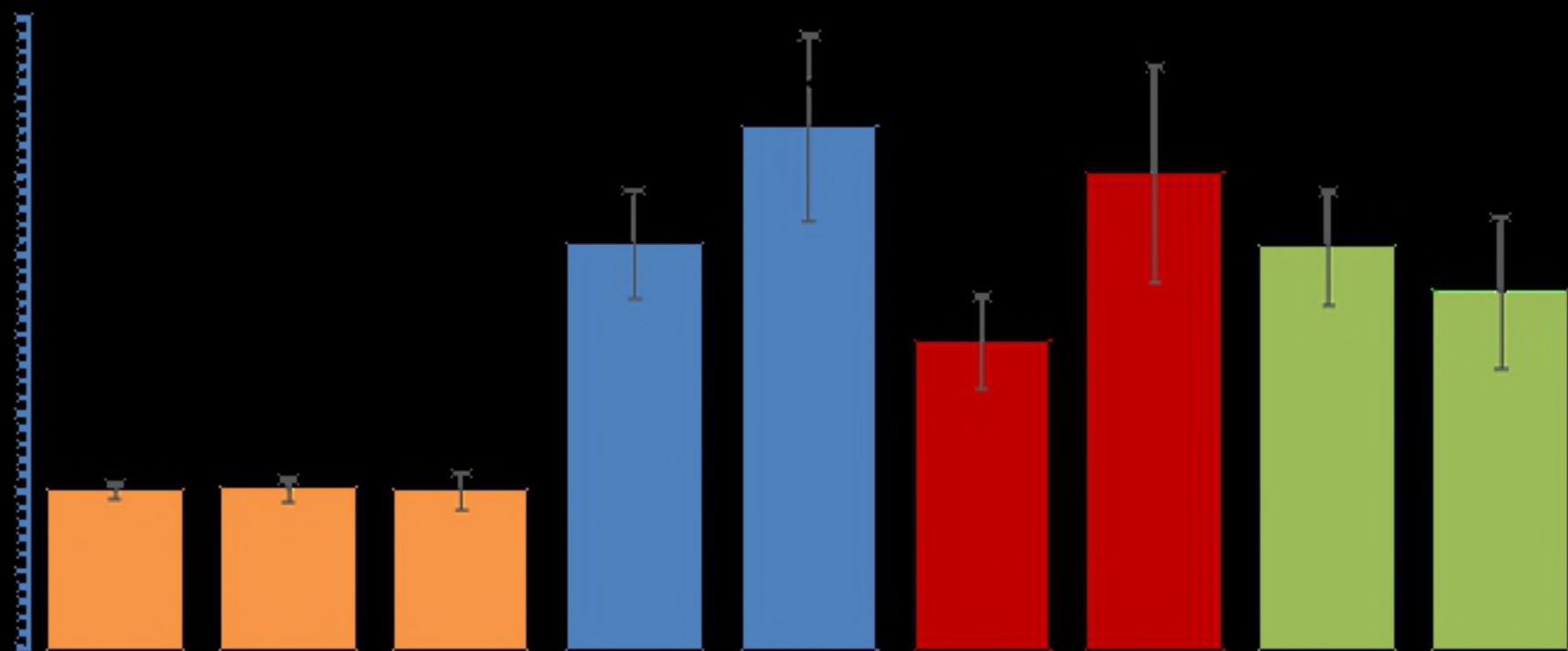
100ppm iron, DSS-treated



200ppm iron, DSS-treated

400ppm iron, DSS-treated





Time	Day-1	Day-8	Day-10	Day-8	Day-10	Day-8	Day-10	Day-8	Day-10
Iron (ppm)	200			100		200		400	
DSS (2%)	-			+		+		+	