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Effects of oral antibiotics and isotretinoin on the murine gut microbiota

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Abstract: Inflammatory bowel disease (IBD) may develop due to an immunogenic response to commensal gut microbiota triggered by environmental factors in the genetically susceptible host. Isotretinoin, applied in the treatment of severe acne, has been variably associated with IBD, but prior treatment with antibiotics, also associated with IBD development, confounds confirmation of this association. This study investigated the effects of doxycycline, metronidazole (frequently used in the treatment of acne and IBD, respectively) and isotretinoin on murine gut (faecal) microbiota after 2 weeks of treatment and after a 4-week recovery period. Faecal microbiota composition was assessed by 16S rRNA gene sequencing on the GS-FLX 454 platform with primers directed against the variable regions V1-V2. Doxycycline had a modest effect on bacterial richness and evenness, but had pronounced persistent and significant effects on the abundance of certain operational taxonomic units compared with the control group. In contrast, metronidazole induced a pronounced reduction in diversity after treatment, but these effects did not persist after the recovery period. This study demonstrates differential effects of antibiotics on the gut microbiota with doxycycline, unlike metronidazole, mediating long-term changes in the murine gut microbiota. Isotretinoin had no significant effect on the faecal microbiota.

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1 **Effects of oral antibiotics and isotretinoin on**
2 **the murine gut microbiota**

3
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22 **Abstract**

23 Inflammatory bowel disease (IBD) may develop due to an immunogenic response to commensal gut
24 microbiota triggered by environmental factors in the genetically susceptible host. Isotretinoin as
25 applied in the treatment of severe acne has been variably associated with IBD but prior treatment
26 with antibiotics, also associated with IBD development, confounds confirmation of this association.
27 We investigated the effects of doxycycline, metronidazole, which are frequently used in the
28 treatment of acne and IBD, respectively, and isotretinoin on murine gut (fecal) microbiota after 2
29 weeks' treatment and after a 4-week recovery period. Fecal microbiota composition was assessed by
30 16S rRNA gene sequencing on the GS-FLX 454 platform with primers directed against the variable
31 regions V1-V2. Doxycycline showed a modest effect on bacterial richness and evenness but
32 pronounced persistent and significant effects on the abundance of certain operational taxonomic
33 units compared to the control group. In contrast, metronidazole induced a pronounced reduction in
34 diversity post treatment but these effects did not persist during the recovery period. This study
35 demonstrates differential effects of antibiotics on the gut microbiota with doxycycline, unlike
36 metronidazole, mediating long-term changes in the murine gut microbiota. Isotretinoin had no
37 significant effect on the fecal microbiota.

38 **Highlights**

- 39 • Medications may modulate the gut microbiota favoring later development of IBD
- 40 • IBD is associated with prior antibiotic use. An impact of isotretinoin is uncertain.
- 41 • Isotretinoin, had no significant impact on community composition or diversity
- 42 • Metronidazole, induced a significant drop in diversity that recovered within 4 weeks.
- 43 • Doxycycline had modest direct effects but a lasting impact on composition at OTU level.

44

45 **Key Words** metronidazole, doxycycline, isotretinoin, dysbiosis, IBD, gut microbiota composition,
46 colitis, antibiotics

47 **Abbreviations**

48 ACE, Chao's Abundance based Coverage Estimator; CD, Crohn's disease; IBD, inflammatory bowel
49 disease; OTU, operational taxonomic unit; UC, ulcerative colitis

50

51 **1. Introduction**

52 The microbiota of the gastrointestinal tract has a profound influence on host physiology and nutrition,
53 including protection of epithelial cell barrier [1] and regulation of host fat storage [2]. Associations
54 between alterations in gut microbiota composition and a wide variety of pathologic conditions
55 including inflammatory bowel disease (IBD), obesity and associated insulin resistance, asthma, allergy,
56 cardiovascular disease and neurologic disorders [3] have been shown over the last decade. Yet, in
57 most cases, it is not clear whether alterations of the gut microbiota are causal or secondary to the
58 disease. However, in recent years an increasing body of evidence rather suggests the former, including
59 IBD-like microbial alterations in healthy siblings [4] as well as an increasing degree of hallmarks of
60 dysbiosis in correlation to the amount of genetic alterations [5]. A breakdown of host-microbial
61 mutualism triggered by environmental factors or genetic predisposition leading to dysbiosis and an
62 inappropriate and progressive immune response to the commensal gut microbiota [2] is assumed to
63 be causal for the pathogenesis of inflammatory bowel diseases (IBD) [6, 7].

64 The specific pathogenesis of IBD remains unclear, so far, but appears to be multifactorial. To
65 date, genome-wide association studies have identified 201 IBD susceptibility loci [8], affecting genes
66 involved in epithelial barrier function, mucosal immune response, autophagy and immune
67 regulation; a major fraction of these genes participate in the sensing of microbial products or affect
68 defense signaling in response to gut microbes [1]. However, host genotype explains only up to 20-
69 25% of IBD heritability overall, and 30-40% of CD and up to 10% of UC incidence [9]. Environmental
70 factors potentially contributing to IBD include diet, appendectomy, smoking, breastfeeding, personal
71 hygiene and medication(s) [6].

72 Evidence is increasing that antibiotics can influence established IBD, as well as on IBD flares,
73 and that they increase the risk of developing IBD in both children and adults [10-12]. However,
74 remarkably few studies have investigated the effect of individual antibiotics, the underlying
75 mechanisms or whether there are any long-term 'persistent' effects of antibiotics on the gut

76 microbiota [13-15]. Furthermore, a number of reports have claimed a potential association between
77 isotretinoin, a non-antimicrobial treatment for severe acne, and development of IBD [16, 17],
78 although a causal role has not been established [18, 19]. Isotretinoin is typically used in patients
79 unresponsive to antibiotics [11], thus, any causal relationship with IBD development is difficult to
80 confirm due to confounding antibiotic treatment.

81 In this study, we investigated the effects of doxycycline (used to treat acne but associated with the
82 development of IBD), metronidazole (one of the preferred antibiotic agents for IBD patients), and
83 isotretinoin on murine gut (fecal) microbiota after 2 weeks' treatment (immediate effects) and after
84 a 4-week recovery period (long-term effects). Our investigations aim at identifying possible
85 environmental stressors that might have an immediate or persistent impact on gut microbiota
86 composition that might have an impact on gut homeostasis and contribute to development of
87 inflammatory bowel disease later on.

88

89

90 **2. Methods**

91 **2.1 Animals and treatment**

92 In total, 164 female BALB/c mice were purchased from Charles River Laboratories (Germany) and
93 housed in individually ventilated cages per treatment in the animal facility of the University Hospital
94 Zurich, with access to rodent chow and water *ad libitum* (Figure 1A). Isotretinoin (30 mg/ml, F-
95 Hoffmann-La Roche Ltd, Basel, Switzerland), vehicle (rapeseed oil, *Brassica rapa*, Sigma-Aldrich, St.
96 Louis, MO, United States), metronidazole (107 mg/kg, Sigma Aldrich), doxycycline (43 mg/kg, Sigma
97 Aldrich) and water were administered orally for two weeks. For details of study design, animals per
98 group and sample collection see Figure 1.

99 **2.2 Sample preparation and 16S rRNA gene sequencing**

100 Total Genomic DNA from fecal samples was extracted using the PowerLyzer® PowerSoil® DNA
101 Isolation Kit (Mo Bio Laboratories, Inc., France) according to the manufacturer's instructions. The
102 hypervariable regions 1 to 2 (V1–2) of the 16S rRNA gene were amplified from isolated genomic DNA
103 using bacterial specific primer Pyro_27F (Adaptor B) and the barcoded reverse primer MIDx_338R
104 (Adaptor A) (Supplementary Table 1). The primer pair had specific 8 base long identifiers (barcode), a
105 linker sequence and sequencing adaptors as described earlier [20] (Supplementary Table 1).

106 Amplification reactions were performed in a total volume of 50 µl containing 5x HF buffer (New
107 England Biolabs, Ipswich, MA, USA), 10 mM deoxynucleotide triphosphate (illustra solution dNTP GE
108 Healthcare, Pittsburgh, PA, USA), 2'000 U/ml Phusion® High-Fidelity DNA Polymerase (New England
109 Biolabs, Ipswich, MA, USA), 10 µM Forward Primer Pyro_27F, 10 µM Reverse Primer MIDx_338R
110 (Metabion, Planegg, Germany) and 50 ng DNA diluted in DNA-free water.

111 PCR amplification was performed on a Thermocycler from SensoQuest with the following
112 cycling conditions employed: 98°C for 3 minutes, 25 cycles at 98°C each for 10 seconds, 55°C for 30
113 seconds, 72°C for 30 seconds and a final extension at 72°C for 10 minutes. Amplicons were run on a

114 2% agarose gel to allow isolation of the bands at 400 base pairs with extraction of amplicons
115 performed with MinElute Gel Extraction Kit (Qiagen AG, Hombrechtikon, Switzerland) and eluted in
116 55 µl DNase-free water. PCR products were pyrosequenced on the GS-FLX 454 platform at the
117 Functional Genomic Center Zurich (Zurich, Switzerland) and at Microsynth (Balgach, Switzerland).

118 **2.3 Bioinformatics**

119 Raw 454 sequencing reads were de-noised and de-multiplexed using the Amplicon Noise software
120 [21] as implemented in mothur [22] and based on mothur's Standard Operating Procedure for 454
121 data [23]. To filter for chimeric sequences, the UCHIME algorithm [24] was run in both *de novo* and
122 *reference-based* mode against a custom, global database of non-chimeric 16S sequences, as
123 described previously [25]. Sequences flagged as 'chimeric' by both algorithms were removed from
124 the dataset. Sequences were aligned against a secondary structure-aware model of the bacterial 16S
125 rRNA gene (provided in the package *ssu-align*) using Infernal [26, 27]. Alignments were pruned to
126 positions 100-357 in the reference model, and sequences that aligned poorly within this range (\geq
127 10% unaligned bases or \geq 20% gaps) were excluded from further analyses. After this filtering, the
128 dataset contained 2'098'361 aligned sequences of length 257 nt.

129 Sequences were clustered into operational taxonomic units (OTUs) at different similarity
130 thresholds according to the *average linkage* algorithms as implemented in hpc-clust [28]. Both
131 algorithms have been shown to provide consistent OTUs that approximate clustering of full-length
132 sequences for the 16S rRNA gene sub-regions targeted in this study [25]. Sequence taxonomy was
133 inferred using the ribosomal database project (RDP) Classifier [29] with default parameters. A
134 maximum likelihood phylogenetic tree of unique sequences was obtained using FastTree2 employing
135 default parameters [30]. Community diversity calculations and statistical analyses were performed in
136 R [31], and in particular using the packages phyloseq [32], vegan [33] and edgeR [34]. All data used in
137 this study are available online (National Center for Biotechnology Information (NCBI) Sequence Read
138 Archive project SRP065320).

139 **2.4 Community richness and evenness**

140 Estimates of inter-group and inter-mouse community richness and evenness were assessed per
141 sample in terms of Chao's Abundance-based Coverage Estimator (ACE), the Gini-Simpson Index and
142 the Shannon entropy index; calculation was based on OTUs clustered at 98% average linkage
143 sequence similarity.

144 **2.5 Detection of individual differentially abundant OTUs (taxonomic analysis)**

145 To investigate the fine-scale community composition at the level of individual OTUs, we used the R
146 package *edgeR* [34], as suggested previously [35]. This approach provides a statistical framework for
147 comparisons between treated and non-treated groups by quantifying the abundances of individual
148 OTUs found in each dataset. Representative sequences for every OTU per treatment and time point
149 that was significantly different from the respective control group was blasted against the NCBI 16S
150 rRNA reference database for fine-scale taxonomic annotation, employing the following thresholds:
151 $|\log_2(\text{fold change})| > 1$, FDR < 0.001 and match-ID $> 97\%$.

152

153

154 **3. Results**

155 **3.1 Immediate and long-term effects on microbiota phylum-level composition**

156 The microbiota composition of all 164 animals at phylum level, across all time points and treatment
157 conditions of the study, were as generally expected for murine gut microbiota (Figure 2A). The
158 dominant phyla were *Bacteroidetes* and *Firmicutes*, with very low abundances of *Proteobacteria* and
159 *Actinobacteria*; approximately 10% of sequences per sample could not be confidently classified at
160 phylum level when using the RDP Classifier with default parameters.

161 In fecal samples collected prior to treatment start, there were no notable differences evident
162 between the treatment and vehicle control groups for either the antibiotics (metronidazole,
163 doxycycline and water; multivariate analysis of variance on phylum-level taxonomic composition, $F =$
164 2.01 , $P\text{-value} = 0.0673$) or isotretinoin (isotretinoin and rapeseed oil; $F = 0.407$, $P\text{-value} = 0.802$)
165 treatment regimen. Following 2 weeks of treatment, microbiota composition at phylum level
166 differed significantly between the antibiotic groups ($F = 3.87$, $P\text{-value} = 0.002$); this difference
167 persisted after the recovery period ($F = 2.62$, $P\text{-value} = 0.016$). In contrast, isotretinoin treatment did
168 not induce significant changes from pre-treatment composition either immediately after treatment
169 ($F = 1.01$, $P\text{-value} = 0.419$), or after the recovery period ($F = 1.55$, $P\text{-value} = 0.25$). Notably, mice
170 treated with water or antibiotics showed generally elevated *Firmicutes* levels after the recovery
171 period when compared to mice treated with isotretinoin or rapeseed oil vehicle.

172 Changes in community composition at phylum level over time within a treatment group were
173 statistically significant for mice treated either with metronidazole (MANOVA, $F = 5.47$, $P\text{-value} < 10^{-5}$)
174 or doxycycline ($F = 3.86$, $P\text{-value} = 0.0013$). Metronidazole treatment induced a transient peak in
175 *Proteobacteria* in two out of five mice immediately after treatment with a relative abundance of up
176 to 34.2%; however, this increase in *Proteobacteria* was not persistent following the recovery period.
177 Interestingly, a mild longitudinal effect on phylum composition was also evident for the water
178 control group ($F = 2.25$, $P\text{-value} = 0.04$), although no individual phyla differed significantly between

179 time points. Isotretinoin ($F = 1.06$, $P\text{-value} = 0.403$) and rapeseed oil ($F = 1.199$, $P\text{-value} = 0.313$) did
180 not induce significant changes over time.

181 **3.2 Immediate and long-term effects on community composition**

182 The trends observed above at a very coarse resolution of phylum-level taxonomic composition were
183 consistent at the level of individual unique sequences, i.e. the highest possible taxonomic resolution.
184 Principal coordinate analysis of weighted UniFrac distances between samples calculated on a
185 maximum likelihood phylogenetic tree at single nucleotide resolution are shown in Figure 3. Before
186 treatment onset, phylogenetic community structure did not differ significantly between groups
187 either for antibiotic (permutational multivariate analysis of variance as implemented in the ‘adonis’
188 function of the R package ‘vegan’; $R^2 = 0.1226$, $P\text{-value} = 0.107$) or isotretinoin treatment ($R^2 =$
189 0.0442 , $P\text{-value} = 0.223$). As expected, both antibiotic treatments led to significant shifts in
190 community composition immediately after treatment relative to controls ($R^2 = 0.4499$, $P\text{-value} \leq$
191 0.001); in particular, metronidazole treatment induced a distinct shift relative to both doxycycline-
192 treated mice ($R^2 = 0.479$, $P\text{-value} \leq 0.001$) and mice administered water only ($R^2 = 0.445$, $P\text{-value} \leq$
193 0.001). The change in community composition of doxycycline-treated mice was less pronounced (R^2
194 $= 0.252$, $P\text{-value} = 0.015$). After the recovery period, community composition in doxycycline-treated
195 mice remained significantly different from control mice ($R^2 = 0.115$, $P\text{-value} = 0.046$). Interestingly,
196 the observed changes in community composition directly after metronidazole treatment did not
197 persist during the recovery period, with metronidazole-treated animals being indistinguishable from
198 controls ($R^2 = 0.025$, $P\text{-value} = 0.73$). In contrast, isotretinoin treatment did not lead to significant
199 alterations in community composition either directly after treatment ($R^2 = 0.0435$, $P\text{-value} = 0.229$)
200 or after the recovery period ($R^2 = 0.0678$, $P\text{-value} = 0.384$).

201 **3.3 Immediate and long-term effects on community richness and evenness**

202 Estimates of community richness and evenness assessed based on 98% average linkage OTUs using
203 Chao’s Abundance based Coverage Estimator (ACE), the Gini-Simpson Index and the Shannon

204 entropy index (Figure 2B-D) supported a generally large intra-group variation in these parameters
205 (Figure 4, left panel), and even shifts in diversity per animal over time based on paired observations
206 were generally unspecific within a given treatment group (Figure 4, right panel). Directly after
207 treatment, metronidazole induced a significant drop in community richness and evenness (ACE,
208 Figure 4A, left panel; Shannon index, Figure 4C, left panel), in comparison to all other groups. The
209 observed shift in Gini-Simpson index was not statistically significant. Similarly, directly after
210 treatment, richness as estimated by ACE showed a significant fall in doxycycline-treated animals
211 compared with isotretinoin-treated animals (P -value = 0.029), the water (P -value = 0.027) and
212 rapeseed oil-treated (P -value = 0.003) controls (Figure 4A). Isotretinoin-treated mice showed
213 significantly increased (P -value = 0.032) richness as estimated by the Gini-Simpson index relative to
214 rapeseed oil-treated controls directly after treatment, which appeared to be due to a decrease in
215 diversity in rapeseed oil-treated controls. No significant differences in richness or evenness were
216 evident between any of the treatment groups after the recovery period.

217 Changes in gut microbiota richness and evenness over time based on paired t-tests for
218 individual animals are shown in Figure 4A-C (right panel). Treatment with metronidazole induced a
219 significant fall in intra-animal evenness directly after treatment (Shannon index, P -value = 0.025),
220 which recovered to pre-treatment richness levels (ACE, P -value = 0.034) and even more diverse
221 composition (Shannon index, P -value \leq 0.001) after the recovery period. Microbiota richness and
222 evenness showed no significant change directly after treatment with doxycycline, but was
223 significantly increased after the recovery period compared with directly after treatment (ACE, P -
224 value = 0.044; Gini-Simpson, P -value = 0.008; Shannon, P -value = 0.006) and pre-treatment (Gini-
225 Simpson, P -value = 0.033). Isotretinoin treatment did not induce any significant changes per animal
226 over time. Interestingly, small but significant changes in gut microbiota evenness and richness were
227 also seen in the vehicle-control groups over time. Relative to pre-treatment baseline, diversity
228 showed a significant drop (Gini-Simpson, P -value = 0.008; Shannon, P -value = 0.013) in rapeseed oil
229 controls directly after treatment, but recovered during the recovery period. In animals administered

230 water, there was a modest but significant increase (P -value = 0.043) in community richness
231 (estimated by ACE) after the recovery period relative to pre-treatment levels, but otherwise there
232 were no significant effects per animal over time.

233 **3.4 Identification of individual OTUs associated to treatment conditions**

234 To identify individual taxa-treatment associations, we quantified significant OTU abundance shifts
235 per treatment relative to the respective vehicle-control group using the *edgeR* statistical framework
236 [34]. The results of this analysis at different levels of taxonomic resolution per treatment group and
237 time points are shown in Figure 5. Within the phylum *Bacteroidetes*, OTUs of the genera *Alistipes sp.*,
238 *Marinilabilia sp.* and 3 other *Bacteroidales* were highly abundant directly after the treatment period
239 and this increase persisted after the recovery period. At the same time, we observed a pronounced
240 decrease in OTUs of *Clostridiales sp.* and *Lachnospiraceae sp.*

241 Treatment with doxycycline led to higher numbers of significantly over- or under-represented
242 taxa in samples taken directly after the treatment period as well as in samples taken after the
243 recovery period. In samples taken directly after the treatment period, 18 OTUs showed a significant
244 increase in abundance and 33 OTUs showed a significant decrease in abundance. The greatest
245 number of taxa with decreased abundance directly after doxycycline treatment was found within the
246 phylum *Firmicutes*. Additionally, OTUs of the genera *Ruminococcus sp.* and *Hespellia sp.* were also
247 less abundant directly after doxycycline exposure, a change that persisted during the recovery
248 period. Moreover, after the recovery period in doxycycline-treated animals, 12 OTUs showed
249 significantly increased abundance and 16 OTUs showed a significantly reduced abundance. Notably,
250 the abundance of individual *Butyrivibrio sp.* and *Proteobacteria sp.* OTUs was found to be decreased
251 after the recovery period, only, but not directly after doxycycline treatment.

252 In metronidazole-treated animals, 20 OTUs (e.g. of *Enterococcus gallianarium* and
253 *Parabacteroides goldsteinii*) were observed to be highly abundant directly after treatment while 11
254 were less abundant (e.g. of *Hespilia sp.* and *Ruminococcus sp.*). Notably, 2 OTUs representing

255 *Proteobacteria sp.* were highly abundant in samples taken directly after treatment, corresponding to
256 findings in this study for taxonomic composition by RDP classifier. Comparable with changes seen in
257 community composition, richness and evenness, only 7 OTUs showed differential abundances during
258 the recovery period.

259 Isotretinoin treatment had a markedly less-pronounced effect on taxonomic composition than
260 the other agents evaluated. Directly after the treatment period, only 8 classifiable OTUs showed a
261 decrease in abundance (e.g. *Bacteroides acidifaciens*, *Ruminococcus sp.*, *Anaerotruncus sp.*) relative
262 to control animals with only a single OTU showing an increase in abundance (*Lachnospiraceae sp.*).
263 After the recovery period, no OTUs in isotretinoin-treated animals showed a significant change in
264 abundance.

265 Further analysis revealed that a number of individual OTUs showed persistent trends (e.g. *B_01*
266 or *F_04*), particularly after treatment with doxycycline but less marked for metronidazole, suggesting
267 an antibiotic-specific effect. Specifically, the abundance of *Bacteroidetes* OTUs (*B_01, 02, 03, 05, 06*)
268 were elevated directly after doxycycline treatment as well as after the recovery period relative to
269 control animals, while OTUs representing *Firmicutes* (*F_03, 04, 07, 11*) were less abundant for both
270 time points. Following treatment with metronidazole, OTUs annotated for *Bacteroidetes* were
271 specific; however, after the recovery period, change in *B_01* and *B_02* (increase) and in *B_07*
272 (decrease) was the same as in doxycycline-treated animals. This finding could either indicate a
273 common effect of both antibiotics or a potential time effect on these OTUs in the common
274 reference, the water-treated mice. Moreover, the abundance of *Firmicutes* (*F_02, 04, 05, 06, 08, 09,*
275 *10*) was decreased directly after the treatment period (comparable with doxycycline) but not after
276 the recovery period. Interestingly, *F_12* (*Lachnospiraceae sp.*) abundance was strongly decreased for
277 both time points and for both antibiotics. Isotretinoin treatment showed no changes in OTUs
278 common with those for the antibiotic groups.

279 **4. Discussion**

280 In this study, we have shown differential effects of antibiotics and isotretinoin on the gut microbiota
281 that support a putative association between doxycycline treatment, but not metronidazole or
282 isotretinoin, and IBD development. Overall, metronidazole induced a significant drop in diversity
283 directly following treatment, which returned to pre-treatment status during the recovery period. In
284 contrast, doxycycline induced only modest effects on diversity directly following treatment but a
285 persistent impact on composition after the recovery period, whereas isotretinoin had no significant
286 impact on community composition, richness or evenness either directly after treatment or after
287 recovery.

288 Distinct changes in gut microbiota composition have been reported in IBD patients [36] and in
289 animal models of colitis alterations in gut microbiota composition are associated with increased
290 disease risk and severity [37]. A history of bacterial gastrointestinal infections and antibiotic
291 treatment is reported to severely affect the intestinal microbiota and to be associated with the
292 development of gastrointestinal disorders such as IBD in children and adults [10, 12].

293 Recent studies have shown metronidazole, a nitroimidazole-based antibiotic frequently
294 prescribed for gastrointestinal-related disorders, including IBD, to be strongly associated with new-
295 onset IBD (odds ratio 5.01) [12, 38] and doxycycline to be associated with CD (odds ratio 2.25) [11]. A
296 putative association to IBD has also been reported for isotretinoin, a treatment for severe acne [11].
297 Confirmation of the putative association between isotretinoin and IBD has proved difficult due to
298 confounding antibiotic treatment and limited evidence on a potential immunogenic response to
299 commensal gut microbiota triggered by isotretinoin, or any long-term persistent effects on the gut
300 microbiota. This led us to investigate mouse fecal microbial composition before and directly after a
301 treatment course with metronidazole, doxycycline or isotretinoin as well as following a recovery
302 period.

303 In animal models, metronidazole treatment has provided contradictory findings, e.g.
304 compromised goblet cell function, decreased mucus layer thickness and increased microbial
305 stimulation of the epithelium as well as increased susceptibility to *Citrobacter rodentium* infection in
306 mice [39], and an increase in mucus layer thickness in rats [38]. In the present study, metronidazole
307 treatment was associated with pronounced changes in community composition and significant
308 reduction in bacterial richness and evenness directly after treatment, which recovered during
309 recovery phase. This finding is seemingly at odds with the reported strong association of
310 metronidazole with new-onset IBD [12]. However, the increases in *Proteobacteria* and the
311 facultative anaerobic species (e.g. *Enterococcus* spp.), marked reduction in *Clostridiales* and
312 relatively modest impact on *Bacteroides* was in accordance with the inherent specificity of
313 metronidazole. Jakobsson *et al.* [15] reported an increase in *Enterococcus* spp. and *Proteobacteria*
314 (*Klebsiella*) and a reduction in *Lachnospiraceae* spp. in human samples, while Sjölund *et al.* [40]
315 showed persistence of resistant *Enterococci* for up to 3 years. A recent study investigating the effect
316 of metronidazole on microbiota composition in a model of elderly colonic fermentation identified a
317 very pronounced shift in gut microbiota composition. *Clostridium* cluster IV, *F. prausnitzii* and
318 *Roseburia* spp. were the bacterial groups particularly affected by metronidazole treatment with
319 incomplete recovery after 10 days without treatment. [41]. Despite the pronounced alteration of gut
320 microbiota composition directly after metronidazole treatment in our study, only seven OTUs
321 remained significantly altered post recovery. Five of these (*B_01*, *B_02*, *F_07*, *F_12*, *F_13*) were
322 similarly impacted after the recovery period in doxycycline-treated animals. It might be
323 hypothesized, therefore, that some strains are particularly sensitive to treatment with antibiotics
324 (*F_07*, *F_12*), or are opportunistic to a disturbed microbiota (*B_01*, *B_02*, *F_13*). However, it is also
325 possible that there is a potential time effect on these OTUs in the common reference, the water-
326 treated animals. An increase or decrease of these OTUs in the water-treated group would skew the
327 abundances in both treatment groups (doxycycline and metronidazole) since they are compared
328 with each other.

329 In the present study, doxycycline was associated with only moderate changes in microbiota
330 richness and evenness immediately after treatment but, based on taxonomic composition analysis, a
331 strong shift mainly in *Clostridiales* (up to 500-fold down-regulation), with only a few strains of the
332 *Firmicutes* phylum showing marked increase in abundance, e.g. *Clostridium fusiformis* and
333 *Lactobacillus murinus*. Directly after treatment with doxycycline, *Firmicutes* strains were reduced
334 whereas *Bacteroidetes* showed a distinct pattern of OTUs that were either increased or decreased in
335 abundance; possibly mirroring the sensitivity of individual strains towards doxycycline. As the gut
336 microbiota is a complex ecosystem with networks of co-dependence between different strains, any
337 niche resulting from a reduction in *Firmicutes*, for example, will very likely be re-occupied by other
338 strains. The findings here, in fact, confirm the overall reduction in *Firmicutes* reported recently in
339 patients suffering from Q-fever endocarditis administered long-term doxycycline and
340 hydrochloroquine [42]. Notably, we did not see the reduction in *Bacteroidetes* reported by Angelakis
341 *et al.*, representing the only other study investigating the effect of doxycycline on the gut microbiota
342 [42]. Tetracyclines have been used as animal growth promoting agents in productive livestock for
343 several decades and the impact on gut microbiota composition has been reviewed recently [43, 44].
344 Most studies investigated the impact of low-dose chlortetracycline in pigs. Zhang *et al.* show an
345 increase in the phylum *Firmicutes* and the genus *Prevotella* [45] while Holman *et al.* observed only
346 minor alterations with sub-therapeutic doses of chlortetracycline [46]. A study investigating the
347 impact of combined administration of chlortetracycline and sulfamethazine on the bovine gut
348 microbiota did not identify any differences in bacterial community fingerprints or bacterial load in
349 comparison to the control group [47]

350 In our study, complete recovery of the microbiota was not observed even by 4 weeks following
351 cessation of doxycycline treatment: The treatment groups showed little differentiation in community
352 richness or evenness but changes in OTU-abundances. Overall community composition remained
353 different from controls, most notably *Bacteroides* OTUs elevated directly after treatment, remained

354 elevated post recovery (*B_01,02,03,05,06*) whereas those *Firmicutes* OTUs, reduced directly after
355 treatment (*F_03,04,07,11,12*) remained so by 4 weeks after cessation of treatment.

356 Such persistent changes at OTU level might be speculated to promote the development of
357 colitis in a susceptible host. To date, reduction in diversity, temporal instability and over- (e.g.
358 *Desulfovibrio*) or under-representation (e.g. *Faecalibacterium prausnitzii*) of individual strains have
359 been shown in UC and new-onset CD In addition, decreased abundance of the genera
360 *Faecalibacterium*, *Roseburia* and *Clostridiales*, and increased abundance of *Enterobacteriaceae*, are a
361 consistent finding in CD patients [36, 48]. Thus, our findings of a persistent reduction of *Clostridiales*
362 OTUs are congruent with these reported alterations in microbiota in CD patients. Nevertheless,
363 further investigations are needed to establish a causal relationship.

364 In summary, these findings demonstrate differential effects of antibiotics on gut microbiota
365 community composition and diversity, with doxycycline mediating long-term changes in the murine
366 gut microbiota. In contrast, the microbiota profile of isotretinoin-treated animals was not
367 significantly affected, providing no evidence that isotretinoin impacts the risk for IBD development
368 through effects on the gut microbiota.

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372 **Competing interest**

373 No author receives stipends or acts in any other consultative capacity for Roche. Roche had no
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376 analyses performed or data interpretation. Decisions as to the data presented were made solely by
377 the investigators with no input from Roche. Manuscript development, review and editing were
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379 resources for medical writing and editorial support, with no access to content.

380 **Ethical approval**

381 All animal experiments were approved by the cantonal veterinary office of Zurich under license
382 numbers ZH-54-2011 and ZH-214-2016. All animal experiments were performed in accordance with
383 Swiss national law for animal welfare and in accordance with the minimal standards for laboratory
384 animals defined by the institute for laboratory animal science of the University of Zurich.

385

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508

509 **Figure Legends**

510 **Figure 1. Experimental design.** (A) BALB/c female mice were treated with isotretinoin, rapeseed oil
511 (isotretinoin vehicle), metronidazole, doxycycline or water (antibiotics vehicle) daily by oral gavage
512 for 2 weeks. Fecal samples were collected before treatment, after two weeks of treatment
513 (immediate effects) and after a recovery phase of four weeks post-treatment cessation (long-term
514 effects). (B) For isotretinoin and rapeseed oil, 16 animals per group were sampled before and
515 immediately after treatment, and 8 animals were sampled after the recovery phase. For
516 metronidazole, doxycycline and water, 5–12 animals were sampled per time point and group. (C) No
517 differences within treatment groups were registered with respect to body weight over all time
518 points.

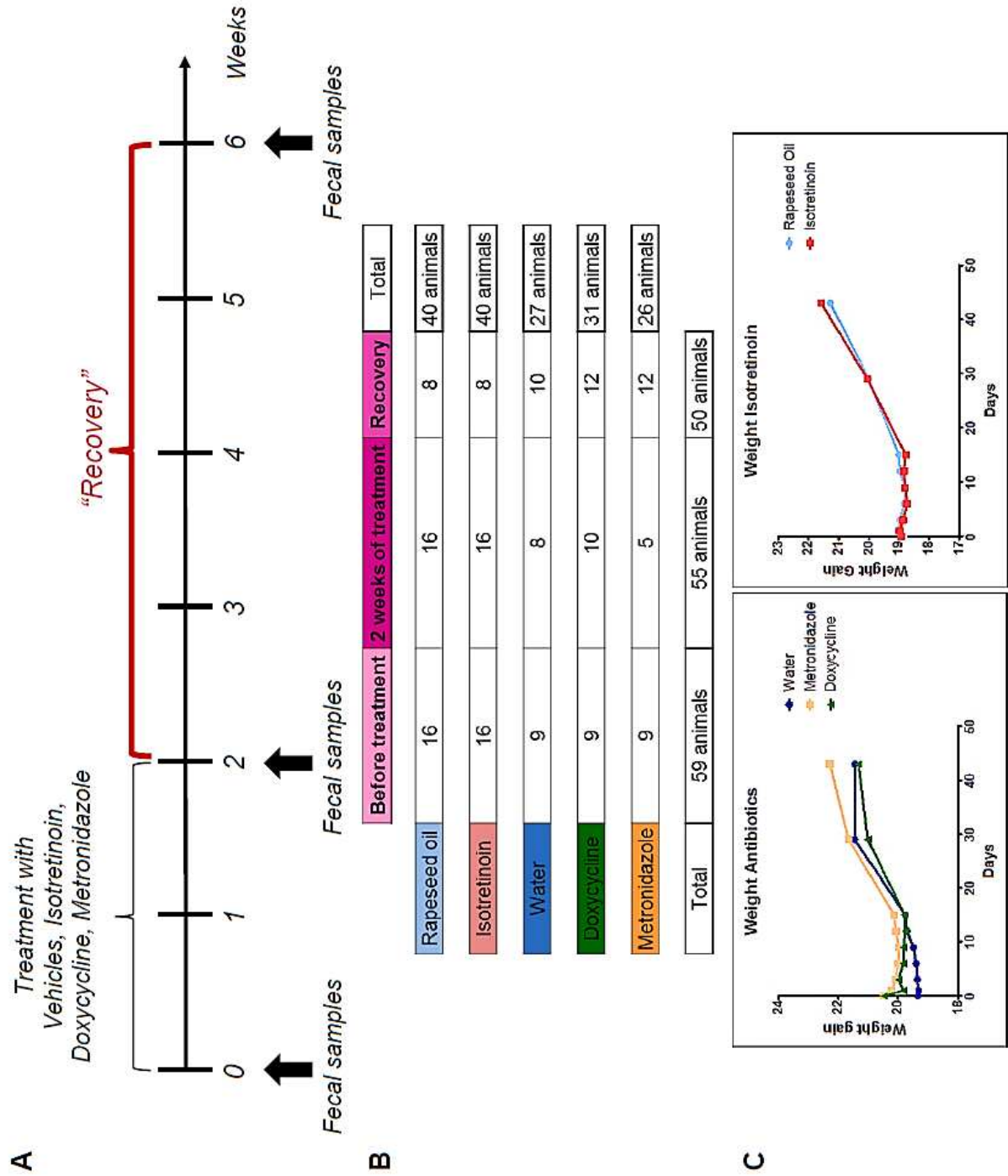
519 **Figure 2. Phylum-level taxonomic composition and diversity of the gut microbiota per mouse.** (A)
520 Relative abundances of the four dominant phyla per animal, treatment and time point, as
521 determined using the ribosomal database project Classifier with default settings. Phylum-level
522 taxonomic composition showed minor variations within groups, where *Bacteroidetes* and *Firmicutes*
523 were the dominant phyla. (B-D) Per-animal diversity (alpha diversity), indicating the differences in
524 community richness and evenness per mouse across time points and treatments. (B) ACE; (C) Gini-
525 Simpson index; (D) Shannon index; all indices calculated based on 98% average linkage OTUs.

526 **Figure 3. Analysis of microbiota community composition.** Pairwise weighted UniFrac distances
527 between samples were mapped to two-dimensional space using principal coordinates analysis for
528 visualization. Individual samples are shown as filled circles, per-group centroids as filled squares; axis
529 labels indicate the percent variance explained per principal coordinate. The upper panel shows
530 animals treated with water (dark blue), doxycycline (green) or metronidazole (orange). The lower
531 panel shows animals treated with isotretinoin (red) or rapeseed oil (light blue). Shifts in community
532 composition between groups per time point were tested using permutational multivariate analysis
533 of variance, as implemented in the 'adonis' function of the R package *vegan*). R2 values correspond

534 to an effect size of between-group differences in community composition, whereas *P*-values indicate
535 significance based on a permutation test ($n = 1'000$).

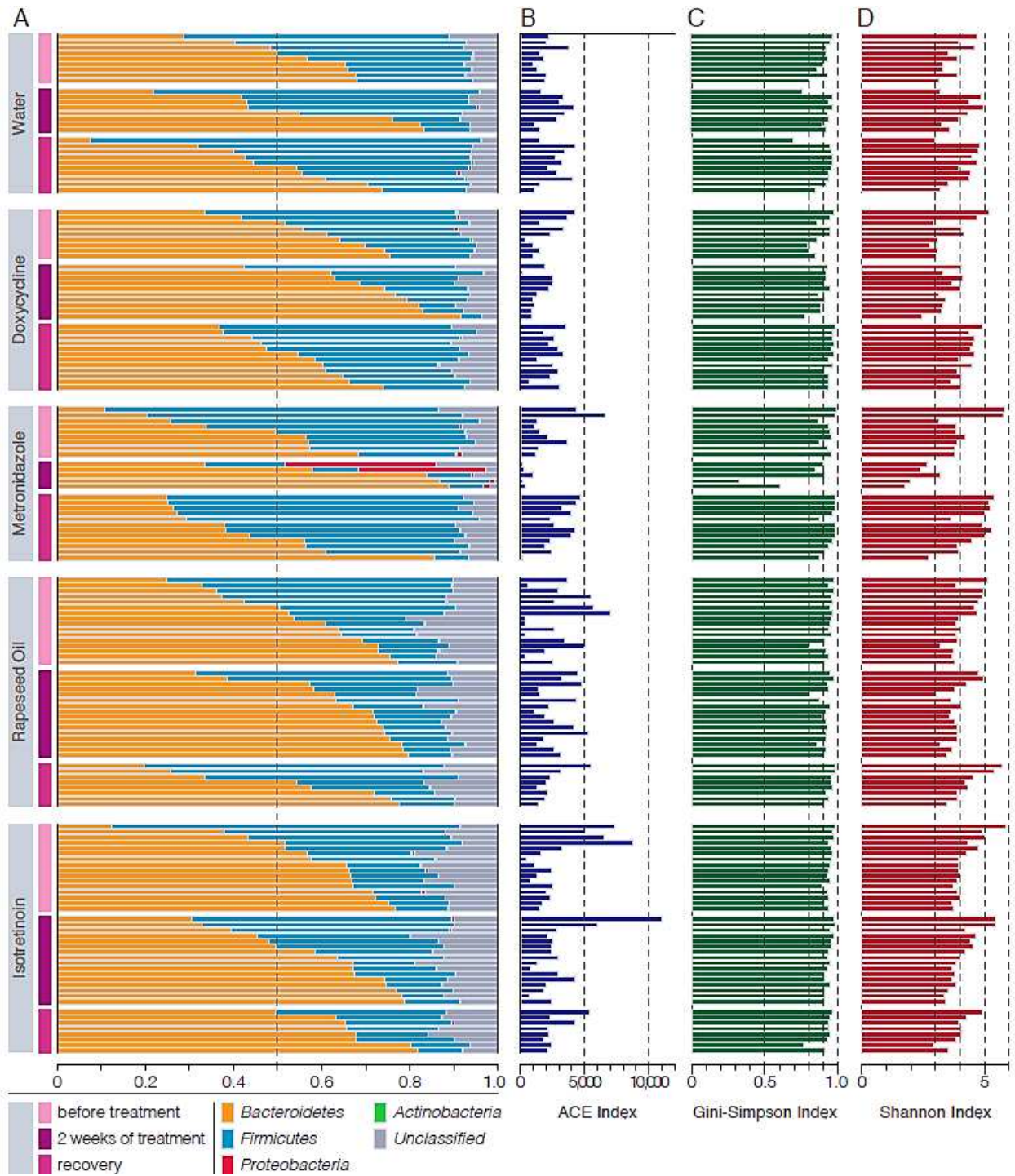
536 **Figure 4. Effects on community richness and evenness ('alpha diversity').** Left panel: ACE (A), Gini-
537 Simpson (B) and Shannon (C) indices are shown per time point and treatment; differences between
538 groups were assessed using one-way analysis of variance (data not shown), followed by unpaired,
539 two-sided t-tests. Right panel: within-group comparisons of per-animal diversity, relative to before-
540 treatment levels; each point indicates the absolute shift of diversity in a given animal with respect to
541 time point 0. Significance of shifts was assessed using paired, two-sided t-tests, also for comparisons
542 of per-mouse levels immediately upon treatment to post-recovery (indicated as dashed brackets).

543 **Figure 5. Identification of individual OTUs associated with treatment conditions using differential**
544 **abundance analysis.** For each time point and treatment group, differentially abundant OTUs with
545 respect to control groups (i.e. animals treated with water or rapeseed oil, respectively) were
546 detected using *edgeR*, at a false discovery rate of $\alpha < 0.001$. Significantly differential OTUs are
547 shown, colored by phylum, sorted by \log_2 (fold change) in abundance relative to control levels (x-
548 axis). Species-level taxonomic annotations were obtained, where possible, by assigning OTU
549 representative sequences to their closest BLAST hit against the National Center for Biotechnology
550 Information's 16S rRNA database, at a tolerance of 97% identity. Individual OTUs with significant
551 abundance shifts for several groups or time points are indicated using running numbers per phylum
552 (*B_01*, *B_02*, etc.).



557 **Figure 2:**

558

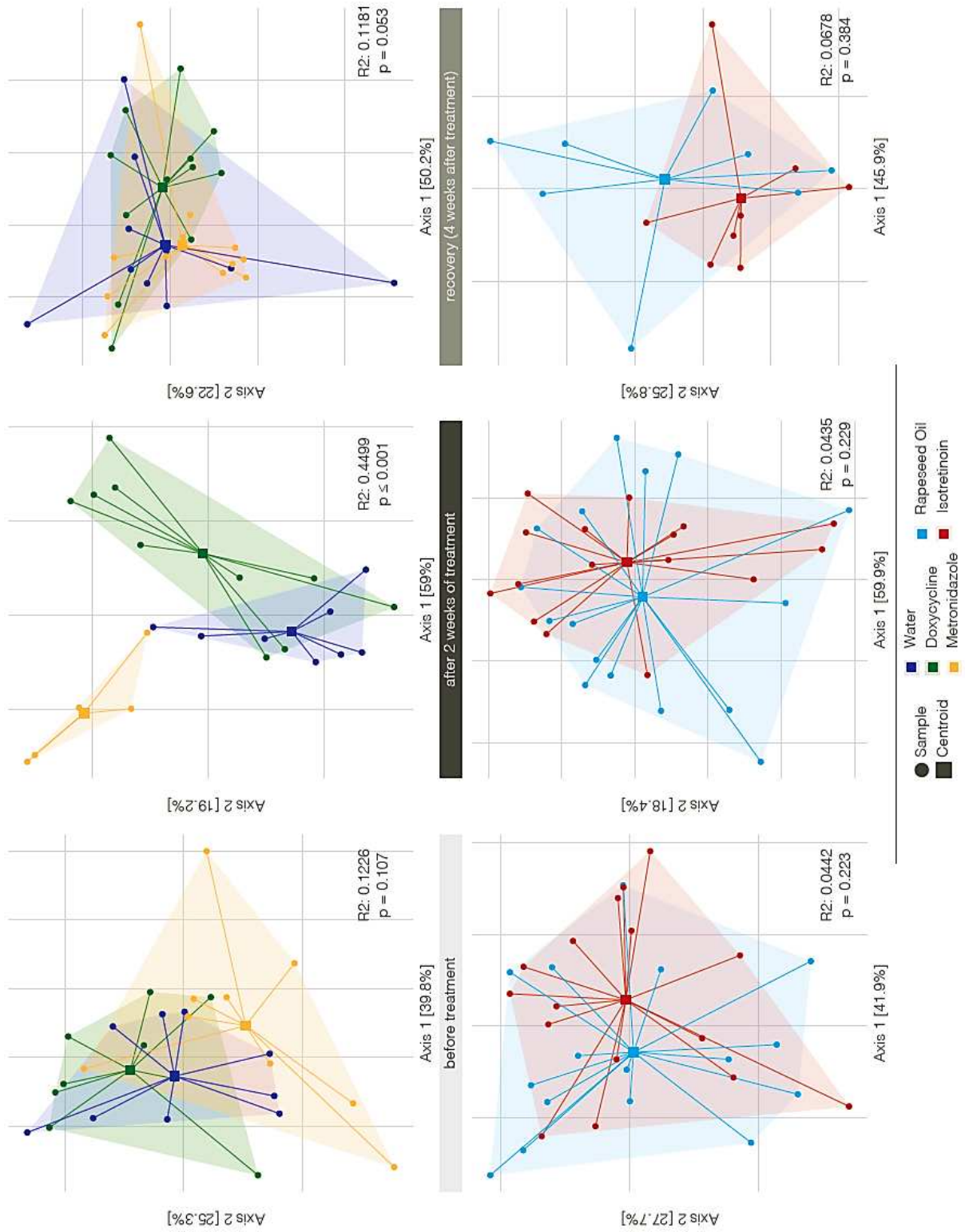


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561 **Figure 3:**

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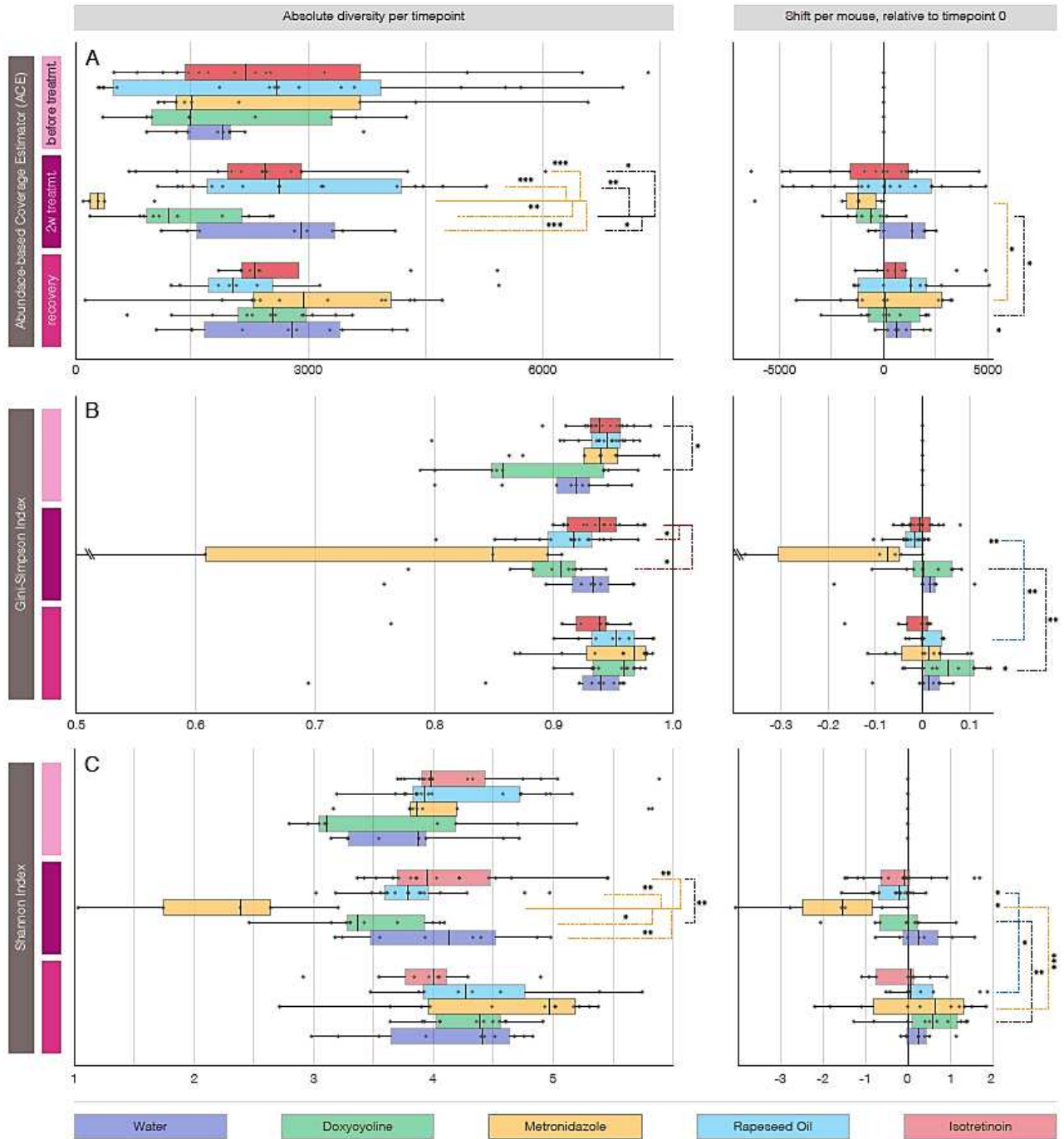


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565 **Figure 4:**

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