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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 as 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. We investigated the effects of doxycycline, metronidazole, which are frequently used in the treatment of acne and IBD, respectively, and isotretinoin on murine gut (fecal) microbiota after 2 weeks' treatment and after a 4-week recovery period. Fecal 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 showed a modest effect on bacterial richness and evenness but pronounced persistent and significant effects on the abundance of certain operational taxonomic units compared to the control group. In contrast, metronidazole induced a pronounced reduction in diversity post treatment but these effects did not persist during 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 fecal microbiota.

Highlights

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

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

Abbreviations

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

1. Introduction

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

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

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

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

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

2. Methods

2.1 Animals and treatment

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

2.2 Sample preparation and 16S rRNA gene sequencing

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

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

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

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

2.3 Bioinformatics

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

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

2.4 Community richness and evenness

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

2.5 Detection of individual differentially abundant OTUs (taxonomic analysis)

To investigate the fine-scale community composition at the level of individual OTUs, we used the R package *edgeR* [34], as suggested previously [35]. This approach provides a statistical framework for comparisons between treated and non-treated groups by quantifying the abundances of individual OTUs found in each dataset. Representative sequences for every OTU per treatment and time point that was significantly different from the respective control group was blasted against the NCBI 16S rRNA reference database for fine-scale taxonomic annotation, employing the following thresholds: $|\log2(\text{fold change})|>1$, FDR <0.001 and match-ID >97%.

3. Results

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

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

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

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

time points. Isotretinoin (F = 1.06, *P-value* = 0.403) and rapeseed oil (F = 1.199, *P-value* = 0.313) did not induce significant changes over time.

3.2 Immediate and long-term effects on community composition

The trends observed above at a very coarse resolution of phylum-level taxonomic composition were consistent at the level of individual unique sequences, i.e. the highest possible taxonomic resolution. Principal coordinate analysis of weighted UniFrac distances between samples calculated on a maximum likelihood phylogenetic tree at single nucleotide resolution are shown in Figure 3. Before treatment onset, phylogenetic community structure did not differ significantly between groups either for antibiotic (permutational multivariate analysis of variance as implemented in the 'adonis' function of the R package 'vegan'; R2 = 0.1226, *P-value* = 0.107) or isotretinoin treatment (R2 = 0.0442, *P-value* = 0.223). As expected, both antibiotic treatments led to significant shifts in community composition immediately after treatment relative to controls (R2 = 0.4499, *P-value* ≤ 0.001); in particular, metronidazole treatment induced a distinct shift relative to both doxycycline-treated mice (R2 = 0.479, *P-value* ≤ 0.001) and mice administered water only (R2 = 0.445, *P-value* ≤ 0.001). The change in community composition of doxycycline-treated mice was less pronounced (R2 = 0.252, *P-value* = 0.015). After the recovery period, community composition in doxycycline-treated mice remained significantly different from control mice (R2 = 0.115, *P-value* = 0.046). Interestingly, the observed changes in community composition directly after metronidazole treatment did not persist during the recovery period, with metronidazole-treated animals being indistinguishable from controls (R2 = 0.025, *P-value* = 0.73). In contrast, isotretinoin treatment did not lead to significant alterations in community composition either directly after treatment (R2 = 0.0435, *P-value* = 0.229) or after the recovery period (R2 = 0.0678, *P-value* = 0.384).

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

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

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

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

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

3.4 Identification of individual OTUs associated to treatment conditions

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

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

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

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

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

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

4. Discussion

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

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

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

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

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

In our study, complete recovery of the microbiota was not observed even by 4 weeks following cessation of doxycycline treatment: The treatment groups showed little differentiation in community richness or evenness but changes in OTU-abundances. Overall community composition remained 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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Competing interest

No author receives stipends or acts in any other consultative capacity for Roche. Roche had no influence on the design of the studies presented but approved the study protocol developed by the investigators. All analyses were performed solely by the investigators; Roche had no influence on the analyses performed or data interpretation. Decisions as to the data presented were made solely by the investigators with no input from Roche. Manuscript development, review and editing were performed without any input from, or sharing with Roche at any point. Roche provided only financial resources for medical writing and editorial support, with no access to content.

Ethical approval

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

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References

[1] Jostins L, Ripke S, Weersma RK, Duerr RH, McGovern DP, Hui KY, et al. Host-microbe interactions have shaped the genetic architecture of inflammatory bowel disease. Nature. 2012;491:119-24.

[2] Backhed F, Ley RE, Sonnenburg JL, Peterson DA, Gordon JI. Host-bacterial mutualism in the human intestine. Science. 2005;307:1915-20.

[3] Blumberg R, Powrie F. Microbiota, disease, and back to health: a metastable journey. Sci Transl Med. 2012;4:137rv7.

[4] Hedin CR, McCarthy NE, Louis P, Farquharson FM, McCartney S, Taylor K, et al. Altered intestinal microbiota and blood T cell phenotype are shared by patients with Crohn's disease and their unaffected siblings. Gut. 2014;63:1578-86.

[5] Imhann F, Vich Vila A, Bonder MJ, Fu J, Gevers D, Visschedijk MC, et al. Interplay of host genetics and gut microbiota underlying the onset and clinical presentation of inflammatory bowel disease. Gut. 2016.

[6] Ananthakrishnan AN. Epidemiology and risk factors for IBD. Nat Rev Gastroenterol Hepatol. 2015;12:205-17.

[7] Khor B, Gardet A, Xavier RJ. Genetics and pathogenesis of inflammatory bowel disease. Nature. 2011;474:307-17.

[8] Liu JZ, van Sommeren S, Huang H, Ng SC, Alberts R, Takahashi A, et al. Association analyses identify 38 susceptibility loci for inflammatory bowel disease and highlight shared genetic risk across populations. Nat Genet. 2015;47:979-86.

[9] Cho JH, Brant SR. Recent insights into the genetics of inflammatory bowel disease. Gastroenterology. 2011;140:1704-12.

[10] Bernstein CN. Antibiotic use and the risk of Crohn's disease. Gastroenterol Hepatol (N Y). 2013;9:393-5.

[11] Margolis DJ, Fanelli M, Hoffstad O, Lewis JD. Potential association between the oral tetracycline class of antimicrobials used to treat acne and inflammatory bowel disease. Am J Gastroenterol. 2010;105:2610-6.

[12] Ungaro R, Bernstein CN, Gearry R, Hviid A, Kolho KL, Kronman MP, et al. Antibiotics associated with increased risk of new-onset Crohn's disease but not ulcerative colitis: a meta-analysis. Am J Gastroenterol. 2014;109:1728-38.

[13] Lofmark S, Jernberg C, Jansson JK, Edlund C. Clindamycin-induced enrichment and long-term persistence of resistant Bacteroides spp. and resistance genes. J Antimicrob Chemother. 2006;58:1160-7.

[14] Jernberg C, Lofmark S, Edlund C, Jansson JK. Long-term ecological impacts of antibiotic administration on the human intestinal microbiota. ISME J. 2007;1:56-66.

425 [15] Jakobsson HE, Jernberg C, Andersson AF, Sjolund-Karlsson M, Jansson JK, Engstrand L.
426 Short-term antibiotic treatment has differing long-term impacts on the human throat and
427 gut microbiome. PLoS One. 2010;5:e9836.

428 [16] Shale M, Kaplan GG, Panaccione R, Ghosh S. Isotretinoin and intestinal inflammation:
429 what gastroenterologists need to know. Gut. 2009;58:737-41.

430 [17] Crockett SD, Porter CQ, Martin CF, Sandler RS, Kappelman MD. Isotretinoin use and the
431 risk of inflammatory bowel disease: a case-control study. Am J Gastroenterol.
432 2010;105:1986-93.

433 [18] Bernstein CN, Nugent Z, Longobardi T, Blanchard JF. Isotretinoin is not associated with
434 inflammatory bowel disease: a population-based case-control study. Am J Gastroenterol.
435 2009;104:2774-8.

436 [19] Etminan M, Bird ST, Delaney JA, Bressler B, Brophy JM. Isotretinoin and risk for
437 inflammatory bowel disease: a nested case-control study and meta-analysis of published
438 and unpublished data. JAMA Dermatol. 2013;149:216-20.

439 [20] Rehman A, Sina C, Gavrilova O, Hasler R, Ott S, Baines JF, et al. Nod2 is essential for
440 temporal development of intestinal microbial communities. Gut. 2011;60:1354-62.

441 [21] Quince C, Lanzen A, Davenport RJ, Turnbaugh PJ. Removing noise from pyrosequenced
442 amplicons. BMC Bioinformatics. 2011;12:38.

443 [22] Schloss PD, Westcott SL, Ryabin T, Hall JR, Hartmann M, Hollister EB, et al. Introducing
444 mothur: open-source, platform-independent, community-supported software for describing
445 and comparing microbial communities. Appl Environ Microbiol. 2009;75:7537-41.

446 [23] Schloss PD, Westcott SL. Assessing and improving methods used in operational
447 taxonomic unit-based approaches for 16S rRNA gene sequence analysis. Appl Environ
448 Microbiol. 2011;77:3219-26.

449 [24] Edgar RC, Haas BJ, Clemente JC, Quince C, Knight R. UCHIME improves sensitivity and
450 speed of chimera detection. Bioinformatics. 2011;27:2194-200.

451 [25] Schmidt TS, Matias Rodrigues JF, von Mering C. Ecological consistency of SSU rRNA-
452 based operational taxonomic units at a global scale. PLoS Comput Biol. 2014;10:e1003594.

453 [26] Nawrocki EP, Kolbe DL, Eddy SR. Infernal 1.0: inference of RNA alignments.
454 Bioinformatics. 2009;25:1335-7.

455 [27] Nawrocki EP, Eddy SR. Infernal 1.1: 100-fold faster RNA homology searches.
456 Bioinformatics. 2013;29:2933-5.

457 [28] Matias Rodrigues JF, von Mering C. HPC-CLUST: distributed hierarchical clustering for
458 large sets of nucleotide sequences. Bioinformatics. 2014;30:287-8.

459 [29] Wang Q, Garrity GM, Tiedje JM, Cole JR. Naive Bayesian classifier for rapid assignment
460 of rRNA sequences into the new bacterial taxonomy. Appl Environ Microbiol. 2007;73:5261-
461 7.

462 [30] Price MN, Dehal PS, Arkin AP. FastTree 2--approximately maximum-likelihood trees for
463 large alignments. PLoS One. 2010;5:e9490.

464 [31] Team RC. R: A Language and Environment for Statistical Computing. Vienna, Austria: R
465 Foundation for Statistical Computing; 2015.

466 [32] McMurdie PJ, Holmes S. phyloseq: an R package for reproducible interactive analysis
467 and graphics of microbiome census data. PLoS One. 2013;8:e61217.

468 [33] Oksanen J. Vegan: ecological diversity. 2015.

469 [34] Robinson MD, McCarthy DJ, Smyth GK. edgeR: a Bioconductor package for differential
470 expression analysis of digital gene expression data. Bioinformatics. 2010;26:139-40.

471 [35] McMurdie PJ, Holmes S. Waste not, want not: why rarefying microbiome data is
472 inadmissible. PLoS Comput Biol. 2014;10:e1003531.

473 [36] Gevers D, Kugathasan S, Denson LA, Vazquez-Baeza Y, Van Treuren W, Ren B, et al. The
474 treatment-naive microbiome in new-onset Crohn's disease. Cell Host Microbe. 2014;15:382-
475 92.

476 [37] Schaubeck M, Clavel T, Calasan J, Lagkouvardos I, Haange SB, Jehmlich N, et al.
477 Dysbiotic gut microbiota causes transmissible Crohn's disease-like ileitis independent of
478 failure in antimicrobial defence. Gut. 2015.

479 [38] Pelissier MA, Vasquez N, Balamurugan R, Pereira E, Dossou-Yovo F, Suau A, et al.
480 Metronidazole effects on microbiota and mucus layer thickness in the rat gut. FEMS
481 Microbiol Ecol. 2010;73:601-10.

482 [39] Wlodarska M, Willing B, Keeney KM, Menendez A, Bergstrom KS, Gill N, et al. Antibiotic
483 treatment alters the colonic mucus layer and predisposes the host to exacerbated
484 Citrobacter rodentium-induced colitis. Infect Immun. 2011;79:1536-45.

485 [40] Sjolund M, Wreiber K, Andersson DI, Blaser MJ, Engstrand L. Long-term persistence of
486 resistant Enterococcus species after antibiotics to eradicate Helicobacter pylori. Annals of
487 internal medicine. 2003;139:483-7.

488 [41] Fehlbaum S, Chassard C, Poeker SA, Derrien M, Fourmestraux C, Lacroix C. Clostridium
489 difficile colonization and antibiotics response in PolyFermS continuous model mimicking
490 elderly intestinal fermentation. Gut Pathog. 2016;8:63.

491 [42] Angelakis E, Million M, Kankoe S, Lagier JC, Armougom F, Giorgi R, et al. Abnormal
492 weight gain and gut microbiota modifications are side effects of long-term doxycycline and
493 hydroxychloroquine treatment. Antimicrob Agents Chemother. 2014;58:3342-7.

494 [43] Angelakis E. Weight gain by gut microbiota manipulation in productive animals. Microb
495 Pathog. 2016.

496 [44] Holman DB, Chenier MR. Antimicrobial use in swine production and its effect on the
497 swine gut microbiota and antimicrobial resistance. Can J Microbiol. 2015;61:785-98.

498 [45] Zhang D, Ji H, Liu H, Wang S, Wang J, Wang Y. Changes in the diversity and composition
499 of gut microbiota of weaned piglets after oral administration of Lactobacillus or an
500 antibiotic. Appl Microbiol Biotechnol. 2016;100:10081-93.

501 [46] Holman DB, Chenier MR. Temporal changes and the effect of subtherapeutic
502 concentrations of antibiotics in the gut microbiota of swine. FEMS Microbiol Ecol.
503 2014;90:599-608.

504 [47] Reti KL, Thomas MC, Yanke LJ, Selinger LB, Inglis GD. Effect of antimicrobial growth
505 promoter administration on the intestinal microbiota of beef cattle. Gut Pathog. 2013;5:8.

506 [48] Manichanh C, Borruel N, Casellas F, Guarner F. The gut microbiota in IBD. Nat Rev
507 Gastroenterol Hepatol. 2012;9:599-608.

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Figure Legends

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

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

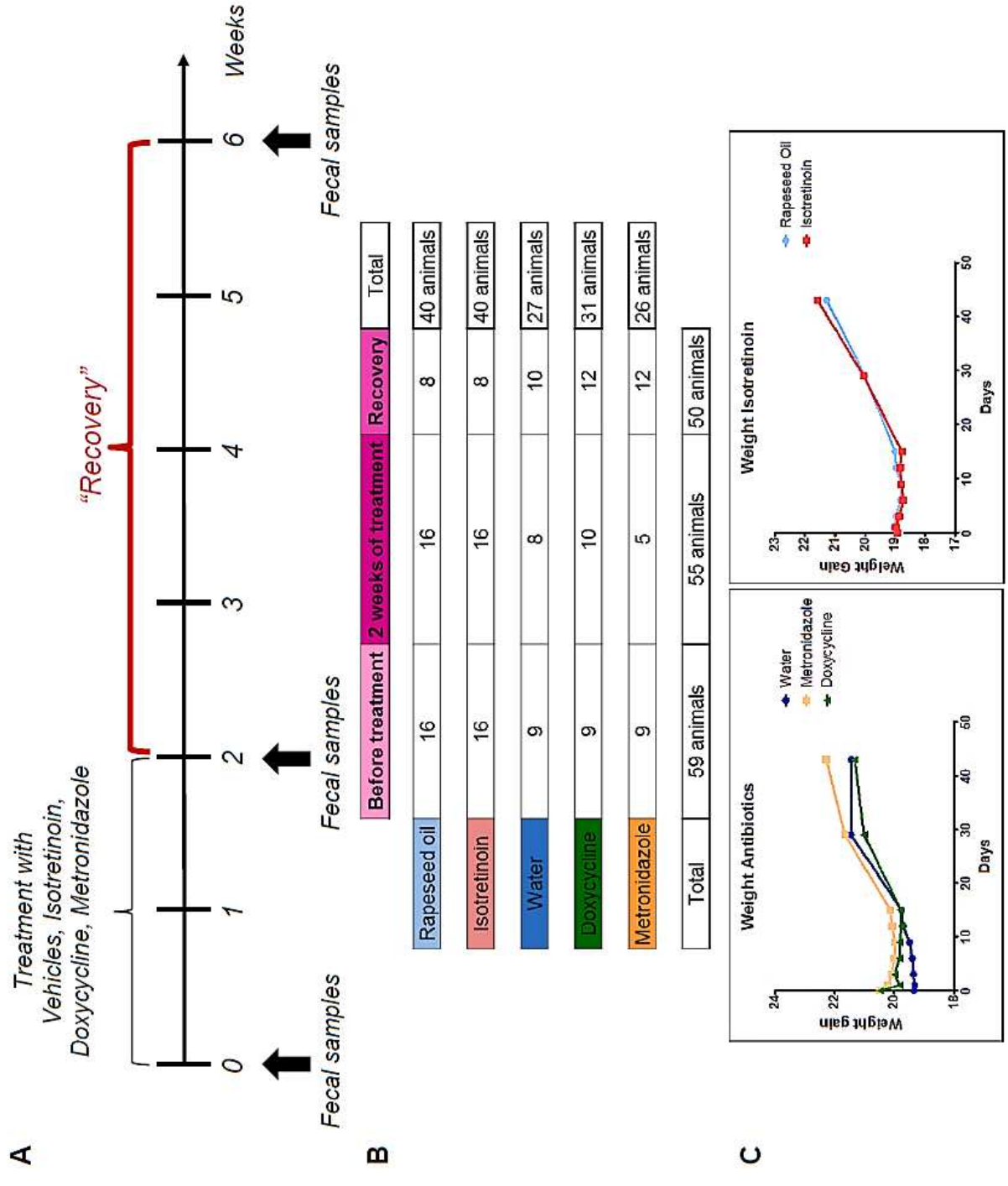
Figure 3. Analysis of microbiota community composition. Pairwise weighted UniFrac distances between samples were mapped to two-dimensional space using principal coordinates analysis for visualization. Individual samples are shown as filled circles, per-group centroids as filled squares; axis labels indicate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

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

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

Figure 5. Identification of individual OTUs associated with treatment conditions using differential abundance analysis. For each time point and treatment group, differentially abundant OTUs with respect to control groups (i.e. animals treated with water or rapeseed oil, respectively) were detected using *edgeR*, at a false discovery rate of $\alpha < 0.001$. Significantly differential OTUs are shown, colored by phylum, sorted by log2 (fold change) in abundance relative to control levels (x-axis). Species-level taxonomic annotations were obtained, where possible, by assigning OTU representative sequences to their closest BLAST hit against the National Center for Biotechnology Information's 16S rRNA database, at a tolerance of 97% identity. Individual OTUs with significant abundance shifts for several groups or time points are indicated using running numbers per phylum (*B_01, B_02, etc.*).

Figure 1:


A
Treatment with
Vehicles, Isotretinoin,
Doxycycline, Metronidazole
"Recovery"
Fecal samples
Fecal samples
Fecal samples
0
1
2
3
4
5
6
Weeks
B
Before treatment
2 weeks of treatment
Recovery
Total
Rapeseed oil 16 16 8 40 animals
Isotretinoin 16 16 8 40 animals
Water 9 8 10 27 animals
Doxycycline 9 10 12 31 animals
Metronidazole 9 5 12 26 animals
Total 59 animals 55 animals 50 animals
C
Weight Antibiotics
Water
Metronidazole
Doxycycline
Weight gain
Days
Weight Isotretinoin
Rapeseed Oil
Isotretinoin
Weight Gain
Days

557 **Figure 2:**

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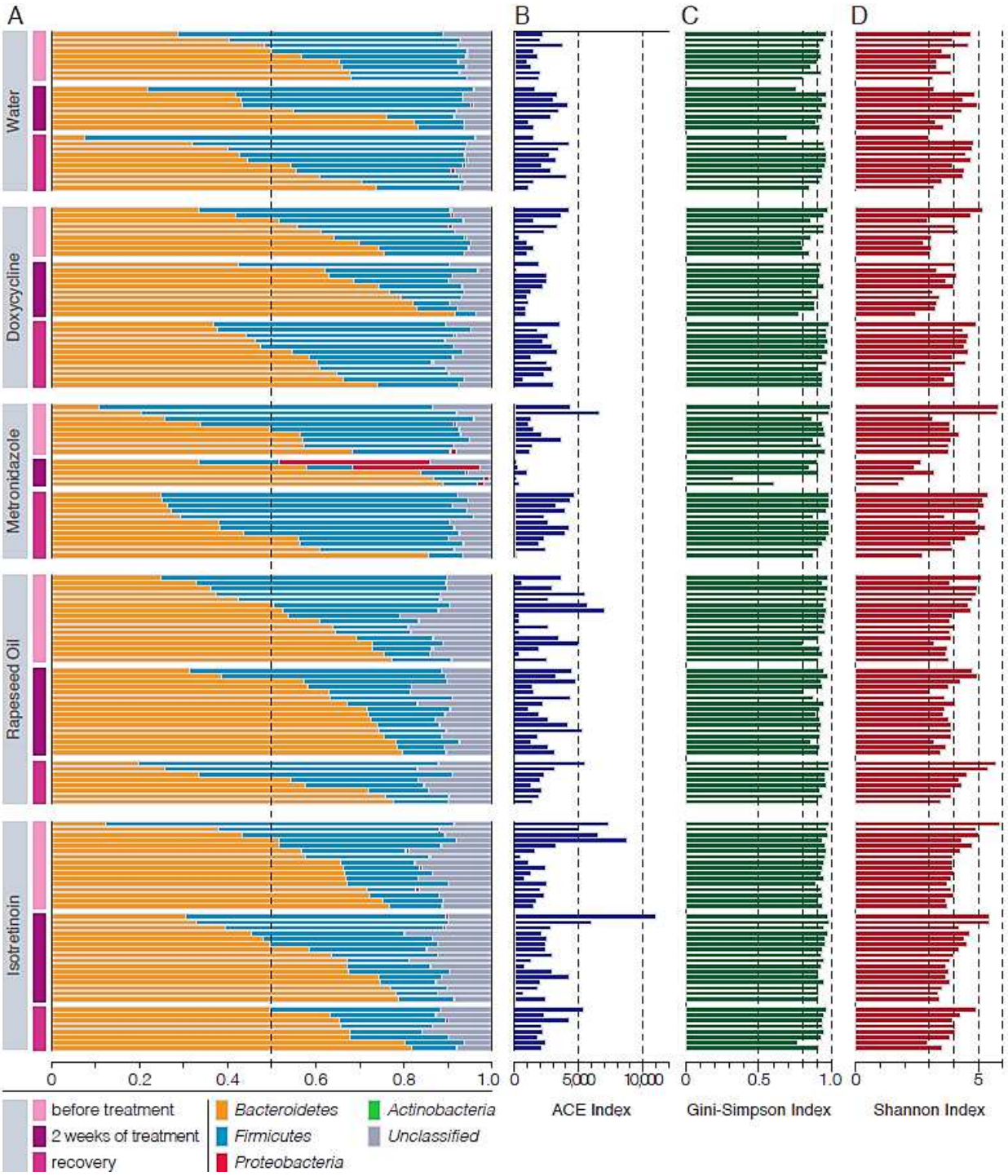

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561 **Figure 3:**

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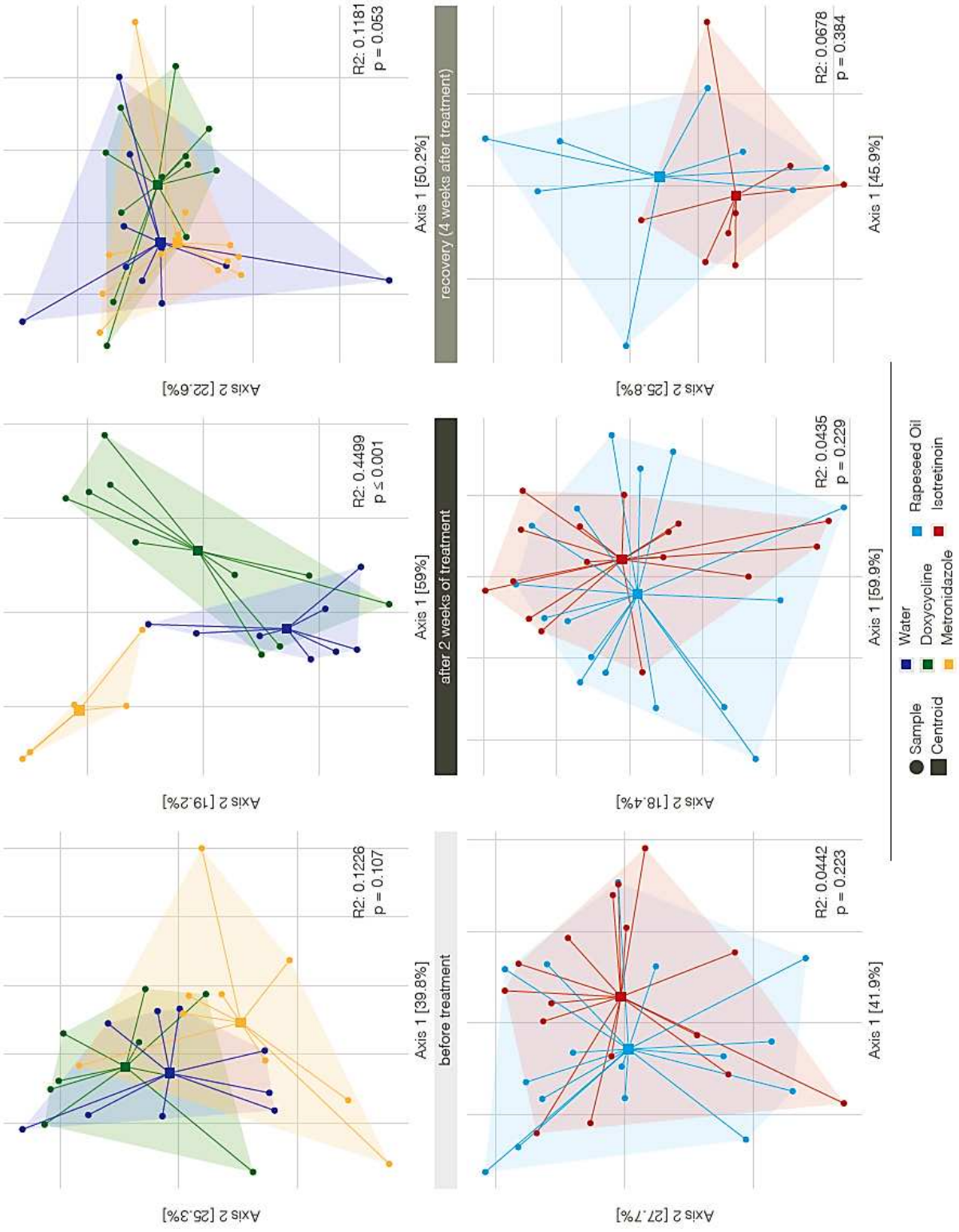

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565 **Figure 4:**

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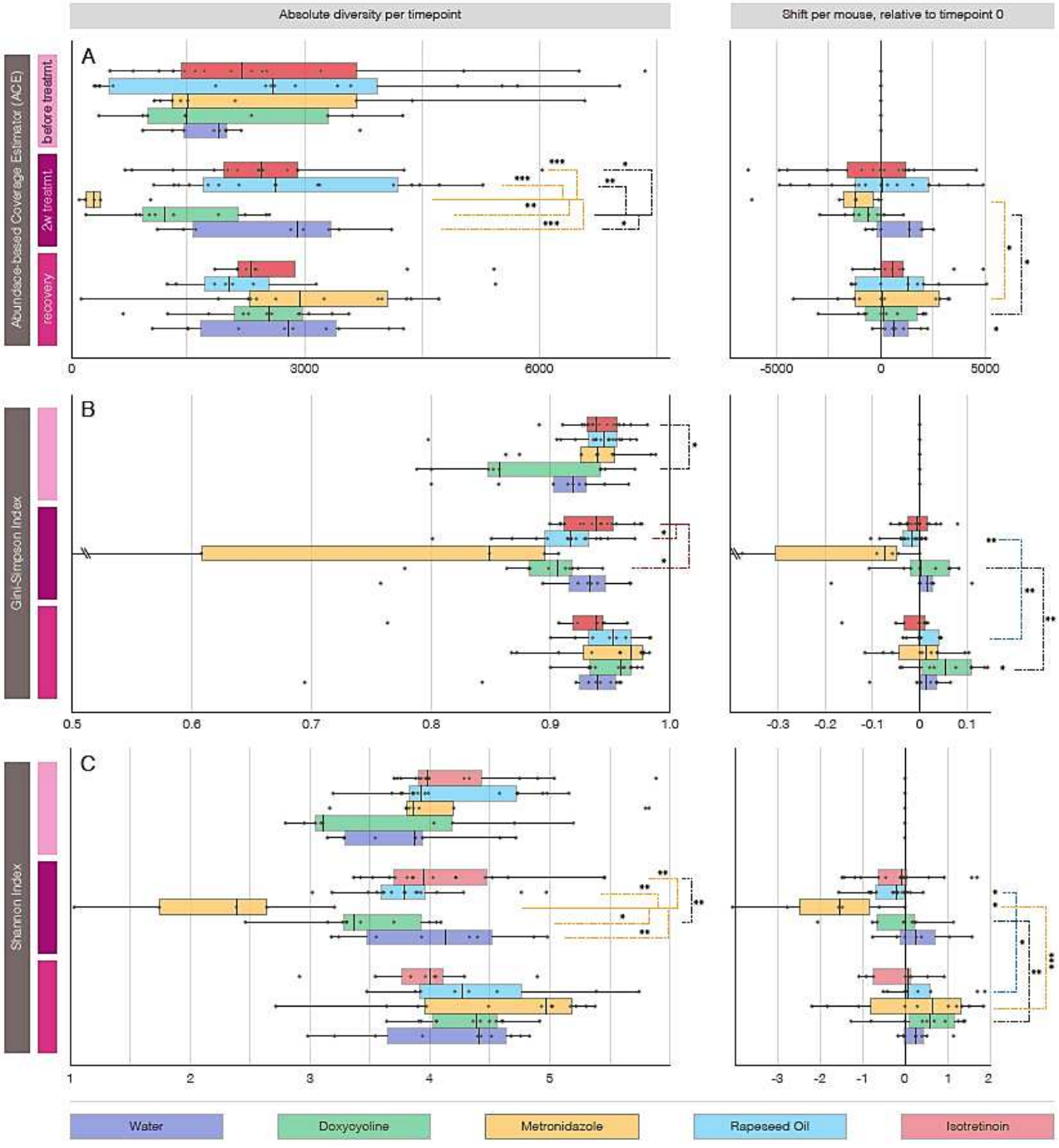

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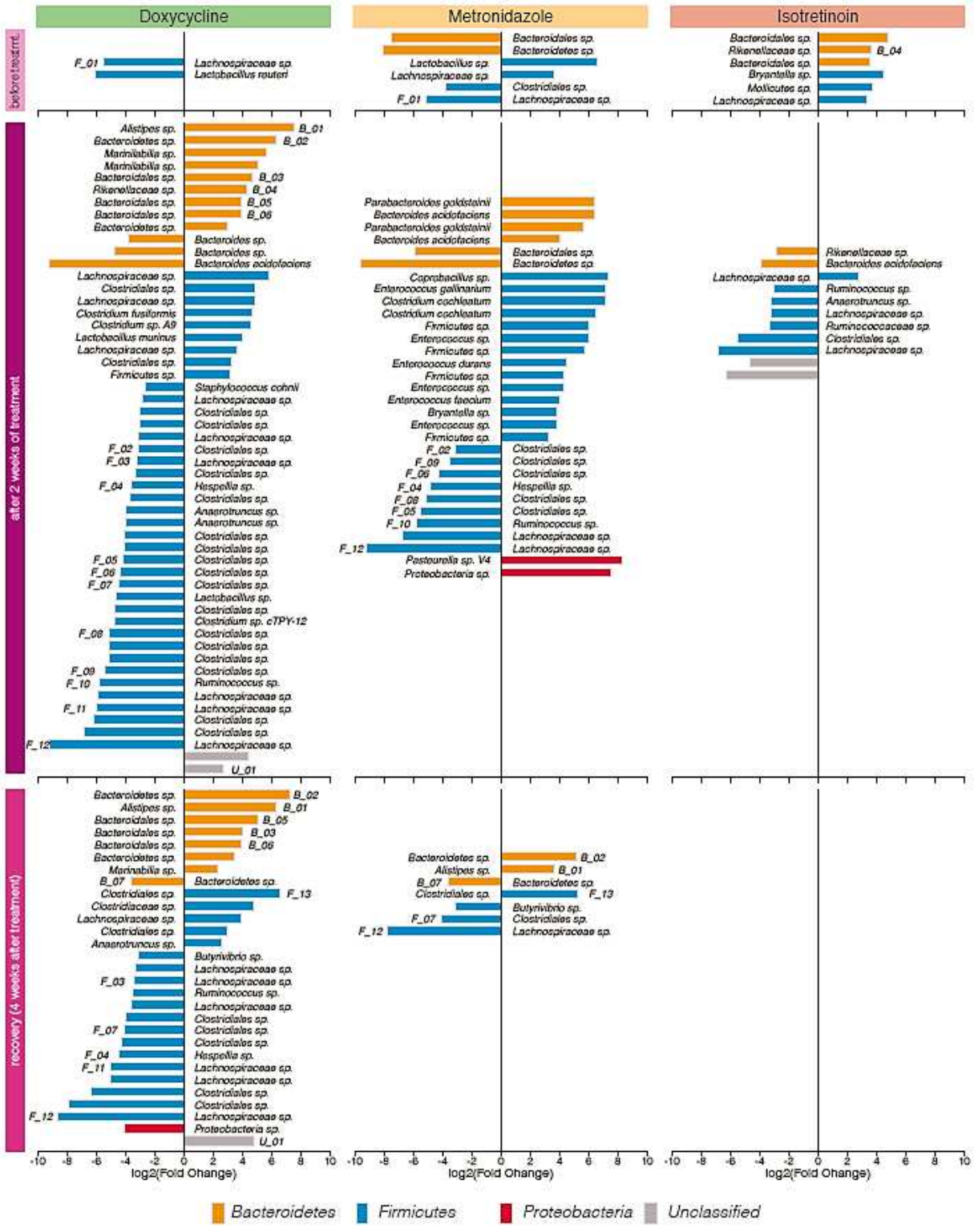
Figure 5:


Doxycycline
Metronidazole
Isotretinoin
before treatment
after 2 weeks of treatment
recovery (4 weeks after treatment)
log2(Fold Change)
Bacteroidetes
Firmicutes
Proteobacteria
Unclassified