1 **Higher alpha diversity and Lactobacillus blooms are associated with better engraftment**

2 **after Fecal Microbiota Transplant in Inflammatory Bowel Disease**

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1   **Abstract**

2   Background: Fecal Microbiota Transplant (FMT) has proven effective in treating recurrent

3   *Clostridioides difficile* infection (rCDI) and has shown some success in treating inflammatory

4   bowel diseases (IBD). There is emerging evidence that host engraftment of donor taxa is a tenet

5   of successful FMT. However, there is little known regarding predictors of engraftment. We

6   undertook a double-blind, randomized, placebo-controlled pilot study to characterize the

7   response to FMT in children and young adults with mild to moderate active Crohn's disease

8   (CD) and ulcerative colitis (UC).

9   Results: Subjects with CD or UC were randomized to receive antibiotics and weekly FMT or

10   placebo in addition to baseline medications. The treatment arm received seven days of

11   antibiotics followed by FMT enema and then capsules weekly for seven weeks. We enrolled four

12   subjects with CD and 11 with UC, ages 14-29 years. Due to weekly stool sampling, we were

13   able to create a time series of alpha diversity, beta diversity and engraftment as they related to

14   clinical response. Subjects exhibited a wide range of microbial diversity and donor engraftment

15   as FMT progressed. Specifically, engraftment ranged from 26% to 90% at week 2 and 3% to

16   92% at two months. Consistent with the current literature, increases over time of both alpha

17   diversity ($p < 0.05$) and donor engraftment ($p < 0.05$) correlated with improved clinical response.

18   Additionally, our weekly time series enabled an investigation into the clinical and microbial

19   correlates of engraftment at various time points. We discovered that the post-antibiotic but pre-

20   FMT time point, often overlooked in FMT trials, was rich in microbial correlates of eventual

21   engraftment. Greater residual alpha diversity after antibiotic treatment was positively correlated

22   with engraftment and subsequent clinical response. Interestingly, a transient rise in the relative

23   abundance of Lactobacillus was also positively correlated with engraftment, a finding that we

24   recapitulated with our analysis of another FMT trial with publicly available weekly sequencing

25   data.

1 Conclusions: We found that higher residual alpha diversity and Lactobacillus blooms after

2 antibiotic treatment correlated with improved engraftment and clinical response to FMT. Future

3 studies should closely examine the host microbial communities pre-FMT and the impact of

4 antibiotic preconditioning on engraftment and response.

5 **Keywords** (3-10): inflammatory bowel disease, fecal microbiota transplant, Crohn's disease,

6 ulcerative colitis, microbiome, microbial therapeutics.

1   **Background**

2   Fecal microbiota transplant (FMT) is the transfer of healthy fecal microbial communities to a

3   patient with an illness associated with gut microbiome perturbances. The best example to date

4   is the use of FMT to treat *Clostridioides difficile* infection, which has proven to be both safe and

5   highly effective for most patients [1–3]. This success has driven an interest in identifying other

6   diseases where FMT may be beneficial. Perturbations of the microbiome contribute to the

7   pathogenesis of IBD, making it a promising candidate for FMT [4].

8

9   Data from several studies, including recent meta-analyses, have demonstrated the efficacy of

10 FMT in some patients with ulcerative colitis (UC) [5–15]. However, the factors that determine

11 responsiveness to FMT are poorly understood. Based on the preliminary data collected,

12 researchers have proposed several theories. Among the proposed factors are the patient

13 characteristics, patient and donor microbial compositions and diversity, degree of donor

14 engraftment, and timing and duration of engraftment [7,12,16,17]. Deeper analyses into the

15 determinants of engraftment have shown highly variable microbe-specific, disease-specific and

16 recipient-specific dynamics. Thus, a generalizable "rule book" for engraftment remains elusive

17 [6,7,12,16,17].

18

19 Given that the mechanisms and predictors of success remain unknown, there are no standard

20 pre-conditioning, treatment, or delivery regimens for FMT in IBD. Studies have employed

21 various pre-conditioning regimens including: no bowel prep, bowel prep with laxatives, dietary

22 changes, proton pump inhibitor use, as well as narrow-spectrum and broad-spectrum antibiotics

23 [16,18,19]. There is some suggestion that antibiotic pre-treatment is favorable for both

24 engraftment and clinical response, though the evidence relies on challenging cross-study

25 comparisons [13,18,20]. Furthermore, other studies have reached conflicting conclusions. Our

26 group recently demonstrated that antibiotic pre-conditioning decreased engraftment after FMT,

1 though in a different disease [21]. The literature also provides conflicting answers for even

2 simple questions, such as the effect of recipient alpha diversity on donor engraftment.

3

4 In this small study of FMT in adolescents and young adults with IBD, we present a weekly

5 microbiome time series. Frequent sampling allowed us to analyze changes in alpha diversity

6 leading to donor engraftment and thereby identify critical time-points in this FMT protocol. With a

7 specific focus on the post-conditioning, pre-FMT time-point, we show that increased diversity

8 and increased abundance of specific taxa in the family Lactobacilliaceae are correlated with

9 engraftment and clinical response.

10

11 **Methods**

12

13 **Study Design**

14 We conducted a single-center, randomized, double-blind, placebo-controlled trial of FMT in

15 patients with colonic or ileocolonic Crohn's Disease (CD) and ulcerative colitis (UC). Subjects

16 were recruited from the Boston Children's Hospital IBD Center as well as through referrals from

17 providers across the country. Standard anthropometric data, past medical and surgical history,

18 and medication history were abstracted from participants' medical records.

19

20 The primary objective of this study was to assess the safety and tolerability of FMT compared to

21 placebo in pediatric and young adult patients (ages 5-30) with IBD that failed first-line

22 maintenance therapy. The secondary objectives were to identify biomarkers in both donors and

23 recipients that correlate with clinical response.

24

25 **Eligibility**

1 Eligible patients were aged 5-30 years with mild to moderate disease activity. Mild to moderate

2 CD Disease Activity was defined as Pediatric Crohn's Disease Activity Index (PCDAI) >10 but

3 ≤30; mild to moderate UC was defined as Pediatric Ulcerative Colitis Activity Index (PUCAI) > 9

4 but < 30. Additional eligibility criteria included the presence of visual or histologic evidence of

5 inflammation no more than 105 days before randomization; negative test results for Hepatitis B

6 (HBV), Hepatitis C (HCV), and Human Immunodeficiency Virus (HIV); negative urine pregnancy

7 test for people of childbearing potential; ability to swallow antibiotic, FMT or placebo capsules;

8 and the absence of any known food allergy.

9

10 Exclusion criteria included extensive and severe CD (i.e. fistulizing disease, abscess, small

11 bowel obstruction, fevers); patients with recent (within four weeks) dosage changes of biologics,

12 5-ASA, steroids or immunomodulators; toxic megacolon; known drug allergy to vancomycin,

13 metronidazole or polymyxin; history of aspiration, gastroparesis, surgery involving the upper

14 gastrointestinal tract (that might affect upper gastrointestinal motility) or unable to swallow pills;

15 esophageal dysmotility or swallowing dysfunction; known food allergies; unable or unwilling to

16 receive a retention enema for purposes of induction therapy; recent (within six weeks) systemic

17 antibiotic use; testing consistent with active clostridium difficile; and known prior experience with

18 FMT.

19 Subjects were maintained on their standard of care medications at the primary provider's

20 discretion. Patients with mild to moderate disease activity were consented and randomized to

21 receive treatment with antibiotics followed by FMT or placebo. This study was approved by the

22 Boston Children's Hospital Institutional Review Board and was registered on

23 https://clinicaltrials.gov (Identifier: NCT02330653).

24 **Randomization**

1 Subjects enrolled were randomized in a 1:1 ratio according to a pre-determined block

2 randomization procedure to receive either the treatment arm or the placebo arm. An unblinded

3 member of the study team maintained the randomization list, ensured subjects were

4 appropriately randomized, and dispensed the correct treatment.

5

6 **Study Groups**

7 Subjects in the treatment arm (hereafter referred to as the FMT arm) received seven days of

8 antibiotic pre-treatment beginning on Day -8. One capsule containing metronidazole (weight-

9 dependent dosing, maximum dose of 500 mg) was administered twice a day. Capsules

10 containing 125 mg of vancomycin and 62.5 mg of polymyxin were administered three times a

11 day. The number of capsules given was based on Body Surface Area parameters.

12

13 Approximately 48 hours after the discontinuation of antibiotic pre-treatment, on Day 0, subjects

14 were given an induction retention enema of FMT over the course of 15-30 minutes. They were

15 encouraged to retain the fecal matter for as long as possible. Subjects were then observed for

16 at least 60 minutes before discharge. Subjects were subsequently treated with a weekly dose of

17 30 FMT capsules for the next seven weeks.

18

19 FMT material was obtained from OpenBiome in Cambridge, MA using established protocols

20 [12,22]. OpenBiome is a non-profit stool bank dedicated to treating and researching the

21 microbiome. Their work focuses on providing safe and affordable FMT material, thereby

22 removing logistical barriers for patients and physicians.

23

24 Subjects in the placebo arm received seven days of corresponding placebo capsules in place of

25 antibiotic treatment. At Day 0, these subjects received a placebo retention enema. Subjects

26 were subsequently treated with a weekly dose of 30 placebo capsules for the next seven weeks.

1

2   Subjects provided weekly stool samples and PCDAI/PUCAI surveys to monitor disease activity

3   regardless of the assigned arm. At Week 4 and Week 8, routine laboratory assessments were

4   collected to measure clinical response.

5

6   **Open-Label Eligibility and Treatment**

7   After eight weeks of treatment and unblinding, subjects in the FMT arm that responded to

8   treatment and all subjects in the placebo arm were eligible to receive an additional eight weeks

9   of open-label FMT. Response to treatment was defined as a decrease in disease activity index

10   score of at least 10 points or a score of 10 points or less.

11

12   **Endpoints/Outcomes**

13   Primary outcome measures included any FMT-related adverse events, grade 2 or above, and

14   the proportion of subjects reporting any FMT-related adverse events of grade 2 or above at

15   eight weeks post-FMT. Patient-related outcomes of abdominal pain and the average daily

16   number of bowel movements were recorded.

17

18   Secondary outcome measures included remission as defined by a disease activity index of <10,

19   improvement of inflammatory biomarkers such as fecal calprotectin, erythrocyte sedimentation

20   rate (ESR), C-reactive protein (CRP), changes in gut microbial composition, improvement of

21   disease activity scores (PCDAI ≥ 12.5, PUCAI ≥ 20), and assessment of engraftment of donor

22   microbes into recipients.

23

24   **Fecal Sample Collection**

1   Subjects submitted stool samples during screening, baseline, after antibiotics but before FMT,

2   then weekly during blinded and open-label treatment, and during follow-up. These samples

3   were stored at -80°C.

4

5   **Microbiome Analysis**

6   We extracted DNA using a Powersoil DNA extraction kit (Qiagen). 16S rDNA libraries were

7   prepared and sequenced by the Broad Institute Genomic Platform, using paired-end 250-bp

8   reads on an Illumina HiSeq. We analyzed 16S data using Qiime2, DADA2, Phyloseq in R, and

9   custom Python scripts [23–25]. We assigned taxonomic labels to 16S sequences using the

10   SILVA database [26]. We used SourceTracker2 to estimate the sources of various bacteria after

11   FMT [27]. We used the pre-antibiotic sample, the post-antibiotics sample, and the known donor

12   as sources for each participant. We also included the other donor, not used in that participant's

13   FMT, as a negative control. To differentiate the participants who achieved high engraftment

14   from those who did not, we used the q2-sample-classifier in Qiime2, using the Random Forrest

15   classifier [28].

16

17   **Results**

18   **Study Enrollment/Patient Characteristics**

19   We selected patients with primarily mild to moderate colonic CD or UC without stricturing or

20   penetrating disease. Four subjects with CD and 11 subjects with UC were consented and

21   randomized to a treatment arm (Figure 1A). The median age at enrollment for CD patients was

22   20 years (range 18-23) and for UC patients was 24 years (range 14-29). All patients were white,

23   and none were Hispanic or Latino. At baseline, all patients had mild or moderate disease activity

24   index scores. All 4 CD subjects had mildly active disease (PCDAI of 11-25). With respect to

25   subjects with UC, three had mildly active disease (PUCAI scores of 10-24), three had mild to

1   moderate disease (PUCAI scores of 25-39) and three had moderate disease activity (PUCAI

2   scores of 40-69) (Supplemental Table 1).

3

4   Eleven (3 CD and 8 UC) subjects completed the blinded phase of the study (Figure 1A). Four (1

5   CD and 3 UC) subjects were ineligible to continue into the open-label phase because they did

6   not meet response criteria (Figure 1A). Seven (two CD and five UC) subjects completed the

7   open-label phase of the study and six (two CD and four UC) subjects completed long term

8   follow up (Figure 1A).

9

10   **Clinical Response**

11   Overall, three patients reported a decrease in IBD-related symptoms. Subjects with UC

12   experienced an average decline in PCDAI score of 5 and an average decline in PUCAI of 7.5

13   while receiving the experimental arm or during open-label therapy (Figure 1B). The FMT was

14   safe and well-tolerated in a majority of the patients. There were two serious adverse events

15   (SAE): one episode of Grade 3 colitis that was determined to be possibly related to the

16   intervention antibiotic treatment (and not FMT) and one hypersensitivity reaction directly after

17   the FMT induction enema that was deemed probably related. Both subjects were removed from

18   the study. There were other adverse events that were determined to be not related to the study

19   intervention (Supplemental Text).

20

21   **Host Microbiome Response**

22   On average, alpha diversity decreased after a week of antibiotics therapy and increased

23   throughout the course of FMT. There were large inter-individual differences in the alpha

24   diversity post-FMT, with Shannon indices ranging from less than 1 to greater than 5 (Figure 2A).

25   We generated a weekly time series of gut microbial changes using serial stool samples for

26   sequencing. The microbial differences between responders and non-responders diverged early.

1 Starting at two weeks post-FMT, we observed higher alpha diversity in the clinical responders

2 compared to the non-responders (t-test, p-value < 0.05) (Figure 2A). The measured difference

3 in alpha diversity between responders and non-responders was significant even when we used

4 a broader definition of clinical response (Supplemental Figure 1).

5

6 We next explored the relationship between alpha diversity and clinical symptoms within

7 individuals. In the three clinical responders we were able to measure a statistically significant

8 relationship (Pearson's adjusted p-values all < 0.05) between the Shannon Index and PUCAI or

9 PCDAI, where higher diversity correlated with lower disease activity (Figure 2B). We did not

10 observe the same relationship in the non-responders (Figure 2B). Finally, we sought to

11 determine at what point in the treatment course microbial diversity was most correlated with

12 symptom severity. There was no correlation between alpha diversity and disease activity index

13 pre- or post-antibiotic treatment, despite a large range of alpha diversity in the participants

14 (Figure 2C, small panels, top). There was a trend towards higher alpha diversity at earlier time

15 points correlated with lower symptom scores after FMT, but this was not statistically significant

16 (Figure 2C, small panels, bottom). On the other hand, starting at 6 weeks post-FMT, the alpha

17 diversity was significantly correlated with PUCAI or PCDAI (Pearson's correlation, r = -0.9,

18 adjusted p-value = 0.0002). Higher alpha diversity similarly demonstrated a strong association

19 with lower disease activity (Figure 2C, left panel).

20

21 **Donor Engraftment**

22 We hypothesized that the relationship between alpha diversity and clinical improvement was

23 driven largely by donor engraftment. There were 2 FMT donors. Each recipient was randomly

24 assigned to receive capsules from a single donor. The donors were healthy, and their

25 microbiomes were easily distinguishable through 16S sequencing and beta-diversity

26 measurements (Supplemental Figure 2). We measured donor engraftment using

1   SourceTracker2, a Bayesian algorithm based on Gibbs sampling that ultimately assigned a

2   predicted proportion of a stool microbial community that originated from a set of input sources

3   [27]. For each stool sample post-FMT, we used 16S sequences from the recipient's pre-

4   antibiotic stool sample, the recipient's post-antibiotic stool sample and the assigned donor's

5   capsules as potential sources. Additionally, we added the other donor as a dummy source as a

6   way of surveying for source misassignment. On average, the dummy donor was assigned a

7   proportion of 0.016, and there were only 3 of 111 instances where the assigned portion was

8   above 0.05 (Supplemental Figure 3 and Supplemental Table 2).

9

10   Starting at two weeks post-FMT, responders had higher levels of donor engraftment compared

11   to non-responders (Figure 3A and Supplemental Table 2). This association was statistically

12   significant at week two and week seven post-FMT (T-test, p-value < 0.05). At weeks six through

13   eight, responder microbiome compositions were much closer to their assigned donor than non-

14   responders (Bray-Curtis dissimilarity) (Figure 3B and 3C). In one case, the pre-FMT microbiome

15   was similar to the donor microbiome in a non-responder. However, the participant's microbiome

16   shifted away from the donor's microbial profile following FMT. (Figure 3C, right-most panel). In

17   this instance, perhaps antibiotics played as big a role in the resultant microbiome community as

18   the FMT itself.

19

20   **Correlates of Engraftment**

21   The degree of engraftment correlated with both restoration of gut microbial diversity and clinical

22   response to FMT. At 3 to 8 weeks post-FMT, there was a wide range of engraftment amongst

23   participants, from less than 0.3% to 91.8% (Figure 3A, inset). We searched for clinical,

24   laboratory and microbial correlates of engraftment by comparing the engraftment at weeks 2

25   and 7 with other features of the participants. These features included symptom severity scores,

26   inflammatory markers in the stool and blood, as well as the baseline diversity of the gut

1   microbiome before and after antibiotics. The strongest correlate of engraftment at seven weeks

2   post-FMT was engraftment at two weeks post-FMT (Pearson R = 0.69, p-value < 0.05),

3   suggesting that early engraftment—or lack thereof—is predictive of late engraftment

4   (Supplemental Figure 4). The only clinical or laboratory measurement significantly correlated

5   with engraftment was fecal calprotectin. After antibiotics, there was a negative correlation

6   between calprotectin and engraftment (Pearson R = 0.72, p-value < 0.05), suggesting that high

7   levels of gut inflammation may prevent engraftment (Supplemental Figure 4).

8

9   Antibiotic pre-conditioning significantly decreases the alpha diversity and changes the ecology

10   of the microbiome (Supplemental Figure 5A and 5B). The unanswered question, however, is

11   whether lower diversity (a "cleaner slate") or higher residual diversity after antibiotics is a more

12   supportive environment for engraftment. In our small study, we found that higher alpha diversity

13   after antibiotics correlated with higher engraftment (Pearson R = 0.69, p-value < 0.05) (Figure

14   4D). We found no such correlation between the alpha diversity prior to antibiotics—the

15   participant's baseline, in other words—and eventual engraftment. Similarly, we were able to

16   separate the post-antibiotic microbial communities in high-engrafting individuals from those in

17   low-engrafting individuals (Bray-Curtis, PERMANOVA p-value < 0.01), whereas pre-antibiotic

18   communities in high engrafting versus low engrafting individuals did not cluster separately

19   (Figure 4E.)

20

21   **Members of Family Lactobacilliaceae are Associated with High Donor Engraftment**

22   We next sought to determine if the presence of specific taxa after antibiotic treatment was

23   correlated with engraftment. To that end, we trained a Random Forest classifier that accurately

24   predicted high engraftment in the test set using relative abundance at the genus level.

25   Interestingly, when ranking feature importance, the top 4 features were genera in the family

26   Lactobacilliaceae (Figure 5A). At the timepoint immediately following antibiotics, 4 of the 12

1   species identified within family Lactobacilliaceae were higher in relative abundance in

2   participants who eventually exhibited high engraftment, including 2 unclassified

3   Lactobacilliaceae species as well as *Lactobacillus zeae* and *Lactobacillus brevis* (Figure 5B).

4   We generated individual taxa time series and compared patients with eventual high versus low

5   engraftment. For the genus Lactobacillus, as well as the species *Lactobacillus zeae*,

6   *Lactobacillus brevis* and an unclassified Lactobacillus species, we observed a spike in the

7   relative abundance of the taxa immediately following antibiotic therapy (Figure 5C and

8   Supplemental Figure 6). As the FMT (and engraftment) progressed, the Lactobacillus taxa

9   decreased in relative abundance. We are aware of only one other study of FMT in patients with

10   IBD with publicly available 16S sequencing data from the post-conditioning pre-FMT time point

11   [7]. In that study, six patients received FMT, three responded clinically and three did not.

12   Interestingly, we found that the responders had a very similar transient spike in the relative

13   abundance of the Genus Lactobacillus and the two Lactobacillus species that were detected in

14   all six patients in the study by Chu et al. (Figure 5D and Supplemental Figure 6).

15

16   **Discussion**

17   The clinical response to FMT in IBD is typically between 20 and 40%, and our study showed

18   similar efficacy [5,8,9,11,13,14]. Three of the 12 patients who completed FMT had a clinical

19   response. In this study, we focused our analysis on what separated eventual success from

20   eventual treatment failure. We found that 1.) engraftment of the donor microbiota was higher in

21   the responders vs. the non-responders, 2.) higher residual alpha diversity after antibiotic therapy

22   was associated with better engraftment and 3.) a relative rise in the abundance of several

23   Lactobacilliaceae taxa after antibiotic therapy correlated with engraftment.

24

25   Previous studies have hinted that antibiotic therapy as a component of pre-FMT conditioning

26   improves efficacy, but conditioning regimens remain highly variable, and their effect on

1 engraftment and clinical response is poorly defined [18]. The small size of our study is a

2 significant weakness. However, weekly sampling throughout the course of FMT, as well as

3 specifically sampling the gut microbial community prior to FMT but after antibiotic conditioning,

4 was a strength. The frequent sampling allowed us to study the determinants and kinetics of

5 engraftment. Among the many unanswered questions about the rules of engraftment, we

6 focused on two questions related to kinetics. First, how quickly does a donor microbiome take

7 hold in patients who eventually engraft? Second, at what point prior to FMT are recipient

8 microbial features correlated with eventual engraftment?

9

10 In patients who engraft and clinically respond, the engraftment takes root within two weeks.

11 Except for one individual, every participant that achieved engraftment of roughly 75% or greater

12 at the end of the FMT period had greater than 75% engraftment at week 2. The other individual

13 achieved greater than 75% engraftment at week 3. This suggests that features supporting

14 engraftment exist early, perhaps even before the initiation of FMT.

15

16 We found no baseline clinical features in our participant pool associated with engraftment. After

17 antibiotics, though, fecal calprotectin was negatively correlated with engraftment, suggesting

18 that decreased gut inflammation after antibiotics supports engraftment. While we could not find

19 any microbial correlates of engraftment at baseline (prior to antibiotics), there were multiple

20 microbial factors *after* antibiotic treatment that correlated with engraftment. We know of only two

21 other studies that sequenced the microbiome after antibiotics but before FMT in patients with

22 IBD. Given the relative importance of this time-limited community to engraftment, we propose

23 that future FMT studies should sample at this point to better understand how to best to prepare

24 a niche for donor engraftment [5,7].

25

1   Pre-FMT antibiotics theoretically help eliminate pathogens and commensals that may

2   outcompete donor microbes as they establish their niche in the recipient's colon. Somewhat

3   contradictory to that theory, our data suggest that maintaining a higher level of diversity after

4   antibiotics predicts better engraftment. While this has not been shown in IBD FMT trials to our

5   knowledge, this is in keeping with two other studies that assessed the determinants of

6   engraftment. In one study of FMT in patients with *Clostridioides difficile* infection, higher

7   recipient alpha diversity was associated with improved donor microbiota engraftment [12]. In a

8   separate study of FMT for patients with irritable bowel syndrome (IBS), antibiotic treatment and

9   the resulting drop in diversity seemed to decrease engraftment after FMT [21]. The discrepant

10 effect of antibiotics in IBD versus IBS (supportive of engraftment in IBD but detrimental in IBS)

11 demonstrates that different diseases may require different pre-FMT conditioning regimens.

12 Perhaps higher pathogen abundance in IBD makes antibiotics more favorable. However, our

13 data shows that there is also high inter-individual variability in the effect of antibiotics, and that

14 even within the same disease, the antibiotics created a supportive milieu for engraftment in

15 some but not in others. Determining who would benefit from antibiotics (and which antibiotics to

16 use) is an area of much-needed continued research.

17

18 The final facet of our analysis aimed to determine the specific taxa associated with engraftment.

19 Separating the participants into high versus low engraftment, we looked back at the baseline gut

20 microbiota and the post-antibiotic microbiota for beta diversity analysis. Similar to alpha

21 diversity, we found no measurable differences in the baseline samples. However, there was a

22 clear separation of the high and low engraftment patients at the time point immediately following

23 antibiotics (but before FMT). The main differences were the relative abundance of commensals

24 from the Lactobacilliaceae family. Patients who eventually had high engraftment tended to have

25 higher relative levels of these taxa. Interestingly, in an FMT trial for *Clostridioides difficile*

26 infection, Lactobacilliaceae had the highest dependency score amongst the taxonomic

1 determinants of engraftment identified [12]. Altogether, these findings suggest that

2 Lactobacilliaceae may promote engraftment, perhaps by creating a more favorable niche for the

3 donor microbiome. Multiple species in the genus Lactobacillus have been shown in pre-clinical

4 models to support intestinal regeneration. More specifically, emerging evidence has

5 demonstrated that Lactobacilli can increase goblet cells and mucin production. This could

6 support the engraftment of a new microbiome [29]. At a broader level, these findings suggest

7 that creating an ideal environment for engraftment is key. Improving FMT engraftment depends

8 on our ability to understand—and maybe one day engineer—the microbial and ecological

9 environment into which FMT microbes enter.

10

11 **Conclusions**

12 Our study demonstrates the need for more inquiry into recipient characteristics that predict FMT

13 engraftment. By analyzing the large inter-individual differences in engraftment, we propose that

14 higher residual microbial diversity after antibiotics supports engraftment, and that several

15 Lactobacilliaceae taxa may mediate that effect. Ultimately, our study is small and underpowered

16 to fully delineate the determinants of engraftment. We argue that the effects of antibiotic pre-

17 conditioning are still largely unknown, and that methodical characterization (microbial

18 sequencing, deeper clinical phenotyping, multi-omics techniques) of the post-antibiotic (but pre-

19 FMT) state will help determine the factors that make FMT in IBD succeed in some but fail in

20 others.

21

22 **List of Abbreviations**

23 CD: Crohn disease; FMT: Fecal Microbiota Transplant; PUCAI: Pediatric Ulcerative Colitis

24 Activity Index; PCDAI: Pediatric Crohn Disease Activity Index; IBD: Inflammatory Bowel

25 Disease; IBS: Irritable Bowel Syndrome; rCDI: recurrent *Clostridiodes difficile* Infection; UC:

26 Ulcerative Colitis

1

Declarations

Ethics approval and consent to participate

The study was conducted in accordance with the protocol, applicable ICH Guidelines, Good

Clinical Practice and the World Medical Association (WMA) Declaration of Helsinki and its

amendments concerning medical research in humans.

In accordance with guidelines and U.S. Code of Federal Regulations applicable to clinical

studies, the protocol and informed consent/assent forms were reviewed and approved by the

Boston Children's Hospital Institutional Review Board (IRB). The investigator informed the IRB

and FDA of subsequent protocol amendments and reportable events as defined by IRB policy

and FDA regulation.

All patients and/or their guardians consented to participate in this study.

Consent for publication

Not applicable

Availability of data and material

The availability of datasets generated during and/or analyzed during the current study is

pending.

Competing Interests

AB receives research support as a subinvestigator on protocols for Janssen, Abbvie, Takeda,

Buhlmann, Arena, Eli Lilly, Bristol Myers Squibb, PROCISE diagnostics. AB receives consulting

revenue from Takeda, Best Doctors, Eli Lilly, and Fresenius Kabi. AB received an honorarium

from Boston University and Royalties from Up To Date.

1

2   No other authors had any competing interests.

3

8

9   Authors' contributions

10   YZ performed the microbiome analysis and wrote the manuscript. AB contributed to trial design,

11   performed clinical trial procedures, and critically reviewed the manuscript. EA and TN helped

12   perform the microbiome analysis and edited the manuscript. AK performed clinical data analysis

13   and helped write the manuscript. MD, MW, PR, ML, LZ, BB and GR recruited patients,

14   performed the clinical tasks related to the trial and edited the manuscript. BB was the research

15   manager and supported the clinical trial procedures. GR designed the trial and edited the

16   manuscript. SK was the primary investigator, contributed to clinical trial design, oversaw clinical

17   trial procedures, analyzed clinical data, and was a major contributor in writing the manuscript.

18

19   **Figure Legends**

20   **Figure 1.** A. Consort Diagram. B. Clinical symptom scores (PUCAI for UC and PCDAI for CD)

21   for individual participants (top) and for responders vs non-responders (bottom).

22   **Figure 2.** A. Stool alpha diversity (Shannon index) time series for all patients, clinical non-

23   responders (blue) vs. clinical responders (red) and for individual patients, each in a unique

24   shade of gray (inset). B. Correlation between clinical symptom score (PUCAI for Ulcerative

25   Colitis, PCDAI for Crohn Disease) and alpha diversity (Shannon index) for clinical responders

26   (top) and non-responders (bottom). C. Correlation between PUCAI and alpha diversity at the

1   post FMT timepoint (left, red, large panel). Smaller panels: top panels show correlation between

2   PUCAI and alpha diversity at the pre- and post-antibiotic timepoints, bottom panels show

3   correlation between the late post-FMT PUCAI and earlier (pre- and post-antibiotic) alpha

4   diversity metrics.

5   **Figure 3.** A. Percent Donor Engraftment, as estimated by SourceTracker, in responders vs.

6   non-responders as well as for each individual patient, each in a unique shade of gray (inset) B.

7   Beta diversity plots (Bray-Curtis) for three example responders. C. Beta diversity plots for three

8   example non-responders. D. Correlation between alpha diversity (Shannon Index) and

9   engraftment at the pre-antibiotic (Left) and post-antibiotic (Right) timepoints. E. Beta-diversity

10 plots comparing eventual high engrafters (blue) and low engrafters (red) at the pre-antibiotic

11 (Left) and post-antibiotic (Right) timepoint.

12 **Figure 4.** A. Important features (on the genus level) from a random forest classifier using the

13 post-antibiotic microbiome to classify high vs. low engrafters (AUC = 1.00). All taxa are genera,

14 lowest identified taxonomic level is labeled. *Asterisks denote members of the Family

15 Lactobacilliaceae. B. Boxplot of relative abundance (proportion of total reads) of species in the

16 Family Lactobacillaceae that were detected in at least 3 individuals. ***, FDR p-value < 0.001;

17 **, FDR p-value < 0.01; *, FDR p-value < 0.05. C. Time series of relative abundance of genus

18 Lactobacillus (top left) and 3 species within genus Lactobacillus, comparing high (each

19 participant represented in a unique shade of blue) vs. low (each participant represented in a

20 unique shade of orange) engrafters in this study. D. Time series of relative abundance of genus

21 Lactobacillus (top) and 2 species within genus Lactobacillus, comparing responders (each

22 participant represented in a unique shade of blue) vs non-responders (each participant

23 represented in a unique shade of orange) in an independent (n = 6) FMT study in IBD (Chu et

24 al.).

25 **Supplemental Figure 1.** Shannon diversity of responders and non-responders using the more

26 inclusive criteria for response for all IBD patients (left) and only UC patients (right).

1 **Supplemental Figure 2.** Principal Coordinate Analysis (Bray Curtis dissimilarity) for multiple

2 stool samples from the two donors used in the study.

3 **Supplemental Figure 3.** Percent of the post-FMT microbiome attributed to the "dummy donor"

4 (the donor not used for FMT for any given subject was used as a potential source in

5 SourceTracker). Each color represents a unique subject.

6 **Supplemental Figure 4.** Correlation matrix of clinical, laboratory and microbiome factors

7 related to engraftment. Pearson R (left) and P (right) values are shown.

8 **Supplemental Figure 5.** A. Shannon index pre- and post-antibiotics. B. Principal Coordinate

9 Analysis of the pre and post antibiotic timepoints.

10 **Supplemental Figure 6.** Lactobacillus relative abundance over time at the species level for

11 both our study (left) and the reanalysis of Chu et al (right).

12 **Supplemental Table 1.** Clinical data table. Subject identifiers were randomly generated for the

13 purposes of this manuscript and are not known to anyone outside the research team.

14 **Supplemental Table 2.** SourceTracker2 results. Subject identifiers were randomly generated

15 for the purposes of this manuscript and are not known to anyone outside the research team.

16

17 **Supplemental Text**

18 **Adverse Event and Serious Adverse Event Reporting**

19 There were 3 serious adverse events (SAE) that occurred. One CD patient had an episode of

20 Grade 3 Colitis that was determined to be possibly related to the intervention. The subject was

21 randomized to the FMT arm and was started on antibiotics. After completing the antibiotic

22 regimen, the subject began experiencing a dramatic worsening of GI symptoms including bloody

23 diarrhea, watery stools, and abdominal pain. The subject declined ED evaluation or

24 hospitalization. Stool testing showed the subject was positive for a toxigenic *Clostridioides*

25 *difficile* infection (CDI). The subject was started on vancomycin. Two days later, the subject's

26 rectal bleeding resolved, but continued to have watery stools and developed nausea. Despite an

increase in vancomycin dosage, the patient had increasing abdominal pain, increased stools, nausea, vomiting, abdominal distention, fever and dehydration. At this time, the subject was evaluated in the ED and was found to have mild tachycardia and hypertension. Intravenous fluids and antibiotics were given and patient was admitted for 8-day inpatient stay until stable. This subject was removed from the study.

One UC patient (had a hypersensitivity reaction from the FMT induction enema. Minutes after the enema was given, the subject had dizziness, flushing, and chest tightness. Shortly after, the subject developed chills and Grade 3 sinus tachycardia. The patient remained tachycardic for 2 hours, at which point they were admitted to the emergency department (ED). In the ED, the subject had a high white blood cell count (WBC) and a low-grade fever. The subject was given antibiotics and admitted to the hospital. He was discharged 2 days later. This SAE was determined to be probably related to the FMT enema. This subject was removed from the study.

Another UC patient presented to the emergency department for worsening UC symptoms including increased abdominal pain, increased stool frequency, vomiting and dehydration. The subject was given intravenous fluids, total parenteral nutrition and an infusion of infliximab before being discharged. This occurred during the follow up period and was determined to be not related to the study intervention.

There were other Grade 1 and 2 adverse events including epiploic appendicitis, decreased white blood cell count, increased bilirubin, sore throat, upper respiratory infection, non-cardiac chest pain, gurgling bowel sounds, eye disorder, rash, eczema, flushing, anemia, increased alkaline phosphatase and an increased lymphocyte count; all were either not determined to be not related to the study intervention or were present at baseline. Most were resolved without sequelae.

1

1 **References**

2 1. Saha S, Mara K, Pardi DS, Khanna S. Long-term Safety of Fecal Microbiota Transplantation
3 for Recurrent Clostridioides difficile Infection. Gastroenterology. 2021;160:1961-1969.e3.

4 2. Kelly CR, Yen EF, Grinspan AM, Kahn SA, Atreja A, Lewis JD, et al. Fecal Microbiota
5 Transplantation Is Highly Effective in Real-World Practice: Initial Results From the FMT National
6 Registry. Gastroenterology. 2021;160:183-192.e3.

7 3. Nicholson MR, Mitchell PD, Alexander E, Ballal S, Bartlett M, Becker P, et al. Efficacy of
8 Fecal Microbiota Transplantation for Clostridium difficile Infection in Children. Clin Gastroenterol
9 H. 2020;18:612-619.e1.

10 4. Cammarota G, Ianiro G. FMT for ulcerative colitis: closer to the turning point. Nat Rev
11 Gastroentero. 2019;16:266–8.

12 5. Ishikawa D, Sasaki T, Osada T, Kuwahara-Arai K, Haga K, Shibuya T, et al. Changes in
13 Intestinal Microbiota Following Combination Therapy with Fecal Microbial Transplantation and
14 Antibiotics for Ulcerative Colitis. Inflamm Bowel Dis. 2017;23:116–25.

15 6. Crothers JW, Chu ND, Nguyen LTT, Phillips M, Collins C, Fortner K, et al. Daily, oral FMT for
16 long-term maintenance therapy in ulcerative colitis: results of a single-center, prospective,
17 randomized pilot study. Bmc Gastroenterol. 2021;21:281.

18 7. Chu ND, Crothers JW, Nguyen LTT, Kearney SM, Smith MB, Kassam Z, et al. Dynamic
19 Colonization of Microbes and Their Functions after Fecal Microbiota Transplantation for
20 Inflammatory Bowel Disease. Mbio. 2021;12:e00975-21.

21 8. Costello SP, Hughes PA, Waters O, Bryant RV, Vincent AD, Blatchford P, et al. Effect of
22 Fecal Microbiota Transplantation on 8-Week Remission in Patients With Ulcerative Colitis: A
23 Randomized Clinical Trial. Jama. 2019;321:156.

24 9. Rossen NG, Fuentes S, Spek MJ van der, Tijssen JG, Hartman JHA, Duflou A, et al. Findings
25 From a Randomized Controlled Trial of Fecal Transplantation for Patients With Ulcerative
26 Colitis. Gastroenterology. 2015;149:110-118.e4.

27 10. Vaughn BP, Vatanen T, Allegretti JR, Bai A, Xavier RJ, Korzenik J, et al. Increased
28 Intestinal Microbial Diversity Following Fecal Microbiota Transplant for Active Crohn's Disease.
29 Inflamm Bowel Dis. 2016;22:2182–90.

30 11. Paramsothy S, Nielsen S, Kamm MA, Deshpande NP, Faith JJ, Clemente JC, et al. Specific
31 Bacteria and Metabolites Associated With Response to Fecal Microbiota Transplantation in
32 Patients With Ulcerative Colitis. Gastroenterology. 2019;156:1440-1454.e2.

33 12. Smillie CS, Sauk J, Gevers D, Friedman J, Sung J, Youngster I, et al. Strain Tracking
34 Reveals the Determinants of Bacterial Engraftment in the Human Gut Following Fecal
35 Microbiota Transplantation. Cell Host & Microbe. 2018;23:229-240.e5.

1   13. Narula N, Kassam Z, Yuan Y, Colombel J-F, Ponsioen C, Reinisch W, et al. Systematic
2   Review and Meta-analysis. Inflamm Bowel Dis. 2017;23:1702–9.

3   14. Moayyedi P, Surette MG, Kim PT, Libertucci J, Wolfe M, Onischi C, et al. Fecal Microbiota
4   Transplantation Induces Remission in Patients With Active Ulcerative Colitis in a Randomized
5   Controlled Trial. Gastroenterology. 2015;149:102-109.e6.

6   15. Costello S, Waters O, Bryant R, Katsikeros R, Makanyanga J, Schoeman M, et al. OP036
7   Short duration, low intensity pooled faecal microbiota transplantation induces remission in
8   patients with mild-moderately active ulcerative colitis: a randomised controlled trial. J Crohn's
9   Colitis. 2017;11:S23–S23.

10 16. Danne C, Rolhion N, Sokol H. Recipient factors in faecal microbiota transplantation: one
11 stool does not fit all. Nat Rev Gastroentero. 2021;18:503–13.

12 17. Kellermayer R, Wu Q, Nagy-Szakal D, Queliza K, Ihekweazu FD, Bocchini CE, et al. Fecal
13 Microbiota Transplantation Commonly Failed in Children With Co-Morbidities. J Pediatr Gastr
14 Nutr. 2022;74:227–35.

15 18. Ianiro G, Punčochář M, Karcher N, Porcari S, Armanini F, Asnicar F, et al. Variability of
16 strain engraftment and predictability of microbiome composition after fecal microbiota
17 transplantation across different diseases. Nat Med. 2022;28:1913–23.

18 19. Smith BJ, Piceno Y, Zydek M, Zhang B, Syriani LA, Terdiman JP, et al. Strain-resolved
19 analysis in a randomized trial of antibiotic pretreatment and maintenance dose delivery mode
20 with fecal microbiota transplant for ulcerative colitis. Sci Rep-uk. 2022;12:5517.

21 20. Keshteli AH, Millan B, Madsen KL. Pretreatment with antibiotics may enhance the efficacy of
22 fecal microbiota transplantation in ulcerative colitis: a meta-analysis. Mucosal Immunol.
23 2017;10:565–6.

24 21. Singh P, Alm EJ, Kelley JM, Cheng V, Smith M, Kassam Z, et al. Effect of antibiotic
25 pretreatment on bacterial engraftment after Fecal Microbiota Transplant (FMT) in IBS-D. Gut
26 Microbes. 2022;14:2020067.

27 22. Fischer M, Sipe B, Cheng Y-W, Phelps E, Rogers N, Sagi S, et al. Fecal microbiota
28 transplant in severe and severe-complicated Clostridium difficile: A promising treatment
29 approach. Gut Microbes. 2017;8:289–302.

30 23. Bolyen E, Rideout JR, Dillon MR, Bokulich NA, Abnet CC, Al-Ghalith GA, et al.
31 Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. Nat
32 Biotechnol. 2019;37:852–7.

33 24. Callahan BJ, McMurdie PJ, Rosen MJ, Han AW, Johnson AJA, Holmes SP. DADA2: High-
34 resolution sample inference from Illumina amplicon data. Nat Methods. 2016;13:581–3.

35 25. McMurdie PJ, Holmes S. phyloseq: An R Package for Reproducible Interactive Analysis and
36 Graphics of Microbiome Census Data. Plos One. 2013;8:e61217.

1   26. Pruesse E, Quast C, Knittel K, Fuchs BM, Ludwig W, Peplies J, et al. SILVA: a
2   comprehensive online resource for quality checked and aligned ribosomal RNA sequence data
3   compatible with ARB. Nucleic Acids Res. 2007;35:7188–96.

4   27. Knights D, Kuczynski J, Charlson ES, Zaneveld J, Mozer MC, Collman RG, et al. Bayesian
5   community-wide culture-independent microbial source tracking. Nat Methods. 2011;8:761–3.

6   28. Bokulich NA, Dillon MR, Bolyen E, Kaehler BD, Huttley GA, Caporaso JG. q2-sample-
7   classifier: machine-learning tools for microbiome classification and regression. J Open Res
8   Softw. 2018;3:934.

9   29. Xie S, Zhao S, Jiang L, Lu L, Yang Q, Yu Q. Lactobacillus reuteri Stimulates Intestinal
10 Epithelial Proliferation and Induces Differentiation into Goblet Cells in Young Chickens. J Agr
11 Food Chem. 2019;67:13758–66.

12

Table 1

	Crohn's Disease Patients (n=4)	UC Patients (n=9)
Sex	3 Male	9 Male
Race	4 Caucasian	9 Caucasian
Age at Diagnosis (Years)	12 (Range: 8-16)	19 (Range: 10-25)
Age at Enrollment (Years)	20 (Range: 18-23)	24 (Range: 14-29)
Disease Index		
Mild	4	3
Mild/Moderate	0	3
Moderate	0	3
Concomitant Medications*		
Amino-salicylates	1	4
Immunomodulators (6MP, MTX)	0	2
Anti-TNF Biologics	3	4
Other Biologics	2	0
Corticosteroids	1	5
Antibiotics	2	2
Baseline Fecal Calprotectin (µg/g)** **Normal: <50**	1440 (Range: 1430-1450) N=2	2190 (Range: 5-5730)
Baseline CRP (mg/L)** **Normal: 0-4.9 mg/L**	2.45 (Range: 0.3-5.2)	1.9 (Range: 0.3-14.7)
Baseline ESR (mm/hr)** **Normal: 0-32 mm/hr**	14.5 (Range: 2-41)	12 (Range: 2-79)
Prior IBD-Related Hospitalization		
Yes	2	6
No	0	1
Unknown	2	2
Arm Assigned		
Treatment	2	6
Placebo	2	3

*total > n because patients may be on multiple medications

**Median values shown

Figure 1

A
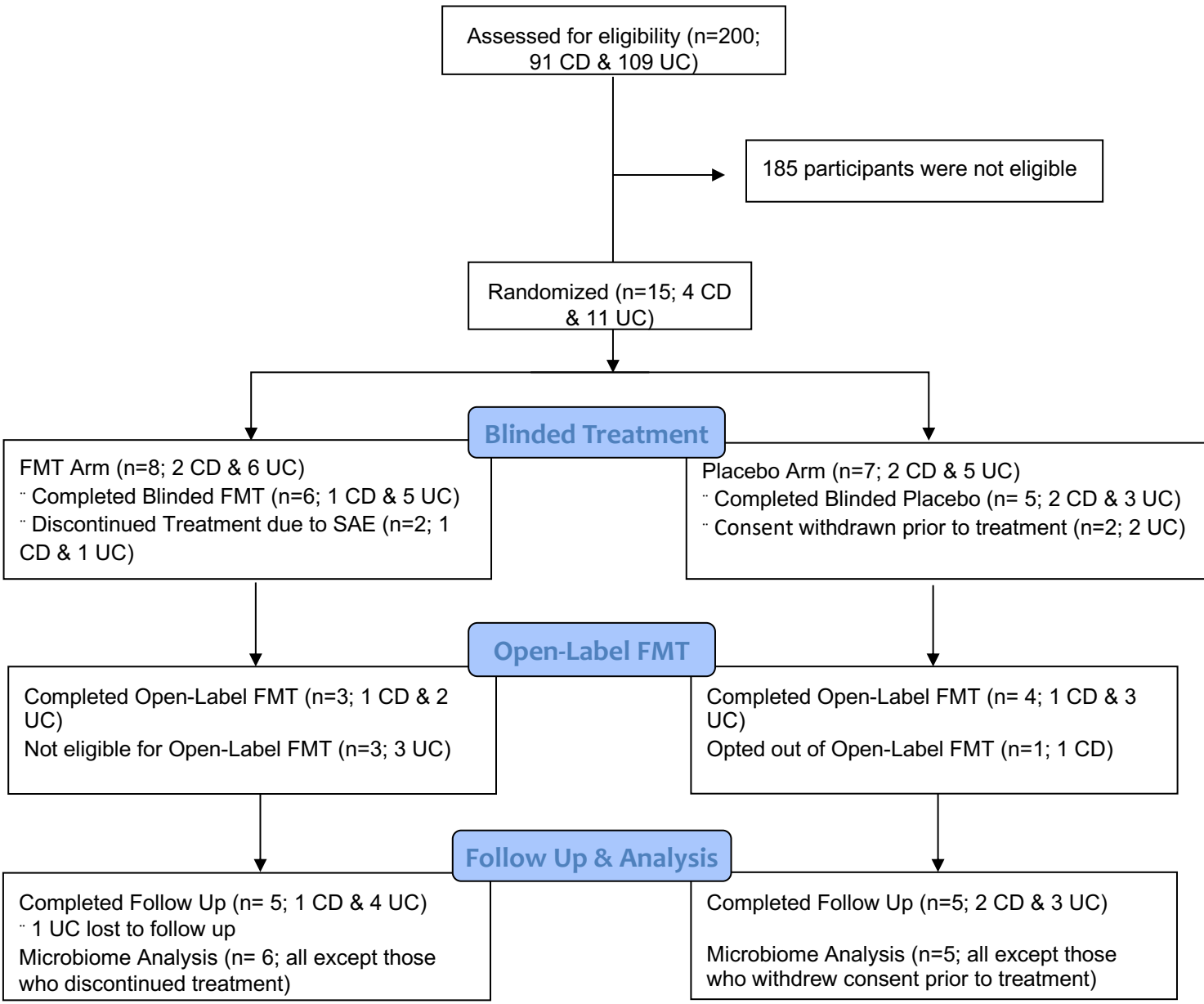
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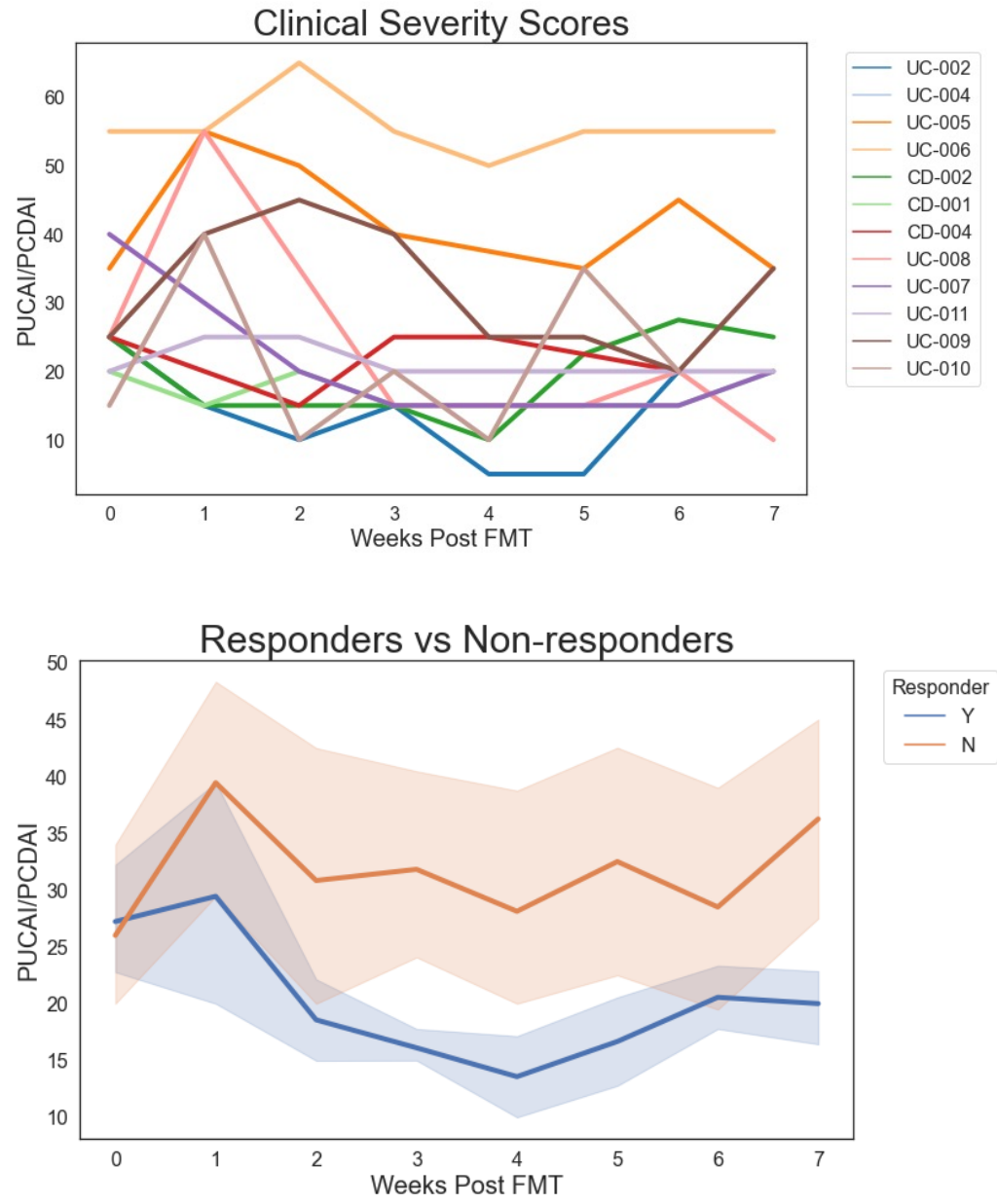
Figure 2

Figure 3

Figure 4

A — Engrafter vs. Non-engrafter Classifier

* = Family Lactobacilliaceae

B — Engraftment after FMT

C — This Study

D — Deep Dive (Chu et al.)
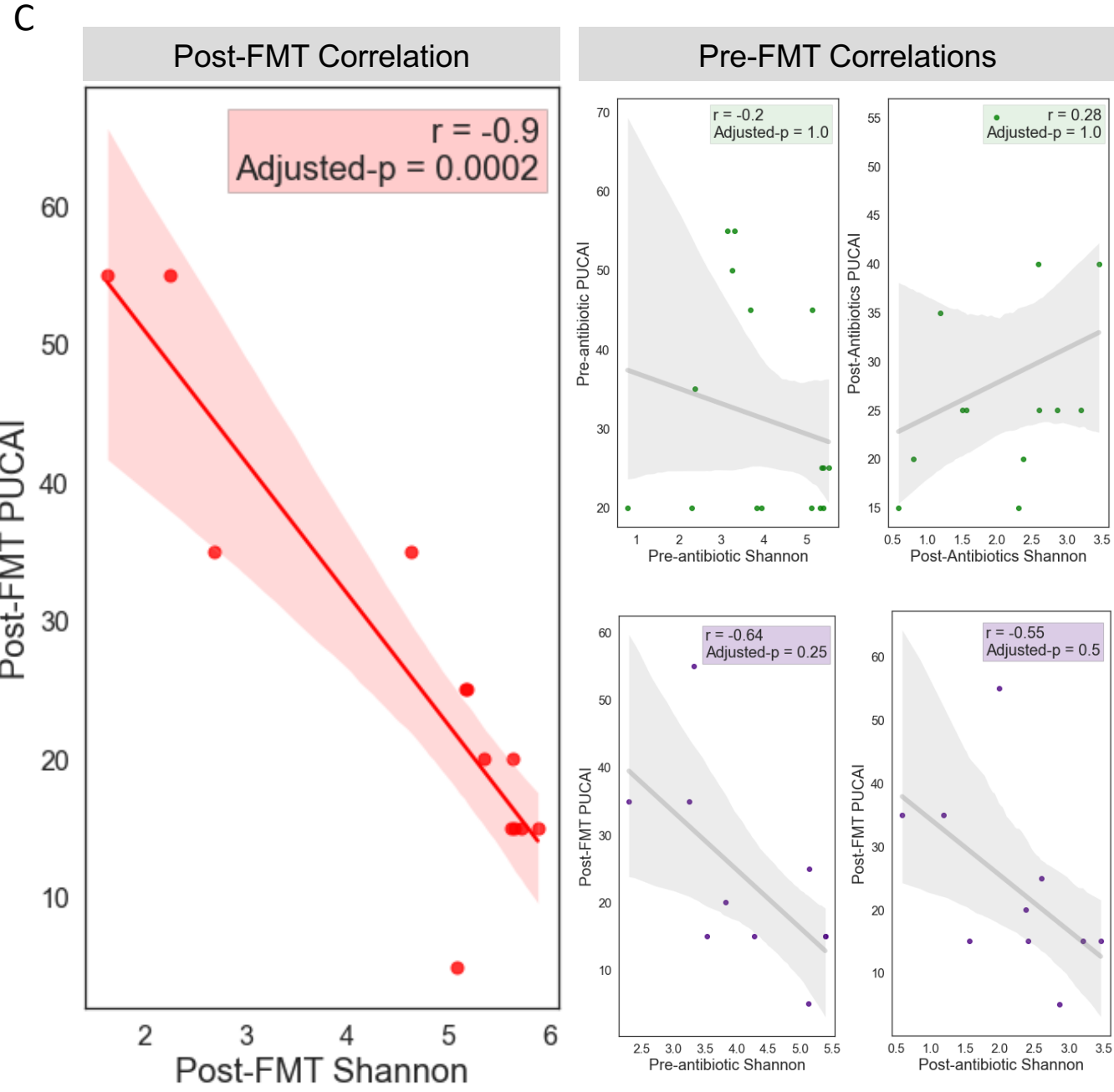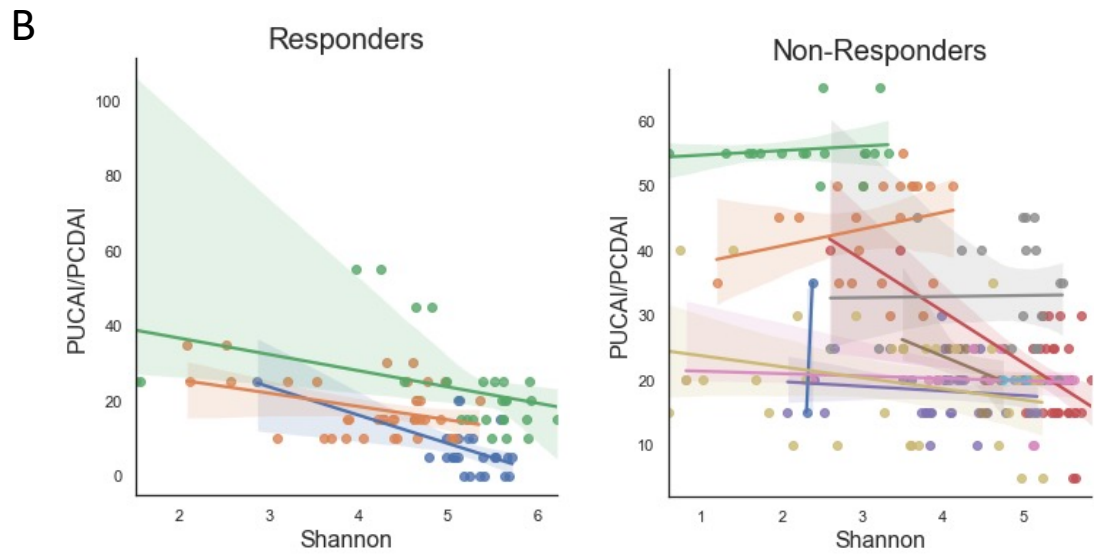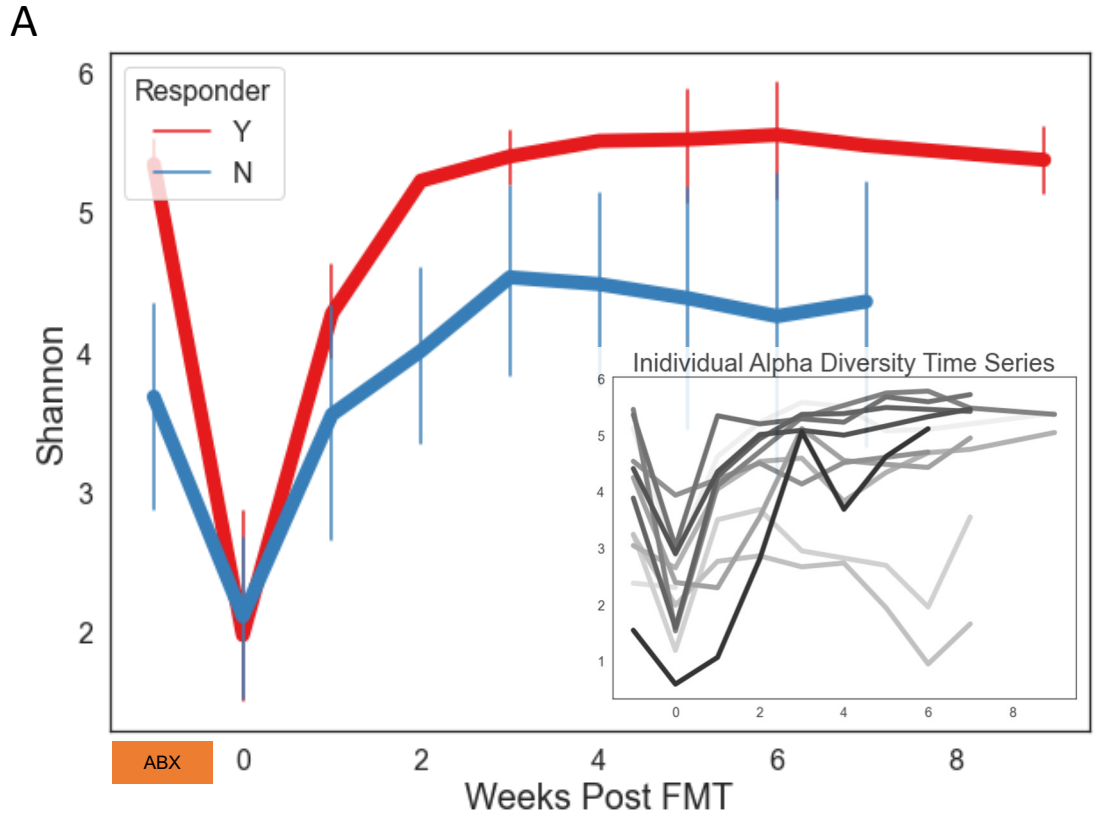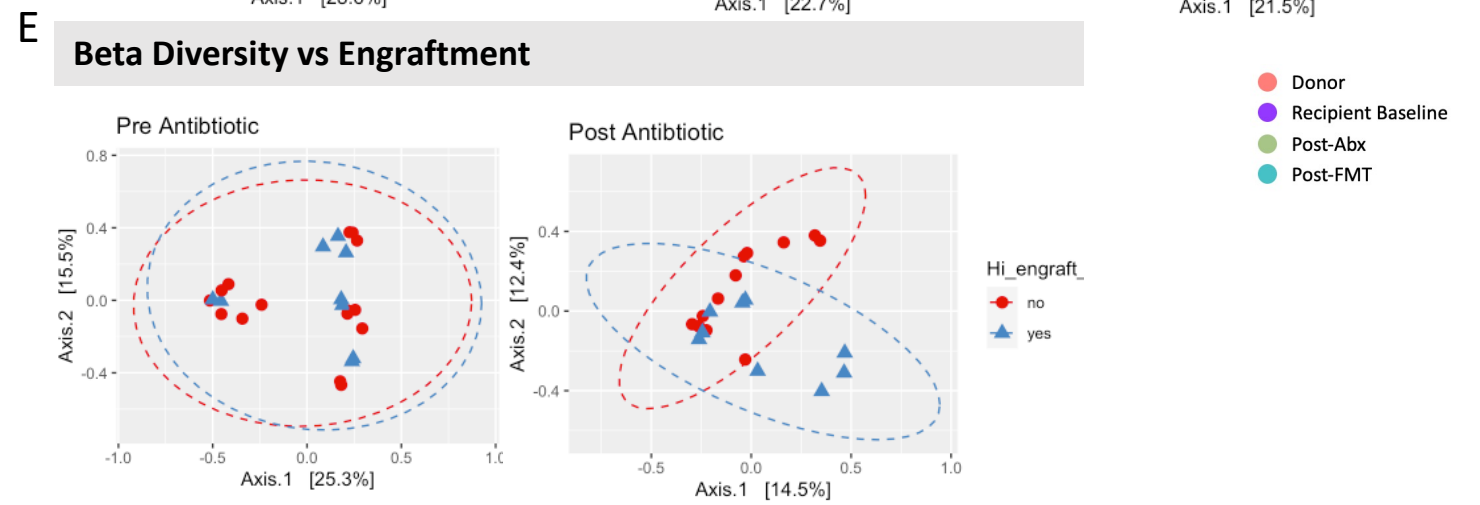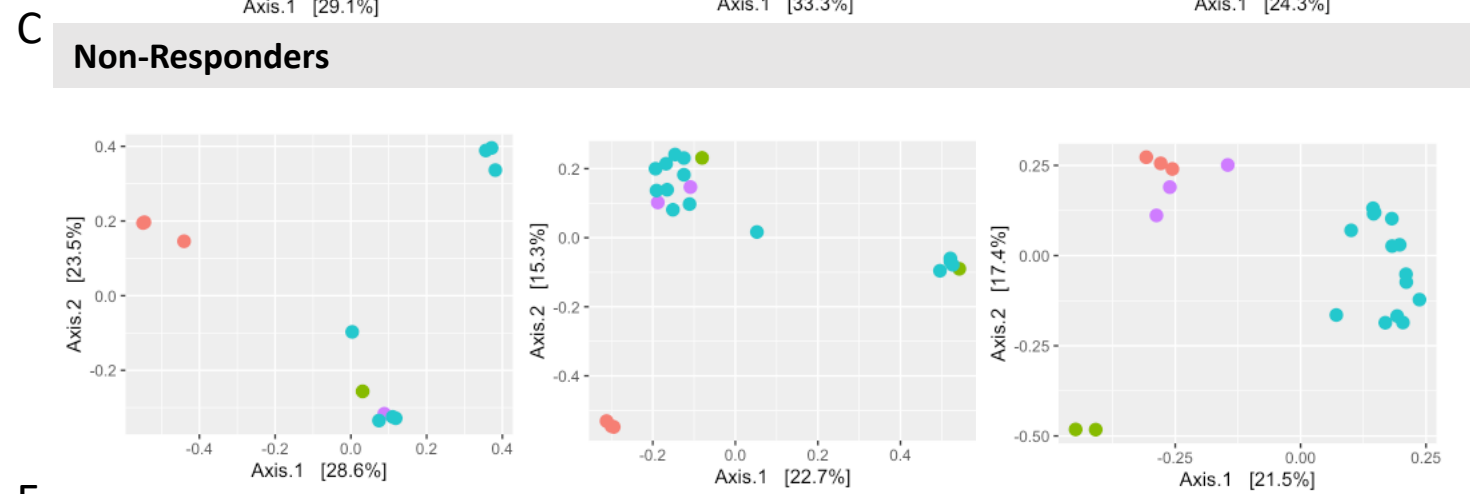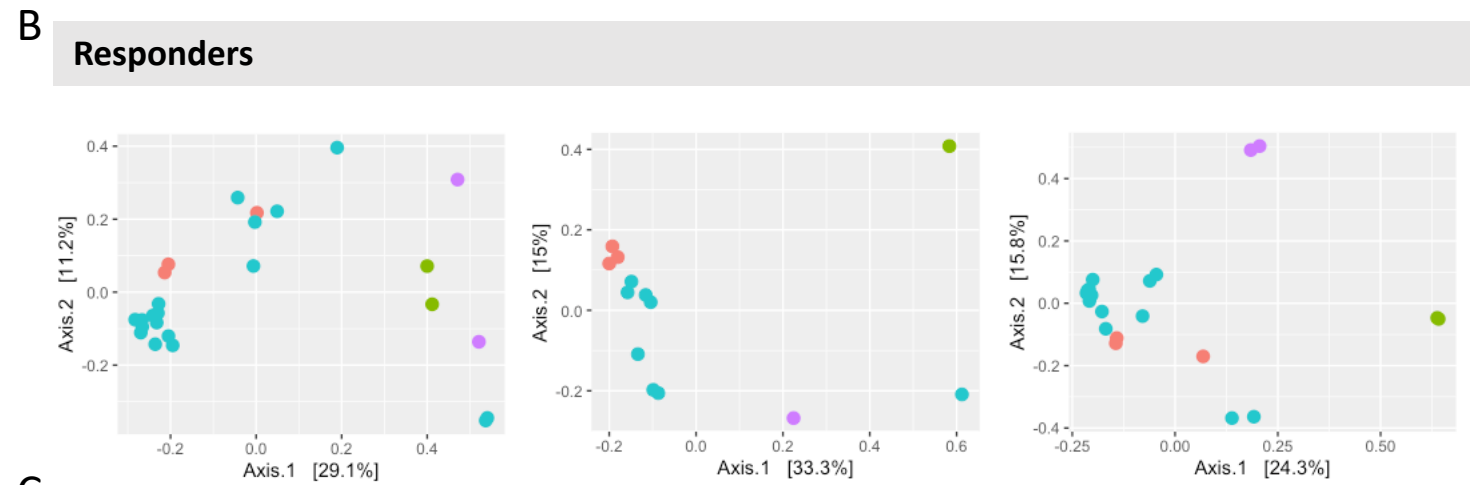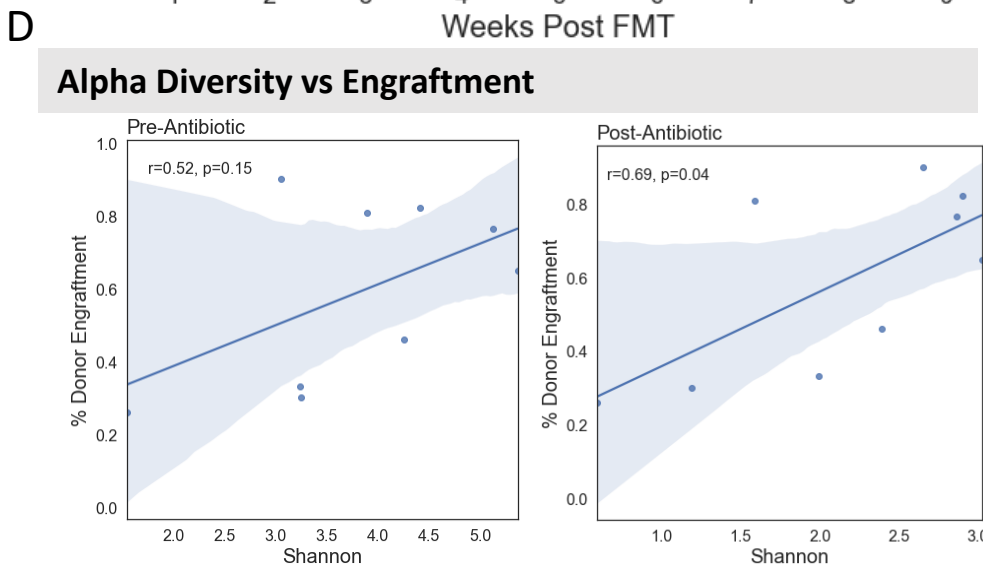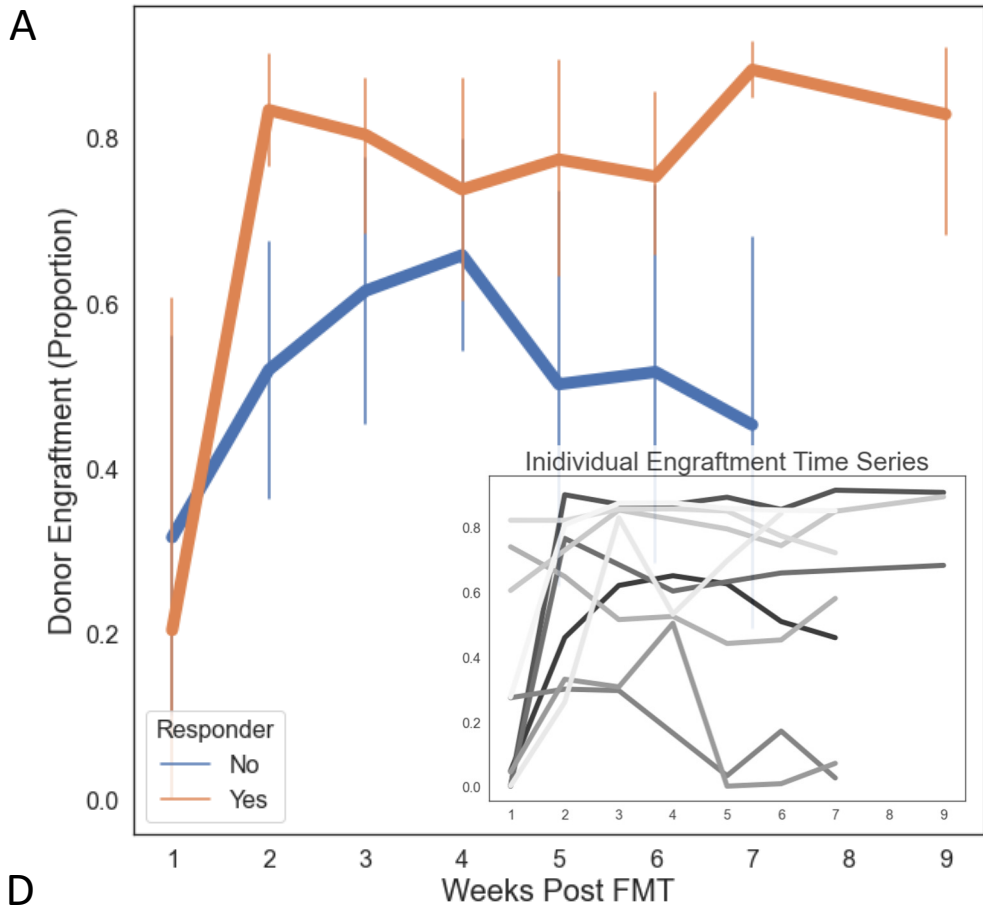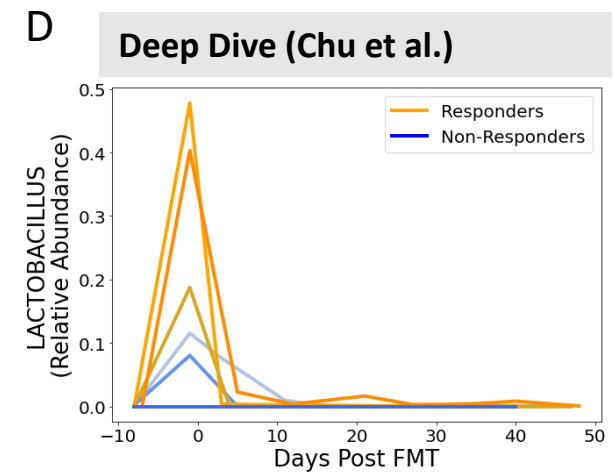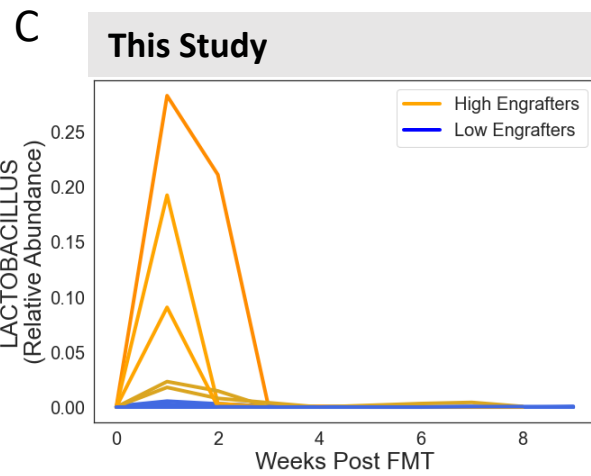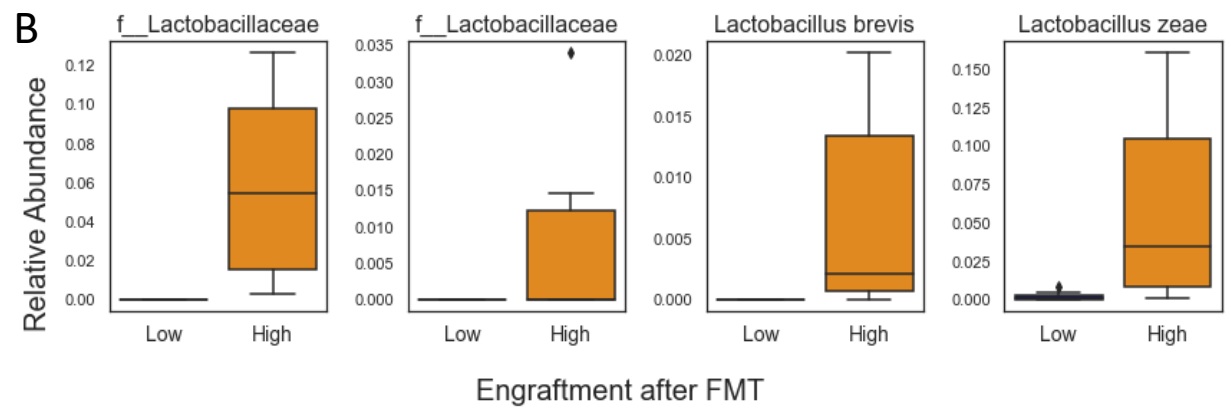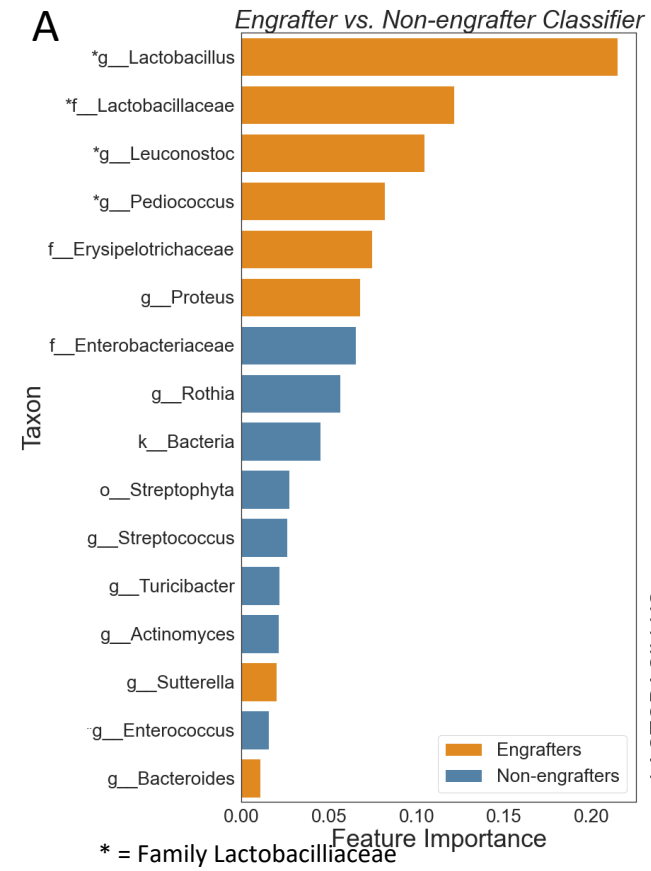
Supplemental Figure 1

Inclusive Responder Definition, all IBD
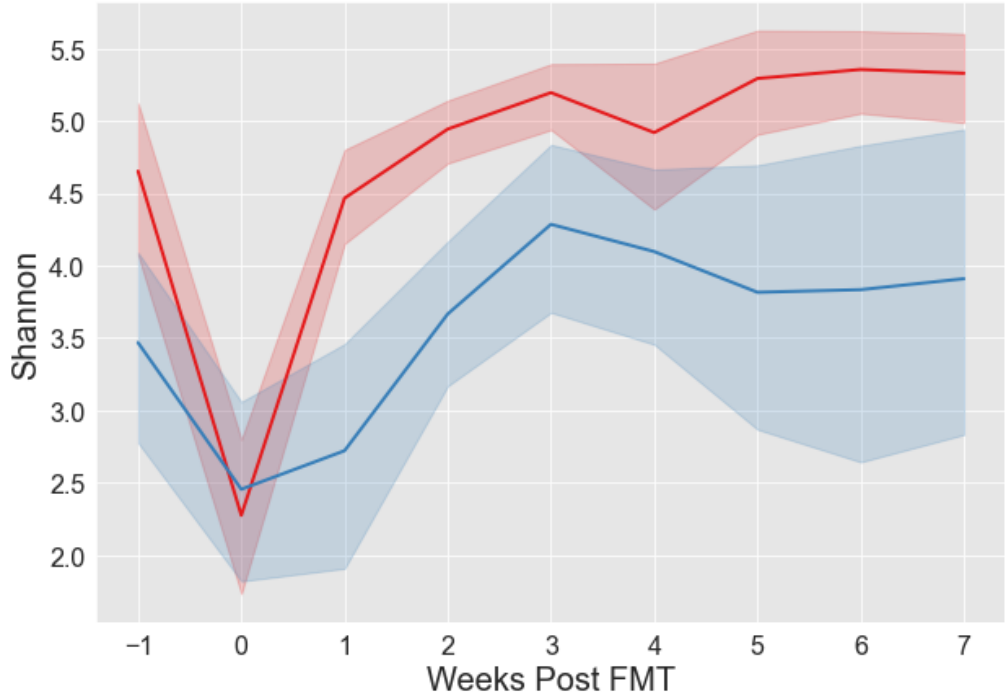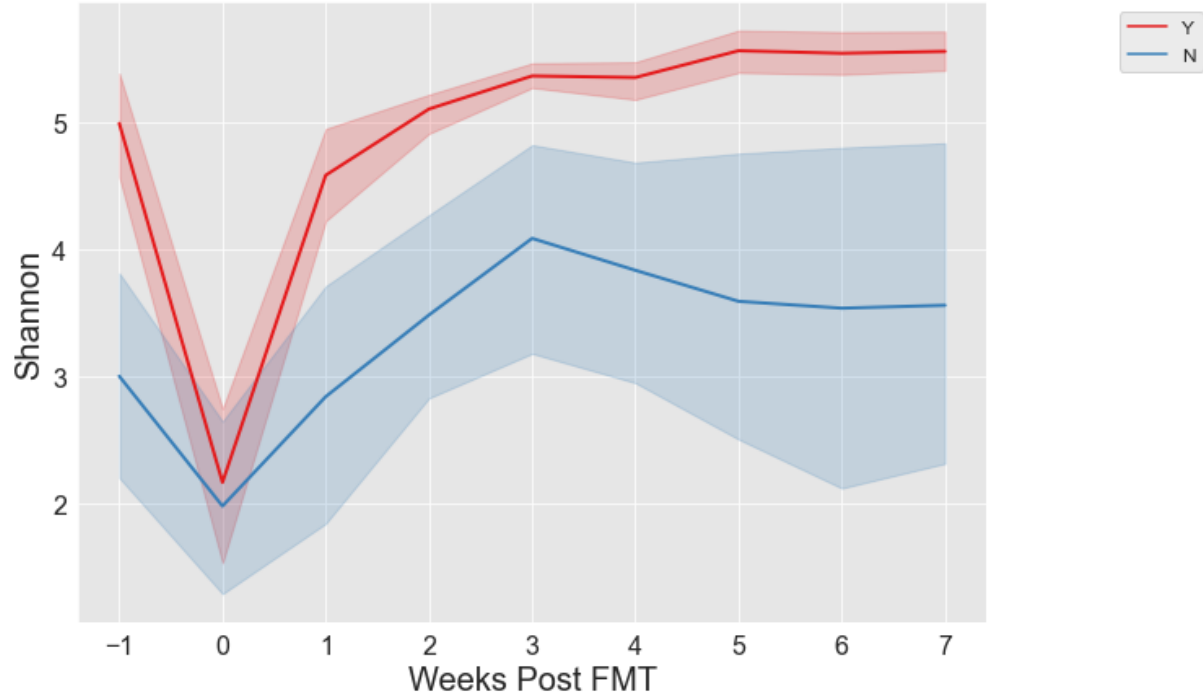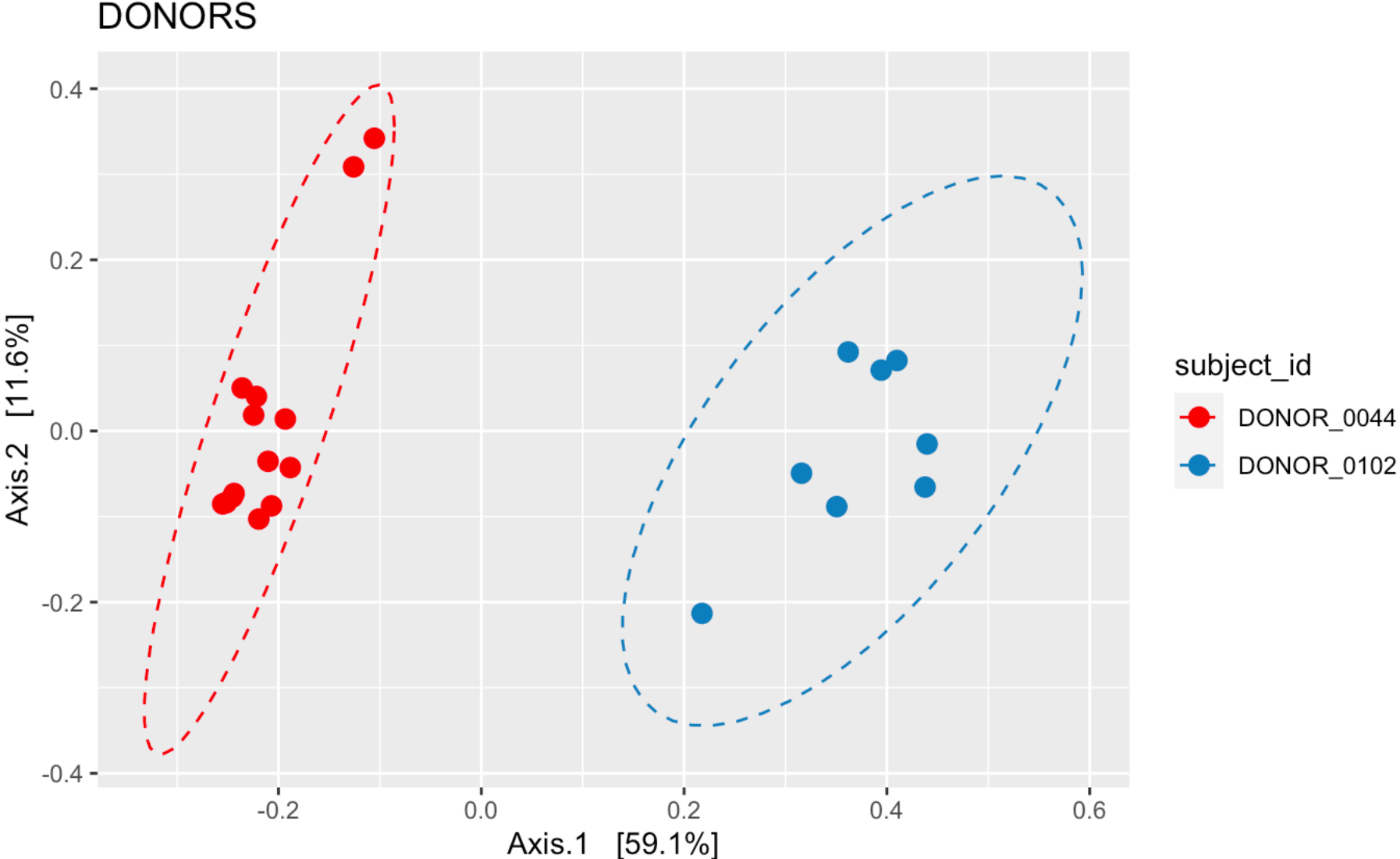
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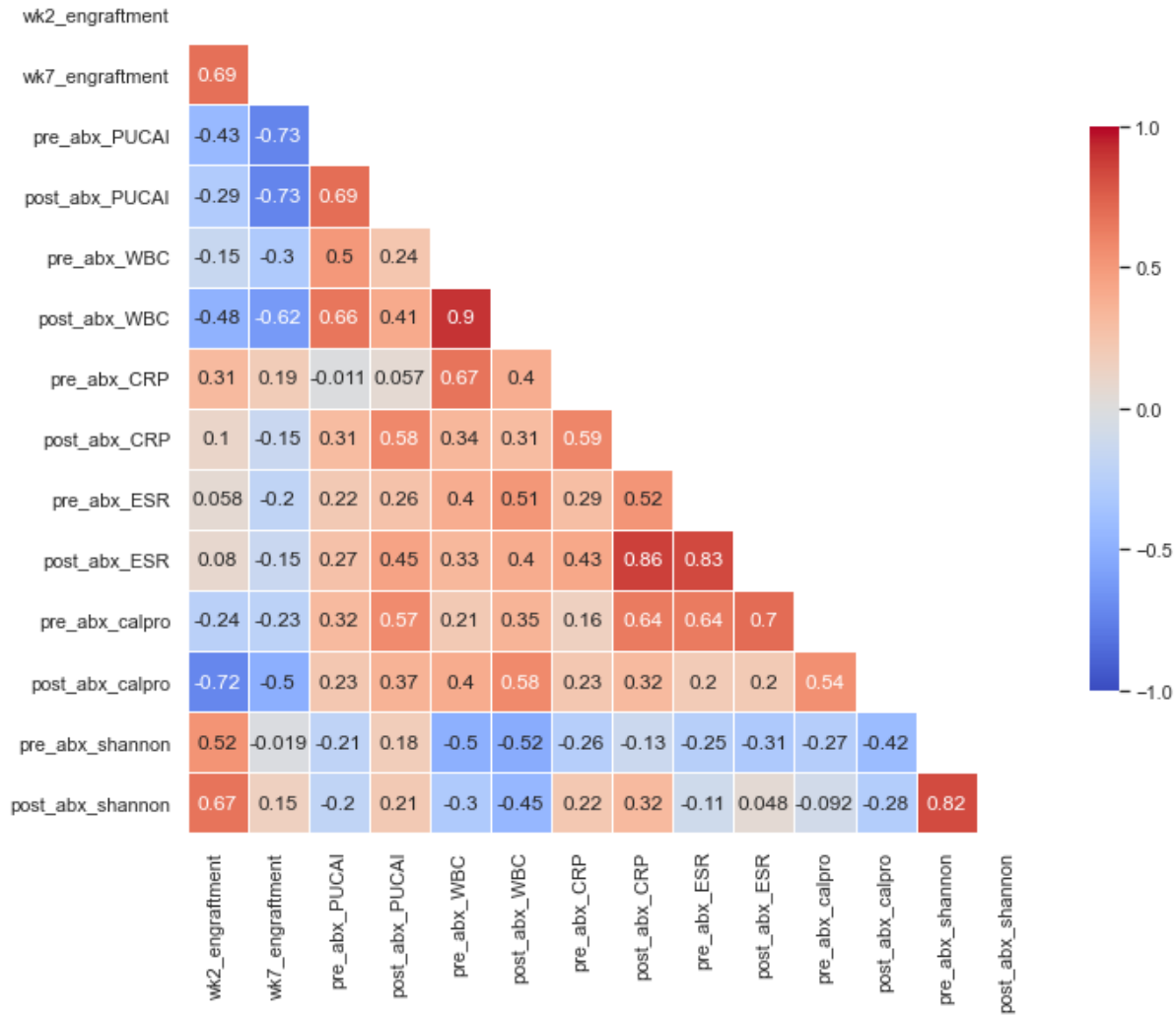
Supplemental Figure 2

Supplemental Figure 3
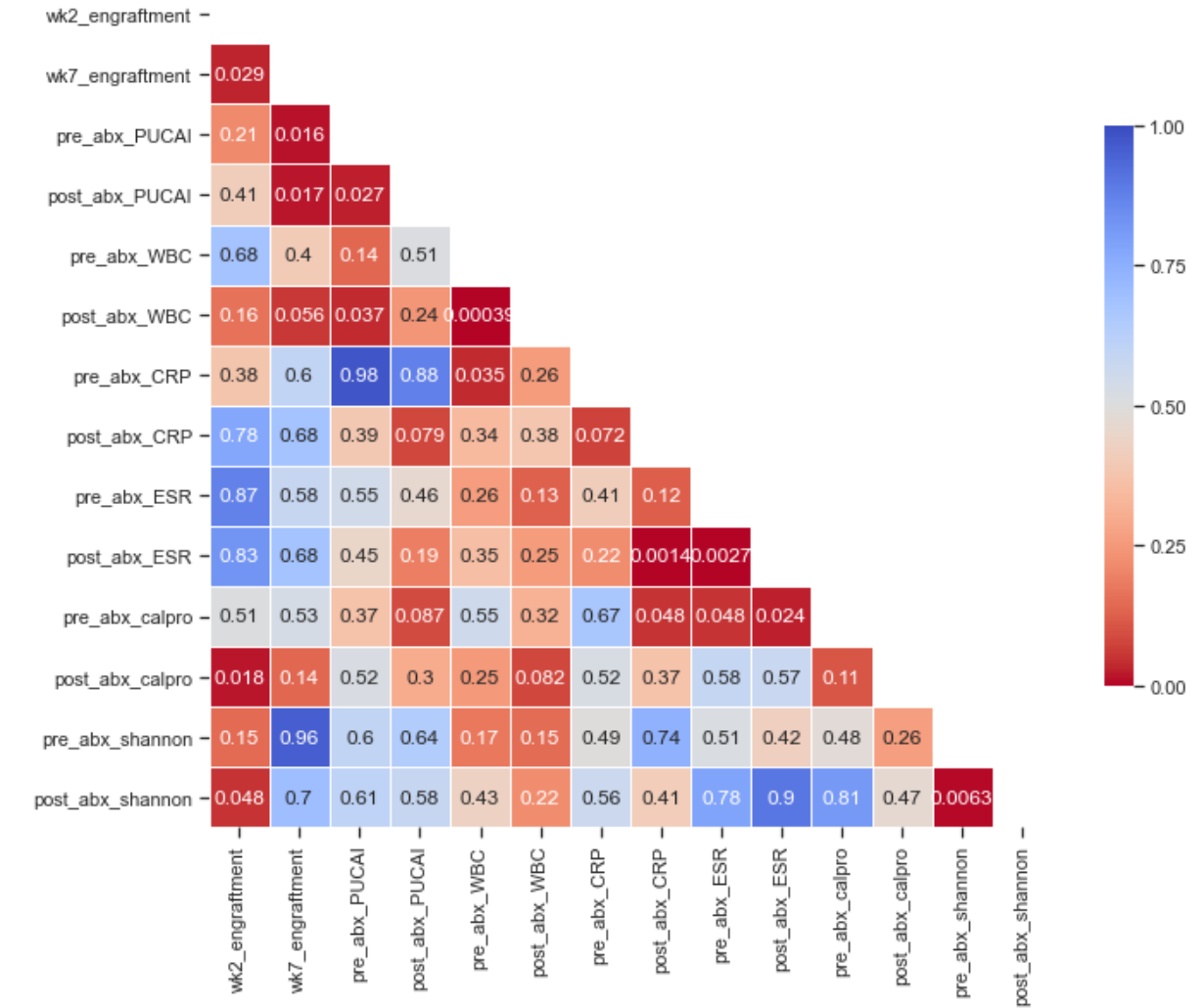
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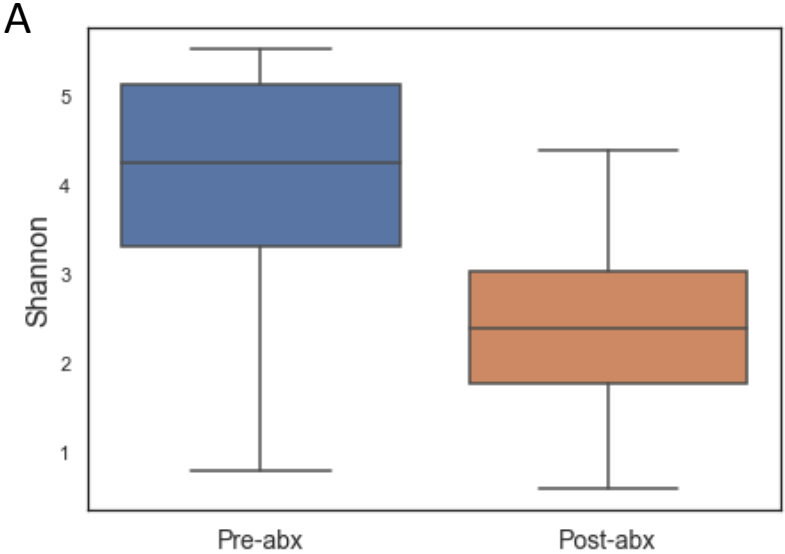### Pearson Correlation

Pearson p value

Supplemental Figure 5

A

B

Pre and Post Antibtiotic

Supplemental Figure 6