Rigorous Donor Selection for Fecal Microbiota Transplantation in Active Ulcerative Colitis: Key Lessons From a Randomized Controlled Trial Halted for Futility

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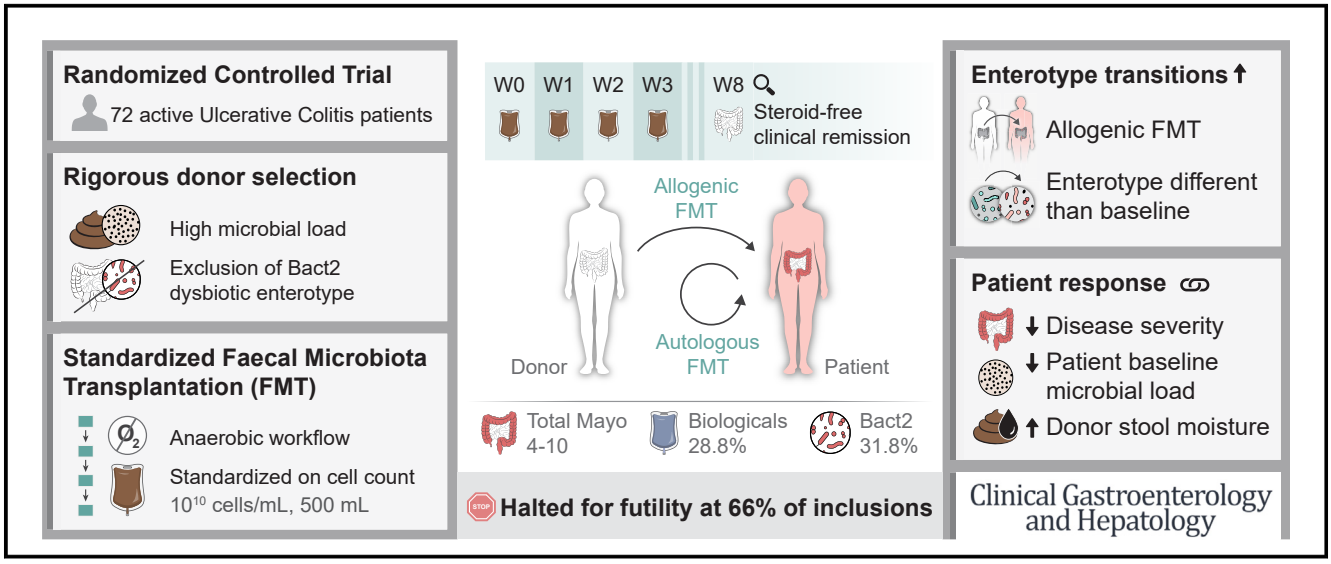

BACKGROUND & AIMS: **Rigorous donor preselection on microbiota level, strict anaerobic processing, and repeated fecal microbiota transplantation (FMT) administration were hypothesized to improve FMT induction of remission in ulcerative colitis (UC).**

*Authors share co-first authorship §Authors share co-senior authorship.

Abbreviations used in this paper: AE, adverse events; Bact2, Bacteriodes 2; CRP, C-reactive protein; FCal, fecal calprotectin; FGFP, Flemish Gut Flora Project; FMT, fecal microbiota transplantation; QMP, quantitative microbiota profiling; UC, ulcerative colitis.

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METHODS: **The RESTORE-UC trial was a multi-centric, double-blind, sham-controlled, randomized trial. Patients with moderate to severe UC (defined by total Mayo 4–10) were randomly allocated to receive 4 anaerobic-prepared allogenic or autologous donor FMTs. Allogenic donor material was selected after a rigorous screening based on microbial cell count, enterotype, and the abundance of specific genera. The primary endpoint was steroid-free clinical remission (total Mayo ≤2, no sub-score >1) at week 8. A pre-planned futility analysis was performed after 66% (n = 72) of intended inclusions (n = 108). Quantitative microbiome profiling (n = 44) was performed at weeks 0 and 8.**

RESULTS: **In total, 72 patients were included, of which 66 received at least 1 FMT (allogenic FMT, n = 30 and autologous FMT, n = 36). At week 8, respectively, 3 and 5 patients reached the primary endpoint of steroid-free clinical remission (P = .72), indicating no treatment difference of at least 5% in favor of allogenic FMT. Hence, the study was stopped due to futility. Microbiome analysis showed numerically more enterotype transitions upon allogenic FMT compared with autologous FMT, and more transitions were observed when patients were treated with a different enterotype than their own at baseline (P = .01). Primary response was associated with lower total Mayo scores, lower bacterial cell counts, and higher Bacteroides 2 prevalence at baseline.**

CONCLUSION: **The RESTORE-UC trial did not meet its primary endpoint of increased steroid-free clinical remission at week 8. Further research should additionally consider patient selection, sterilized sham-control, increased frequency, density, and viability of FMT prior to administration. ClinicalTrials.gov, Number: NCT03110289.**

Keywords: Fecal Microbiota Transplantation; IBD; Microbiome; Ulcerative Colitis.

The human gut microbiota has been identified as a key mediator in the pathogenesis of ulcerative colitis (UC), with patients displaying a low bacterial load, low microbial richness, higher prevalence of the dysbiotic enterotype Bacteroides 2 (Bact2), and reduced abundance of anti-inflammatory and butyrate-producing taxa such as *Faecalibacterium spp*.[1] Despite these findings, UC therapies primarily aim to attenuate inflammation by targeting the host response, leading to 1-year remission rates ceiling at 30%. Therefore, (complementary) strategies to modulate the microbiota away from UC-associated dysbiosis have gained attention.[2]

Fecal microbiota transplantation (FMT) is a radical approach to restore eubiosis in patients harboring dysbiotic gut microbial communities. Several randomized clinical trials have investigated FMT's therapeutic potential for UC,[3–8] but heterogeneity in study design limits generalization of results. A trend towards donor-dependent FMT success[3] suggests an association between donor microbiota richness and positive treatment outcomes.[9,10] Moreover, preserving the viability of oxygen-sensitive colonic bacteria by anaerobic FMT preparation has been hypothesized to be associated with increased efficacy,[5] with aerobic processing affecting specifically Clostridiales abundances.[11]

With respect to standardization of FMTs, a key aspect that is frequently overlooked concerns the microbial density of the fecal slurries administered. Aside from some commendable exceptions,[12,13] it appears common practice to standardize the latter based on the weight of the fecal material used for the preparation of a predefined FMT volume.[14] However, quantitative microbiome profiling demonstrated up to 10-fold differences in microbial load between stools of healthy individuals.[15] Using weight-based methods of standardization, these differences prevail in the microbial cell density of FMTs, generating a currently uninvestigated confounder affecting treatment outcome.

Here, we present the results of a multi-center, double blind, sham-controlled, randomized clinical trial (RESTORE-UC) with repeated FMTs to induce clinical remission in patients with active UC through rigorous donor screening and by applying an anerobic workflow to create cell-density-standardized FMT preparations. Thereby, we targeted the identification and characterization of potentially highly effective donors (also referred to as 'superdonors') for treatment of UC.

Methods

Study Design

The RESTORE-UC trial (NCT03110289) was a multicentric, double-blind, sham-controlled randomized clinical trial performed in Belgium, to evaluate the efficacy and safety of rigorously screened allogenic donor FMT in patients with active UC.

Ethical compliance. The study protocol was approved by the ethical committee of UZ/KU Leuven (Commissie Medische Ethiek, S59525/B322201732687). Study design complied with all relevant ethical regulations

(Declaration of Helsinki and Belgian privacy). All participants provided a signed informed consent. All authors had access to the study data, and reviewed and approved the final manuscript.

Allogenic donor screening. Eligible donors were recruited locally, according to international consensus guidelines,[14] based on a general health questionnaire and blood and fecal parameters (Supplementary Table 1). All potential donors were tested for transmittable diseases by blood and fecal examination (Supplementary Table 2), maximum 4 weeks before donation started and a second time at the end of the donation period. Potential 'superdonors' were further selected based on 3 criteria: microbial cell counts ($>1.75 \times 10^{11}$ cells/g), enterotype and the abundance ($>1\%$) of the genera *Fusobacterium*, *Escherichia/Shigella*, and *Veillonella*. Also, samples belonging to the Bact2 enterotype were excluded, even if they were not low in bacterial cell count.

Patient recruitment. Patients were required to have active UC (Total Mayo score 4–10) confirmed by endoscopy (Mayo endoscopic sub-score ≥ 2) (Supplementary Table 3).

Study design and futility analysis. Patients were randomized to receive 4 infusions of allogenic donor or autologous FMT (Figure 1; Supplementary Methods). Fecal, blood, and (partial) Mayo scores were collected at each study visit, and endoscopy was performed at week 8 (primary endpoint). A safety analysis was conducted after 33% and 66% of inclusions, complemented with a futility analysis (Supplementary Methods) after 66% of projected inclusions (n = 72).

Primary and secondary outcomes. The primary endpoint was steroid-free clinical remission at week 8, defined as a total Mayo score of ≤ 2, with no individual sub-score >1. Secondary endpoints included steroid-free PRO-2 remission (with partial Mayo score for rectal bleeding and stool frequency combined ≤ 1), steroid-free clinical response (defined as a decrease of ≥ 3 points in the partial Mayo score or a $\geq 50\%$ reduction from baseline in combined rectal bleeding plus stool frequency Mayo sub-scores, or both), endoscopic remission (Mayo endoscopic sub-score of 0), and endoscopic improvement (Mayo endoscopic sub-score <2). In addition, changes in C-reactive protein (CRP) and fecal

What You Need to Know

Background

Generalization of findings on fecal microbiota transplantation (FMT) in ulcerative colitis (UC) is hampered by heterogenous study designs, including major differences in patient populations, donor selection, preparation methods, dosage, frequency, and administration protocol.

Findings

FMT standardization including rigorous allogenic donor screening based on microbial cell counts, enterotype, and abundance of dysbiosis-related genera, anaerobic preparation, and multiple administrations were insufficient to increase efficacy in patients with moderate to severe UC.

Implications for patient care

Future study design should consider only patients with mild to moderate UC, opt for a sterilized sham treatment, reduce volume and increase density of FMTs, increase the number of administrations, prescreen patients for dysbiosis, and assess viability of FMT prior to treatment.

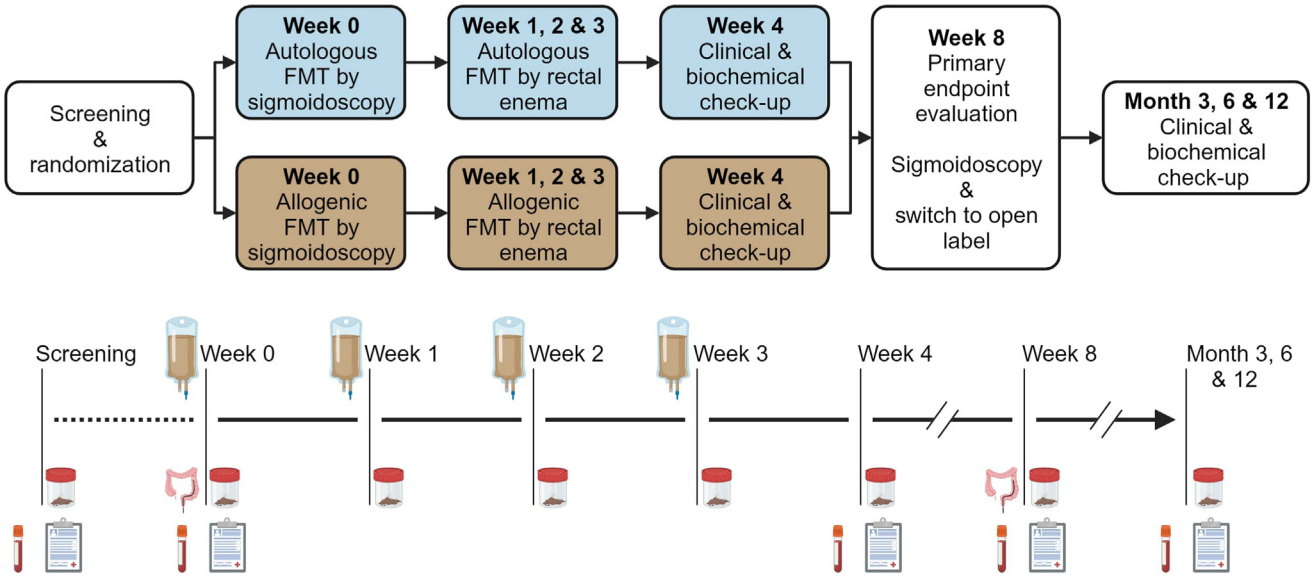

Figure 1. Study design of the RESTORE-UC trial.

calprotectin (FCal) before and after FMT were analyzed. The microbial endpoint was defined as a shift away from the Bact2 enterotype. An interim futility analysis at 66% of inclusions (n = 72) was performed, requiring a treatment difference of at least 5% in favour of allogenic FMT.

Characterization of Fecal Microbial Communities

Fecal microbiota were characterized (Supplementary Methods) by microbial load measurement through flow cytometry, fecal moisture and FCal, and 16S sequencing followed by quantitative microbiota profiling and enterotyping.

Faecal Microbiota Transplantation Preparation

Allogenic FMT. From August to September 2017, 57 healthy volunteers were invited to participate in a rigorous screening effort to identify potentially highly effective FMT donors (Supplementary Figure 1). After a medical interview and parasite screening, the 15 individuals with highest fecal cell counts (Supplementary Methods) were selected as allogenic donors for the RESTORE-UC trial (Supplementary Table 4). From October to December 2017, donors provided up to 40 fecal samples that were used to generate 500 mL FMT preparations with standardized cell density of 10^{10} cells/mL (Supplementary Methods). Additionally, samples containing the Bact2 enterotype (observed in 3 donors [4%]) were excluded for administration to patients.

Autologous FMT. During the screening period, each patient with UC delivered 4 fresh fecal samples for preparation of the autologous FMTs, regardless of the treatment arm allocation. Autologous FMT preparation followed the same anerobic procedure as for the allogenic donor FMTs, except for diluting, since none of the patients reached the microbial load barrier that was set for allogenic FMTs.

FMT procedure. FMTs were administered at baseline and weeks 1, 2, and 3. Before administration, the FMT was thawed at 37 °C for 30 minutes in a circulating water bath (Lauda-Brinkmann, VWR). Patients were instructed to take standard polyethylene glycol electrolyte solution prior to the baseline endoscopy. The first FMT was always administrated through sigmoidoscopy upon bowel cleansing, and the following FMTs were applied via rectal enemas, without prior cleansing.

Statistical Analyses

Statistical analyses were performed using the statistical software R version 4.3.0. *P*- or *q*-values smaller than .05 were considered statistically significant.

Results

Patient Inclusion and Randomization

Between March 2018 and March 2021, 72 UC patients were screened and 70 subjects randomized to allogenic (n = 33) or autologous (n = 37) FMT treatment (Figure 2). Four patients dropped out prior to the administration of the first FMT (withdrawal of consent [n = 2], cytomegalovirus colitis [n = 1], inability to attend the study visits due to injury [n = 1]), resulting in a final cohort composition of, respectively, 30 and 36 patients in the allogenic and autologous intervention arm (Table 1; Figure 3*A*).

No Significant Differences in Baseline Microbiome Composition Between Treatment Arms

Limited by sample availability, a microbiome RESTORE-UC sub-cohort (mRESTORE-UC; n = 44) was compiled, comprising those patients for whom a full triade of quantitative microbiome profiling (QMP) profiles could be generated, including samples from donor, baseline, and week 8. No significant differences in baseline demographic or clinical characteristics were observed between the mRESTORE-UC allogenic (n = 20) and autologous (n = 24) subsets and the respective treatment groups from which they were drawn (Supplementary Tables 4 and 5). Analysis of quantitative genus-level patient microbiome community variation at baseline revealed no significant difference between treatment groups (Bray-Curtis distance on QMP matrix, Adonis test; $P = .89$) (Figure 3*B*). Additionally, no significant differences in taxon abundances (Supplementary Table 6) and richness, diversity, or evenness indicators were observed between patients randomized to both intervention arms (Supplementary Figure 2).

Microbiome community-typing identified 14 of 44 (31.8%) (Figure 3*C*) mRESTORE-UC participants as carriers of the Bact2 enterotype, which largely exceeded the 12.9% observed in a large cross-sectional cohort recruited in the same region (n = 1164; Fisher exact test, $P = .002$), but remained significantly lower than the 57.1% recently reported for a UC cohort (n = 108; Fisher exact test, $P = .0006$).[16] Analyses of baseline Bact2 configurations confirmed previous findings (Supplementary Results).

No Significant Impact of Allogenic FMT on Primary Endpoint - Steroid-free Clinical Remission At Week 8

After 66% of intended inclusions (n = 72) (Figure 2), a predefined futility analysis was performed, applying a modified intention-to-treat approach (excluding subjects that dropped out before the start of the treatment). This

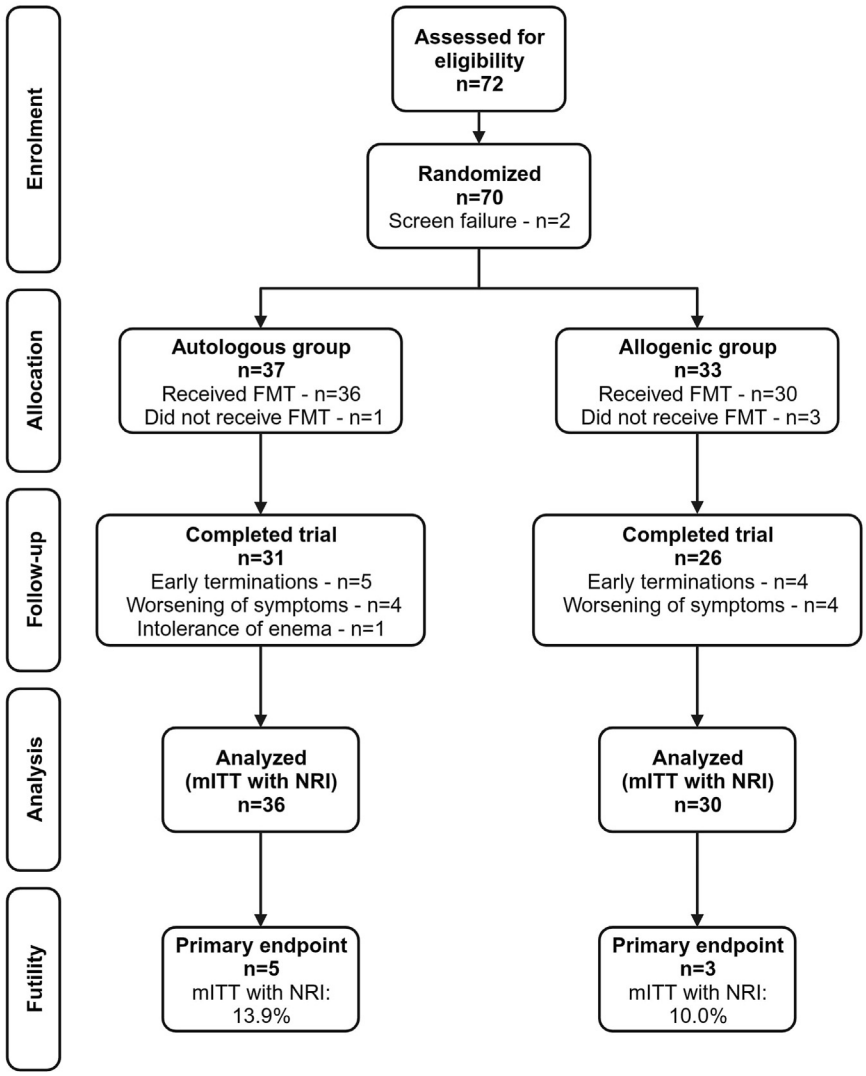

Figure 2. Consolidated Standards of Reporting Trials (CONSORT) flowchart of the RESTORE-UC study.

analysis did not show a significant difference in steroid-free clinical remission rates at week 8 between the allogenic (3/30; 10.0%) and autologous (5/36; 13.9%) treatment groups (Fisher exact test, $P = .72$) (Figure 2; Figure 4*A*; Table 2). The per protocol analysis confirmed these results with clinical remission rates of 11.5% (3/26) and 16.1% (5/31) for allogenic and autologous treatment groups, respectively (Fisher exact test, $P = 0.72$). Failing to meet the predefined criteria requiring a treatment difference in favor of allogenic FMT of at least 5%, the study was halted due to futility. In line with the primary endpoint findings, none of the secondary endpoints reached significant differences between treatment groups (Table 2). Furthermore, no new FMT-related signals were observed (Supplementary Results).

Higher Frequency of Enterotype Transitions Upon Allogenic Treatment

In both treatment groups, no significant shifts in microbiome-derived features occurred between week 0 and 8 (Supplementary Results; Supplementary Tables 11–14). In terms of microbiome community types, 18 patients (40.9%, including 4 randomized to autologous FMT) were treated with an FMT preparation enterotyped differently than their own baseline configuration (Supplementary Table 7). Among the latter, 67% transitioned to another community type (vs 27% of patients receiving a preparation matching their baseline enterotype; n = 44; Fisher exact test, $P = .01$), with 58% transitioning towards the donor enterotype. In line with these observations, a trend to more frequent enterotype transitions was observed in the allogenic treatment group (55% vs 33% of patients transitioning; n = 44; Fisher exact test, $P = .22$) (Figure 4*B,C*). When zooming in on Bact2 communities, this difference became even more pronounced (62% vs 34%, with all carriers randomized into the autologous treatment group effectively receiving a Bact2 FMT); however, given the relatively low number of Bact2 carriers recruited into the cohort, statistical significance was not reached (n = 14; χ^2 test, $P = .62$). Moreover, notwithstanding the differences in enterotype mobility observed, no significant differences in Bact2 prevalence between treatment groups were

Table 1. Baseline Demographic and Clinical Characteristics of Patients

		Autologous FMT (n = 36)	Allogenic FMT (n = 30)	*P*-value
Biological sex	Female	19 (52.8)	12 (40.0)	.431
Age at inclusion, *years*	Mean (SD)	43.31 (11.7)	44.40 (14.1)	.731
Disease duration, *years*	Mean (SD)	9.36 (6.7)	11.00 (9.6)	.418
BMI, kg/m^2	>25	16 (44.4)	11 (36.7)	.698
Endoscopic Mayo score	2	21 (58.3)	16 (53.3)	.874
	3	15 (41.7)	14 (46.7)	.874
Total Mayo score	Mean (SD)	7.9 (1.6)	7,8 (2.0)	.797
Disease extent	E1	6 (16.7)	3 (10.0)	.196
	E2	24 (66.7)	16 (53.3)	.196
	E3	6 (16.7)	9 (30.0)	.196
	NA	0 (0)	2 (6.7)	.196
Smoking	Active	1 (2.8)	2 (6.7)	.871
	Ex	18 (50)	7 (23.3)	**.049**
Concomitant therapy	Mesalamine	17 (48.6)	18 (60.0)	.431
	Steroids	13 (36.1)	8 (26.7)	.579
	Thiopurine	5 (15.2)	3 (10.3)	.918
	Biologicals - all	7 (19.4)	12 (40.0)	.118
	Biologicals - anti-TNF	3 (8.3)	6 (20.0)	.310
	Biologicals - vedolizumab	5 (16.1)	9 (31.0)	.196
Previous exposure	Any biological	21 (58.3)	20 (66.7)	.660
FCal, $\mu g/g$[a]	Median (range)	1470.5 (30.0–1800)	811.6 (30.0–1800)	.100
	>150	32 (97.0)	21 (84.0)	.154
	>250	31 (94.0)	20 (80.0)	.221
CRP, mg/L[b]	Median (IQR)	3.4 (1.3–10.1)	6.35 (2.4–15.5)	.359
	>5	13 (43.3)	12 (54.5)	.575

Note: Data are presented as number (%) except where indicated.
BMI, body mass index; CRP, C-reactive protein; FCal, fecal calprotectin; FMT, fecal microbiota transplantation; IQR, interquartile range; SD, standard deviation.
[a]n = 33 and n = 25 for autologous and allogenic FMT treatment, respectively.
[b]n = 30 and n = 22 for autologous and allogenic FMT treatment, respectively.

detected at week 8 (n = 44; Fisher exact test, $P = .97$) (Supplementary Figure 3; Supplementary Table 8).

Lower Total Mayo Score and Fecal Cell Count at Baseline are Associated With Success

A responder analysis did not reveal significant associations between treatment success and changes in clinical parameters or microbiome-derived features, nor was the restoration of eubiosis linked to remission (Supplementary Results). When looking at patient baseline characteristics across both treatment groups, a lower total Mayo score (n = 44; Wilcoxon test, $P = .015$) and lower fecal cell counts ($P = .024$) were associated with successful intervention outcome, although not significantly after correction for multiple testing (both adjusted $P = .097$) (Figure 4*D,E*; Supplementary Table 9). Of note, smoking status (n = 44; Fisher exact test, $P = .41$) and concomitant biological treatment ($P = .17$), variables distributed respectively significantly and markedly uneven over intervention arms, were not linked with treatment success. Additionally, patients reaching the primary endpoint did not differ significantly from those not achieving clinical remission in baseline genus abundances (Supplementary Table 10) or richness, evenness, and diversity indicators (Supplementary Figure 4).

No Highly Effective 'Superdonor' Profile Could be Identified

At the allogenic donor side, a positive association was observed between stool moisture and treatment success (n = 20; Wilcoxon test, $P = .057$) (Figure 4*F*; Supplementary Table 11). However, also here, statistical significance could no longer be established after correction for multiple testing (adjusted $P = .229$). Within the limitations of the amplicon sequencing approach applied (not allowing strain-level nor functional analyses), no differences were identified between effective and ineffective donors with respect to quantitative genus abundances (Supplementary Table 12) and richness, evenness, or diversity (Supplementary Figure 5). For autologous stool donations, no features could be linked with reaching the primary endpoint (Supplementary

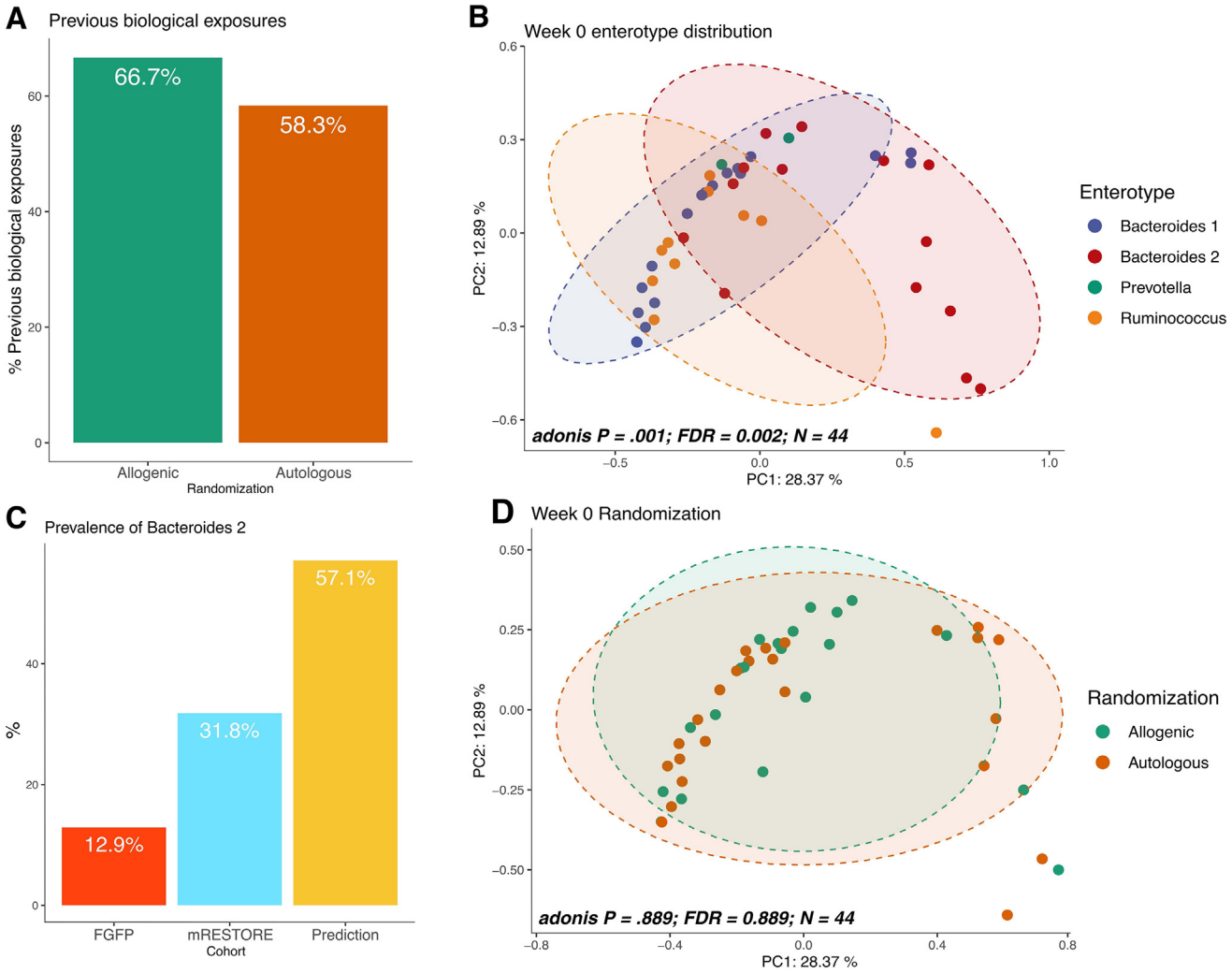

Figure 3. (*A*) Proportions of previously exposed patients to biologicals. (*B*) Prevalence of Bact2 in different cohorts: Flemish Gut Flora Project (FGFP), prediction-paper,[16] and the mRESTORE. (*C*) Principal coordinates analysis (PCoA) plot of quantitative microbiota profiling (QMP, Bray-Curtis distance) at baseline (*left*: enterotype distribution; *right*: treatment arms). (*D*) Differential abundant taxa in Bact2 enterotype vs other enterotype.

Tables 13 and 14; Supplementary Figure 8). In addition, with 26 subjects effectively having received allogenic FMTs from 15 donors at the time of futility assessment, several patients were treated with fecal material from the same host. However, a highly effective 'superdonor' profile could not be identified (Supplementary Results).

Discussion

The RESTORE-UC trial, a double-blind, randomized study, evaluated the impact of donor screening and repeated FMT administration on clinical remission rates in active UC. Although it confirmed the safety of allogenic FMTs, the trial was halted at 66% of intended inclusions due to futility. Building further on a recent meta-analysis,[17] a mechanistic post-hoc analysis identified several potential factors contributing to the negative outcome, which are critically discussed below.

A first aspect potentially contributing to failure to meet endpoints concerns the donor selection. Three previous trials[3,4,6] had mixed results, with one suggesting a donor effect.[3] In addition, donor bacterial richness was shown to be associated with FMT treatment success.[9,10] Therefore, a single-donor approach was employed to identify effective donor profiles, selecting only those with high fecal microbial load and excluding Bact2 enterotype samples—two features associated with microbiome richness.[18] Despite these efforts, clinical remission was only achieved in 10% of patients randomized into the allogenic group. Consequently, administering multi-donor FMTs[5,6,19] could be considered to mitigate the risk of selecting ineffective or non-compatible donors. Accordingly, only one double-blind randomized controlled trial[8] has unequivocally demonstrated the efficacy of single-donor FMTs. This additional disappointing outcome may prompt a rethinking of the donor selection, but single-donor approaches should not

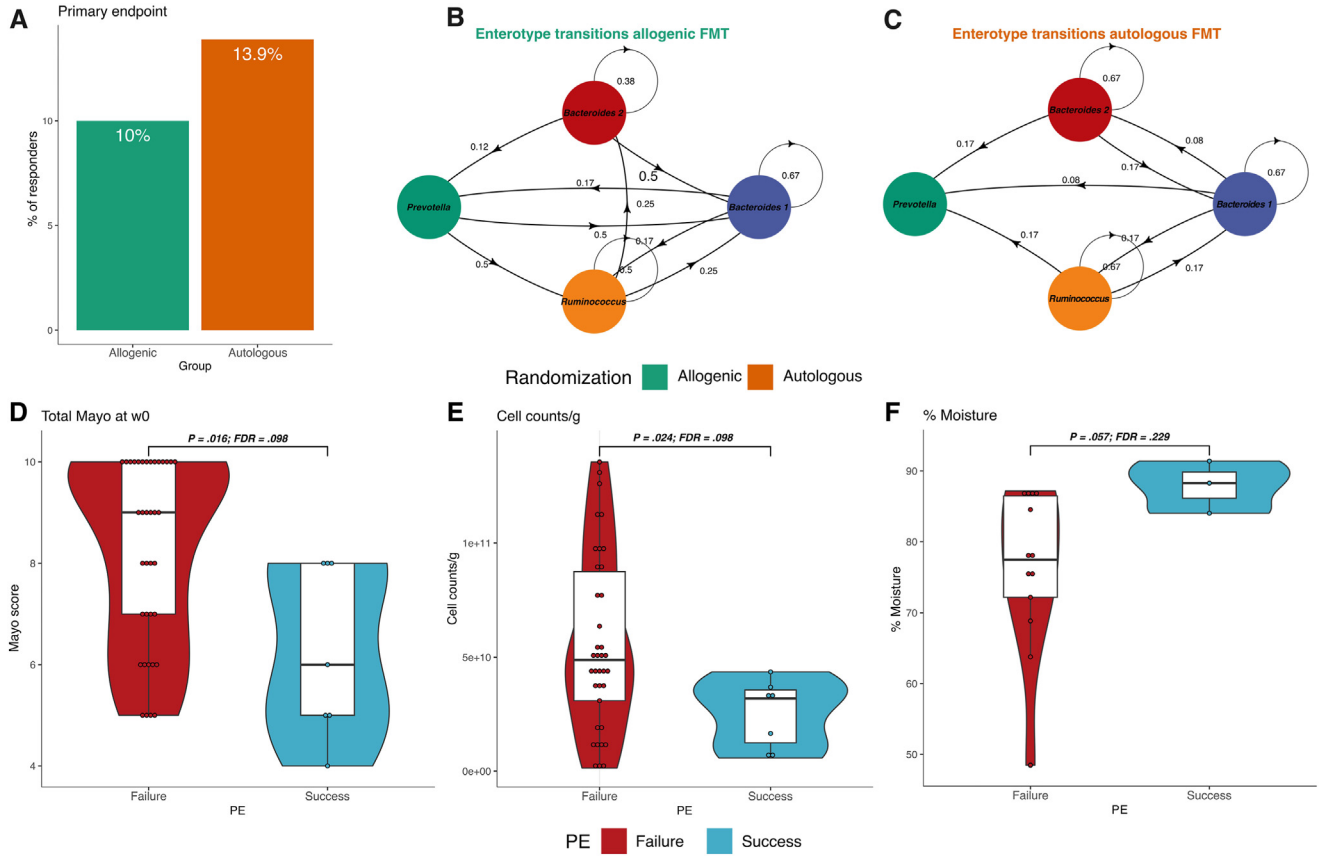

Figure 4. (*A*) Percentage of patients in each treatment arm reaching the primary endpoint. (*B*) Enterotype transitions in the autologous FMT group. (*C*) Enterotype transitions in the allogenic FMT group. (*D*) Lower total Mayo score at baseline is associated with reaching the primary endpoint. (*E*) Lower cell count at baseline is associated with reaching the primary endpoint. (*F*) A positive association could be observed between stool moisture and allogenic treatment success.

Table 2. Primary and Secondary Endpoints and Changes in Biomarkers Over the 8-week Treatment Period

Outcome at week 8	Autologous FMT (n = 36)	Allogenic FMT (n = 30)	*P*-value
Primary outcome			
Steroid-free clinical remission[a]	5 (13.90)	3 (10.00)	.72
Secondary outcomes			
Steroid-free PRO-2 remission[b]	10 (27.8)	7 (23.3)	.78
Steroid-free clinical response[c]	12 (33.3)	9 (30.0)	.79
Steroid-free endoscopic remission[d]	7 (19.4)	5 (16.7)	1.00
Steroid-free endoscopic response[e]	7 (19.4)	5 (16.7)	1.00
Inflammatory markers			
CRP, *mg/L*[f]	1.95 (0.93–3.50)	2.8 (1.5–8.9)	.24
CRP >5 mg/L[f]	6 (20.0)	9 (34.6)	.21
FCal, *μg/g*[g]	1003.2 (30.0–1800.0)	992.7 (30.0–1800.0)	.42
FCal >150 μg/g[g]	28 (93.3)	18 (75.0)	.12
FCal >250 μg/g[g]	25 (83.3)	17 (70.8)	.33

Note: Data are presented as number (%) or median (interquartile range).
CRP, C-reactive protein; FCal, fecal calprotectin; FMT, fecal microbiota transplantation; PRO, patient-reported outcome.
[a]Total Mayo score ≤2, with all sub-scores ≤1.
[b]Combined Mayo sub-scores of ≤1 for rectal bleeding and stool frequency.
[c]Decrease of ≥3 points or ≥50% reduction from baseline in combined Mayo sub-scores for rectal bleeding and stool frequency.
[d]Mayo endoscopy sub-score 0.
[e]Mayo endoscopy sub-score ≤1.
[f]n = 30 and n = 23 for autologous and allogenic FMT treatment, respectively.
[g]n = 30 and n = 24 for autologous and allogenic FMT treatment, respectively.

be abandoned, as this method is crucial for identifying donor features associated with restoring eubiosis and clinical remission.

A second aspect that should be taken into consideration when contrasting RESTORE-UC findings with those of trials meeting the primary endpoint relates to patient characteristics. The patient cohort in the present study was found to be more refractory than those studied in all positive FMT trials, with longer disease durations and higher previous exposure to biologicals.[5,6,8,19–21] Over 62% of participants reported prior exposure and 28.8% continued treatment during the intervention. Although no patient on concomitant biological therapy met the primary endpoint, no impact of impact of biological history on outcomes was identified. Nonetheless, baseline total Mayo scores and remission rates were negatively associated, which is in line with recent guidelines[22] advising to reserve FMT treatment for patients with mild to moderate disease.

A third matter of interest regards the use of autologous feces to prepare FMTs for sham treatment, as it has shown higher steroid-free remission rates than water[20] or saline.[6] Potentially as a consequence, 2 of 3 studies[4,5] using autologous FMTs could not establish a significant difference between sham and allogenic treatment. The exception[5] had a limited 9% success rate in the autologous arm, potentially due to aerobic workflow applied for autologous FMT preparation. As for allogenic FMTs, it remains unclear whether and how autologous preparations could induce an effective positive response. If confirmed, such effect would confound futility analyses, leading to an underestimation of the impact of allogenic treatment. Although autologous preparations have advantages with respect to full blinding, the latter would make them unsuited for evaluating the efficacy of FMT in UC. The requirement of live bacteria for successful FMT remains to be established; therefore, the application of sterilized autologous solutions as sham intervention could be considered as an alternative. Research regarding potential parallel mechanisms inducing clinical response following allogenic and autologous treatment should be considered as secondary, requiring prior (currently lacking) insights in donor/patient features determining FMT efficacy, and a specific study design.

A fourth set of factors that need to be considered concerns methodological differences in FMT preparation and administration. Because the current hypothesis assumes a mediating effect of live bacteria, an anaerobic workflow remains an absolute requirement. Also, keeping track of bacterial load, either for standardization purposes or to account for the confounding effects of weight-based FMTs, should be adopted as common practice by the scientific community. Nonetheless, more successful trials[5,6] used smaller volumes and more dense solutions, together with more intensive treatment regiments. Moreover, a successful trial[8] using oral FMT capsules settled on a daily intake over an 8-week intervention period. Taking these findings into consideration, a more frequent administration of smaller FMT volumes, potentially using oral capsules or applying more proximal administration of preparations (through transcolonic or terminal ileal infusion), with a higher microbial load would be an option for future trials. With respect to the latter, we acknowledge that the predefined concentration of density of 10^{10} cells/mL for FMT preparations might not have been sufficient. Additionally, in hindsight, standardization based on the concentration of viable cells might have been a more suited approach. On the longer term, response surface analyses to determine optimal dosage can be envisaged.

Finally, a fifth aspect concerns the microbiota of patients and donors. The hypothesis that FMTs would have the largest impact on subjects with a dysbiotic gut ecosystem at baseline was not confirmed due to the low proportion of Bact2 carriers recruited. However, baseline Bact2 configurations appeared more closely linked to response rates than other enterotypes. Moreover, lower microbial load at baseline was associated with positive treatment outcomes. These findings suggest to include microbial load and dysbiosis to patient inclusion criteria or considering pre-FMT antibiotic treatment[22,23] to increase therapeutic efficacy. For donors, samples harbouring the Bact2 enterotype were excluded, hypothesizing that eubiosis could not be restored by treating dysbiotic patients with an equally dysbiotic FMT. Accordingly, FMTs with a distinct enterotype from patient baseline configuration indeed increased community transition rates, particularly with respect to resolving Bact2-defined dysbiosis (in healthy individuals, both short- and longer-term enterotype stability has been estimated $>80\%$,[24–26] with Bact2 showing lowest transition rates).[26] However, it should be noted that no allogenic Bact2 donations were included in the study as a reference and that a shift away from a dysbiotic Bact2 community could not significantly be linked to treatment success. Additionally, although FMTs were anaerobically prepared and stored at $-80\ ^{\circ}C$ containing 10% glycerol as cryoprotectant, viability of the bacteria was not evaluated prior to transfer—which should be evaluated in future studies. Combined with standardization of preparation based on the number of viable cells, this approach would allow evaluation of the shelf life of FMTs. Here, also the observed association with donor stool moisture could be taken into account: higher fecal water contents have been associated with higher proportions of fast-growing taxa,[18] which could contribute to a more efficient colonization of the patient's large-intestinal habitat.

Conclusion

In conclusion, strict allogenic donor selection could not increase the efficacy of FMT in active UC. Nevertheless, key lessons for future research were learned, being to include only patients with mild to moderate inflammation, opt for a sterilized sham treatment, increase the

frequency and density and lowering the volume, prescreen patients for dysbiosis and microbial load, and assess viability of FMTs prior to administration.

Supplementary Material

Note: To access the supplementary material accompanying this article, visit the online version of *Clinical Gastroenterology and Hepatology* at www.cghjournal.org, and at https://doi.org/10.1016/j.cgh.2024.05.017.

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Conflicts of interest

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Data transparency

The data that support the findings of this study are available from the corresponding author (Séverine Vermeire) upon reasonable request. The study protocol is included as a data supplement available with the online version of this article.

Supplementary Methods

Randomization, Masking, and Study Design

Patients were randomized 1:1 to receive four infusions of allogenic donor or autologous fecal microbiota transplantation (FMT). Randomization was performed using a pre-established computer-generated randomization tool with permutated blocks of 2 and 4. Stratification for weight (body mass index $\leq$25kg/m^2 or $>$25 kg/m^2), concomitant corticosteroid use (yes/no), and therapy refractoriness (previous biological therapies $\leq$1 or $>$1) was applied. Both patients and investigators were unaware of treatment allocation. Fecal, blood, and (partial) Mayo scores were collected at each study visit (Figure 1). Endoscopy was performed at week 8 (primary endpoint). At this time point, non-responders randomized to autologous FMT had the possibility to switch to open label allogenic FMT after unblinding.

Sample Size Assumptions and Futility Analysis

The trial involved a sample size of 49 patients per arm, allowing to significantly identify a 25% difference between treatment groups as observed in previous trials.[3,4,6] Given an estimated dropout rate of 10%, inclusion of 108 patients was targeted. A safety analysis was conducted after 33% and 66% of inclusions, complemented with a futility analysis after 66% of projected inclusions (n = 72). The intention-to-treat analysis included all patients who received at least 1 FMT dose (n = 66). Treatment failures included those in need of rescue therapy, breaching the study protocol, failing to taper corticosteroids by week 8, or terminating the study. In addition, per-protocol analysis included patients who completed the 8 weeks without protocol breach (n = 57).

Faecal Microbiota Transplantation Preparation

Allogenic donor selection and FMT preparation. The selected donors (Supplementary Table 15) provided a fecal sample daily or at every bowel movement if less than daily. Each donor delivered approximately 40 fecal samples, which were stored immediately under anaerobic conditions using an anaerobic patch (Anaerogen compact) at 4 °C. Fecal samples were transported cooled (4 °C) to the research facility, and further processing was performed within 5 hours in an anaerobic chamber (Whitley A35 Workstation, Don Whitley Scientific), following guidelines regarding FMT preparation.[24] A minimum of 50 grams stool was requested. Depending on quantity and fecal cell counts, donations were used to generate 1 or more preparations, but distinct samples were never combined into a single FMT. Aliquots of donations were subjected to microbiome analysis and determination of fecal calprotectin (FCal) and moisture.

Thereafter, 500 mL 0.9% saline (Baxter) was added, and the sample was stirred for 10 minutes. The suspension was diluted twice (1:100) and filtered (Minisart syringe filter, Sartorius, pore size: 5 μm). One milliliter was taken from the filtrate (referred to as processed fecal samples) to determine the bacterial concentration using flow cytometry (BD Accuri C6). The same technique was used as described above (Microbial load measurement by flow cytometer). During flowcytometric analyses, all donor suspensions were stored at 4 °C until further processing. Based on the flowcytometric results, fecal infusion bags were further diluted in the anaerobic chamber, with 0,9% saline (Baxter), until a bacterial load of 10^{10} cells/*mL*. Moreover, 10% glycerol (Sigma > 99%) was added as cryoprotectant. All FMTs were stored at −80 °C until dispensation to the patients. All donor samples (N = 384) underwent 16S rDNA sequencing, so the exact microbial composition of each FMT was known before administration. Finally, batches of 4 FMT preparations generated from faecal material of a single donor were randomly assigned to patients in the allogenic treatment group.

Faecal Microbiota Characterization

Microbial load measurement by flow cytometry. The microbial load was determined from all eligible donors and patients' samples using flow cytometry (BD Accuri C6). Therefore, a 0.2 g frozen (−80 °C) aliquot from each eligible donor was dissolved in physiological solution to a total volume of 100 mL (8.5 g/L NaCl; VWR International). Subsequently, the fecal slurry was diluted 1000 times. Samples were filtered using a sterile syringe filter (pore size of 5 μm; Sartorius Stedim Biotech GmbH). Next, 1 mL of the microbial cell suspension obtained was stained with 1 μL SYBR Green I (1:100 dilution in DMSO; shaded 15 minute incubation at 37 °C; 10,000 concentrate, Thermo Fisher Scientific). The flow cytometry analysis was performed using a C6 Accuri flow cytometer (BD Biosciences).[1] Fluorescence events were monitored using the FL1 533/30 nm and FL3 >670 nm optical detectors. In addition, also forward and sideward-scattered light was collected. The BD Accuri CFlow software was used to gate and separate the microbial fluorescence events on the FL1/FL3 density plot from the fecal sample background. A threshold value of 2000 was applied on the FL1 channel. The gated fluorescence events were evaluated on the forward/sideward density plot, as to exclude remaining background events. Instrument and gating settings were kept identical for all samples (fixed staining/gating strategy[1]). Based on the exact weight of the aliquots analyzed, cell counts were converted to microbial loads per gram of fecal material. All measurements were performed in duplicate.

Fecal moisture and calprotectin measurement. Moisture content was determined as the percentage of mass loss after lyophilization of frozen aliquots of non-

homogenized faecal material (−80 °C). Fecal calprotectin concentrations were determined using the fCAL ELISA kit (Bühlmann) according to the manufacturer's protocol.

DNA extraction, 16S rRNA gene amplicon sequencing, and data pre-processing. Fecal DNA extraction and microbiota profiling was performed as described previously.[2] Briefly, DNA was extracted from faecal material using the MoBio PowerMicrobiome DNA/RNA KF isolation kit (Qiagen) with addition of 10 minutes incubation at 90 °C after the initial vortex step. The V4 region of the 16S rRNA gene was amplified with primer pair 515F/806R.[3] Sequencing was performed on the Illumina MiSeq platform with sequencing kit MiSeq v2, to generate paired-end reads of 250 bases in length in each direction. Fecal samples were processed altering the protocol above to dual-index barcoding as described by Tito and colleagues.[4] After de-multiplexing using LotuS (version 1.565),[5] sequencing data pre-processing was performed using the DADA2 pipeline v1.6.0.,[6] including trimming, quality control, merging of pairs, and taxonomic annotation using GTDB with default parameters.

Quantitative microbiome profiling and enterotyping. The quantitative microbiome profiling (QMP) matrix was obtained combining sequencing data and microbial load assessment by flow cytometry.[7] In short, samples were downsized to even sampling depth, defined as the ratio between sampling size (16S rRNA gene copy number corrected sequencing depth) and microbial load (average total cell count per gram of frozen fecal material). 16S rRNA gene copy number correction was based on the ribosomal RNA operon copy number database rrnDB3332. The copy number corrected sequencing depth of each sample was rarefied to the level necessary to equate the minimum observed sampling depth in the cohort. Diversity analysis was performed using the R statistical software (v4.3.1). The Bray-Curtis index (library "Vegan," function "vegdist") was used to estimate the dissimilarities between samples in the QMP even sampling depth Genus table. The low frequent genera (80% of zero data) were removed before the dissimilarity estimation. A distance-based redundancy analysis (dbRDA) (library "Vegan" function "capscale") was performed to reduce dimensionality in the taxonomic and functional distance matrix. The significant association between the microbial communities and the FMT donations, the time-points, and the response was assessed using the Permutational Multivariate Analysis of Variance Using Distance Matrices (ADONIS test) (library "vegan" function "adonis"). The observed richness, the Shannon and the Inverse Simpson index (library "phyloseq"[8] function "estimate_richness") and Pielou's evenness (library "microbiome" function "evenness") was estimated at the genus level for each sample of the cohort. Enterotyping (or community typing) was performed over the 16s rRNA bacterial profiles aggregated at the genus level and integrated with the Flemish Gut Flora Project (FGFP) cohort. Briefly, the genus-level count matrix was rarefied to 10000 reads and merged alongside the 2998 samples of the FGFP cohort, adding the estimated fraction of unobserved genera (n = 265) according to the asymptotic maximum number of species inferred from the Lomolino model[9,10] (R package vegan, function = "fitspecaccum," model = "lomolino"). The identification of the enterotypes was accomplished with the Dirichlet-multinomial Model (DMM) approach (R library "DirichletMultinomial" function "dmn").[11] The optimal number of enterotypes was the one that minimized the BIC score.

Supplementary Results

Analyses of Baseline Bact2 Configurations Confirmed Previous Findings

Our analyses confirmed Bact2 to be characterized by lower microbial richness (n = 44; Wilcoxon test, adjusted $P = 2.5 \times 10^{-5}$) and diversity (adjusted $P = .004$) (Supplementary Figure 6) and associated with higher faecal moisture levels (Wilcoxon test, adjusted $P = .018$) and lower microbial loads (adjusted $P = .001$) (Supplementary Table 16). Here, patients harboring Bact2 microbiota were characterized as younger than individuals hosting eubiotic communities (n = 44; Wilcoxon test, adjusted $P = .068$) (Supplementary Table 16), but no differences in disease duration (adjusted $P = .938$) or total Mayo score (adjusted $P = 1.00$) were detected. Distribution of Bact2 carriers over treatment groups was not significantly uneven (n = 44; Fisher exact test, $P = .34$) (Supplementary Figure 7).

No Significant Changes in CRP or FCal Were Observed

Over the course of the intervention period, an overall decrease in CRP, but not FCal levels, was noted (CRP, week 0 vs week 8, 4.8 vs 2.0 mg/L; n = 47; paired Wilcoxon test, $P = .01$; FCal, n = 51, 1353.9 vs 1063.5 μg/g; $P = .069$). However, this decline in systemic inflammatory tone did not differ significantly between patients receiving allogenic vs autologous FMT preparations (n = 45; paired Wilcoxon test, $P = .40$) (Supplementary Table 17).

No New FMT-Related Signals Were Observed

In total, 78 adverse events (AEs; including for example, insect bites) were reported. Twenty-six of these (16 unique patients) were identified as potentially related to treatment, without significant difference between study arms (6 AEs in 5 patients for allogenic FMT vs 20 in 11 for autologous FMT; n = 66; Fisher exact test, $P = .253$) (Supplementary Table 18). However, as all patients suffered from active ulcerative colitis (UC), no

categorical discrimination between disease- and treatment-related AE could be made. Two severe AEs were registered after autologous FMT, being 1 case of dysuria and constipation requiring hospitalization and 1 patient exhibiting worsening of UC resulting in total colectomy.

No Significant Impact of Allogenic FMT on Primary Endpoint in mRESTORE

Also for the mRESTORE-UC sub-cohort, no significant differences in primary/secondary endpoints and inflammation markers were observed between treatment groups at week 8 evaluation (Supplementary Table 19). In both the allogenic and autologous treatment group, no significant shifts in microbiome community composition occurred between week 0 and 8 (Adonis test, $P = .98$ and $P = .95$, respectively). Accordingly, no differences in quantitative genus abundances could be established between baseline and endpoint evaluation (Supplementary Table 20). Similar to baseline observations, no significant differences between study groups were detected post-treatment, neither in terms of community composition (n = 44; Adonis test, $P = .87$), genus abundances (Supplementary Table 21), nor quantitative changes of the latter over the course of the intervention (Supplementary Table 22). Additionally, changes in observed richness (n = 44; Wilcoxon test, adjusted $P = .56$), evenness (adjusted $P = .17$), or diversity (adjusted $P = .56$) between week 0 and 8 did not differ significantly between patients receiving allogenic or autologous FMTs.

A Responder Analysis Did Not Indicate Significant Associations Among Host and Microbiota Readouts

To identify changes in host (CRP, fecal calprotectin), stool (moisture, microbial load), and microbiome (taxa abundances, diversity indices, Bray-Curtis distance, Bact2 carrier status) readouts potentially associated with clinical remission, a responder analysis was performed (Supplementary Figure 8; Supplementary Tables 23 and 24). No significant associations were detected. Reversely, from a microbial point of view and zooming in on those patients hosting a Bact2 community at baseline, restoration of eubiosis did not translate in a significantly higher clinical remission rate compared to stable dysbiosis (n = 14; Fisher exact test, $P = 1.00$).

No Highly Effective 'Superdonor' Profile Could Be Identified

Given the design of the RESTORE-UC study, with 26 subjects effectively having received allogenic FMTs from 15 donors at the time of futility assessment, several patients were treated with fecal material from the same host. Feces from 1, 2, and 5 allogenic donors were respectively used for the treatment of 5, 3, and 2 individuals each. Two of 3 successful remissions in the allogenic treatment group were achieved with FMTs from the donor providing fecal material for 5 interventions; the third one resulted from treatment with FMTs from a volunteer donating for 2. Overall, this observation did not allow to identify and characterize a highly effective 'superdonor' profile.

Supplementary References

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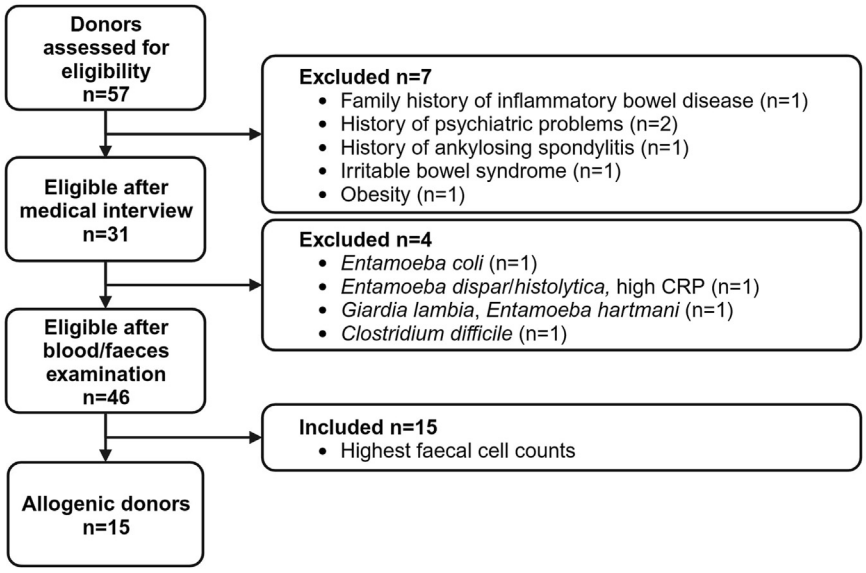
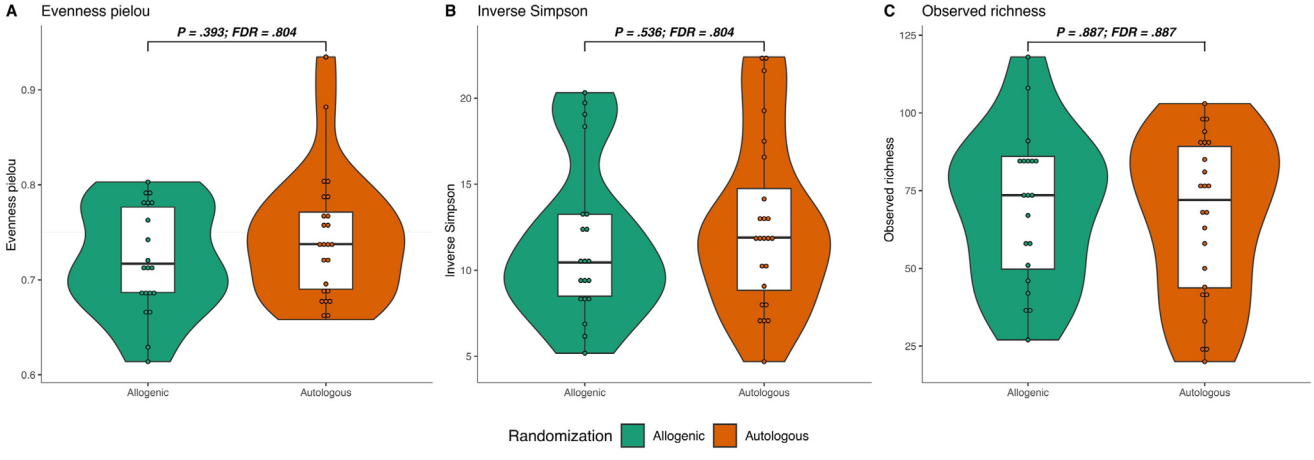
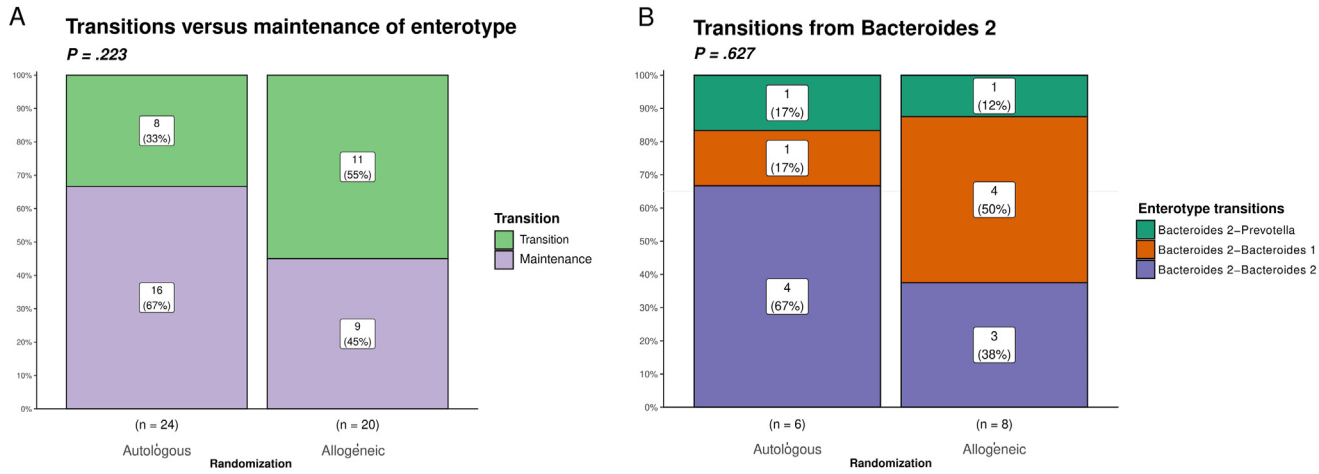


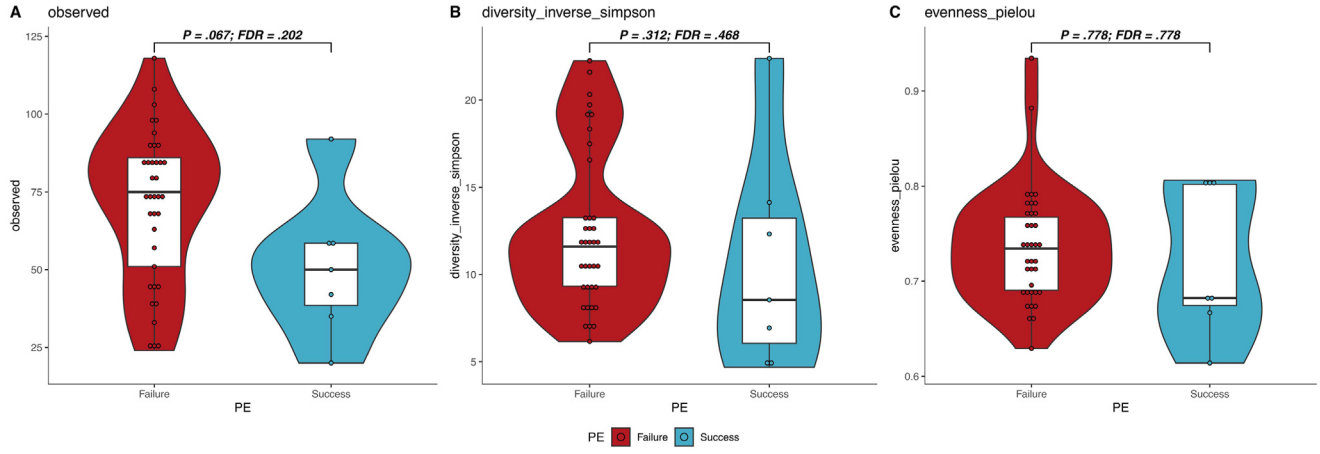
Supplementary Figure 1. Flowchart of allogenic donor selection for the RESTORE-UC trial.



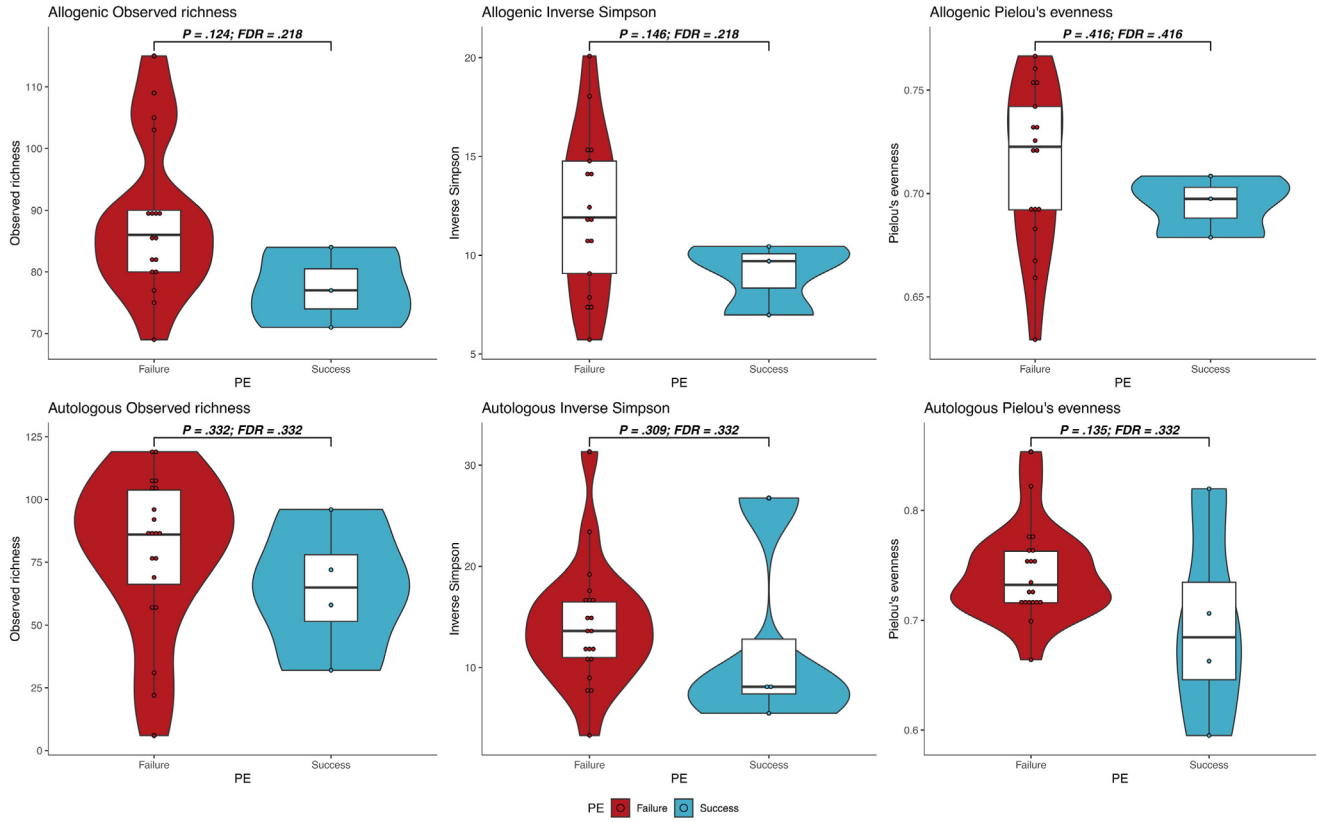
Supplementary Figure 2. Pielou evenness, diversity (inverse Simpson) and observed richness at baseline over both treatment arms.



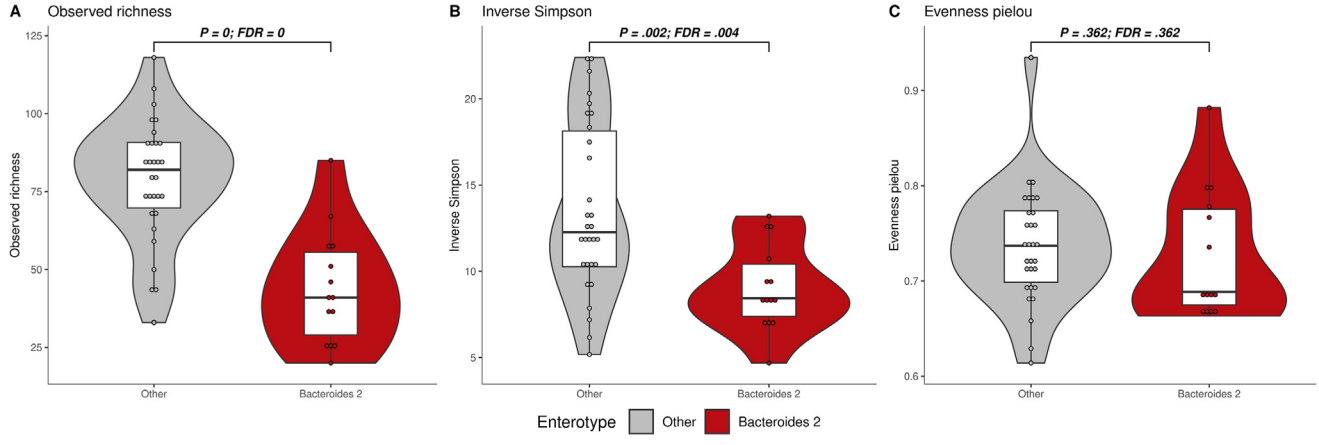
Supplementary Figure 3. Proportion of changes in enterotype after FMT. (*A*) All transitions vs maintenance of enterotype in both study arms. (*B*) Transitions for those patients harboring the Bacteroides 2 enterotype at baseline.



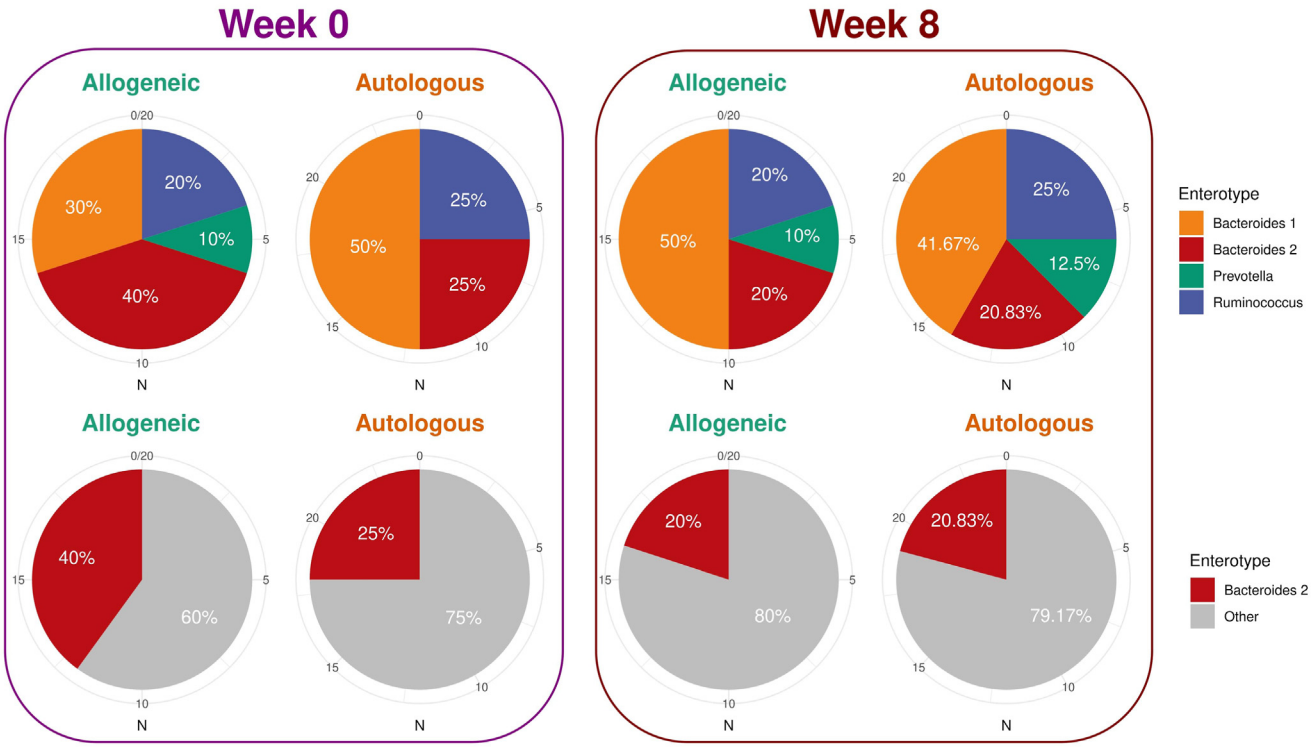
Supplementary Figure 4. Overview of observed richness, diversity, and evenness of patients independent from treatment and association with primary response.



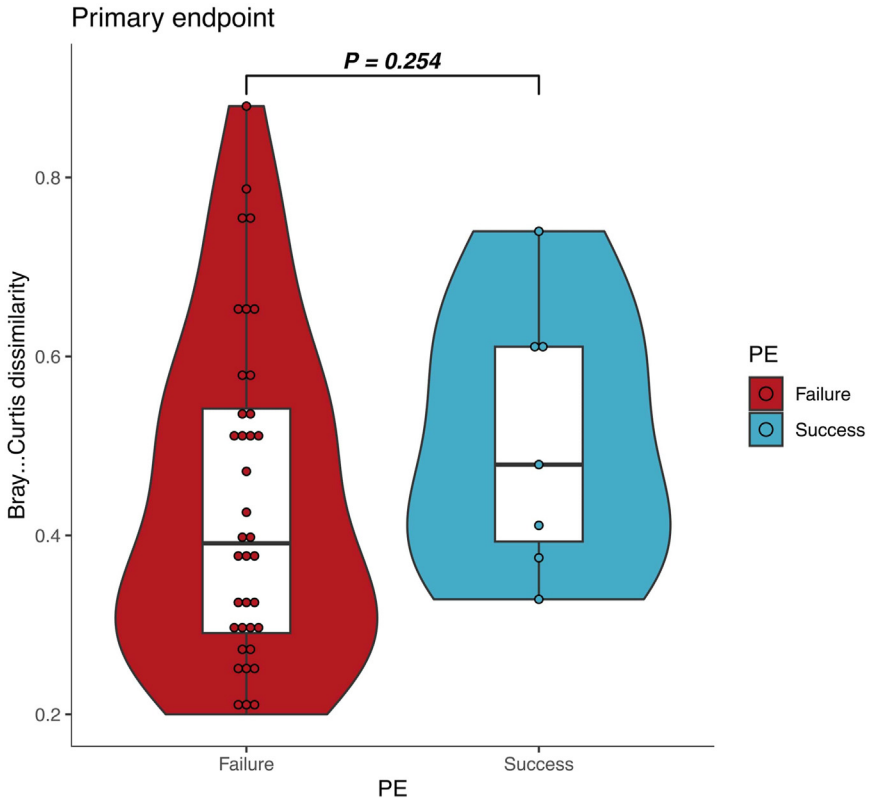
Supplementary Figure 5. Overview of observed richness, diversity, and evenness of donors and association with primary response.



Supplementary Figure 6. Baseline diversity in patients harbouring the Bact2 enterotype vs any other enterotype.



Supplementary Figure 7. Distribution of Bact2 vs other enterotypes at baseline and week 8.



Supplementary Figure 8. Bray-Curtis distance from week 0 to week 8 in relation to response.