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RIGOROUS DONOR SELECTION FOR FMT IN ACTIVE ULCERATIVE COLITIS: KEY LESSONS FROM A RANDOMIZED CONTROLLED TRIAL HALTED FOR FUTILITY

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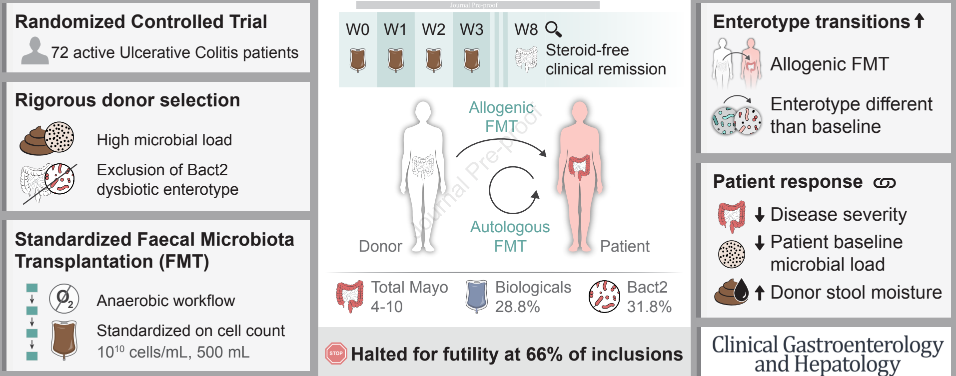

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RIGOROUS DONOR SELECTION FOR FMT IN ACTIVE ULCERATIVE COLITIS: KEY LESSONS FROM A RANDOMIZED CONTROLLED TRIAL HALTED FOR FUTILITY

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Short title: Faecal microbiota transplantation in active ulcerative colitis

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Author Contributions

CC*, GF#, JR#, and SV# conceived the presented study design. CC*, SD*, KA, SB, KM, FB, FM, LP, PH, TL, EL, DF, BV, MF, and JS, contributed to donor or patient inclusions and data collection. SVS and GF#, designed the microbiome analysis. CC*, SD*, and JFVC* performed the data-analysis. CC*, SD*, and JFVC* wrote the manuscript under the supervision of GF#, JR#, and SV#. All authors had access to the study data, reviewed and approved the final manuscript.

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132

ABSTRACT

Background and aims. Rigorous donor preselection on microbiota level, strict anaerobic processing, and repeated FMT administration were hypothesized to improve FMT induction of remission in UC.

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 four 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 one 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=0.72), indicating no treatment difference of at least 5% in favour of allogenic-FMT. Hence, the study was stopped due to futility. Microbiome analysis showed numerically more enterotype transitions upon allogenic-FMT compared to autologous-FMT and more transitions were observed when patients were treated with a different enterotype than their own at baseline (p=0.01). 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.

Clinical trial registry. NCT03110289 ()

Keywords. Microbiome, ulcerative colitis, IBD, faecal microbiota transplantation

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161 **WHAT YOU NEED TO KNOW - CGH**

162 **BACKGROUND.** Generalization of findings on faecal microbiota transplantation (FMT) in
163 ulcerative colitis is hampered by heterogenous study designs, including major differences in
164 patient populations, donor selection, preparation methods, dosage, frequency, and
165 administration protocol.

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

170 **IMPLICATIONS FOR PATIENT CARE.** Future study design should consider only patients with
171 mild to moderate UC, opt for a sterilized sham treatment, reduce volume and increase density
172 of FMTs, increase the number of administrations, pre-screen patients for dysbiosis, and assess
173 viability of FMT prior to treatment.

174 **Abbreviations (in alphabetical order)**

175 5-ASA 5-aminosalicylates
176 AE Adverse events
177 CRP C-reactive protein
178 DMM Dirichlet Multinomial Mixtures
179 DMSO Dimethyl sulfoxide
180 DSMB Data safety monitoring board
181 FDR False discovery rate
182 FGFP Flemish gut flora project
183 FMT Faecal microbiota transplantation
184 IBD Inflammatory bowel disease
185 IBS Irritable bowel syndrome
186 IQR Interquartile range
187 ITT Intention-to-treat
188 NRI Non-responder imputation
189 PE Primary endpoint
190 PEG Polyethylene glycol
191 QMP Quantitative microbiota profiling
192 RCT Randomized controlled trial
193 SAE Serious adverse event
194 SE Secondary endpoints
195 UC Ulcerative colitis

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197 **INTRODUCTION**

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

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

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

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

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229 **METHODS**

230 **Study design**

231 The RESTORE-UC trial [NCT03110289] was a multi-centric, double-blind, sham-controlled
232 randomized clinical trial performed in Belgium, to evaluate the efficacy and safety of rigorously
233 screened allogenic donor FMT in patients with active UC.

234 **Ethical compliance.** The study protocol was approved by the ethical committee of UZ/KU
235 Leuven (Commissie Medische Ethiek, S59525/B322201732687). Study design complied with
236 all relevant ethical regulations (Declaration of Helsinki and Belgian privacy). All participants
237 provided a signed informed consent. All authors had access to the study data, reviewed and
238 approved the final manuscript.

239 **Allogenic donor screening.** Eligible donors were recruited locally, according to international
240 consensus guidelines[14], based on a general health questionnaire, blood and faecal parameters
241 (Supplementary Table S1). All potential donors were tested for transmittable diseases by blood
242 and faecal examination (Supplementary Table S2), maximum four weeks before donation
243 started and a second time at the end of the donation period. Potential 'superdonors' were
244 further selected based on three criteria: microbial cell counts (>1.75 x10^{11} cells/*g*), enterotype
245 and the abundance (>1%) of the genera *Fusobacterium*, *Escherichia/Shigella*, and *Veillonella*.
246 Also, samples belonging to the Bact2 enterotype were excluded, even if they were not low in
247 bacterial cell count.

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248 **Patient recruitment.** Patients were required to have active UC (Total Mayo score 4-10)
249 confirmed by endoscopy (Mayo endoscopic sub-score ≥ 2; Supplementary Table S3).

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

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

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263 changes in C-reactive protein (CRP) and faecal calprotectin (FCal) before and after FMT were
264 analysed. The microbial endpoint was defined as a shift away from the Bact2 enterotype. An
265 interim futility analysis at 66% of inclusions (n=72) was performed requiring a treatment
266 difference of at least 5% in favour of allogenic FMT.

267 **Characterization of faecal microbial communities**

268 Faecal microbiota were characterized (Supplementary Methods) by microbial load
269 measurement through flow cytometry, faecal moisture and calprotectin, and 16S sequencing
270 followed by quantitative microbiota profiling and enterotyping.

271 **Faecal microbiota transplantation preparation**

272 **Allogenic FMT.** From August to September 2017, 57 healthy volunteers were invited to
273 participate in a rigorous screening effort to identify potentially highly effective FMT donors
274 (Supplementary Figure S1). After a medical interview and parasite screening, the 15
275 individuals with highest faecal cell counts (Supplementary Methods) were selected as allogenic
276 donors for the RESTORE-UC trial (Supplementary Table 4). From October to December 2017,
277 donors provided up to 40 faecal samples that were used to generate 500 mL FMT preparations
278 with standardized cell density of 10^{10} cells/mL (Supplementary Methods). Additionally, samples
279 containing the Bact2 enterotype (observed in three donors [4%]) were excluded for
280 administration to patients.

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281 **Autologous FMT.** During the screening period, each UC patient delivered four fresh faecal
282 samples for preparation of the autologous FMTs, regardless of the treatment arm allocation.
283 Autologous FMT preparation followed the same anaerobic procedure as for the allogenic donor
284 FMTs, except for diluting, since none of the patients reached the microbial load barrier that
285 was set for allogenic FMTs.

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

292 **Statistical analyses**

293 Statistical analyses were performed using the statistical software R version 4.3.0. *P*- or *q*-
294 values smaller than 0.05 were considered statistically significant.

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296 **RESULTS**

297 **Patient inclusion and randomisation**

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

304 **No significant differences in baseline microbiome composition between treatment arms**

305 Limited by sample availability, a microbiome RESTORE-UC sub-cohort (mRESTORE-UC;
306 n=44) was compiled, comprising those patients for whom a full triade of QMP profiles could be
307 generated, including samples from donor, baseline, and week 8. No significant differences in
308 baseline demographic or clinical characteristics were observed between the mRESTORE-UC
309 allogenic (n=20) and autologous (n=24) subsets and the respective treatment groups from
310 which they were drawn (Supplementary Table S4, S5). Analysis of quantitative genus-level
311 patient microbiome community variation at baseline revealed no significant difference between
312 treatment groups (Bray-Curtis distance on QMP matrix, Adonis test, p=0.89; Figure 3b).
313 Additionally, no significant differences in taxon abundances (Supplementary Table S6) and
314 richness, diversity, or evenness indicators were observed between patients randomized to
315 both intervention arms (Supplementary Figure S2).

316 Microbiome community-typing identified 14 out of 44 (31.8%, Figure 3c) mRESTORE-UC
317 participants as carriers of the Bact2 enterotype, which largely exceeded the 12.9% observed
318 in a large cross-sectional cohort recruited in the same region (n=1,164, Fisher's exact test,
319 p=0.002), but remained significantly lower than the 57.1% recently reported for a UC cohort
320 (n=108, Fisher's exact test, p= 0.0006)[16]. Analyses of baseline Bact2 configurations confirmed
321 previous findings (Supplementary Results).

322 **No significant impact of allogenic FMT on primary endpoint - steroid-free clinical**
323 **remission at week 8**

324 After 66% of intended inclusions (n=72, Figure 2), a predefined futility analysis was performed,
325 applying a modified intention-to-treat approach (mITT; excluding subjects that dropped out
326 before the start of the treatment). This analysis did not show a significant difference in steroid-
327 free clinical remission rates at week 8 between the allogenic (3/30, 10.0%) and autologous
328 (5/36, 13.9%) treatment groups (Fisher's exact test, p=0.72; Figure 2, 4a; Table 2). The *per*
329 *protocol* analysis confirmed these results with clinical remission rates of 11.5% (3/26) and
330 16.1% (5/31) for allogenic and autologous treatment groups, respectively (Fisher's exact test,

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331 p=0.72). Failing to meet the predefined criteria requiring a treatment difference in favour of
332 allogenic FMT of at least 5%, the study was halted due to futility. In line with the primary
333 endpoint findings, none of the secondary endpoints reached significant differences between
334 treatment groups (Table 2). Furthermore, no new FMT-related signals were observed
335 (Supplementary Results).

336 **Higher frequency of enterotype transitions upon allogenic treatment**

337 In both treatment groups, no significant shifts in microbiome-derived features occurred
338 between week 0 and 8 (Supplementary Results; Tables S11-14). In terms of microbiome
339 community types, 18 patients (40.9%, including four randomized to autologous FMT) were
340 treated with an FMT preparation enterotyped differently than their own baseline configuration
341 (Supplementary Table S7). Among the latter, 67% transitioned to another community type (vs.
342 27% of patients receiving a preparation matching their baseline enterotype; n=44, Fisher exact
343 test, p=0.01), with 58% transitioning towards the donor enterotype. In line with these
344 observations, a trend to more frequent enterotype transitions was observed in the allogenic
345 treatment group (55 vs. 33% of patients transitioning; n=44, Fisher's exact test, p=0.22; Figure
346 4b,c). When zooming in on Bact2 communities, this difference became even more pronounced
347 (62 vs. 34%, with all carriers randomized into the autologous treatment group effectively
348 receiving a Bact2 FMT), however, given the relatively low number of Bact2 carriers recruited
349 into the cohort, statistical significance was not reached (n=14, Chi square test, p=0.62).
350 Moreover, notwithstanding the differences in enterotype mobility observed, no significant
351 differences in Bact2 prevalence between treatment groups were detected at week 8 (n=44,
352 Fisher's exact test, p=0.97; Supplementary Figure S3; Table S8).

353 **Lower total Mayo score and faecal cell count at baseline are associated with success**

354 A responder analysis did not reveal significant associations between treatment success and
355 changes in clinical parameters or microbiome-derived features, nor was the restoration of
356 eubiosis linked to remission (Supplementary Results). When looking at patient baseline
357 characteristics across both treatment groups, a lower total Mayo score (n=44, Wilcoxon test,
358 p=0.015) and lower faecal cell counts (p=0.024) were associated with successful intervention
359 outcome, although not significantly after correction for multiple testing (both adj.p=0.097;
360 Figure 4d,e; Supplementary Table S9). Of note, smoking status (n=44, Fisher's exact test,
361 p=0.41) and concomitant biological treatment (p=0.17), variables distributed respectively
362 significantly and markedly uneven over intervention arms, were not linked with treatment
363 success. Additionally, patients reaching the PE did not differ significantly from those not
364 achieving clinical remission in baseline genus abundances (Supplementary Table S10) or
365 richness, evenness, and diversity indicators (Supplementary Figure S4).

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366 **No highly effective 'superdonor' profile could be identified**

367 At the allogenic donor side, a positive association was observed between stool moisture and
368 treatment success (n=20, Wilcoxon test, p=0.057; Figure 4f; Supplementary Table S11).
369 However, also here, statistical significance could no longer be established after correction for
370 multiple testing (adj.p=0.229). Within the limitations of the amplicon sequencing approach
371 applied (not allowing strain-level nor functional analyses), no differences were identified
372 between effective and ineffective donors with respect to quantitative genus abundances
373 (Supplementary Table S12) and richness, evenness, or diversity (Supplementary Figure S5).
374 For autologous stool donations, no features could be linked with reaching the primary endpoint
375 (Supplementary Table S13, S14; Supplementary Figure S8). In addition, with 26 subjects
376 effectively having received allogenic FMTs from 15 donors at the time of futility assessment,
377 several patients were treated with faecal material from the same host. However, a highly
378 effective 'superdonor' profile could not be identified (Supplementary Results).

379 **DISCUSSION**

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

386 A first aspect potentially contributing to failure to meet endpoints concerns the donor selection.
387 Three previous trials[3,4,6] had mixed results, with one suggesting a donor effect[3]. In addition,
388 donor bacterial richness was shown to be associated with FMT treatment success[9,10].
389 Therefore, a single-donor approach was employed to identify effective donor profiles, selecting
390 only those with high faecal microbial load and excluding Bact2 enterotype samples - two
391 features associated with microbiome richness[18]. Despite these efforts, clinical remission was
392 only achieved in 10% of patients randomized into the allogenic group. Consequently,
393 administering multi-donor FMTs[5,6,19] could be considered to mitigate the risk of selecting
394 ineffective or non-compatible donors. Accordingly, only one double-blind RCT[8] has
395 unequivocally demonstrated the efficacy of single-donor FMTs. This additional disappointing
396 outcome may prompt a rethinking of the donor selection, but single-donor approaches should
397 not be abandoned, as this method is crucial for identifying donor features associated with
398 restoring eubiosis and clinical remission.

399 A second aspect that should be taken into consideration when contrasting RESTORE-UC
400 findings with those of trials meeting the primary endpoint relates to patient characteristics. The

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401 patient cohort in the present study was found to be more refractory than those studied in all
402 positive FMT trials, with longer disease durations and higher previous exposure to
403 biologicals[5,6,8,19–21]. Over 62% of participants reported prior exposure and 28.8% continued
404 treatment during the intervention. Although no patient on concomitant biological therapy met
405 the primary endpoint, no impact of impact of biological history on outcomes was identified.
406 Nonetheless, baseline total Mayo scores and remission rates were negatively associated,
407 which is in line with recent guidelines[22] advising to reserve FMT treatment for patients with mild
408 to moderate disease.

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

425 A fourth set of factors that need to be considered concerns methodological differences in FMT
426 preparation and administration. Since the current hypothesis assumes a mediating effect of
427 live bacteria, an anaerobic workflow remains an absolute requirement. Also, keeping track of
428 bacterial load, either for standardization purposes or to account for the confounding effects of
429 weight-based FMTs, should be adopted as common practice by the scientific community.
430 Nonetheless, more successful trials[5,6] used smaller volumes and more dense solutions,
431 together with more intensive treatment regiments. Moreover, a successful trial[8] using oral FMT
432 capsules settled on a daily intake over an eight-week intervention period. Taking these findings
433 into consideration, a more frequent administration of smaller FMT volumes, potentially using
434 oral capsules or applying more proximal administration of preparations (through trans-colonic
435 or terminal ileal infusion), with a higher microbial load would be an option for future trials. With
436 respect to the latter, we acknowledge that the predefined concentration of density of 10^{10}

437 cells/mL for FMT preparations might not have been sufficient. Additionally, in hindsight,
438 standardization based on the concentration of viable cells might have been a more suited
439 approach. On the longer term, response surface analyses to determine optimal dosage can be
440 envisaged.

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

463 In conclusion, strict allogenic donor selection could not increase the efficacy of FMT in active
464 UC. Nevertheless, key lessons for future research were learnt being include only patients with
465 mild to moderate inflammation, opt for a sterilized sham treatment, increase the frequency and
466 density and lowering the volume, pre-screen patients for dysbiosis and microbial load, and
467 assess viability of FMTs prior to administration.

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469 **LEGENDS**

470 **Table 1.** Baseline demographic and clinical characteristics of patients.

471 **Table 2.** Primary and secondary endpoints and changes in biomarkers over the 8-week treatment
472 period.

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

474 **Figure 2**. CONSORT flowchart of the RESTORE-UC study. mITT, modified intention-to-treat analysis;
475 NRI, non-responder imputation.

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

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

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563 ***Designated authors in bold share first authorship.***

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TABLES

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%)	0.431
Age at inclusion (years)	Mean (SD)	43.31 (11.7)	44.40 (14.1)	0.731
Disease duration (years)	Mean (SD)	9.36 (6.7)	11.00 (9.6)	0.418
BMI	>25 kg/m^2	16 (44.4%)	11 (36.7%)	0.698
Endoscopic Mayo score	2	21 (58.3%)	16 (53.3%)	0.874
	3	15 (41.7%)	14 (46.7%)	0.874
Total Mayo score	Mean (SD)	7.9 (1.6)	7,8 (2.0)	0.797
Disease extent	E1	6 (16.7%)	3 (10.0%)	0.196
	E2	24 (66.7%)	16 (53.3%)	0.196
	E3	6 (16.7%)	9 (30.0%)	0.196
	NA	0 (0%)	2 (6.7%)	0.196
Smoking	Active	1 (2.8%)	2 (6.7%)	0.871
	Ex	18 (50%)	7 (23.3%)	**0.049**
Concomitant therapy	Mesalamine	17 (48.6%)	18 (60.0%)	0.431
	Steroids	13 (36.1%)	8 (26.7%)	0.579
	Thiopurine	5 (15.2%)	3 (10.3%)	0.918
	Biologicals - all	7 (19.4%)	12 (40.0%)	0.118
	Biologicals - anti-TNF	3 (8.3%)	6 (20.0%)	0.310
	Biologicals - vedolizumab	5 (16.1%)	9 (31.0%)	0.196
Previous exposure	Any biological	21 (58.3%)	20 (66.7%)	0.660
Faecal calprotectin (µg/g)*	Median (range)	1470.5 (30.0-1800)	811.6 (30.0-1800)	0.100
	>150 µg/mg	32 (97.0%)	21 (84.0%)	0.154
	>250 µg/mg	31 (94.0%)	20 (80.0%)	0.221
C-reactive protein (mg/L)**	Median (IQR)	3.4 (1.3-10.1)	6.35 (2.4-15.5)	0.359
	>5 mg/L	13 (43.3%)	12 (54.5%)	0.575

*, n=33 and 25 for autologous and allogenic FMT treatment; **, n=30 and 22 for autologous and allogenic FMT treatment.

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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*	5 (13.90%)	3 (10.00%)	0.72
Secondary outcomes			
Steroid-free PRO-2 remission**	10 (27.8%)	7 (23.3%)	0.78
Steroid-free clinical response***	12 (33.3%)	9 (30.0%)	0.79
Steroid-free endoscopic remission****	7 (19.4%)	5 (16.7%)	1.00
Steroid-free endoscopic response*****	7 (19.4%)	5 (16.7%)	1.00
Inflammatory markers			
CRP (mg/L; median [IQR])#	1.95 (0.93-3.50)	2.8 (1.5-8.9)	0.24
CRP >5 mg/L #	6 (20.0%)	9 (34.6%)	0.21
Faecal calprotectin (µg/g; median [range])##	1003.2 (30.0-1800.0)	992.7 (30.0-1800.0)	0.42
Faecal calprotectin >150 µg/g ##	28 (93.3%)	18 (75.0%)	0.12
Faecal calprotectin >250 µg/g ##	25 (83.3%)	17 (70.8%)	0.33

*, Total Mayo score ≤2, with all sub-scores ≤1; **, Combined Mayo sub-scores of ≤1 for rectal bleeding and stool frequency; ***, Decrease of ≥3 points or ≥50% reduction from baseline in combined Mayo sub-scores for rectal bleeding and stool frequency; ****, Mayo endoscopy sub-score 0; *****, Mayo endoscopy sub-score ≤1; #, n=30 and 23 for autologous and allogenic FMT treatment; ##, n=30 and 24 for autologous and allogenic FMT treatment.

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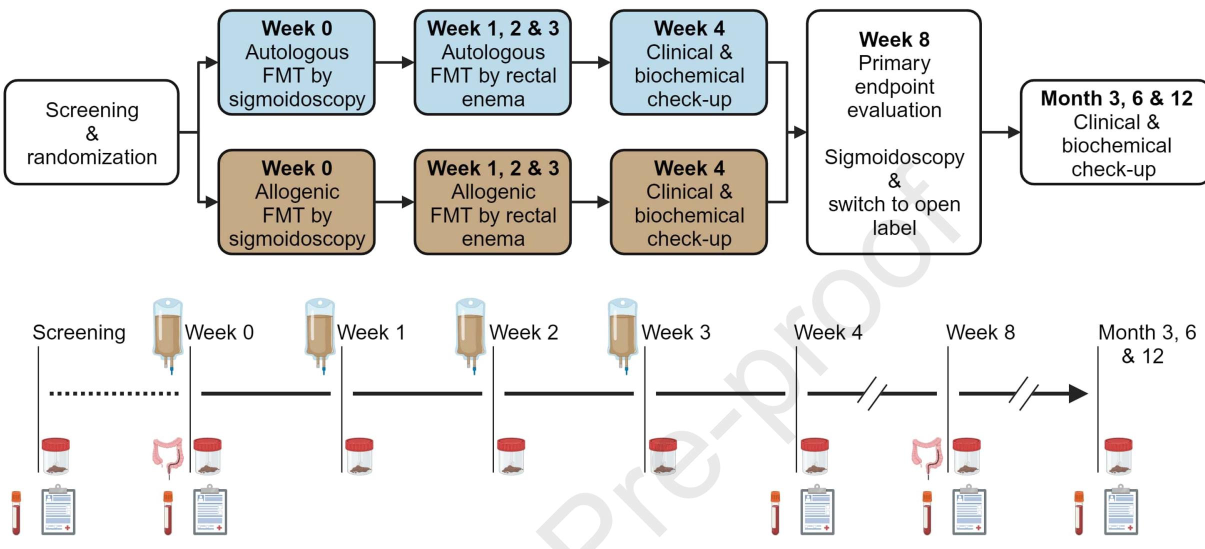

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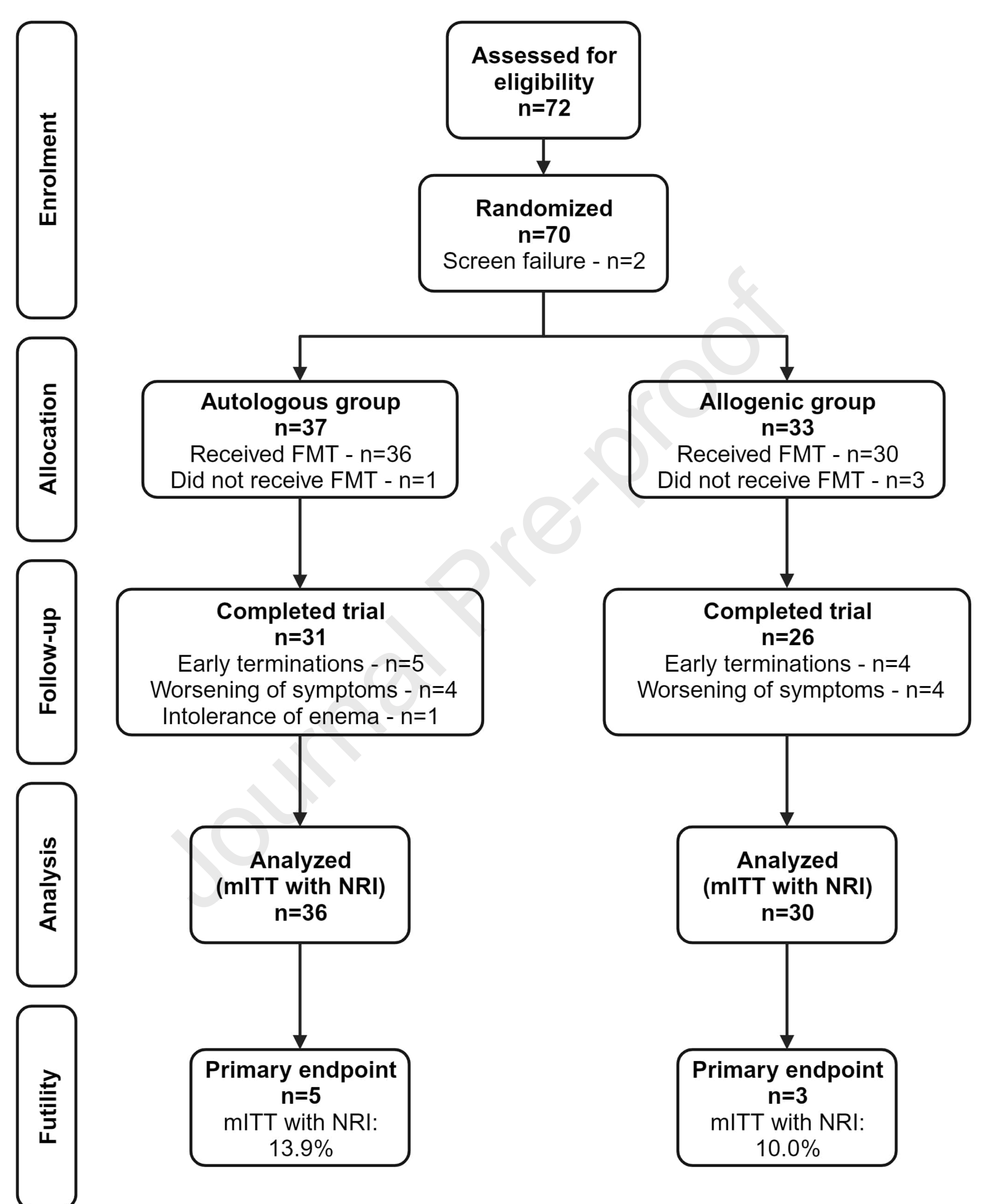

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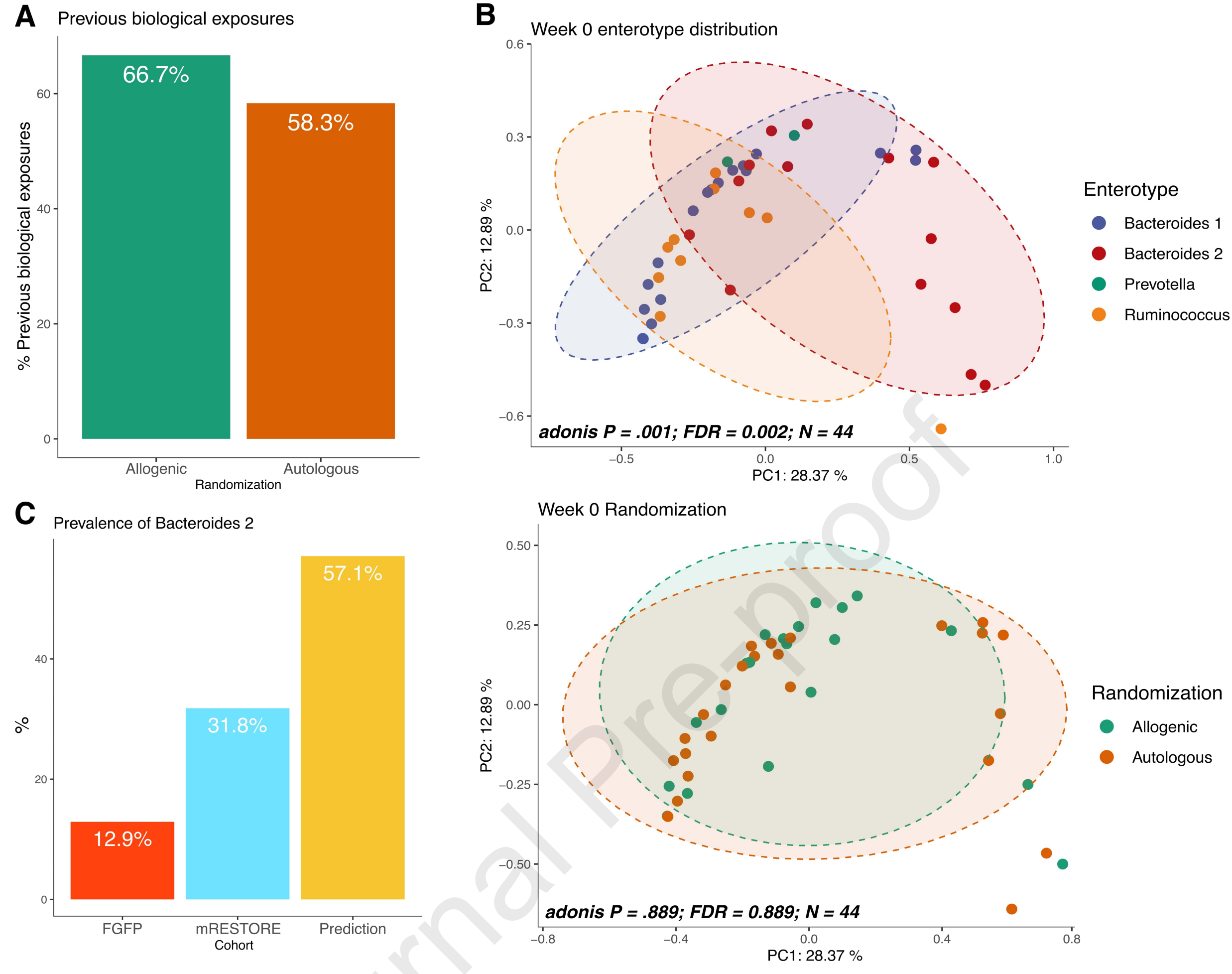

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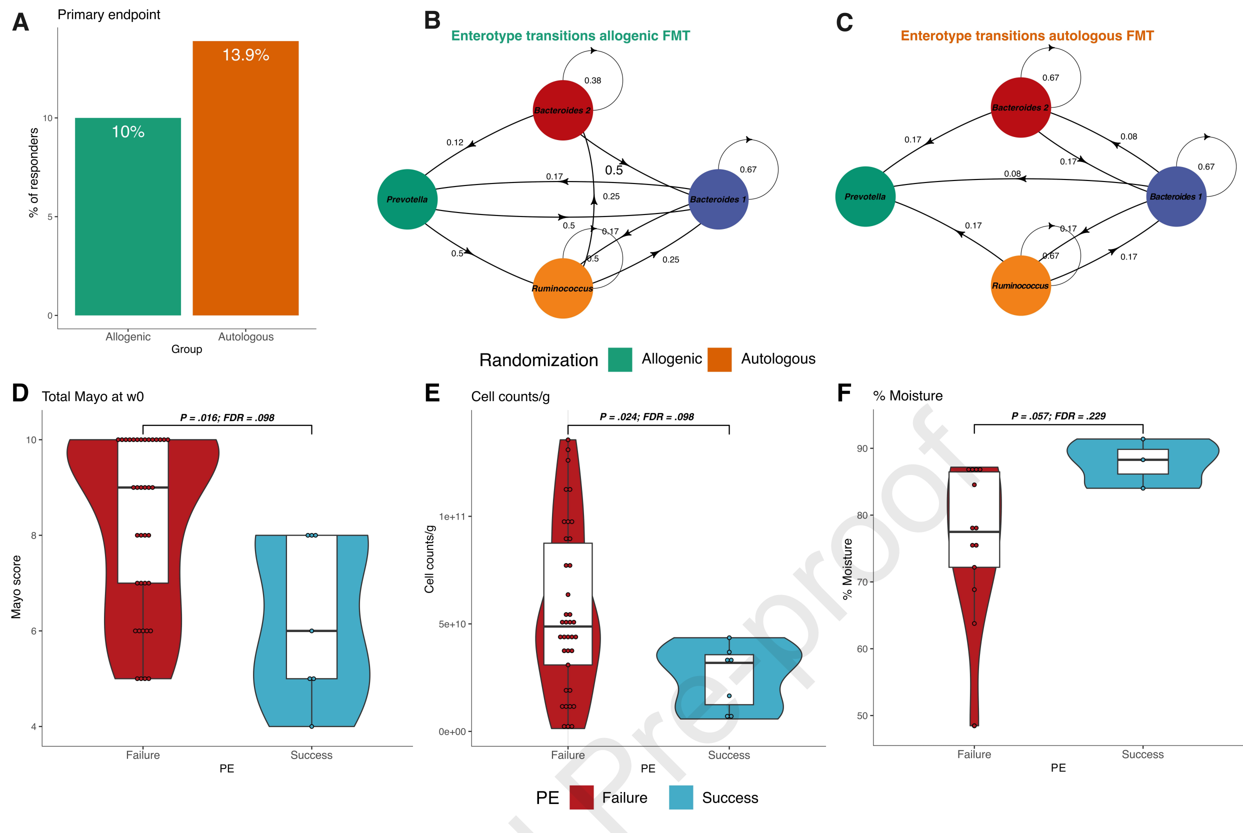

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WHAT YOU NEED TO KNOW - CGH

BACKGROUND. Generalization of findings on faecal microbiota transplantation (FMT) in ulcerative colitis 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 moderate to severe patients.

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, pre-screen patients for dysbiosis, and assess viability of FMT prior to treatment.

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SUPPLEMENTARY METHODS

Randomization, masking, and study design. Patients were randomized 1:1 to receive four infusions of allogenic donor or autologous FMT. Randomization was performed using a pre-established computer-generated randomization tool with permutated blocks of two and four. Stratification for weight (body mass index [BMI] ≤25kg/m2 or >25 kg/m2), concomitant corticosteroid use (yes/no), and therapy refractoriness (previous biological therapies ≤1 or >1) was applied. Both patients and investigators were unaware of treatment allocation. Faecal, 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 one 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 S15) provided a faecal sample daily or at every bowel movement if less than daily. Each donor delivered approximately 40 faecal samples which were stored immediately under anaerobic conditions using an anaerobic patch (Anaerogen compact) at 4°C. Faecal samples were transported cooled (4°C) to the research facility and further processing was performed within five hours in an anaerobic chamber (Whitley A35 Workstation, Don Whitley Scientific, UK), following guidelines regarding FMT preparation [24]. A minimum of 50 grams stool was requested. Depending on quantity and faecal cell counts, donations were used to generate one or more preparations, but distinct samples were never combined into a single FMT. Aliquots of donations were subjected to microbiome analysis and determination of 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: 5μm). One millilitre was taken from the filtrate (referred to as processed faecal samples) to determine the bacterial concentration using flow cytometry (BD Accuri™

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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, faecal 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 four 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, Germany). Subsequently, the faecal slurry was diluted 1,000 times. Samples were filtered using a sterile syringe filter (pore size of 5 µm; Sartorius Stedim Biotech GmbH, Germany). Next, 1 mL of the microbial cell suspension obtained was stained with 1 µL SYBR Green I (1:100 dilution in DMSO; shaded 15 min incubation at 37°C; 10,000 concentrate, Thermo Fisher Scientific, Massachusetts, USA). The flow cytometry analysis was performed using a C6 Accuri flow cytometer (BD Biosciences, New Jersey, USA)[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 faecal 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 analysed, cell counts were converted to microbial loads per gram of faecal material. All measurements were performed in duplicate.

Faecal 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). Faecal calprotectin concentrations were determined using the fCAL ELISA kit [Bühlmann, Schönenbuch, Switzerland] according to the manufacturer's protocol.

DNA extraction, 16S rRNA gene amplicon sequencing, and data pre-processing. Faecal 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

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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 (San Diego, California, USA) with sequencing kit MiSeq v2, to generate paired-end reads of 250 bases in length in each direction. Faecal 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 faecal 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 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 minimised the BIC score.

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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, adj.p=2.5x10-5) and diversity (adj.p=0.004; Supplementary Figure S6 and associated with higher faecal moisture levels (Wilcoxon test, adj.p=0.018) and lower microbial loads (adj.p=0.001; Supplementary Table S16). Here, patients harbouring Bact2 microbiota were characterized as younger than individuals hosting eubiotic communities (n=44, Wilcoxon test, adj.p=0.068; Supplementary Table S16), but no differences in disease duration (adj.p=0.938) or total Mayo score (adj.p=1.00) were detected. Distribution of Bact2 carriers over treatment groups was not significantly uneven (n=44, Fisher's exact test, p=0.34; Supplementary Figure S7).

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=0.01; FCal, n=51, 1353.9 vs. 1063.5 µg/g, n=51, p=0.069). 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=0.40; Supplementary Table S17).

No new FMT-related signals were observed

In total, 78 adverse events (AEs; including e.g. 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 AE in 5 patients for allogenic FMT vs. 20 in 11 for autologous FMT; n=66, Fisher's exact test, p=0.253; Supplementary Table S18). However, as all patients suffered from active UC, no categorical discrimination between disease- and treatment-related AE could be made. Two severe AEs were registered after autologous FMT, being one case of dysuria and constipation requiring hospitalization and one 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 S19). In both the allogenic and autologous treatment group, no significant shifts in microbiome community composition occurred between week 0 and 8 (Adonis test, p=0.98 and p=0.95, respectively). Accordingly, no differences in quantitative genus abundances could be established between baseline and endpoint evaluation (Supplementary Table S20). Similar to baseline observations, no significant differences

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between study groups were detected post-treatment, neither in terms of community composition (n=44, Adonis test, p=0.87), genus abundances (Supplementary Table S21), nor quantitative changes of the latter over the course of the intervention (Supplementary Table S22). Additionally, changes in observed richness (n=44, Wilcoxon test, adj. p=0.56), evenness (adj.p=0.17), or diversity (adj.p=0.56) between week 0 and 8 did not differ significantly between patients receiving allogenic or autologous FMTs.

A responder analysis did not indicate significant associations amongst host and microbiota readouts

In order to identify changes in host (CRP, faecal 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 S8; Supplementary Table S23, S24). 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's 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 faecal material from the same host. Faeces from one, two, and five allogenic donors were respectively used for the treatment of five, three, and two individuals each. Two out of three successful remissions in the allogenic treatment group were achieved with FMTs from the donor providing faecal material for five interventions; the third one resulted from treatment with FMTs from a volunteer donating for two. Overall, this observation did not allow to identify and characterize a highly effective 'superdonor' profile.

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LEGENDS SUPPLEMENTARY FIGURES

Supplementary figure S1 Flowchart of allogenic donor selection for the RESTORE-UC trial. *IBD: Inflammatory bowel disease; IBS: Irritable bowel syndrome; CRP: C-reactive protein.*

Supplementary figure S2 Pielou evenness, diversity (inverse Simpson) and observed richness at baseline over both treatment arms.

Supplementary figure S3 Proportion of changes in enterotype after FMT. (A) *All transitions versus maintenance of enterotype in both study arms* ***(B)*** *Transitions for those patients harboring the Bacteroides 2 enterotype (Bact2) at baseline.*

Supplementary figure S4 Overview of observed richness, diversity and evenness of patients independent from treatment and association with primary response.

Supplementary figure S5 Overview of observed richness, diversity and evenness of donors and association with primary response.

Supplementary figure S6 Baseline diversity in patients harbouring the Bacteroides 2 enterotype versus any other enterotype.

Supplementary figure S7 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.

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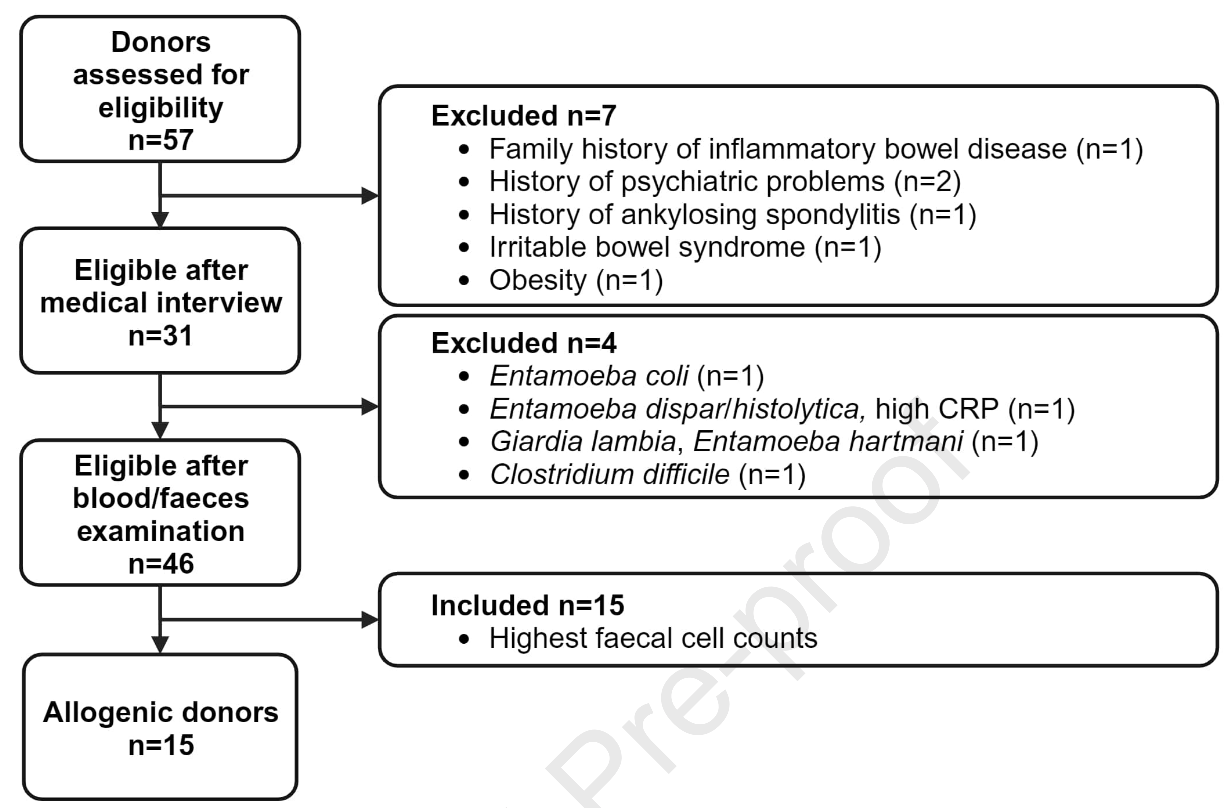

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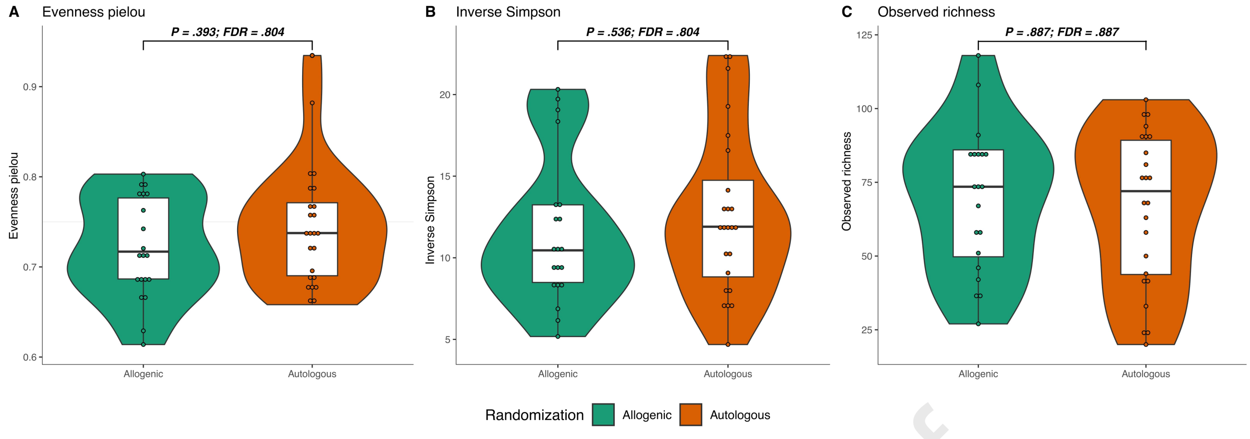

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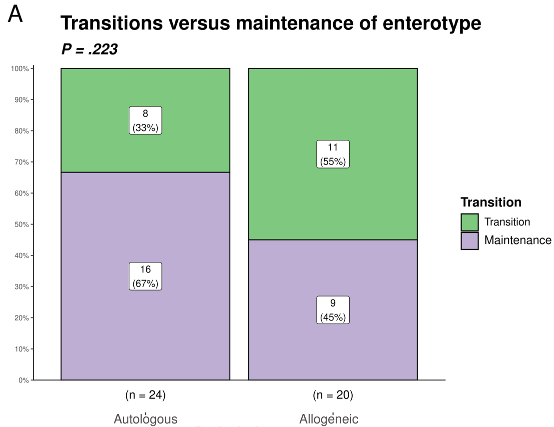

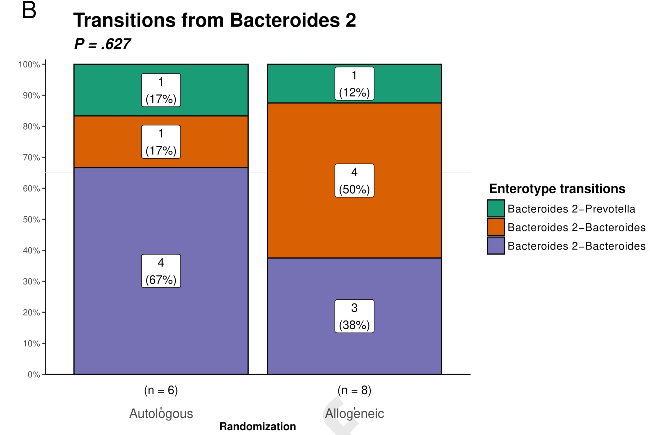

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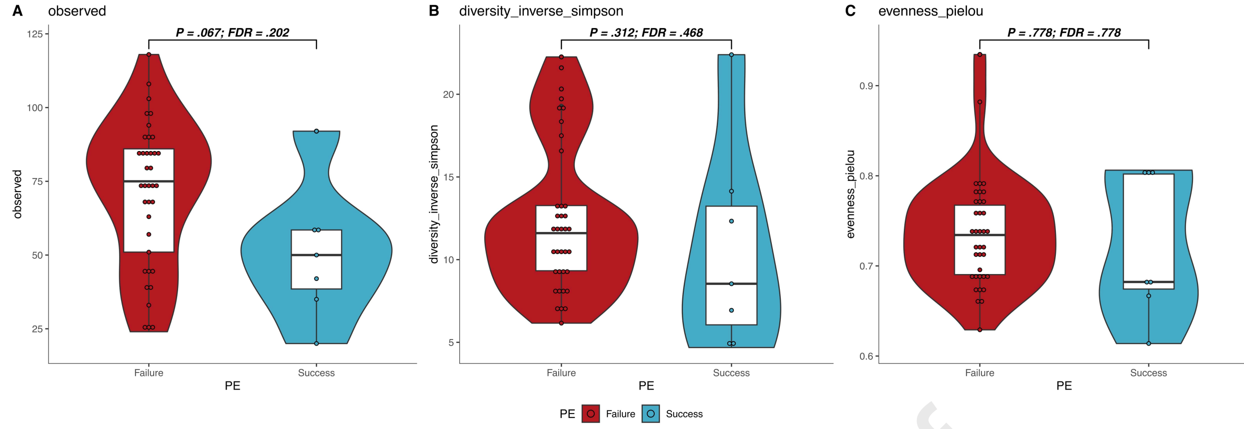

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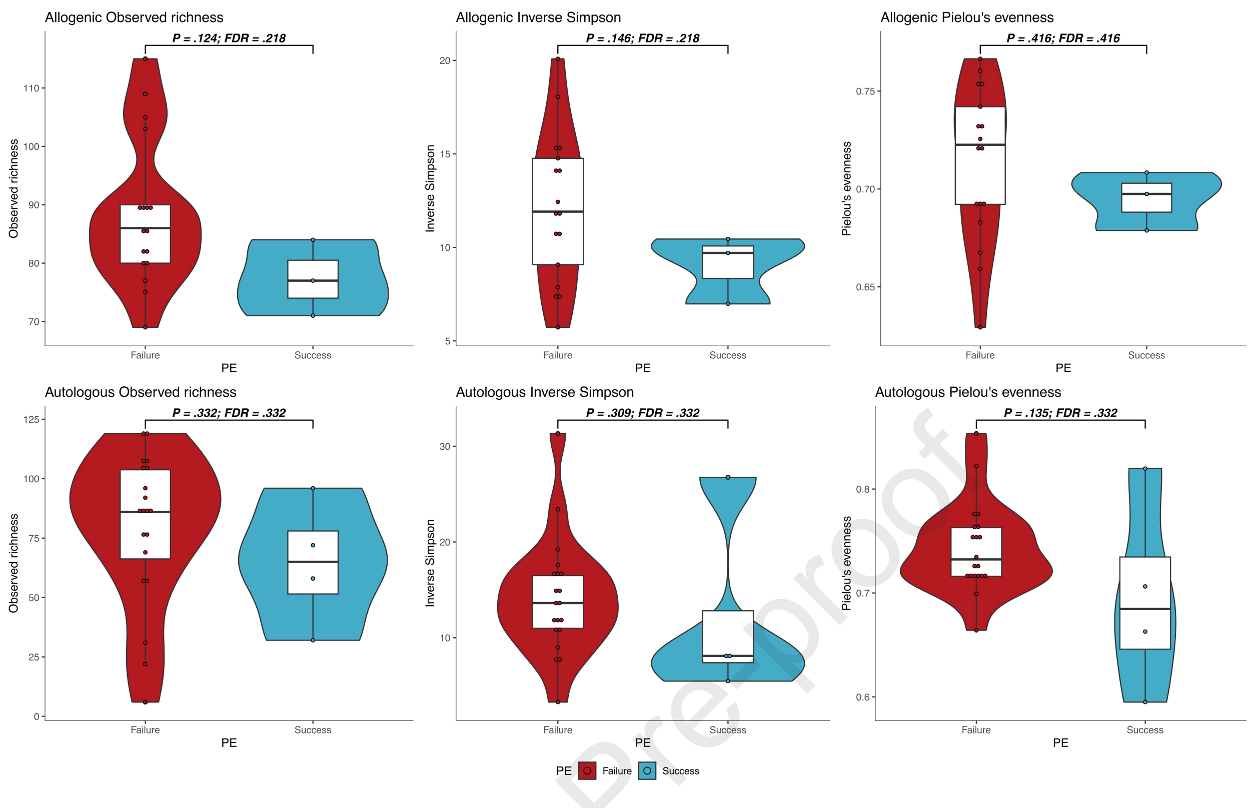

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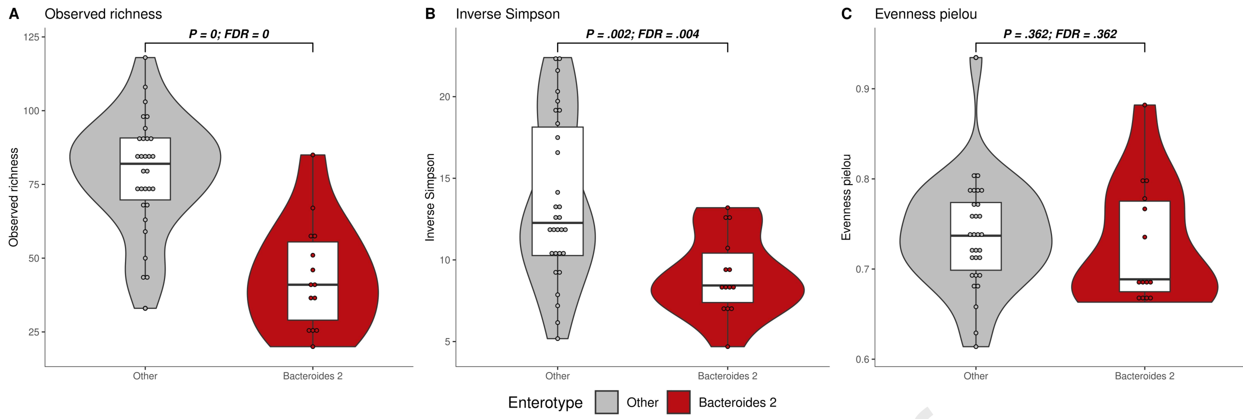

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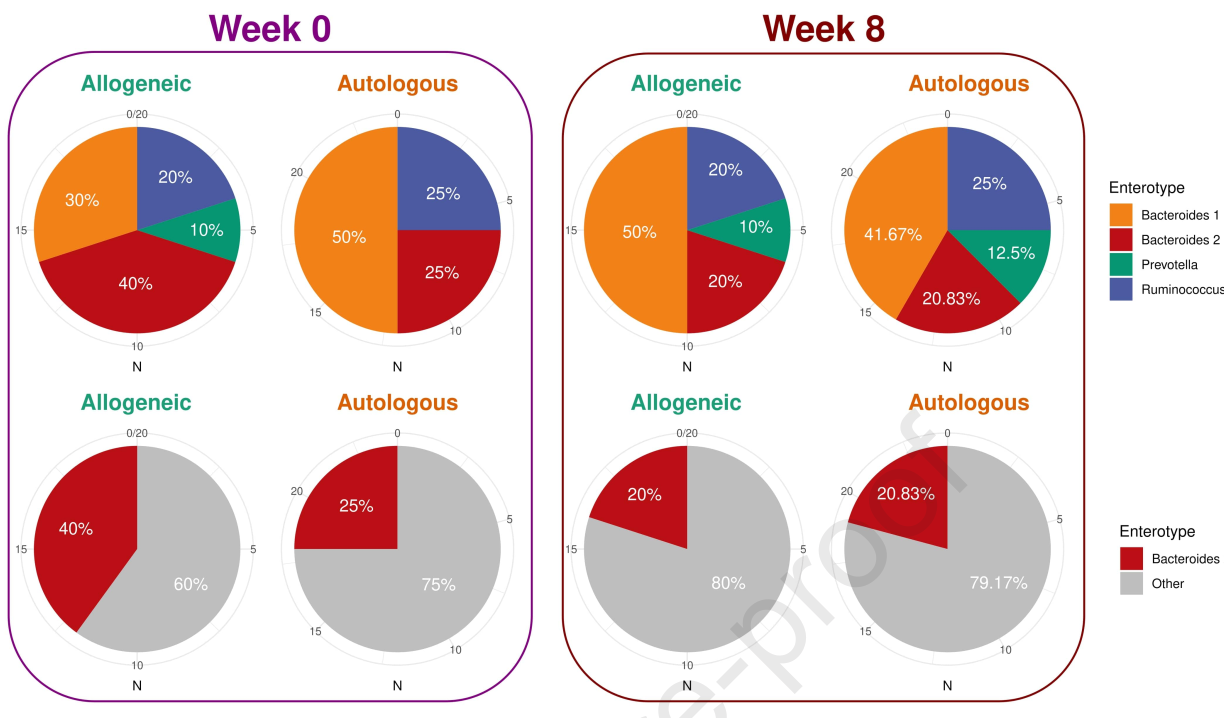

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