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Depletion of butyrate-producing microbes of the Firmicutes predicts nonresponse to FMT therapy in patients with recurrent *Clostridium difficile* infection

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ABSTRACT

Approximately 10% of individuals diagnosed with *Clostridium difficile* infection (CDI) show the resistance to fecal microbiota transplantation (FMT), with the underlying mechanisms remaining elusive. Deciphering the intricate microbiome profile within this particular subset of FMT-refractory patients via clinical FMT investigations assumes paramount importance, as it holds the key to designing targeted therapeutic interventions tailored for CDI, particularly recurrent CDI (rCDI). A cohort of twenty-three patients afflicted with rCDI, exhibiting congruent clinical baselines, was meticulously selected for FMT. Rigorous screening of thousands of healthy individuals identified ten FMT donors who met stringent health standards, while a total of 171 stool samples were collected to serve as healthy controls. To assess the influence of microbiome dynamics on FMT efficacy, fecal samples were collected from four donors over a continuous period of twenty-five weeks. After FMT treatment, seven individuals exhibited an inadequate response to FMT. These non-remission patients displayed a significant reduction in α-diversity indexes. Meanwhile, prior to FMT, the abundance of key butyrate-producing Firmicutes bacteria, including *Christensenellaceae_R_7_group*, *Ruminococcaceae_unclassified*, *Coprococcus_2*, *Fusicatenibacter*, *Oscillospira*, and *Roseburia*, were depleted in non-remission patients. Moreover, *Burkholderiales_unclassified*, *Coprococcus_2*, and *Oscillospira* failed to colonize non-remission patients both pre- and post-treatment. Conversely, patients with a favorable FMT response exhibited a higher relative abundance of *Veillonella* prior to treatment, whereas its depletion was commonly observed in non-remission individuals. Genera interactions in lower effectiveness FMT donors were more similar to those in non-remission patients, and *Burkholderiales_unclassified*, *Coprococcus_2*, and *Oscillospira* were frequently depleted in these lower effectiveness donors. Older patients were not conducive to the colonization of *Veillonella*, consistent with their poor prognosis after FMT. FMT non-remission rCDI patients exhibited distinct characteristics that hindered the colonization of beneficial butyrate-producing Firmicutes microbes. These findings hold promise in advancing the precision of FMT therapy for rCDI patients.

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Clostridium difficile often colonizes healthy individuals, especially infants and the older adult.[1] Abuse of such medications as immunosuppressive drugs and broad-spectrum antibiotics can trigger its rapid proliferation and subsequent toxin release, leading to severe, potentially life-threatening gastrointestinal *C. difficile* infections (CDI).[2] In clinical practice, 20%-30% of antibiotic-associated diarrhea, 50%-75% of antibiotic-associated colitis, and 95%-100% of pseudomembranous colitis are associated with CDI.[3] CDI also contributes to higher mortality in inflammatory bowel disease.[4] Mechanistically, *C. difficile* secretes enterotoxin A and cytotoxin B, leading to the destruction of

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the intestinal epithelium and the massive release of inflammatory mediators, causing severe diarrhea, toxic megacolon, colon perforation, septic shock, and even death.[5] Over the past decade, with the global prevalence of high-level toxin-producing *C. difficile*, recurrent *C. difficile* infections (rCDI) have caused the mortality rate to soar.[6]

Antibiotic therapy is typically favored for CDI treatment, but it exacerbates the disturbance of intestinal microbiota, and the recurrence rate after antibiotic withdrawal is pretty high (20%-60%) and may accompany with serious antibiotic resistance problems.[7–10] In contrast, fecal microbiota transplantation (FMT) therapy does not have these disadvantages; the effective rate for CDI treatment is 91.2%, and the recurrence rate is much lower (5.5%).[11,12] However, for approximately 10% of FMT-refractory CDI patients, the specific reasons for the ineffectiveness of treatment are still unclear. Given the vast global prevalence of CDI, this portion of FMT-refractory CDI patients can reach hundreds of thousands of individuals.[13] How to accurately classify and treat these patients is an important clinical problem that requires immediate resolution.

Several potential factors affecting the efficacy of FMT on CDI treatment include donor selection, choice of transplantation route, and method of FMT execution. To this end, we have established stringent and effective donor selection criteria,[14] and comprehensive FMT treatment criteria, in an effort to alleviate these technical constraints[15]. Although the overall effective rate has increased, it is still insufficient to adequately explain the prognosis of these FMT-refractory CDI patients.[14,15] Therefore, it is necessary to further explore the microbiota characteristics of the donors and CDI recipients, as well as their impact on patient's treatment response under the execution of standard FMT treatment.

Since most FMT-refractory CDI patients are rCDI patients, we recruited 23 rCDI patients to this study with parallel clinical baselines. Following FMT treatment, 7 cases were non-remission and 16 cases were cured. Ten donors who met health criteria were selected from thousands of healthy people, and 171 stool samples were collected as healthy controls. Among them, fecal samples were collected from 4 donors continuously for 25 weeks to evaluate the impact of microbiota fluctuation on FMT application. Microbiome data mining was performed to find clinical factors and microbiome characteristic of donors and recipients associated with poor rCDI prognosis following FMT.

Methods and materials

Study population

23 rCDI patients who received FMT therapy at Shanghai Tenth People's Hospital between January 2021 and January 2022 were recruited. Inclusion criteria included individuals (a) aged over 18 years old; (b) positive in stool test for *C. difficile* gene; (c) who can tolerate nasojejunal tube and complete full course of FMT treatment; and (d) with complete clinical baseline data. Exclusion criteria excluded those who (a) suffering from concomitant chronic wasting diseases, such as malignant tumor and hyperthyroidism; (b) had gastrointestinal organic diseases, including short bowel syndrome and intestinal fistula; (c) had severe destruction of the intestinal mucosa, severe immunosuppression, combined with severe systemic infection; (d) were subject to antibiotic intervention during treatment; (e) had severe neuropsychiatric disorders; and (f) experienced difficulty in cooperating with treatment and follow-up procedures.

Donor recruitment

The current standardized donor screening program adheres to the *Chinese Expert Consensus FMT Guideline,*[14,16] which recommends the evaluation of donor screening across the following six dimensions: physiology, psychology, personal history, stability, persistence, and tolerance to dietary restriction. Donors should meet the criteria of the above six dimensions, without any other illnesses, especially gastrointestinal disease or motility disorders, and not have been hospitalized for at least 3 months before FMT donation, and not received antibiotics or proton pump inhibitors for at least 6 months before FMT donation.

Recipient preparation

Before patients received FMT treatment, we had the following requirements and preparations. Patients had normal vital signs, as well as the absence of fever, severe infection, sepsis, SIRS, or other inflammatory diseases. Antibiotic preparation (oral vancomycin) was given to patients with rCDI. An initial oral antibiotic (vancomycin, 500 mg orally, twice per day) was given for 6 consecutive days. Then, a nasojejunal tube was placed in the patient's proximal jejunum, and the position of the tube was verified by abdominal radiography. Then, donor fecal microbiota was infused through the nasojejunal tube for 6 consecutive days. During FMT treatment, antibiotics, hormones, and immunosuppressants were generally not recommended.[15]

Outcome

The outcome was combined clinical status (24-hour stool frequency ≤ 3 with formed stools) and a negative PCR test for *C. difficile* and its toxin genes 8 weeks after the assigned treatment.[17]

PCR detection for C. difficile and its toxin genes

C. difficile 16S rDNA and toxin A/B gene were tested using quantitative real-time PCR method. The primer sequence are as follows:

16S-F: GCAAGTTGAGCGATTTACTTCGGT;
16S-R:GTACTGGCTCACCTTTGATATTC AAGAG;
16S-P:TGCCTCTCAAATATATTATCCCGT ATTAG;
tcdA-F:CAGTCGGATTGCAAGTAATTGA CAAT;
tcdA-R:AGTAGTATCTACTACCATTAACAGT CTGC;
tcdA-P:TTGAGATGATAGCAGTGTCAGGA TTG;
tcdB-F:TACAAACAGGTGTATTTAGTA CAGAAGATGGA;
tcdB-R:CACCTATTTGATTTAGACCTTTAA AAGC;
tcdB-P: TTTTCCAGTAAAATCAATTGCTTC.

Microbiome sequencing

For CDI patients, the first feces sample was collected after diagnosis, and the others were collected 8 weeks after FMT. Microbial DNA was extracted from 200 mg fecal sample using the QIAamp PowerFecal Pro DNA Kit (Qiagen), which contains a bead-beating step. Briefly, we added 200 mg of fecal sample and 800 mL of lysis buffer to a bead-containing tube and vortexed at maximum speed for 10 min. We took 350 mL of the supernatant after 1 min centrifugation at 15,000 × *g* and used it in subsequent steps according to the kit instructions. DNA was finally eluted in 100 mL elution buffer for downstream applications and amplified using primers targeting the V4 region of the 16S rRNA gene (515F 5′-GTGYCAGC MGCCGCGGTA-3′, 806 R 5′-GGACTACNVGGGTWTCTAAT-3′). PCR was run in a VeritiTM 96-Well Thermal Cycler PCR system (Thermo Fisher Scientific) using the following program: 95°C for 3 min, followed by 21 cycles of 95°C for 30 s, 56°C for 30 s, 72°C for 30 s, with a final extension at 72°C for 5 min. Mixed amplicons were pooled, and sequencing was conducted at Shanghai Biotecan Pharmaceuticals Co., Ltd. (Shanghai, China) using an Illumina Novaseq 6000 Sequencing system (Illumina, USA) according to the manufacturer's instructions. Sequences were assigned to operational taxonomic units (OTUs) with 97% similarity (Greengenes database: http://greengenes.lbl.gov) in mothur (v.1.39.5). OTU taxonomy was assigned via comparisons with data in the Greengenes database using the Quantitative Insights into Microbial Ecology (QIIME 1.9.1) software package, which will facilitate cross-cohort comparisons of analytical results. Abundance profiles of butyrate synthesis genes (*Hbd*, *Bcd*, *Thl*, *CroR*, *Buk* and *But*) were calculated using picrust2 (https://github.com/picrust/picrust2). To identify taxa that differed in relative abundances between two groups, linear discriminant analysis (LDA) effect size (LEfSe) analyses were performed on the website (http://huttenhower.sph.harvard.edu/galaxy).

The cutoff value was the absolute LDA score (log10) >3.0 with a $p < 0.05$. For the alpha diversity, a "summary.single" script was used to calculate ACE, Chao1, Shannon and Simpson indexes with the mothur software package. A co-occurrence network was established based on MB distance matrix using R package SPIEC-EASI. Enterotype identification was performed on the website (http://enterotypes.org/).

Statistical analysis

SPSS 19.0 software was used for statistical analysis. Continuous variables are presented as the median (interquartile range). Statistical differences between two or more groups of variables were analyzed using ANOVA design with a post hoc test. The chi-squared test was used for comparative analysis of discrete variables among groups. Only p-values < 0.05 were considered statistically significant. The data were plotted using the online tool Chiplot (chiplot.online). The schematic diagrams were drawn using the online tool MedPeer (image.medpeer.cn).

Results

Cohort characteristics

Twenty-three patients who met the inclusion criteria were included, including 15 males and 8 females (Table 1). After FMT treatment, 16 patients achieved clinical remission, and 7 patients did not respond to treatment. The *C. difficile* burden, immune factor levels, and genetic testing results of each patient before and after FMT treatment are shown in Tables S1-S2. The specific donor who was matched to each recipient and the prognosis of each recipient are shown in Figure 1a. The median chronological age of the non-remission group was 57 years, and that of the remission group was 49.5 years, with no significant difference between the two groups. In addition to the age factor, there was also no statistical difference in BMI, Bristol stool scale, or defecation frequency between the two groups of rCDI patients. Moreover, after FMT treatment in both groups, IL-8 was significantly down-regulated, while IL-17 was obviously up-regulated (Table S1 and Figure S4A-B). For the non-remission group, 3 patients recently had gastrointestinal surgery history, and 4 patients had long-term antibiotic use. In the remission group, 5 patients recently had gastrointestinal surgery history, and 2 patients had long-term antibiotic use. Three non-remission patients had concomitant Crohn's disease, and 1 had a long-term medication history of PPI use. Two remission patients had coronary heart disease, and 1 had long-term use of hormones. In general, under the control of strict inclusion and exclusion principles, all enrolled patients had a relatively parallel baseline, which is crucial for subsequent microbiome analysis.

Values are median (interquartile range). The *p* value was obtained by Chi-square test or ANOVA design.

Trajectory of the microbiome of rCDI patients after receiving FMT treatment

Previous studies have found that the response to FMT therapy in CDI patients is related to the enterotype matching between donors and recipients.[18] In accordance with the enterotype theory proposed by Peer Bork,[19] we clustered the gut microbiota of rCDI patients and healthy

Table 1. Characteristics of patients in remission group and non-remission group.

Variables	Remission (*n*=16)	Non-remission (*n*=7)	p-value
Gender (M:F)	10:6	5:2	0.679
Age (y)	57.0 (28.5–68.0)	49.5 (28.0–60.8)	0.930
BMI (kg/m^2)	17.7 (14.9–21.0)	21.1 (18.1–21.8)	0.252
Stools/day (times)	4.0 (3.5–7.0)	2.5 (1.0–6.0)	0.470
Bristol stool scale	6.0 (5.5–6.0)	6.0 (5.5–6.0)	0.694
Medications history	3 patients had a history of gastrointestinal surgery; 4 patients had long-term antibiotic use; 3 patients had Crohn's disease; 1 patient with long-term medication history of PPI use.	5 patients had a history of gastrointestinal surgery; 2 patients had long-term antibiotic use; 2 patients had coronary heart disease; 1 patient with long-term use of hormones.	

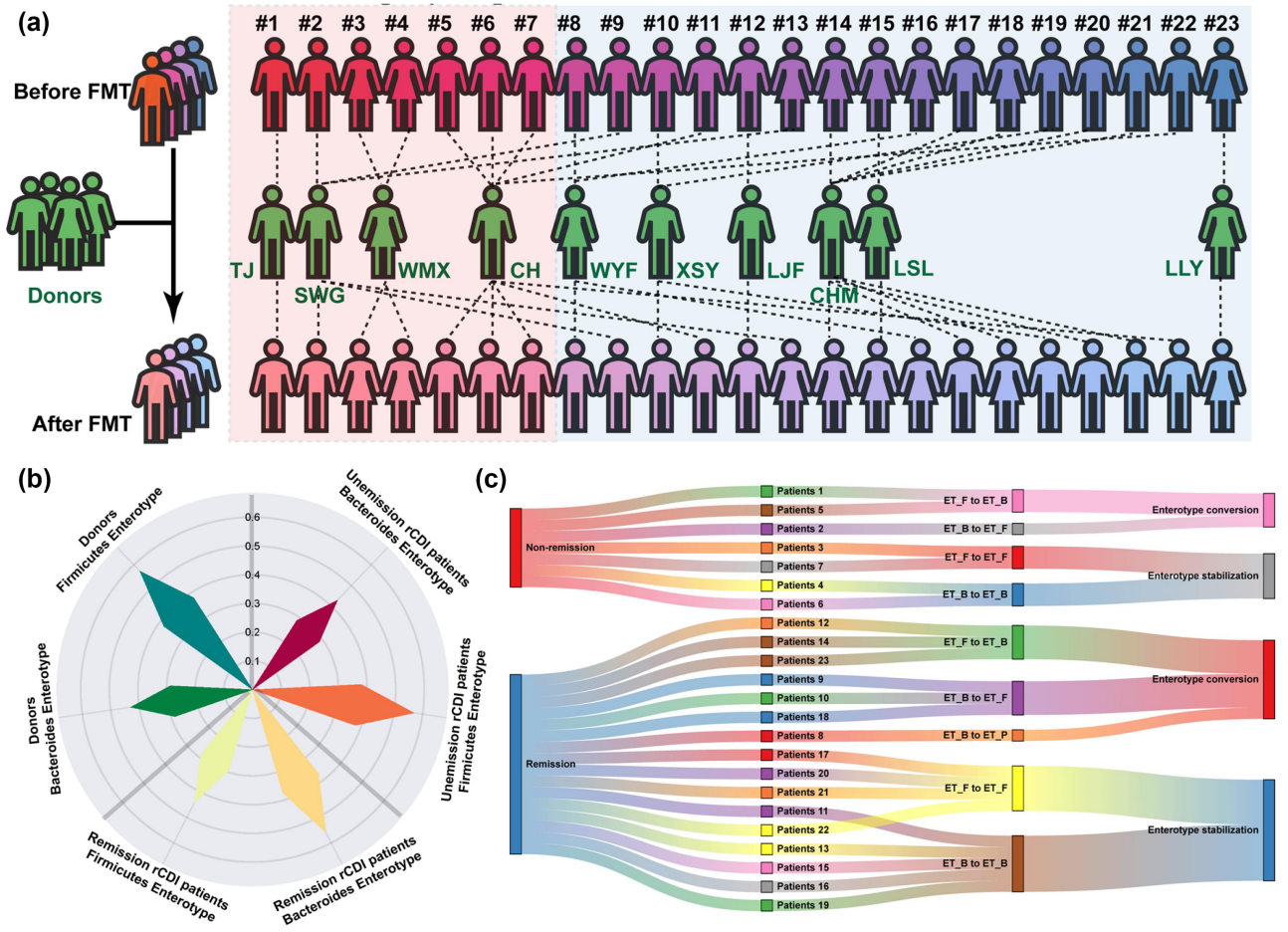

Figure 1. (a) Donor-recipient matching and prognostic information for the cohort. Acronyms stands for the donor code. Seven individuals highlighted with a red background displayed ineffective responses to FMT treatment. Another sixteen patients denoted with a blue background experienced alleviation of symptoms following FMT intervention. The gender annotations of both patients and donors were accurately aligned with real-world cases. (b) Enterotype distribution in the donor group and rCDI patient group before receiving FMT therapy. The ordinate indicates the percentage distribution of certain enterotype individuals in each group. (c) Treatment response and enterotype switching outcomes for each rCDI patient after FMT treatment.

donors into three enterotypes, ET_B, ET_F, or ET_P, representing the *Bacteroides*, Firmicutes, and *Prevotella* enterotypes. Consequently, 57% of the healthy donor samples were classified as ET_F, and the remaining 43% as ET_B. Among the remission group of rCDI patients, 56% were ET_B (9/16), and 44% were ET_F (7/16). For refractory rCDI patients, 43% were ET_B (3/7), and 57% were ET_F (Figure 1b). The enterotype conversion rate in the remission group was 44% and, in the non-remission group, it was 57% after FMT treatment (Figure 1c). This high frequency of enterotype switching reflected the high degree of microbiota dysbiosis in the rCDI patients and suggests that use of vancomycin before transplantation may be more favorable for colonization by donor microbes. β-diversity analysis further revealed that rCDI patients, either before and after FMT management, in symptom remission or non-remission, and regardless of the kind of enterotype classified, could not match the principal component dimension of healthy donors (Figure 2a,b). Microbiome data before and after treatment, regardless of whether treatment was effective, were dispersed to areas far from healthy donors. According to the marginal density curve, we divided the two-dimensional plane of PCoA into three regions: ET_B donor region, ET_F donor region, and CDI dysregulation region. PCoA represents a data projection of the differences in microbial composition (i.e., Bray-Curtis distance) between different samples in the direction of the largest variance and the second largest variance. In addition, rCDI patients and healthy donors were significantly

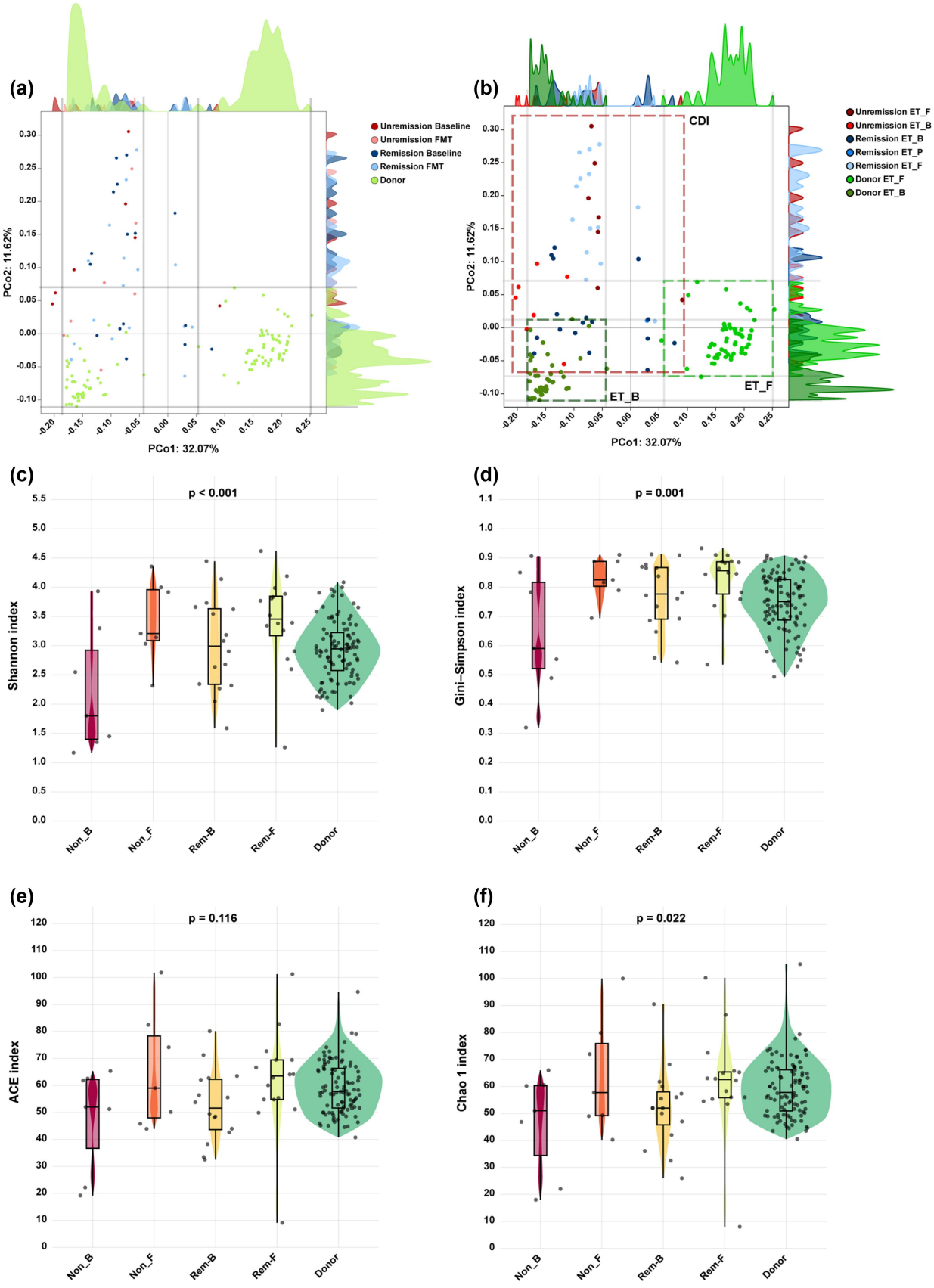

Figure 2. PCoA plot based on Bray-Curtis dissimilarity matrices of (A) FMT outcome and (b) enterotype. Marginal densities were used to show the separation of microbiome data. α-diversity indices, including (c) Shannon index, (d) Gini-Simpson index, (e) ACE index, and (f) Chao 1 index, show that rCDI patients in the FMT-ineffective group had lower bacterial diversity. The *p* value was obtained by ANOVA design.

different in the direction of the first principal component, indicating that there was a large difference in the composition of the microbiota between the two groups. The differences among rCDI patients mainly manifested in the direction of the second principal component, indicating that the differences in the composition of the microbiota among them were small, but the presence or relative abundance of a small number of genera varied significantly. The microbiome data of the remission group and non-remission group could not be separated in either the first principal component or second principal component (Figure 2), and the change in enterotype was not enough to explain the prognosis of FMT (Figure 2b), so we combined the data on different enterotypes for subsequent analysis.

To more accurately define FMT-induced changes in intestinal microbiota, we next performed α-diversity analysis. ACE and Chao 1 indexes were selected to evaluate the richness of the species number. Shannon and Simpson indexes were selected to reflect the evenness of microbial communities among patients before and after receiving FMT treatment (Figure 2c–f). Compared with the other groups, the non-remission group's four baseline α-diversity indexes were lowest. After FMT treatment, the Shannon index of the non-remission patients reached that of healthy donors. Meanwhile, other α-diversity indexes, including ACE, Chao 1, and Simpson index, also increased after FMT in these non-remission patients. These results suggest that FMT-refractory rCDI patients had fewer bacterial species, lower bacterial abundance, and more severe dysbiosis at baseline. Although FMT therapy can improve this microbiota defect, it does not seem to be able to induce immediate symptomatic remission.

Specifically, at the phylum level, the baseline microbiota of FMT-refractory rCDI patients consisted mainly of Firmicutes (32.3%), Bacteroidetes (36.9%), Proteobacteria (24.5%), Actinobacteria (1.5%), and Fusobacteria (4.5%). Among these, a decrease in the relative abundance of Bacteroidetes and Firmicutes, and an increase in the relative abundance of Proteobacteria and Fusobacteria, indicating comprehensive microbiota dysbiosis, were the predominant characteristics of FMT-refractory rCDI patients (Figure 3a). Furthermore, a decreased abundance of Bacteroidetes and increased abundance of Proteobacteria appeared to be common features of all rCDI patients, and these failed to return to donor levels even after symptom remission. Further genus-level analyses revealed that the baseline microbiota of FMT-refractory rCDI patients consisted

mainly of *Bacteroides*, *Escherichia_Shigella*, *Bacteroidales_unclassified*, *Fusobacterium, Prevotella_9*, *veillonellaceae_unclassified*, *Veillonella*, and *Faecalibacterium*. Other genera in non-remission patients at baseline accounted for only 17.5% of relative abundance, lower than 24.8% in the donor group and 37.8% in the remission group (Figure 3b). A reduction in the abundance of *Fusobacterium* and *Prevotella_9* appeared to be a common feature of the rCDI group, and these were difficult to restore to healthy levels with FMT therapy. An enrichment of *Escherichia-Shigella* and a depletion of *Faecalibacterium* were characteristic of patients in the FMT-refractory group, suggesting that there may be an antagonistic relationship between the two microbes.

To find the hallmark microbes of rCDI patients before and after FMT treatment, we performed LEfSe analysis and focused on genus-level variations between the different groups. Eleven microbes were differentially enriched in the rCDI and donor groups (Figure 3c and Figure S1). Among them, *Coprococcus_2*, *Fusicatenibacter*, *Roseburia*, *Oscillospira*, and *Burkholderiales _unclassified* were significantly enriched in donors; *Bacteroidetes_ unclassified* were hallmark microbes of the non-remission FMT group; *Veillonella* was a characteristic microbe of the remission group at baseline; and *Bifidobacterium*, *Christensenellaceae_ R_7_group*, *Dorea*, and *Ruminococcaceae_ unclassified* were enriched in the remission FMT group (Figure 3c). All 11 of these genera were depleted in non-responding patients at baseline (Figure S1). Recently, colonization antagonism has been postulated as an important reason for poor prognosis after FMT therapy.[20] We found that *Oscillospira*, *Burkholderiales_unclassified*, and *Coprococcus_2* indeed colonized FMT-refractory patients with difficulty (Figure 3d). These three genera were generally depleted before and after treatment in FMT-refractory patients (Figure 3e–g). In our cohort, five patients, #5, #6, #7, #11, and #22, received

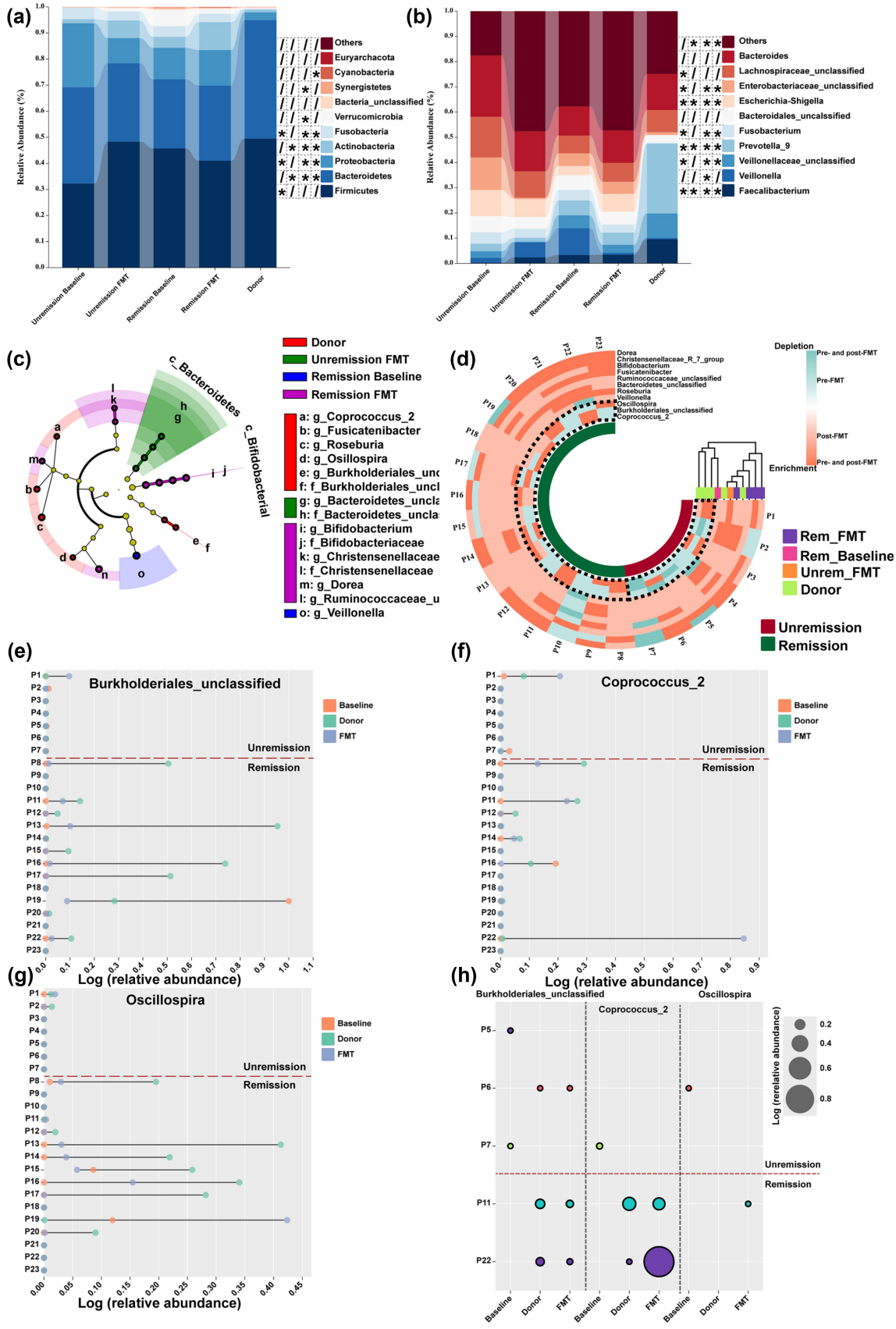

Figure 3. The composition of bacterial (a) phyla and (b) genera in the donor group and rCDI patient group before and after receiving FMT treatment. *Indicates a significant difference compared with the donor group ($p < 0.05$), and the p value was obtained by ANOVA design. (c) LEfSe cladogram presented the characteristic microbes of donors and patients with or without FMT response. (d) Heatmap showed the colonization antagonism of characteristic microbes. The relative abundance of (e) *Burkholderiales_unclassified*, (f) *Coprococcus_2*, and (g) *Oscillospira* in rCDI patients and their donors. (h) Five patients received enterobacteria transplantation from donor CH.

transplantations of microbiota from the same donor. *Oscillospira* and *Burkholderiales_ unclassified* were generally of low abundance prior to treatment in these five patients, and they were depleted only in donor samples from the non-remission group. As a result, patients in the non-remission group were also deficient in these two microbes after FMT treatment, whereas these genera recovered or even reached donor levels in patients in the remission group (Figure 3h).

Based on the results of LEfSe analysis, we found that these characteristic bacteria were mostly classic butyrate-producing bacteria and their commensal, so we next performed Spearman correlation analysis on the abundance of these genera and butyrate-producing bacteria genes. The result presented that the abundance of butyrate-producing bacteria, *Roseburia*, *Dorea*, *Fusicatenibacter*, *Christensenellaceae_R_7_group* and *Oscillospira*, was significantly positively correlated with the abundance of butyrate-producing genes (Figure S2A). Among them, the abundance of four butyrate-producing genes *Hbd*, *Bcd*, *Thl*, and *CroR* were all positively correlated with *Fusicatenibacter*, and the abundance of *Buk* gene was only positively correlated with *Oscillospira*. Patients in FMT remission groups had a higher abundance of *Oscillospira* and relatively elevated levels of the *Buk* and *Bcd* genes (Figure S2B-C), which may confer a higher capacity for butyrate synthesis in the gut.

Potential impact of donor microbiota on treatment response to FMT therapy

In clinical practice, the donor selection does have an important impact on the efficacy of FMT treatment.[14] By screening the donor's medical history and microbial structure, the overall effective rate of FMT in our department can reach 68.7%.[14] In the donor cohort, the overall effective rate of CH and SWG was about 60%, while that of CHM and XSY was higher at 80% (this result is a comprehensive statistic of the overall effective rate for 2 years, including but not limited to CDI treatment). CH, SWG, and XSY were all ET_B, and CHM was ET_F. These four donors were also the main FMT donors for the rCDI patients, so we wanted to further explore the relationship between their microbiota fluctuations and the result of their FMT applying. We collected stool samples from these donors for 25 consecutive weeks. Donors with low FMT efficiency had similar characteristics, but the fluctuation range of their microbiota was relatively small (Figure S3). Although there was a significant difference between donors with high FMT efficiency (regardless of whether they were in ET_B or ET_F) and donors with low efficiency, there was also significant variation among the samples from each high-efficiency FMT donor (Figure S3). The α-diversity results showed that the donor CHM had a lower Gini-Simpson index, and donor XSY had lower ACE index and Chao1 index (Figure 4a–d).

Of the genera associated with good FMT prognosis, *Bifidobacterium* and *Dorea* were significantly upregulated in high-efficacy FMT donors; *Christensenellaceae_R_7_group* was only enriched in CHM samples of ET_F, and *Ruminococcaceae_unclassified* was depleted in XSY samples (Figure 5a–d). With the exception of *Dorea*, the other three genera are widely reported probiotics, and their relative abundances are host-specific. The genera associated with poor FMT prognosis, *Bacteroidetes_unclassified* and *Veillonella*, were depleted in XSY samples, but were relatively more abundant in the other three donors, especially the low-efficiency FMT donors CH and SWG (Figure 5e–f). Two donor-characteristic genera, *Fusicatenibacter* and *Roseburia*, were present in all four donors, but their relative abundances were individual-specific (Figure 5g–h). However, *Burkholderiales_unclassified*, *Coprococcus_2*, and *Oscillospira* were sporadically depleted in specific donors (Figure 5i–k).

As noted above, *Burkholderiales_unclassified*, *Coprococcus_2*, and *Oscillospira* were generally depleted before and after treatment in rCDI patients with poor FMT outcomes (Figure 3e–h). These three genera also showed large fluctuations in their relative abundance in the donor cohort (Figure 6a–c). *Burkholderiales_unclassified* was occasionally depleted in SWG, XSY, and CHM donors, but the frequency was relatively low (Figure 6d). *Coprococcus_2* was frequently depleted in

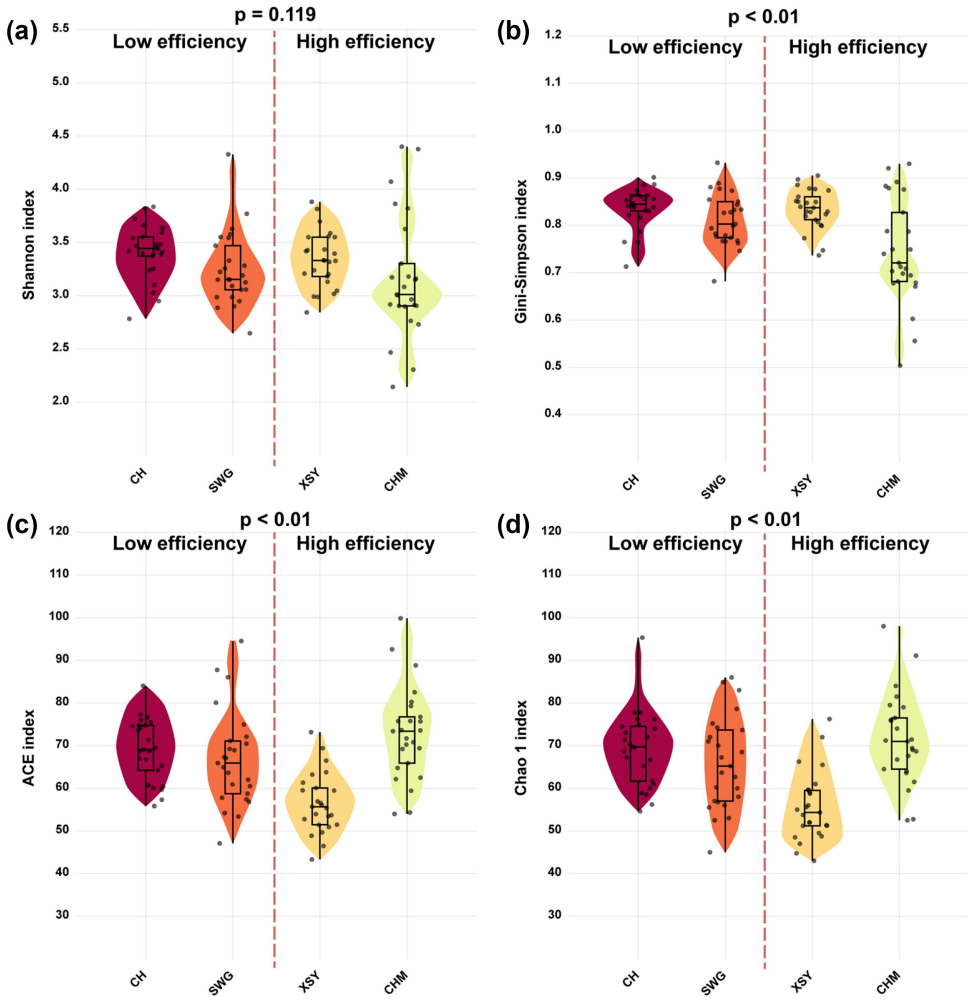

Figure 4. (a) Shannon index, (b) Gini-Simpson index, (c) ACE index, and (d) Chao 1 index for samples from 4 donors. Donor CH and SWG had low average FMT effectiveness levels, and XSY and CHM had high average FMT effectiveness levels. The *p* value was obtained by ANOVA design.

donor SWG and XSY (Figure 6e). *Oscillospira* was depleted in almost all samples of donor XSY (Figure 6f). Therefore, the low abundance of these genera after treatment of rCDI patients is likely due to their low abundance in both donors and recipients at baseline.

Through co-occurrence network analysis, we found that the deletion of *Oscillospira* and *Burkholderiales_unclassified* caused widespread cascading effects (Figure 6g–h). Moreover, the intestinal microbiota of non-remission patients and low FMT efficiency donors had similar regulatory network structures (Figure 6g–h). However, these regulatory relationships were relatively weak in patients with a better prognosis and donors with high FMT efficacy rates (Figure 6i–j).

Effect of clinical factors on the colonization by butyrate-producing microbes

Colonization by transplanted microbiota is inevitably affected by the recipient's physical state. In patients who have recently undergone abdominal surgery especially, the process will be accompanied by the use of a large number of antibiotics. We found that the abundance of *Bifidobacterium* was downregulated before FMT in both patients with a history of recent abdominal surgery and those who had not undergone surgery but received antibiotics (Figure 6k). It is noteworthy that abdominal surgery and the use of antibiotics did not affect colonization by *Bifidobacterium* after FMT. However, patients' BMI values were significantly positively correlated with the relative

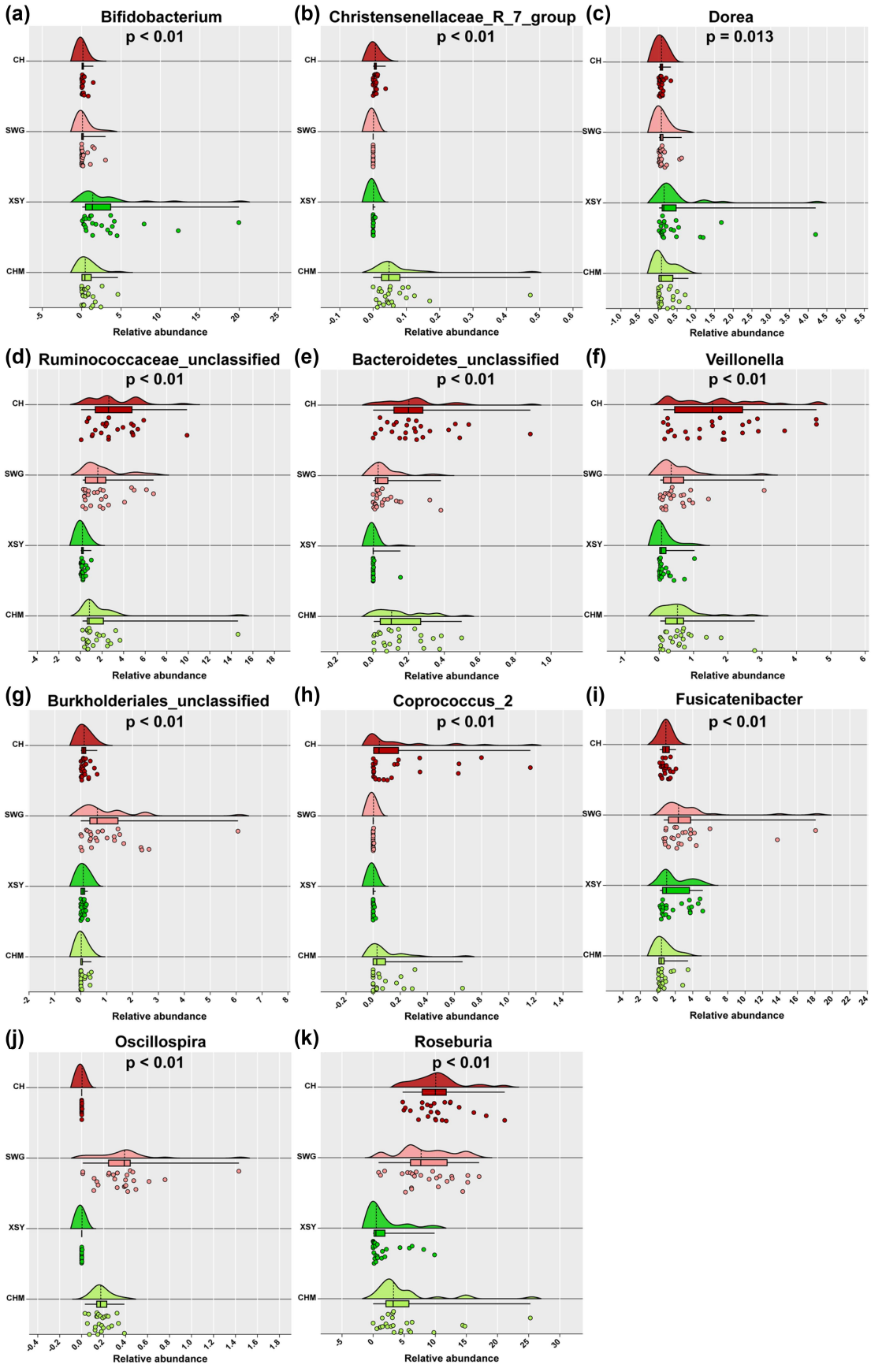

Figure 5. The relative abundance of (a) *Bifidobacterium*, (b) *Christensenellaceae_r_7_group*, (c) *Dorea*, (d) *Ruminococcaceae_unclassified*, (e) *Bacteroidetes_unclassified*, (f) *Veillonella*, (G) *Burkholderiales_unclassified*, (h) *Coprococcus_2*, (I) *Fusicatenibacter*, (j) *Oscillospira*, and (k) *Roseburia* from the four donors. The *p* value was obtained by ANOVA design.

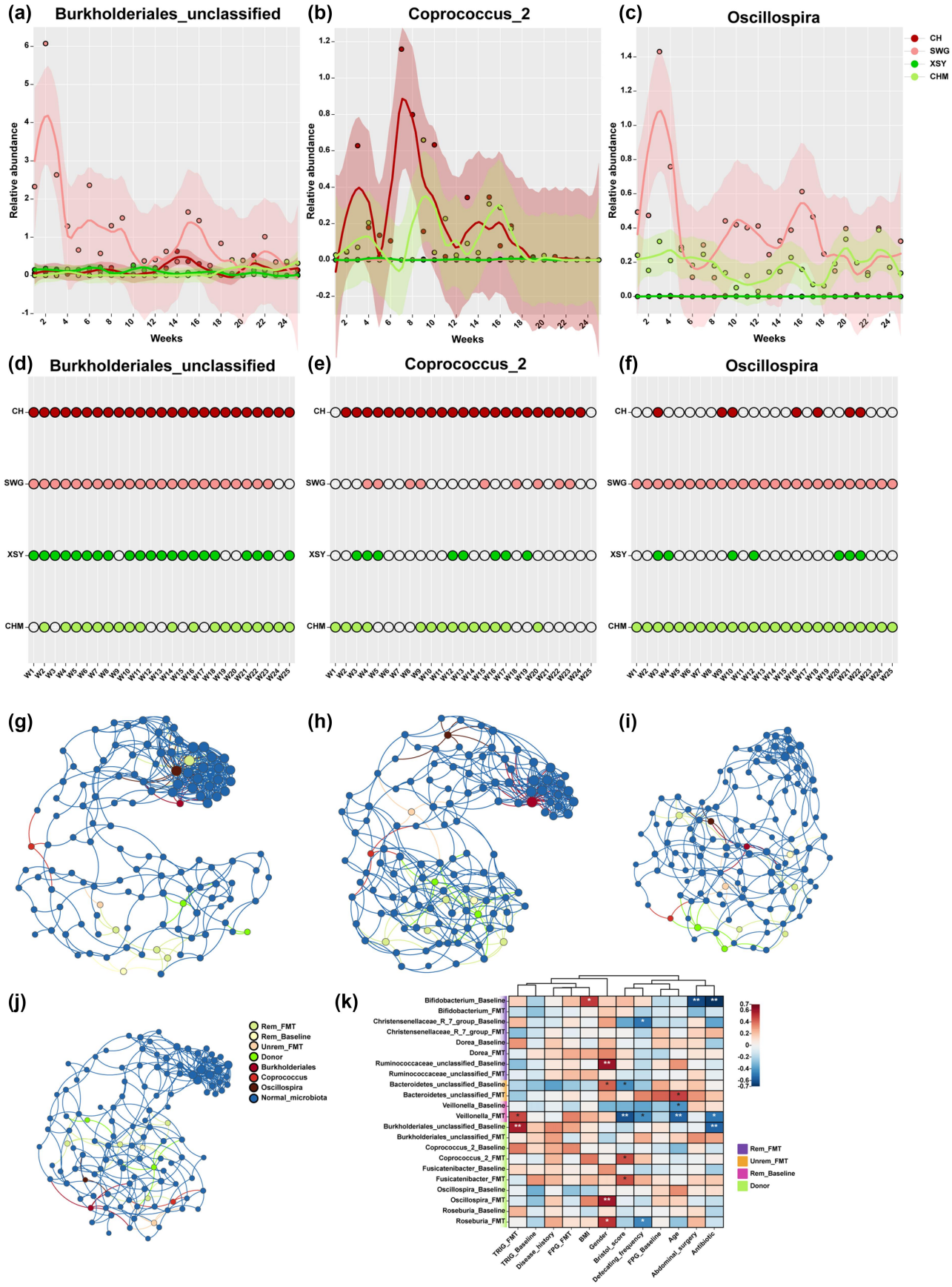

Figure 6. The relative abundance fluctuation of (a) *Burkholderiales_unclassified*, (b) *Coprococcus_2*, (c) *Oscillospira* for the four donors. The presence or absence of (d) *Burkholderiales_unclassified*, (e) *Coprococcus_2*, and (f) *Oscillospira* in the four donors. Co-occurrence network of microbes in (g) rCDI patients with poor FMT prognosis, (h) low-effectiveness FMT donors, (i) rCDI patients with good FMT prognosis, and (j) high-effectiveness FMT donors. (k) Spearman correlation between the relative abundance of 11 characteristic genera in rCDI patients before and after treatment and the clinical factors of the patients. * indicates p-value <0.05. ** indicates p-value <0.01.

abundance of *Bifidobacterium*, but BMI did not affect the proportion of *Bifidobacteria* after FMT treatment. The patient's autoimmunity was also found to be related to changes in the microbiome: the down-regulation of IL-8 was associated with the up-regulation of the abundance of *Christensenellaceae_R_7_group*, while the up-regulation of IL-17A was associated with the up-regulation of the abundance of *Bacteroidetes_unclassified* (Figure S4C). In addition, female patients tend to have a higher relative abundance of *Ruminococcaceae_unclassified* and *Bacteroidetes_unclassified* before receiving FMT treatment and were more likely to be colonized by *Oscillospira* and *Roseburia* after receiving FMT treatment. Elderly patients were less likely to be colonized by *Veillonella*, which affected their prognosis following FMT treatment. Both improved stool frequency and improved stool morphology were significantly associated with a higher relative abundance of *Veillonella* after FMT treatment, emphasizing the importance of this genus in the treatment of rCDI.

Discussion

rCDI is considered to be the most successful clinical application of FMT, with a success rate of 91.2%, and is safe and effective with a low recurrence rate.[12] Recently, a phase III clinical trial once again proved that intervention with an intestinal microecology of Firmicutes spores can effectively treat CDI with a recurrence rate of only 12%.[8] Encouraged by this clinical study, more and more microbial products have been included in clinical trials for CDI and rCDI treatment, and these are vying for regulatory approval.[21] However, the mechanism of FMT in the treatment of rCDI is still unclear.[22] The donor-recipient matching criteria and microbial genera that play roles in the treatment are also far from clearly demonstrated.[22] Answers to these questions are essential for the development of microbial products related to rCDI treatment and a necessary prerequisite for efforts to further improve the response rate and safety of FMT for rCDI.

In this study, we recruited 23 rCDI patients with parallel clinical baseline and complete pre- and post-FMT information. In addition, we enrolled 10 donors, 4 of whom provided stool samples for 25 consecutive weeks. In the patient group, the symptoms of 16 patients were effectively relieved after receiving FMT, and the remaining 7 patients did not respond to treatment. The FMT-refractory patients generally had lower α-diversity indices, including Shannon index, Gini-Simpson index, ACE index, and Chao 1 index (Figure 2c–f). Although FMT therapy effectively restored the diversity of their gut microbiota, it seems that severe microbiota dysbiosis had affected the FMT therapeutic response of patients. This situation has also been reported in a recent FMT-treated RCT cohort of rCDI,[23] so it could be a general rule for FMT therapy non-responsiveness in rCDI patients. However, through β-diversity analysis, we found that, even if the number and uniformity of the microbiota were restored, the microbiota composition of rCDI patients was difficult to restore to the level of healthy donors (Figure 2a–b). Based on LEfSe analysis, we found that *Coprococcus_2*, *Fusicatenibacter*, *Roseburia*, *Oscillospira*, and *Burkholderiales_unclassified* were enriched in the donor group. *Bacteroidetes_unclassified* was significantly enriched in the non-remission FMT group. *Veillonella* was a characteristic microbe of the remission baseline group; and *Bifidobacterium*, *Christensenellaceae_R_7_group*, *Dorea*, and *Ruminococcaceae_unclassified* were enriched in the remission FMT group (Figure 3c). These genera were all depleted in non-responding patients at baseline (Figure S1).

Among the above 11 microbes screened by LEfSe analysis, *Bacteroidetes_unclassified* was an important depleted genus of Bacteroidetes. Bacteroidetes were also generally less abundant before and after FMT treatment in rCDI patients (Figure 3a), and this appears to be a common phenomenon that has been reported several times in previous studies.[24–26] Bacteroidetes are strictly anaerobic bacteria that are sensitive to changes in the intestinal microenvironment.[25] A noteworthy phenomenon demands attention – the enrichment of *Bacteroidetes_unclassified* in non-remission patients after FMT therapy (Figure 3c). This could be attributed to the microenvironment of *C. difficile*, as the metabolic activities of the latter demand the consumption of succinic acid

produced by *Bacteroides*.[27] Therefore, the enrichment of *Bacteroidetes_unclassified* could potentially furnish the requisite ecological milieu for the resurgence of *C. difficile*.

In addition, enrichment of Proteobacteria was also a typical feature of rCDI patients (Figure 3a) and was reported in previous rCDI research.[28] This is a type of endotoxin-bearing microbial phylum[29] positively correlated with fecal calprotectin levels.[30] However, the relative abundance of *Burkholderiales_unclassified* within the Proteobacteria phylum was generally lower in rCDI patients, both before and after FMT treatment. Moreover, in the rCDI non-remission group, this microbe was almost completely depleted before and after treatment (Figure 3d and Figure S1). In fact, *Burkholderia* has an extremely important inhibitory effect on the pathogenesis of CDI. It can inhibit the Rho-glucosylation activity of *C. difficile* virulence factor TcdB, thereby activating Pyrin inflammation.[31] Moreover, upregulation of *Burkholderia cenocepacia* abundance was reported to be associated with reduced lung inflammation in mice.[31] In our cohort, 5 rCDI patients were matched with the same donor, and when *Burkholderiales_unclassified* was reduced or depleted in both patients and their donor samples, the patients had a high probability of treatment failure (Figure 3h).

Firmicutes was one of the phyla with the most severe disorder in the invalid rCDI treatment group (Figure 3a), and the above genera, except *Bacteroidetes_unclassified* and *Burkholderiales_unclassified*, all belonged to Firmicutes. Of significant importance, *Coprococcus_2*, *Fusicatenibacter*, *Roseburia*, *Oscillospira*, *Veillonella*, *Bifidobacterium*, *Christensenellaceae_R_7_group*, and *Ruminococcaceae_unclassified* are renowned as producers of short-chain fatty acids (SCFA), with a primary focus on butyrate production. Butyrate can effectively improve intestinal inflammation by stabilizing the expression level of hypoxia-inducible factor-1 (HIF1) in the intestine, thereby improving the intestinal barrier, preventing bacterial translocation, and reducing the local inflammatory response and systemic consequences of infection.[32] Furthermore, *Roseburia* can alleviate colitis symptoms by balancing Treg/Th17 proportions and protecting the intestinal epithelial barrier.[33] *Roseburia*-produced butyrate was previously found to be depleted in CDI patients,[34] and its recovery is usually positively correlated with the response to FMT treatment.[35] *Veillonella*, both a butyrate producer and a pro-inflammatory bacterium, was highly enriched in patients with various gastrointestinal tumors.[36,37] However, accumulating evidence indicates that an elevated abundance of *Veillonella* is associated with recurrence-resistance in CDI.[38,39] In our cohort, patients in the remission group also had a higher relative abundance of *Veillonella*, much higher than that in the non-remission group, before treatment (Figure 3b–c). In addition, *Bifidobacterium*, *Dorea*, and *Ruminococcaceae* have mucinophilic properties, which are also important features of *Bacteroides* and *C. difficile*.[40] This suggests a strong possibility of niche competition between these two bacterial groups. FMT not only facilitates the introduction of butyrate-producing bacteria to restore intestinal mucosal health but also promotes the displacement of *C. difficile* through niche competition, thus preventing recurrence.

Through the paired microbiome analysis of patients and their donors, we found that the selection of donors has an important guiding significance in restoring patient's intestinal microbiota (Figure 3d), as shown by the general absence of *Burkholderiales_unclassified*, *Coprococcus_2*, and *Oscillospira* before and after FMT treatment in ineffective patients. This is consistent with previous findings related to FMT therapy. In fact, successful colonization of recipient guts by transplanted microbiota is largely determined by the phylogeny of the microbes in the donor and pre-FMT patients. Moreover, the engraftment of donor strains into a species is usually performed in an all-or-nothing manner.[41] One possible explanation is that the microbes are linked by ecological functions, and the loss of some important microbes in the functional linkage leads to a failure in colonization by other microbes.[42]

At the level of the donors, we tested the microbiota of 4 donors for 25 consecutive weeks and found that the above-mentioned 11 microbes had significant individual differences (Figure 5). Furthermore, *Burkholderiales_unclassified*, *Coprococcus_2*, and *Oscillospira* were occasionally completely depleted in the donor population (Figure 6a–f). Of particular importance is *Oscillospira*, whose loss has

recently been reported to be associated with overall colonization-resistance of the donor microbiota.[20] A lot of evidence shows that *Oscillospira* is generally less abundant in gastrointestinal inflammatory diseases, such as inflammatory bowel disease.[43] In addition, a high abundance of *Oscillospira* was associated with dry and hard stools, which can lead to a constipation phenotype, and its abundance was significantly upregulated in women with chronic constipation.[43,44] This evidence was consistent with the microbiota phenotype of rCDI patients with poor prognosis in FMT (Figure S1). It should be noted that, in our cohort, 5 patients were matched with CH donors, but CH donors had a high frequency of *Oscillospira* deletion (Figure 6c,f). As a result, none of the 5 patients were colonized by *Oscillospira*. This may be one important reason for the low FMT effectiveness of this donor.

Driven by a series of factors, such as environment, diet, and disease, the intestinal microbiota in healthy individuals is constantly undergoing adaptive changes.[45–47] A longitudinal cohort study lasting 512 days showed that only about 60% of bacterial genera in the intestinal tract can exist for a long time, and they are occasionally depleted. Most of the bacterial genera are absent for a long time in the intestinal tract, with occasional appearances.[47] More importantly, intestinal colonization by transplanted microbiota is largely related to the niche adaptability of the microbes.[48] Some microbes need to invade the epithelial mucus layer and reside deep in the intestinal tissue, and for this reason, some Bacteroidetes have evolved specialized proteins for assisting colonization.[48–51] Large-sample population studies have also confirmed that Bacteroidales has the highest transmission efficiency.[52] This evidence can partly explain why *Bacteroidetes_unclassified* varied less among the 4 donors (Figure 5e). It may also explain the lower proportion of Bacteroidetes among the genera associated with FMT prognosis.

In contrast to Bacteroidetes, intestinal colonization by Firmicutes is slower and more difficult.[53,54] The host colonization by Firmicutes is more clearly reflective of the specialization of co-metabolic behavior and the active expression of genes related to the regulation of metabolic behaviors. However, this adaptive process occurs at the expense of spore-forming activity, and the proportion of spore-forming Firmicutes was much lower than that of non-spore-forming Firmicutes. Thus, Firmicutes are highly population-specific.[53] It is not difficult to understand why most of the genera associated with FMT prognosis in our cohort belonged to Firmicutes. Both *Coprococcus_2* and *Oscillospira* belong to genera that do not produce spores or have weak spore-forming abilities. In addition, the lack of motility of *Coprococcus_2* and the slow growth of *Oscillospira* make them less able to colonize in the diarrheal guts of rCDI patients. Given that the depletion of these two genera is associated with poor prognosis in FMT, particular attention should be paid to their abundance in patients and their matched donors during microbiota transplantation. Moreover, the microbiota capsules used for transplantation, rather than the donor feces, should be sequenced, and the microbiota structure and abundance should be recorded, and donors and recipients should be matched using this information.

Returning to the recipients themselves, pretreatment of physical fitness or disease severity may also affect colonization by transplanted microbiota. We found that a recent history of abdominal surgery or antibiotic use can affect the relative abundance of *Bifidobacterium* in patients before treatment, but not its colonization when transplanted by FMT therapy (Figure 6k). Nonetheless, the potential impact of antibiotics use on long-term *Bifidobacterium* colonization still needs to be addressed. Several recent studies have shown that the use of antibiotics can significantly reduce the abundance of *Bifidobacterium* and induce long-term colonization resistance.[55–57] *Bifidobacterium* can protect intestinal health, support the immune system to fight infection, and inhibit *C. difficile* infection, which are necessary for the treatment of rCDI.[55,58]

In addition, the relative abundance of the genera *Oscillospira* and *Roseburia* was generally higher in female patients after FMT treatment (Figure 6k). It should be noted that the enrichment of *Roseburia* in female patients was likely to be caused by the higher proportion of female patients in the rCDI remission group (Table 1). *Roseburia* generally

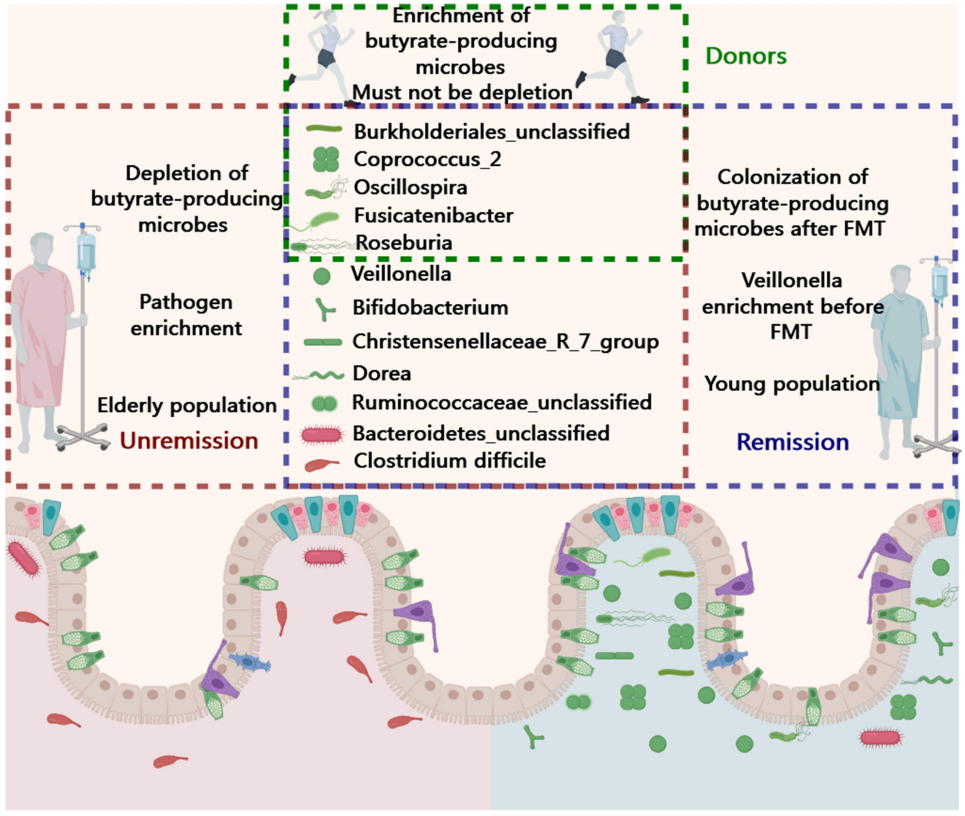

Figure 7. Microbiota characteristics of donor and recipient associated with FMT therapy prognosis in rCDI patients. The group of genera marked in green encompass butyrate-producing bacteria and their commensal microbes. Conversely, the genera highlighted in red include pathogenic bacteria and their associated commensal microbes. Except for *Bacteroidetes_unclassified* and *Burkholderiales_unclassified*, all genera belong to Firmicutes.

correlates positively with blood testosterone concentrations and is, therefore, more abundant in healthy men.[59] Therefore, for rCDI patients with *Roseburia* depletion, male donor microbiota may be more suitable from the perspective of supplementing *Roseburia*.

The effect of age on microbiota transplantation was mainly reflected in the levels of *Veillonella*; specifically, it was manifested in the lower abundance of *Veillonella* in elderly patients before and after treatment (Figure 6k). In recent years, the *Veillonella* population has been frequently reported to be gradually reduced with age.[59,60] Although the specific mechanism is unclear, it seems that the intestinal microenvironment of the elderly is not suitable for the colonization and growth of *Veillonella*. As we have mentioned earlier, intestinal colonization by *Veillonella* can inhibit *C. difficile* infection,[38,39] and depletion of *Veillonella* in the elderly may be related to the infections and recurrences of *C. difficile*, which are also consistent with previous reports that older adults are more susceptible to *C. difficile* infections.[61] Therefore, in the FMT treatment of elderly CDI patients, the transplant of *Veillonella* should not be ignored.

This study had several limitations. First, this was a single-center treatment intervention study. Multi-center parallel verification and long-time follow-ups would further validify the results from this study. Second, due to the need for baseline balance, there were only 7 non-responders in this study. Although most of the conclusions of this study have been verified in previous reports, cross-cohort analysis is still needed. Third, this study used 16S rRNA gene sequencing technology. Future investigations should incorporate targeted metabolomics detection and metagenomic sequencing, with particular emphasis on third-generation sequencing, to achieve species-level or even strains-level resolution of the microbiome. It is crucial to delve into the genetic alterations within *C. difficile*

itself, comparing the remission and non-remission groups, in order to establish a more precise understanding of the causal relationship between FMT technology and the cure of CDI. This approach will contribute to refining FMT techniques and advancing our comprehension of CDI treatment.

Conclusions

In this study, we retrospectively analyzed more than 200 microbiome data from 23 rCDI patients and 10 donors, and revealed the key microbiota factors of non-remission patients treated with FMT. From the perspective of the microbiota, patients with poor prognosis had a significantly reduced α-diversity index; butyrate-producing genera were easily depleted; and *Burkholderiales_unclassified*, *Coprococcus_2*, and *Oscillospira* could not colonize guts after treatment. Patients with good prognosis were characterized by a high relative abundance of *Veillonella* before treatment, relative to the generalized depletion in patients with poor prognosis. From the perspective of donors, genus interactions in lower-effectiveness FMT donors were more similar to those in patients with a poor prognosis. *Burkholderiales_unclassified*, *Coprococcus_2*, and *Oscillospira* were frequently depleted in these donors. From the perspective of the recipients' clinical factors, the intestines of older patients were not conducive to colonization by *Veillonella*, which may be related to their poor prognosis. These conclusions, which are summarized in Figure 7, should improve the application of FMT therapy in the field of rCDI treatment.

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Disclosure statement

Authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Authors' contribution

HT, XW, QC and HQ conceived the ideas and experimental design. NL was responsible for patient treatment and follow-up. HT, JC, BY and XL provided clinical samples. JC, CY, LW, CM, JZ, YX and SZ collected the baseline information of our cohort. XW and SJ analyzed the data. HT and XW wrote the manuscript. All authors read, reviewed this final version for publication. All authors read and approved the final manuscript.

Availability of data and material

The detail baseline information of our cohort is available in the *Supplementary information*. Raw sequencing data are available at the National Center for Biotechnology Information server under study accession number PRJNA940621.

Ethics approval and consent to participate

The study was approved by the Ethics Committee of the Shanghai Tenth People's Hospital. All patients provided written informed consent for this study. All methods in this study carried out in accordance with the *Declaration of Helsinki*. Permission to use the patient's samples was obtained from the Ethics Committee of the Shanghai Tenth People's Hospital. ClinicalTrials ID is NCT05703477.

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