Open Forum Infectious Diseases

MAJOR ARTICLE

Gut Microbial and Metabolic Features Associated With *Clostridioides difficile* Infection Recurrence in Children

Xiaolu Li,[1,a] Fangfei Xiao,[1,a] Xufei Wang,[1] Lin Ye,[1] Yongmei Xiao,[1] Dan Li,[1] Ting Zhang,[1,2] and Yizhong Wang[1,2]

[1]Department of Gastroenterology, Hepatology and Nutrition, Shanghai Children's Hospital, School of Medicine, Shanghai Jiao Tong University, Shanghai, China, and [2]Gut Microbiota and Metabolic Research Center, Institute of Pediatric Infection, Immunity and Critical Care Medicine, School of Medicine, Shanghai Jiao Tong University, Shanghai, China

Background. Recurrent *Clostridioides difficile* infection (CDI) is a critical clinical issue due to the increase in incidence and difficulty in treatment. We aimed to identify gut microbial and metabolic features associated with disease recurrence in a group of pediatric CDI patients.

Methods. A total of 84 children with primary CDI were prospectively enrolled in the study. Fecal samples collected at the initial diagnosis were subjected to 16S rRNA gene sequencing and targeted metabolomics analysis to profile the bacterial composition and metabolome.

Results. Twenty-six of 84 (31.0%) pediatric CDI patients experienced recurrence. The alpha diversity of the fecal microbiota was significantly lower in the recurrent group than in the nonrecurrent group, and the beta diversity was different from that of the nonrecurrent group. Taxonomic profiles revealed that the relative abundances of multiple bacterial taxa significantly differed between the recurrent and nonrecurrent groups. Linear discriminant analysis effect size analysis identified several bacterial genera that discriminated between recurrent and nonrecurrent groups, including *Parabacteroides*, *Coprococcus*, *Dialister*, and *Clostridium*. Recurrent bacteria presented lower abundances of several short-chain fatty acid (SCFA)–producing bacteria (*Faecalibacterium*, *Butyricicoccus*, *Clostridium*, *Roseburia*, and *Ruminococcus*), which were correlated with reduced fecal SCFA levels. In addition, several bile acids, including lithocholic acid (LCA), 12-ketoLCA, trihydroxycholestanoic acid, and deoxycholic acid, were decreased in recurrent patients.

Conclusions. Our study suggests that the differing gut microbiota profiles in pediatric CDI patients may contribute to disease recurrence by modulating SCFA concentrations and bile acid profiles. The gut microbiota and metabolite signatures may be used to predict disease recurrence in children with CDI.

Keywords. bile acids; gut microbiota; metabolites; recurrent *Clostridioides difficile* infection; short-chain fatty acids.

Clostridioides difficile is the most common opportunistic pathogen responsible for healthcare-associated infection in both adults and children [1]. The symptoms of *C difficile* infection (CDI) range from self-resolving diarrhea to life-threatening fulminant colitis and toxic megacolon [2]. The disease is attributed to the production of 2 major virulence factors, toxins A (TcdA/enterotoxin) and B (TcdB/cytotoxin), by toxigenic *C difficile* strains, which can bind to intestinal epithelia to induce cell death and severe inflammation [3]. Due to increasing morbidity and mortality, CDI is a serious substantial health and economic burden on the healthcare system worldwide [4–6].

The development of CDI is associated with increased susceptibility to *C difficile* caused by alterations in the gut microbiota composition and function [7, 8]. Previous studies have revealed that either asymptomatic colonization or infection with *C difficile* in the human gut is associated with a loss of gut microbiota diversity [7, 8]. In CDI patients, the relative abundances of taxa of the phyla Bacteroidetes and Firmicutes generally decrease, whereas those of species belonging to the phylum Proteobacteria increase [8]. Specific changes in gut microbial populations increase susceptibility to *C difficile* colonization [7]. Therefore, factors resulting in gut microbiota disruption, such as advanced age, hospitalization, antibiotic exposure, proton pump inhibitor (PPI) use, immunosuppression, and comorbidities (inflammatory bowel diseases, etc), are recognized as risk factors for CDI [6, 7].

Current clinical practice guidelines recommend vancomycin as the first-line option for pediatric CDI and fidaxomicin for

Received 06 April 2024; editorial decision 29 August 2024; accepted 02 September 2024; published online 3 September 2024

[a]X. L. and F. X. contributed equally to this work.

Correspondence: Yizhong Wang, PhD, Department of Gastroenterology, Hepatology and Nutrition, Shanghai Children's Hospital, School of Medicine, Shanghai Jiao Tong University, 355 Luding Road, Shanghai 200062 China (wangyz@shchildren.com.cn); Ting Zhang, MD, PhD, Department of Gastroenterology, Hepatology, and Nutrition, Shanghai Children's Hospital, School of Medicine, Shanghai Jiao Tong University, 355 Luding Road, Shanghai 200062, China (zhangt@shchildren.com.cn).

Open Forum Infectious Diseases®

https://doi.org/10.1093/ofid/ofae506

adult CDI [2, 9]. However, conventional antibiotic treatment is limited by a high risk of recurrence, which is defined as an episode of symptom onset and positive assay result following an episode with positive assay result in the previous 2–8 weeks [2]. Recurrent CDI (rCDI) occurs in 15%–30% of patients who respond to first-round antibiotic treatment, and the subsequent recurrence rate can reach 45%–65% after the first and second recurrences [10]. Our previous study revealed that rCDI occurred in 48.53% (33/68) and 46.33% (19/41) of pediatric CDI patients treated with metronidazole and vancomycin, respectively [11]. Fecal microbiota transplantation (FMT) has been used to manage rCDI, with high cure rates in both adults and children [12–15].

To further explore the roles of the gut microbiota in primary and rCDI, several studies have compared the differences in the gut microbiota composition at the initial CDI diagnosis between individuals with and without recurrence. Khanna et al reported that, compared with patients without recurrence, patients with rCDI presented significant increases in *Veillonella*, Enterobacteriaceae, streptococci, *Parabacteroides*, and Lachnospiraceae before treatment [16]. Pakpour et al identified *Veillonella dispar* as a candidate organism for reducing risk of CDI recurrence [17]. Seekatz et al reported a less diverse microbial community in patients with rCDI [18]. Furthermore, a recent study conducted by Dawkins et al revealed that gut metabolites can predict disease recurrence at 1 week and 2 weeks posttreatment for primary CDI [19]. Taken together, these studies indicate that changes in the gut microbiota composition and metabolites are involved in rCDI and that specific microbiota-derived characteristics may be used to predict disease recurrence in adults. However, studies investigating the roles of the gut microbiota and metabolites in predicting rCDI in pediatric patients are still limited.

In this study, we aimed to investigate the characteristics of the fecal microbiota and metabolites in a group of pediatric patients at the initial diagnosis of CDI and to explore their relationships with disease recurrence.

MATERIALS AND METHODS

Study Design

A total of 84 pediatric patients with CDI were prospectively recruited to the study cohort from the Department of Gastroenterology, Hepatology and Nutrition of Shanghai Children's Hospital between September 2019 and August 2022. The inclusion criteria were children aged <16 years and diagnosed with primary (no episodes of CDI within the past 6 months), uncomplicated CDI by a positive *C difficile* test with either enzyme immunoassay toxin or polymerase chain reaction (PCR) and who were being treated for clinical symptoms. Patients with comorbidities of other gastrointestinal diseases, inherited or acquired immunodeficiencies, hepatic or renal dysfunction, hematologic malignancy, allergy, or fulminant colitis were excluded. Participant demographic and clinical data were extracted from the electronic medical records and systematically analyzed. Stool samples were collected at the time of initial diagnosis before antibiotic treatment for CDI. All the stool samples were stored at −80°C until bacterial 16S ribosomal RNA (rRNA) gene sequencing and fecal metabolite quantification were performed.

This study was conducted in accordance with the Declaration of Helsinki (as revised in 2013) and was approved by the Ethical Review Board of the Shanghai Children's Hospital (2022R043-E01). Written informed consent was obtained from the parents or legal guardians of all the participants.

DNA Extraction and 16S rRNA Sequencing

Total fecal DNA was extracted from 250 μL of each fecal sample using a QIAamp DNA Stool Mini Kit (Qiagen, Germany) following the manufacturer's protocol. The eluted DNA was quantified using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific) and agarose gel electrophoresis. The isolated DNA was used for bacterial 16S rRNA V3–V4 hypervariable region amplification using the primer set 341F 5′-CCTACGGGAGGCAGCAG-3′/806R 5′-GGACTACHVG GGTWTCTAAT-3′ as previously described [20]. The purified PCR amplicons were subjected to barcoded library construction using the TruSeq Nano DNA LT Library Prep Kit (Illumina) according to the manufacturer's instructions. Library quality was assessed and quantified on an Agilent 2100 Bioanalyzer (Agilent Technologies). Each library was diluted to a final concentration of 12.5 nM and sequenced on an Illumina NovaSeq platform (Illumina).

Sequencing Data Processing and Analysis

The raw sequence data were checked using FastQC (Babraham Bioinformatics), and forward and reverse reads were joined and quality trimmed using *fastq_maxee* 1.0. Paired-end reads were de-noised using DADA2 software and further analyzed using the QIIME2 platform (v.2020.2) [21, 22]. The taxonomic assignment of amplicon sequence variants (ASVs) was classified using the Greengenes (v.13.8) reference database implemented in QIIME2 (v.2020.2). To reduce the effect of spurious sequences, ASVs with <0.005% of the total number of sequences were removed. The alpha diversity was evaluated using the observed species, abundance-based coverage estimator (ACE), and Chao1, Shannon, and Simpson indices. The beta diversity was calculated using principal coordinate analysis (PCoA) of the Bray-Curtis distance based on the ASV abundance. The significant differences in alpha diversity and ASV abundances between groups were analyzed using the Wilcoxon rank-sum test, with P values adjusted for the false discovery rate. Permutational multivariate analysis of variance was used to estimate the significant differences in the gut microbiota

structure. For the relative abundance ratio of bacteria at the phylum, class, order, and family levels, rare taxa of ASVs present with <10 counts and in <10% of the fecal samples were removed prior to differential abundance analysis. The linear discriminant analysis (LDA) effect size (LEfSe) [23] method was used to assess the significance and biological relevance of the enriched taxa in different groups.

Quantification of Fecal Metabolites

Targeted metabolomic profiling was performed to quantify fecal metabolites as previously described [24]. In brief, 5 mg of each lyophilized fecal sample was dissolved in 25 μL of sterile water and homogenized with zirconium oxide beads for 3 minutes, after which 120 μL of methanol containing internal standards was added. After homogenization for 3 minutes, the sample was centrifuged at 18 000*g* at 4°C for 20 minutes. A 20-μL supernatant aliquot of each sample was transferred to a 96-well plate, and 20 μL of freshly prepared derivative reagents was added to each well. After 60 minutes of incubation at 30°C for derivatization, the sample was diluted with 330 μL of ice-cold 50% methanol solution. The plate was then incubated at −20°C for 20 minutes and subsequently centrifuged at 4000*g* at 4°C for 30 minutes. A volume of 135 μL of supernatant per sample was transferred to another 96-well plate with 10 μL of internal standards in each well. A 5-μL aliquot of each sample was injected to quantify fecal metabolites using ultraperformance liquid chromatography coupled with a tandem mass spectrometry (UPLC-MS/MS) system (ACQUITY UPLC-Xevo TQ-S) at a flow rate of 0.4 mL/minute.

The UPLC-MS/MS raw data were processed using TMBQ software (Metabo-Profile, China) to perform peak integration, calibration, and quantitation for each metabolite. iMAP (Metabo-Profile, China) was used for statistical analysis and feature selection of the metabolomic data, including principal component analysis and partial least squares discriminant analysis (PLS-DA). PLS-DA was used to analyze the variable importance in projection (VIP) values of each metabolite between groups. The Wilcoxon rank-sum test with *P* values adjusted with the Bonferroni correction procedure was performed for univariate statistical analysis. Differentially abundant metabolites with VIP scores of PLS-DA >1, $P < .05$, and $\log_2$ (fold change) >1 were selected. Volcano plots and heatmaps were generated via R software.

Statistical Analyses

The demographic and clinical data are presented as numbers (percentages) for dichotomous variables and medians with interquartile ranges (IQRs [25th to 75th percentiles]) for continuous variables. The χ^2 test and the Mann-Whitney *U* test were used for statistical analysis of categorical and continuous variables, respectively. Statistical analysis was performed using SPSS software (version 25.0), and a *P* value of <.05 was considered statistically significant.

RESULTS

Participant Characteristics

The median age of the 84 enrolled pediatric patients with primary CDI was 4.5 years (IQR, 2.4–7.8 years), and 49 (58.3%) were boys (Supplementary Table 1). A history of exposure to antibiotics and PPI use occurred in 64 (76.2%) and 21 (25.0%) patients, respectively. The main clinical symptoms were diarrhea (60/84 [17.4%]), abdominal pain (42/84 [50.0%]), and hematochezia (35/84 [41.7%]). For initial antibiotic treatment, 46 (54.8%) patients received metronidazole, and 38 (45.2%) patients received vancomycin (Supplementary Table 1). As shown in Table 1, 58 (69.0%) patients were cured by 1 round of conventional antibiotic treatment, whereas 26 (31.0%) patients experienced disease recurrence. The ratios of female sex and PPI exposure were greater in recurrent patients than in nonrecurrent patients. There were no significant differences in clinical symptoms between recurrent and nonrecurrent patients. In addition, the disease recurrence rate was comparable in patients treated with either metronidazole or vancomycin.

Gut Microbiota Analysis

The average number of raw reads per sample generated by sequencing was 95 720, ranging from 83 354 to 111 770. A total of 6 534 142 high-quality bacterial 16S rRNA gene sequences spanning 2280 ASVs were obtained after sequence processing and filtering. Alpha diversity analysis revealed that the richness calculated for the observed species ($P < .001$), ACE ($P < .01$), and

Table 1. Demographic and Clinical Data of Participants With *Clostridioides difficile* Infection

Characteristic	Nonrecurrers (n = 58)	Recurrers (n = 26)	*P* Value
Sex, male, No. (%)	38.0 (65.5)	11.0 (42.3)	.05
Age, y, median (IQR)	4.7 (2.5–7.5)	4.0 (2.0–7.7)	.58
BMI, kg/m^2, median (IQR)	14.8 (13.1–16.8)	14.3 (13.1–16.4)	.77
Exposure history, No. (%)			
Antibiotics	44.0 (75.9)	20.0 (76.9)	.91
PPI	10.0 (17.2)	11.0 (42.3)	.02
Symptoms, No. (%)			
Fever	11.0 (20.0)	3.0 (11.5)	.40
Vomiting	11.0 (20.0)	8.0 (30.8)	.24
Diarrhea	39.0 (67.2)	21.0 (80.8)	.21
Abdominal pain	28.0 (48.3)	14.0 (53.8)	.64
Hematochezia	21.0 (36.2)	14.0 (53.8)	.13
Pseudomembrane	9.0 (15.5)	7.0 (26.9)	.22
Initial treatment antibiotic used, No. (%)			
Metronidazole	35.0 (60.3)	11.0 (42.3)	.23
Vancomycin	23.0 (39.7)	15.0 (57.7)	.23

Abbreviations: BMI, body mass index; IQR, interquartile range; PPI, proton pump inhibitor.

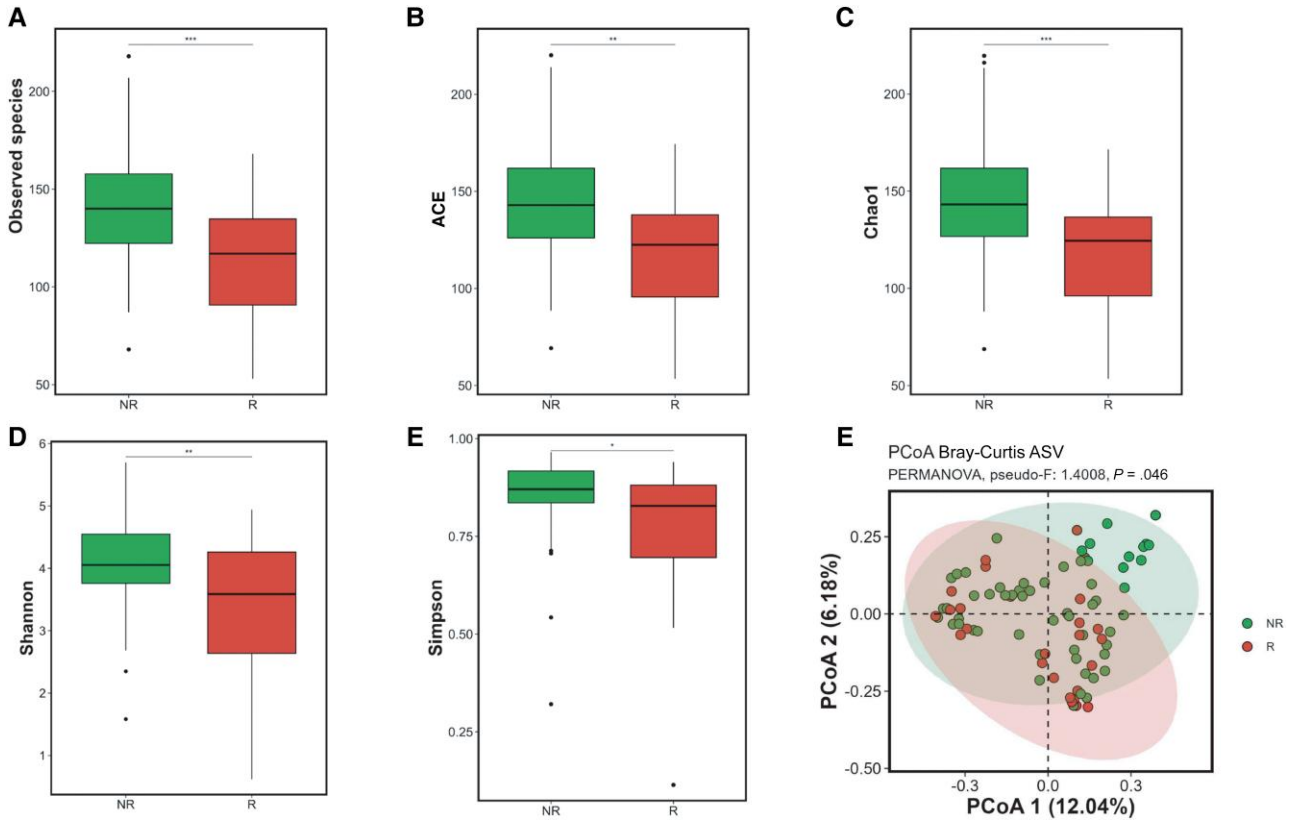

Figure 1. Fecal microbiota biodiversity of children with *Clostridioides difficile* infection (CDI). *A–E,* Alpha diversity measured in the observed species, abundance-based coverage estimator (ACE), Chao1, Shannon, and Simpson indices. Significance was determined using the Wilcoxon rank-sum test, with *$P < .05$, **$P < .01$, and ***$P < .001$. *F,* Beta diversity analyzed by principal coordinate analysis (PCoA) of the Bray-Curtis distance on amplicon sequence variants (ASVs). The permutational multivariate analysis of variance (PERMANOVA) method was used to compare the differences between groups. NR indicates nonrecurrent; R indicates recurrent.

Chao1 indices ($P < .001$) and the Shannon ($P < .01$) and Simpson indices ($P < .05$) were significantly lower in the recurrent samples than in the nonrecurrent samples (Figure 1*A*–*E*). PCoA of beta diversity using the Bray-Curtis distance revealed that the microbial community of the recurrent group was separated from that of the nonrecurrent group (Figure 1*F*). The bacterial microbiota was dominated by the phyla Firmicutes, Proteobacteria, Actinobacteria, Bacteroidetes, and Verrucomicrobia in both groups (Figure 2*A*).

Taxonomic profiles revealed the relative abundances of the phyla, class, order, and family between the 2 groups. Phyla Firmicutes, class Clostridia, order Clostridiales, and family Lachnospiraceae were mostly decreased, whereas the abundances of the phyla Proteobacteria, class Gammaproteobacteria, order Enterobacterales, and family Enterobacteriaceae were mostly increased in the recurrent group compared with those in the nonrecurrent group (Figure 2*A*–*D*). Intergroup comparisons revealed significantly lower relative abundances of the genera *Blautia*, *Clostridium*, *Coprococcus*, *Dorea*, *Eggerthella*, *Eubacterium*, and *Subdoligranulum* and significantly greater abundances of the genus *Ethanoligenens* and unidentified Synergistaceae in the recurrent versus nonrecurrent group (Figure 2*E*, Supplementary Table 2).

LEfSe analysis identified multiple taxa at different levels that were differentially abundant between recurrent and nonrecurrent patients. The class Clostridia, order Clostridiales; family Coriobacteriaceae; and genera *Adlercreutzia*, *Eggerthella*, *Parabacteroides*, *Blautia*, and *Clostridium* were enriched in nonrecurrers, whereas the classes Chthonomonadetes and Bacilli, order Bacillales, family Helicobacteraceae, and genera *Ethanoligenens* and *Helicobacter* were enriched in recurrers (Supplementary Figure 1*A*). Specifically, the classes Bacilli, Clostridia, and Erysipelotrichi; orders Clostridiales, Lactobacillales, and Erysipelotrichales; families Prophyromonadaceae, Erysipelotrichaceae, Rumincoccaceae, and Lachnospiraceae; and genera *Parabacteroides*, *Coprococcus*, *Dialister*, *Clostridium*, *Faecalibacterium*, *Blautia*, *Eubacterium*, and *Ruminococcus* were differentially abundant between the 2 groups, with LDA scores >3.0 (Supplementary Figure 1*B*).

Metabolic Profiling

A total of 250 metabolites were detected and quantified by targeted metabolomics analysis in all the fecal samples. Amino acids,

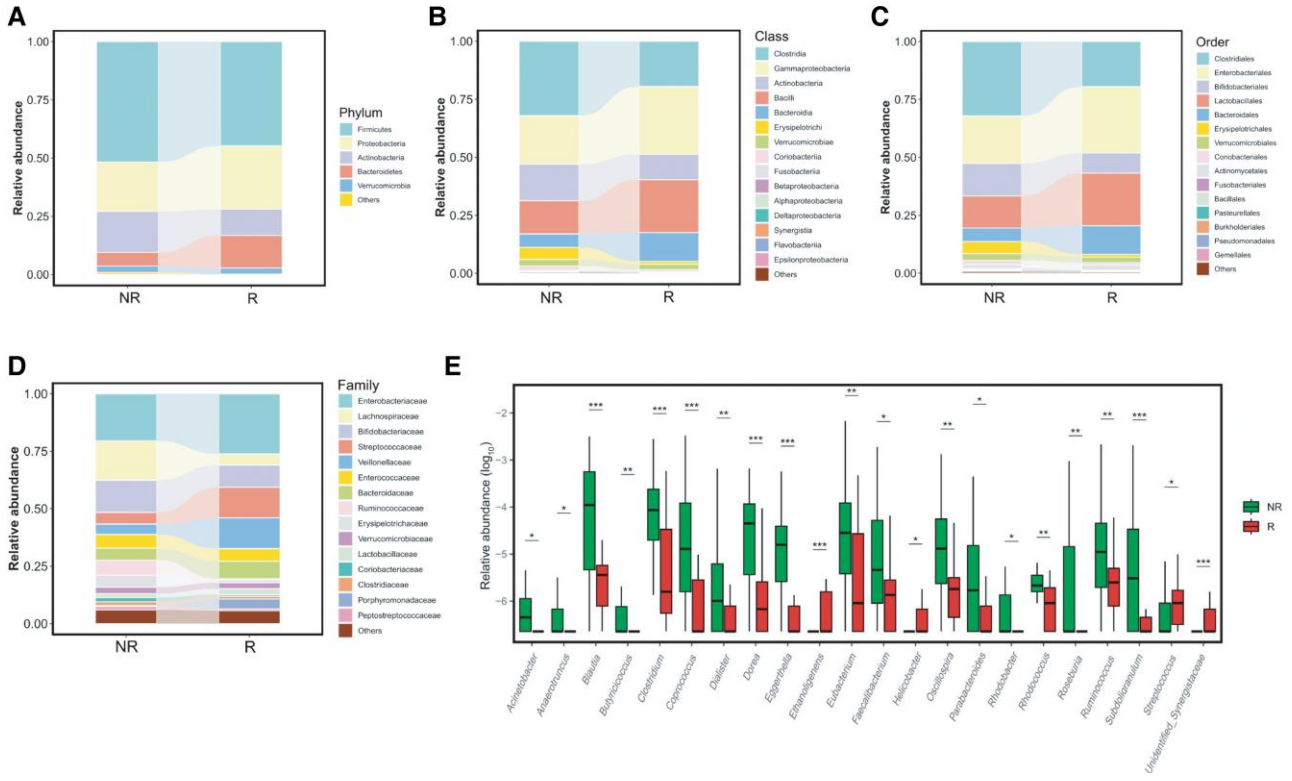

Figure 2. Fecal microbiota composition of children with *Clostridioides difficile* infection (CDI). *A–D*, Relative abundance ratios of bacteria at the phylum, class, order, and family levels between the nonrecurrent (NR) and recurrent (R) groups. The top 5 abundant phyla and the top 15 abundant classes, orders, and families are indicated. *E*, Genera with significant differences between the NR and R groups. The relative abundances are shown in $\log_{10}$ values. Statistical significance was assessed via the Wilcoxon rank-sum test, with $^{*}P < .05$, $^{**}P < .01$, and $^{***}P < .001$.

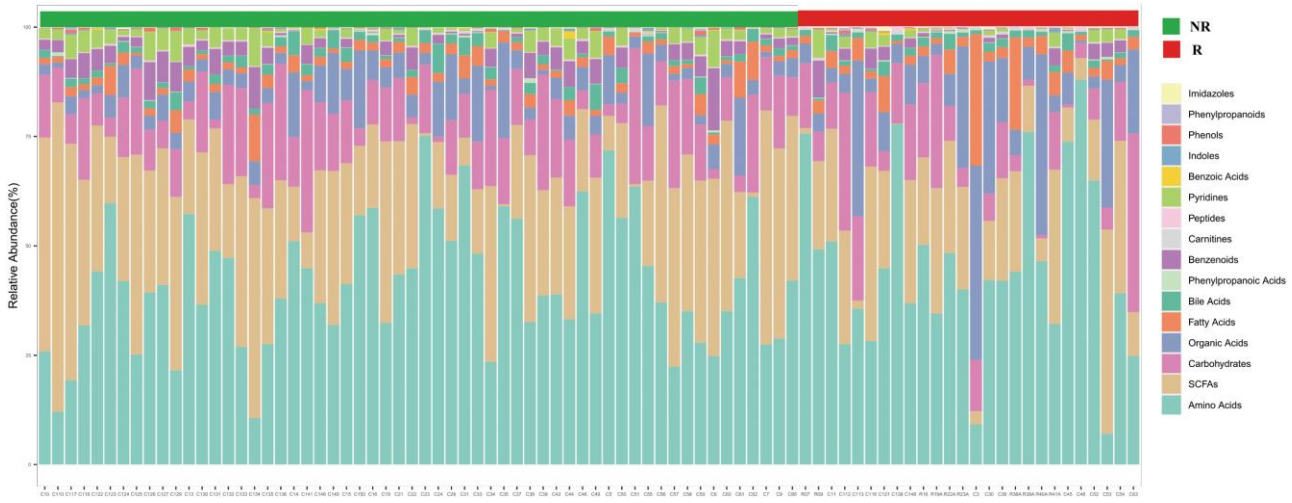

Figure 3. Relative abundance of each metabolite class in fecal samples from patients with nonrecurrent and recurrent *Clostridioides difficile* infection. Abbreviations: NR, nonrecurrent; R, recurrent; SCFA, short-chain fatty acid.

short-chain fatty acids (SCFAs), carbohydrates, bile acids (BAs), and organic acids were the dominant fecal metabolites in both groups (Figure 3). Different distribution patterns of metabolites between recurrent and nonrecurrent patients were observed using both PCoA (Figure 4*A*) and PLS-DA (Figure 4*B*). Among the 19 metabolites that were the most discriminative (VIP score >1.5) for classification between the 2 groups, the metabolites of the recurrent group presented higher

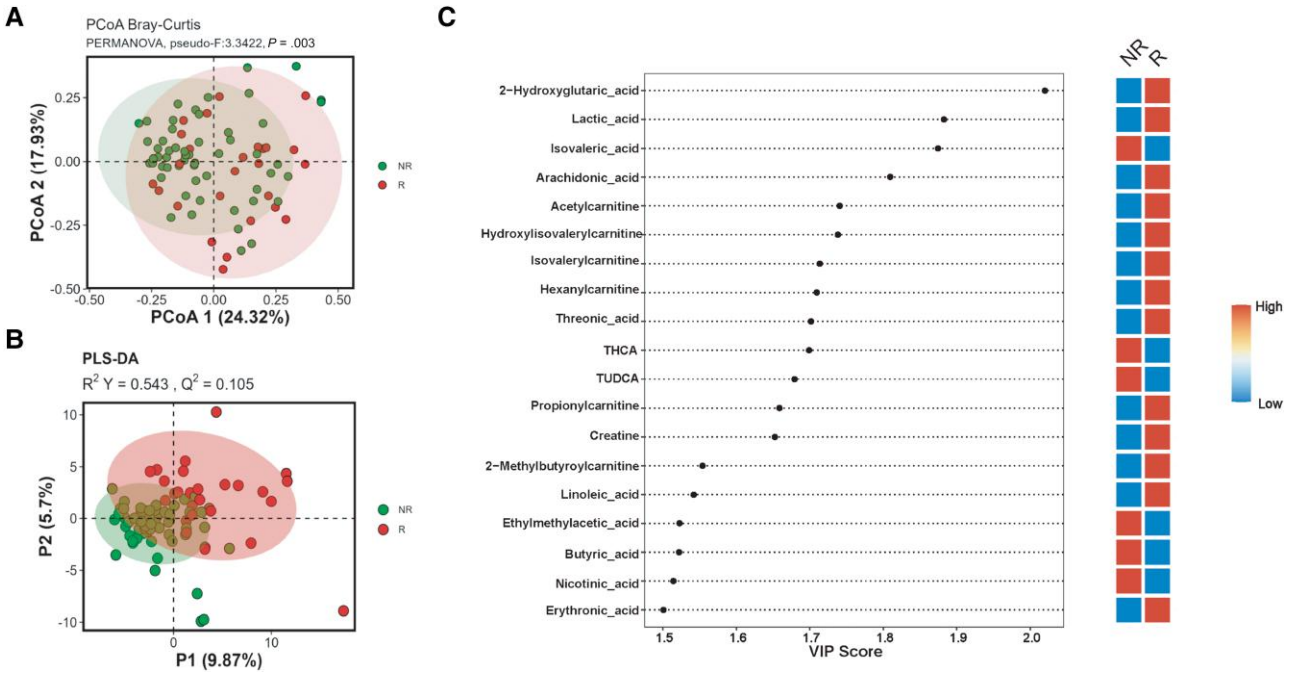

Figure 4. Fecal metabolome of children with *Clostridioides difficile* infection (CDI). *A*, Principal coordinate analysis plot of fecal metabolites of patients with nonrecurrent (NR) and recurrent (R) CDI according to the Bray-Curtis distance. The permutational multivariate analysis of variance method was used to compare the differences between groups. *B*, Partial least squares discriminant analysis models discriminated fecal metabolites between the NR and R groups. *C*, The top metabolites ranked by variable importance in projection scores. Abbreviations: NR, nonrecurrent; PCoA, principal coordinate analysis; PERMANOVA, permutational multivariate analysis of variance; PLS-DA, partial least squares discriminant analysis; R, recurrent; THCA, thiophenecarboxylic acid; TUDCA, tauroursodeoxycholic acid; VIP, variable importance in projection.

fecal levels of 2-hydroxyglutaric acid, lactic acid, arachidonic acid, acetylcarnitine, hydroxylisovalerylcarnitine, isovalerylcarnitine, hexanylcarnitine, threonic acid, propionylcarnitine, creatine, 2-methylbutyroylcarnitine, linoleic acid, and erythronic acid, and lower levels of isovaleric acid, thiophenecarboxylic acid, tauroursodeoxycholic acid, ethylmethylacetic acid, butyric acid, and nicotinic acid (Figure 4*C*).

As shown in Figure 4 and Supplementary Table 3, the concentrations of 35 unique fecal metabolites differed significantly between the recurrent and nonrecurrent samples. Among them, the concentrations of lithocholic acid (LCA), 12-ketoLCA, trihydroxycholestanoic acid, butyric acid, deoxycholic acid (DCA), ethylmethylacetic acid, glutaric acid, isobutyric acid, isoLCA, isovaleric acid, methylsuccinic acid, *N*-methylnicotinamide, nicotinic acid, phenylacetic acid, pipecolic acid, and valeric acid decreased, whereas the concentrations of 2-hydroxyglutaric acid, 2-methylbutyroylcarnitine, 3-hydroxylisovalerylcarnitine, acetylcarnitine, α-linolenic acid, arachidonic acid, butyrylcarnitine, carnitine, creatine, docosahexaenoic acid, eicosapentaenoic acid, erythronic acid, γ-linolenic acid, hexanylcarnitine, isovalerylcarnitine, lactic acid, linoleic acid, propionylcarnitine, and threonic acid increased significantly in patients with recurrence compared with nonrecurrence (Supplementary Figure 2).

Covariance Between Gut Bacteria and Metabolites

Associations between the differential gut bacteria and metabolites in fecal samples were coupled by Spearman rank correlations. Strong correlations between 22 bacterial genera and 35 metabolites were observed in nonrecurrent and recurrent samples (Figure 5). A total of 19 metabolites that increased in the recurrent group were positively correlated with the enriched genera in the recurrent group and negatively correlated with the genera that were enriched in the nonrecurrent group. For example, the decreased levels of butyric acid, isobutyric acid, isovaleric acid, and valeric acid in the recurrent group were associated with decreased abundances of SCFA producers, such as *Faecalibacterium*, *Clostridium*, *Butyricicoccus*, *Roseburia*, and *Ruminococcus*. Conversely, the enriched SCFA producers were positively correlated with high concentrations of fecal SCFAs in nonrecurrent patients. Furthermore, the genera enriched in the recurrent group, including *Helicobacter*, *Ethanoligenens*, and *Streptococcus*, were negatively correlated with the levels of isoLCA, 12-ketoLCA, and DCA.

DISCUSSION

Owing to the increase in community-acquired infections and the emergence of hypervirulent *C difficile* strains, the incidence and severity of pediatric CDI have increased in recent decades

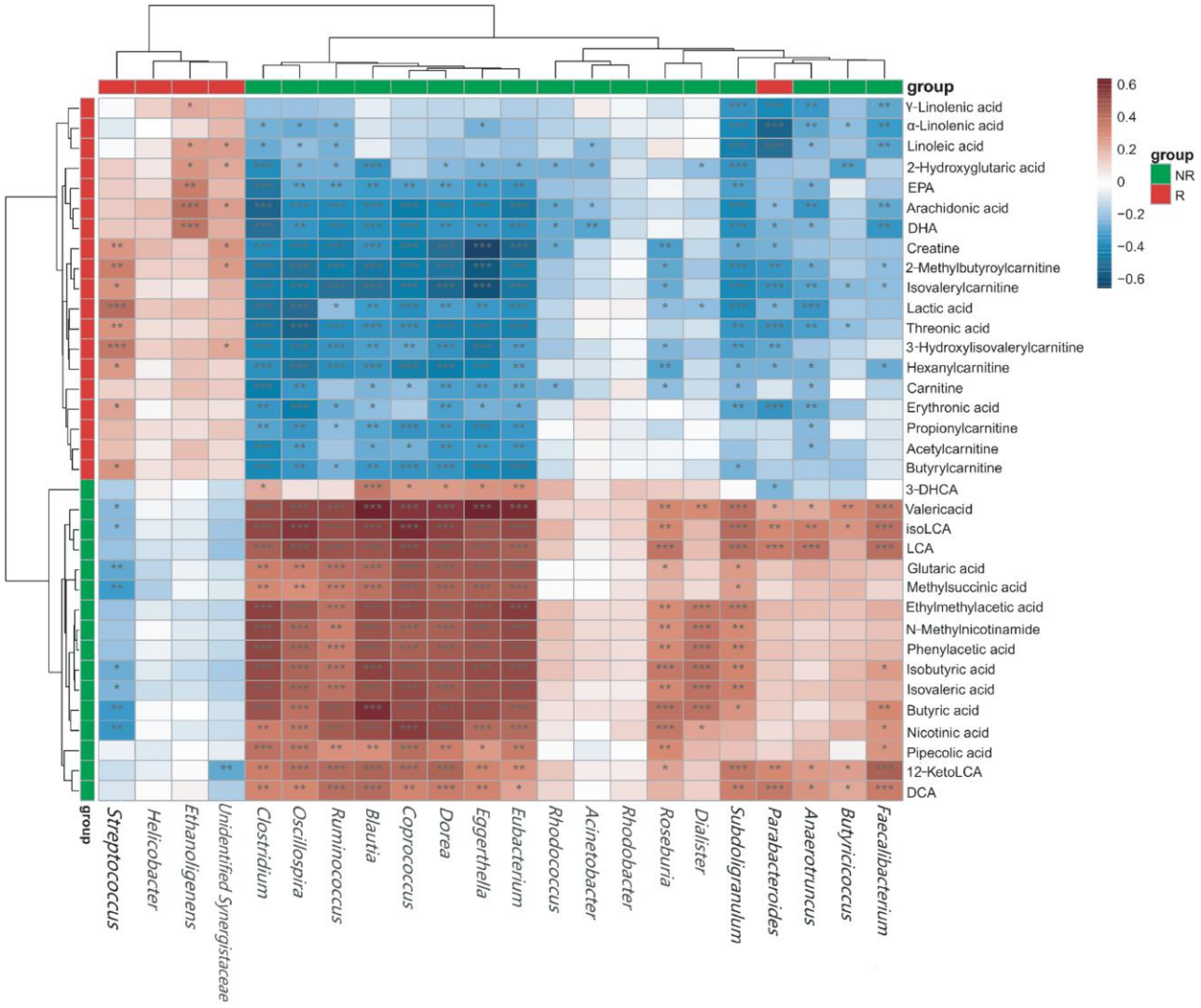

Figure 5. Heatmap of the correlation matrix of different fecal bacterial genera and metabolites between patients with nonrecurrent and recurrent *Clostridioides difficile* infection. $^{*}P < .05$, $^{**}P < .01$, $^{***}P < .001$. Abbreviations: 3-DHCA, trihydroxycholestanoic acid; DCA, deoxycholic acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; LCA, lithocholic acid; NR, nonrecurrent; R, recurrent.

[2, 25–27]. Although conventional antibiotic-based treatments are generally effective for managing pediatric CDI, a high risk of disease recurrence is challenging [2]. The treatment of rCDI is more difficult than the initial episode because of the significantly increased rate of further recurrence [10]. Identification of risk factors related to rCDI is vital for early detection, treatment, and prevention of disease recurrence. Early alternative therapies, such as FMT, could be used to treat CDI patients who are likely to experience recurrence.

To our knowledge, our study represents the first prospective study focused on the role of the gut microbiota in the occurrence of rCDI in pediatric patients, employing both fecal microbiome sequencing and targeted metabolomics analyses. With respect to the results of the fecal microbiome analysis, we reported a significantly lower alpha diversity of the fecal microbiota in pediatric CDI patients than in nonrecurrent patients prior to antibiotic treatment, which was consistent with the findings of previous studies conducted in adult CDI patients [16–19]. The gut microbiota signatures at the initial diagnosis were characterized by increasing abundances of taxa belonging to the phyla Proteobacteria and Bacteroidetes but decreasing abundances of species of the phyla Firmicutes and Actinobacteria. Specifically, recurrent patients had significantly greater abundances of Enterobacteriaceae, Streptococcaceae, Veillonellaceae, Porphyromonadaceae, Prevotellaceae, *Shigella*, *Streptococcus*, *Veillonella*, *Prevotella*, and *Dysgonomonas* than nonrecurrent patients. Similarly, Enterobacteriaceae, *Veillonella*, and streptococci abundances were significantly increased in pre-antibiotic-treated fecal samples from adult CDI patients with recurrence [16]. The persistent expansion of Enterobacteriaceae may increase

susceptibility to *C difficile* colonization [28]. However, Pakpour et al reported that *Veillonella dispar* is a candidate organism for preventing CDI recurrence, which was contrary to our study [17]. A study in 2023 indicated that *Fusobacterium* increased during rCDI, whereas *Collinsella*, *Senegalimassilia*, *Prevotella*, and *Ruminococcus* decreased, findings that were partly similar to our data [29]. We found that the genus *Parabacteroides* was differentially abundant between the 2 groups, with LDA scores >3.0, which means that it may be a key microbiota for preventing rCDI. *Parabacteroides distasonis*, which can produce succinate, has been reported to be associated with CDI. Many studies have shown that *Parabacteroides distasonis* is inversely related to rheumatoid arthritis, obesity, and diabetes mellitus [30, 31]. It may also play an important role in *C difficile* expansion [16, 32]. In addition, the decreased abundances of Lachnospiraceae, *Blautia*, and *Faecalibacterium* reported in adults with recurrent CDI were also decreased in our pediatric patients [19]. Future studies are needed to investigate the mechanisms of these bacteria that may be involved in rCDI after successful antibiotic treatment.

Gut-associated metabolites are key mediators of microbiota–host interactions. A variety of host- and microbial-derived metabolites play important roles in *C difficile* pathogenesis, including BAs, SCFAs, organic acids, and amino acids [33, 34]. Certain BAs have been shown to either promote or inhibit *C difficile* germination or affect *C difficile* growth and colonization [33, 34]. For example, both taurocholic acid and cholic acid initiate *C difficile* spore germination in the small intestine, whereas chenodeoxycholic acid suppresses the germination of *C difficile* spores [35–37]. IsoalloLCA inhibited *C difficile* growth both in vitro and in vivo [34]. DCA is known to decrease the sporulation efficiency of *C difficile* by downregulating the expression of the master regulator of sporulation, Spo0A [35, 37]. DCA can inhibit *C difficile* growth and repress toxin activity [38]. SCFAs and organic acids are small molecules derived from the bacterial fermentation of dietary carbohydrates. SCFAs of acetate and propionate had inhibitory effects on toxin production and the growth of *C difficile* [39]. Furthermore, several organic acids and amino acids have been reported to be involved in CDI, such as succinate and sialic acid, which promote *C difficile* expansion [40, 41], whereas glycine, proline, and cysteine inhibit the toxin yield of *C difficile* [42–44]. Our data revealed that the fecal levels of several BAs (12-ketoLCA, isoLCA, LCA, and DCA), SCFAs (butyric acid, glutaric acid, isobutyric acid, isovaleric acid, and valeric acid), and organic acids (methylsuccinic acid, nicotinic acid, phenylacetic acid, and amino acids [pipecolic acid]) were significantly decreased, suggesting that these metabolites may be involved in the disease recurrence of pediatric CDI. Nevertheless, future studies are warranted to further investigate the mechanisms of interactions between gut-associated metabolites and *C difficile* pathogenesis.

There are several limitations to our study. First, this was a single-center study with a limited number of enrolled participants. Studies with large populations of pediatric CDI patients are needed. Second, only fecal samples before treatment were collected for gut microbiota and metabolite analysis. Longitudinal studies dedicated to the dynamics of the microbiome and metabolome are needed to advance our understanding of the interactions between the gut microbiota/metabolites and *C difficile* pathogenesis. Third, we only evaluated bacterial composition changes using 16S rRNA sequencing; other microbiota components, such as viruses and fungi, need to be investigated in future studies. Finally, the mechanisms of the potential gut microbiota and metabolite changes involved in CDI recurrence are underexplored in the current study.

In summary, our data indicate that the disturbed gut microbiota in pediatric CDI patients may contribute to disease recurrence by modulating microbial metabolism characterized by altered BA profiles and reduced SCFA concentrations. The gut microbiota associated with BA and SCFA signatures may be used to predict disease recurrence in pediatric CDI patients. In the future, more studies on the microbiota and BA and SCFA in children with CDI should be conducted to determine their beneficial effects on the development of methods for diagnosing and treating CDI recurrence.

Supplementary Data

Supplementary materials are available at *Open Forum Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

Notes

Author contributions. Y. W. and T. Z. conceived the study. X. L. and F. X. performed the experiment. Y. W., X. L., and F. X. analyzed the data and created figures. L. Y., X. W., and Y. X. contributed to data curation, formal analysis, and software. D. L. contributed to stool sample collection. Y. W. and X. L. interpreted the data and wrote the manuscript. T. Z. edited the manuscript. All authors read and approved the final manuscript.

Data availability. Raw sequencing data have been deposited in the National Center for Biotechnology Information Sequence Read Archive database under the BioProject ID PRJNA1020794 and BioSamples ID SUB13861569.

Financial support. This work was supported by the National Natural Science Foundation of China (grant numbers 32370951 and 81900472); the Natural Science Foundation of Shanghai (grant number 22ZR1451800); the Shanghai Municipal Health Commission (grant number 20214Y0349); the training program for young talents of Shanghai Children's Hospital (grant number 2021YQ03); and 2023 Xinyi Digestive Disease Research Foundation, School of Digestive Science, Shanghai Jiao Tong University School of Medicine (grant number KY-2023-08-01).

Potential conflicts of interest. All authors: No potential conflicts of interest.

References

1. Rupnik M, Wilcox MH, Gerding DN. *Clostridium difficile* infection: new developments in epidemiology and pathogenesis. Nat Rev Microbiol **2009**; 7:526–36.
2. McDonald LC, Gerding DN, Johnson S, et al. Clinical practice guidelines for *Clostridium difficile* infection in adults and children: 2017 update by the Infectious Diseases Society of America (IDSA) and Society for Healthcare Epidemiology of America (SHEA). Clin Infect Dis **2018**; 66:e1–48.

3. Buddle JE, Fagan RP. Pathogenicity and virulence of *Clostridioides difficile*. Virulence **2023**; 14:2150452.
4. De Roo AC, Regenbogen SE. *Clostridium difficile* infection: an epidemiology update. Clin Colon Rectal Surg **2020**; 33:49–57.
5. Balsells E, Shi T, Leese C, et al. Global burden of *Clostridium difficile* infections: a systematic review and meta-analysis. J Glob Health **2019**; 9:010407.
6. Kelly CR, Fischer M, Allegretti JR, et al. ACG clinical guidelines: prevention, diagnosis, and treatment of *Clostridioides difficile* infections. Am J Gastroenterol **2021**; 116:1124–47.
7. Samarkos M, Mastrogianni E, Kampouropoulou O. The role of gut microbiota in *Clostridium difficile* infection. Eur J Intern Med **2018**; 50:28–32.
8. Gonzales-Luna AJ, Carlson TJ, Garey KW. Gut microbiota changes associated with *Clostridioides difficile* infection and its various treatment strategies. Gut Microbes **2023**; 15:2223345.
9. Johnson S, Lavergne V, Skinner AM, et al. Clinical practice guideline by the Infectious Diseases Society of America (IDSA) and Society for Healthcare Epidemiology of America (SHEA): 2021 focused update guidelines on management of *Clostridioides difficile* infection in adults. Clin Infect Dis **2021**; 73: e1029–44.
10. Song JH, Kim YS. Recurrent *Clostridium difficile* infection: risk factors, treatment, and prevention. Gut Liver **2019**; 13:16–24.
11. Li X, Xiao F, Li Y, et al. Characteristics and management of children with *Clostridioides difficile* infection at a tertiary pediatric hospital in China. Braz J Infect Dis **2022**; 26:102380.
12. Cammarota G, Ianiro G, Tilg H, et al. European consensus conference on faecal microbiota transplantation in clinical practice. Gut **2017**; 66:569–80.
13. Jorgensen SMD, Hansen MM, Erikstrup C, Dahlerup JF, Hvas CL. Faecal microbiota transplantation: establishment of a clinical application framework. Eur J Gastroenterol Hepatol **2017**; 29:e36–45.
14. Drekonja D, Reich J, Gezahegn S, et al. Fecal microbiota transplantation for *Clostridium difficile* infection: a systematic review. Ann Intern Med **2015**; 162: 630–8.
15. Li X, Gao X, Hu H, et al. Clinical efficacy and microbiome changes following fecal microbiota transplantation in children with recurrent *Clostridium difficile* infection. Front Microbiol **2018**; 9:2622.
16. Khanna S, Montassier E, Schmidt B, et al. Gut microbiome predictors of treatment response and recurrence in primary *Clostridium difficile* infection. Aliment Pharmacol Ther **2016**; 44:715–27.
17. Pakpour S, Bhanvadia A, Zhu R, et al. Identifying predictive features of *Clostridium difficile* infection recurrence before, during, and after primary antibiotic treatment. Microbiome **2017**; 5:148.
18. Seekatz AM, Rao K, Santhosh K, Young VB. Dynamics of the fecal microbiome in patients with recurrent and nonrecurrent *Clostridium difficile* infection. Genome Med **2016**; 8:47.
19. Dawkins JJ, Allegretti JR, Gibson TE, et al. Gut metabolites predict *Clostridioides difficile* recurrence. Microbiome **2022**; 10:87.
20. Wang Y, Gao X, Zhang X, et al. Microbial and metabolic features associated with outcome of infliximab therapy in pediatric Crohn's disease. Gut Microbes **2021**; 13:1–15.
21. Bolyen E, Rideout JR, Dillon MR, et al. Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. Nat Biotechnol **2019**; 37:852–7.
22. Callahan BJ, McMurdie PJ, Rosen MJ, Han AW, Johnson AJ, Holmes SP. DADA2: high-resolution sample inference from Illumina amplicon data. Nat Methods **2016**; 13:581–3.
23. Segata N, Izard J, Waldron L, et al. Metagenomic biomarker discovery and explanation. Genome Biol **2011**; 12:R60.
24. Xie G, Wang L, Chen T, et al. A metabolite array technology for precision medicine. Anal Chem **2021**; 93:5709–17.
25. Alvarez AM, Rathore MH. *Clostridium difficile* infection in children. Adv Pediatr **2019**; 66:263–80.
26. Malmqvist L, Ullberg M, Hed Myrberg I, Nilsson A. *Clostridium difficile* infection in children: epidemiology and trend in a Swedish tertiary care hospital. Pediatr Infect Dis J **2019**; 38:1208–13.
27. Miranda-Katz M, Parmar D, Dang R, Alabaster A, Greenhow TL. Epidemiology and risk factors for community associated *Clostridioides difficile* in children. J Pediatr **2020**; 221:99–106.
28. Schubert AM, Rogers MA, Ring C, et al. Microbiome data distinguish patients with *Clostridium difficile* infection and non-*C. difficile*-associated diarrhea from healthy controls. mBio **2014**; 5:e01021–01014.
29. Vazquez-Cuesta S, Garcia NL, Fernandez AI, et al. Microbiome profile and calprotectin levels as markers of risk of recurrent *Clostridioides difficile* infection. Front Cell Infect Microbiol **2023**; 13:1237500.
30. Sun H, Guo Y, Wang H, et al. Gut commensal *Parabacteroides distasonis* alleviates inflammatory arthritis. Gut **2023**; 72:1664–77.
31. Wang K, Liao M, Zhou N, et al. *Parabacteroides distasonis* alleviates obesity and metabolic dysfunctions via production of succinate and secondary bile acids. Cell Rep **2019**; 26:222–35.e5.
32. Lawley TD, Clare S, Walker AW, et al. Targeted restoration of the intestinal microbiota with a simple, defined bacteriotherapy resolves relapsing *Clostridium difficile* disease in mice. PLoS Pathog **2012**; 8:e1002995.
33. Aguirre AM, Sorg JA. Gut associated metabolites and their roles in *Clostridioides difficile* pathogenesis. Gut Microbes **2022**; 14:2094672.
34. Gao Y, Ma L, Su J. Host and microbial-derived metabolites for *Clostridioides difficile* infection: contributions, mechanisms and potential applications. Microbiol Res **2022**; 263:127113.
35. Sorg JA, Sonenshein AL. Bile salts and glycine as cogerminants for *Clostridium difficile* spores. J Bacteriol **2008**; 190:2505–12.
36. Sorg JA, Sonenshein AL. Chenodeoxycholate is an inhibitor of *Clostridium difficile* spore germination. J Bacteriol **2009**; 191:1115–7.
37. Sorg JA, Sonenshein AL. Inhibiting the initiation of *Clostridium difficile* spore germination using analogs of chenodeoxycholic acid, a bile acid. J Bacteriol **2010**; 192:4983–90.
38. Thanissery R, Winston JA, Theriot CM. Inhibition of spore germination, growth, and toxin activity of clinically relevant *C. difficile* strains by gut microbiota derived secondary bile acids. Anaerobe **2017**; 45:86–100.
39. Fachi JL, Secca C, Rodrigues PB, et al. Acetate coordinates neutrophil and ILC3 responses against *C. difficile* through FFAR2. J Exp Med **2020**; 217: jem.20190489.
40. Ferreyra JA, Wu KJ, Hryckowian AJ, Bouley DM, Weimer BC, Sonnenburg JL. Gut microbiota-produced succinate promotes *C. difficile* infection after antibiotic treatment or motility disturbance. Cell Host Microbe **2014**; 16:770–7.
41. Ng KM, Ferreyra JA, Higginbottom SK, et al. Microbiota-liberated host sugars facilitate post-antibiotic expansion of enteric pathogens. Nature **2013**; 502:96–9.
42. Karlsson S, Burman LG, Akerlund T. Suppression of toxin production in *Clostridium difficile* VPI 10463 by amino acids. Microbiology (Reading) **1999**; 145(Pt 7):1683–93.
43. Karlsson S, Burman LG, Akerlund T. Induction of toxins in *Clostridium difficile* is associated with dramatic changes of its metabolism. Microbiology (Reading) **2008**; 154:3430–6.
44. Karlsson S, Lindberg A, Norin E, Burman LG, Akerlund T. Toxins, butyric acid, and other short-chain fatty acids are coordinately expressed and down-regulated by cysteine in *Clostridium difficile*. Infect Immun **2000**; 68:5881–8.