Risk factors and Intestinal Microbiota: *Clostridioides difficile* Infection in Patients Receiving Enteral Nutrition at Intensive Care Unit

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Research

Keywords: Clostridioides difficile infection, Enteral nutrition, Intensive unit care, Intestinal microbiota, Risk factors, Bacteroides

Posted Date: March 13th, 2020

DOI: https://doi.org/10.21203/rs.3.rs-16899/v1

Version of Record: A version of this preprint was published at Critical Care on July 13th, 2020. See the published version at https://doi.org/10.1186/s13054-020-03119-7.

Abstract

Background: Clostridioides difficile infection (CDI) is a leading cause of nosocomial diarrhea. Patients receiving enteral nutrition (EN) in the intensive care unit (ICU) are potentially at high risk of CDI. Presently, we assessed the risk factors and intestinal microbiome of these patients to better understand the occurrence and development of CDI.

Methods: Patients were screened for C. difficile every week after EN started and their clinical records were collected for risk factor identification. Feces were analyzed for 16S rRNA sequencing to evaluate the intestinal microbiota.

Results : Overall incidence of CDI was 10.7% (18/168 patients). History of cerebral infarction was associated with CDI occurrence (OR, 9.759; 95% CI, 2.140-44.498) and treatment with metronidazole could be protective (OR, 0.287; 95% CI, 0.091-0.902). Patients with EN had lower microbial richness and diversity, accompanied by reduced abundance of Bacteroides , Prevotella_9, Ruminococcaceae and Lachnospiraceae. Of these patients, acquisition of C. difficile resulted in a transient increase in microbial diversity, along with consistent alterations in the proportion of some bacterial taxa, especially Ruminococcaceae and Lachnospiraceae. At the initiation of EN, patients who were positive for C. difficile later had enhanced abundance of Bacteroides , which was negatively correlated with C. difficile load when CDI developed.

Conclusion : ICU patients receiving EN had a high prevalence of CDI, and a fragile intestinal microbial environment. Alteration of microbiota composition could be vital in the process of CDI development, bringing new insights in the interaction between C. difficile and host microbiome.

Background

Clostridioides difficile, a Gram-positive, spore-forming anerobic bacterium in the colon, can cause a wide range of illnesses from diarrhea to more severe pseudomembranous colitis [1, 2]. C. difficile infection (CDI) is closely related to antibiotic exposure, which disrupts the endogenous intestinal microbiota and promotes proliferation of C. difficile [1, 3]. In recent years, there has been a dramatic increase in the incidence and severity of CDI, leading to prolonged hospital stays and significant increased economic burdens, which have aroused worldwide concern [4].

Besides antibiotics, risk factors for CDI include advanced age, underlying disease, admission to the intensive care unit (ICU), proton pump inhibitor (PPI) treatment and enteral nutrition (EN) [5–8]. EN, also known as tube feeding, is widely used among patients admitted to ICUs. Contributed by access for inoculation with C. difficile spores provides by tubes and plenty of prophylactic treatments with antibiotics and PPIs, patients receiving EN at ICU are potentially more vulnerable to CDI [9]. However, the incidence and specific risk factors for CDI in patients with EN have not been comprehensively investigated. Moreover, structure of the intestinal microbiota is also reported to be closely bound up with receive consistent diets, and most of them are

exposed to broad-spectrum antibiotics and PPIs. The elemental diets as well as the medication used may contribute to the alteration of intestinal microbiome and further affect the incidence of CDI, which needs to be further explored.

Currently, we conducted the prospective study on patients admitted to the ICU who were receiving EN. Their feces were collected and screened for C. difficile from the start of EN. 16S rRNA sequencing was performed to characterize the intestinal microbiota. This study aimed to evaluate the incidence and risk factors for CDI in patients with EN, describe the microbial characteristics to specify gut microbiota alterations in CDI, and ultimately gain a better understanding of the interaction between the host microbiome and C. difficile.

Methods

Study design and clinical data collection

We conducted a prospective study on adult patients admitted to the ICU in Ruijin Hospital (Shanghai, China) between July 2018 and December 2019. All patients who had received EN for at least 1 week were included. Fecal specimens were obtained from each patient at the beginning of EN, every week during EN, and at the onset of diarrhea (if applicable). According to European guidelines [11], CDI was defined as the presence of diarrhea (at least three episodes of unformed stools within 24 hours) and a positive toxigenic C. difficile detection test.

Clinical epidemiological information for all eligible patients was extracted from the patients' medical records, including demographics, duration of hospitalization, surgical intervention (in the previous 6 months), mortality, comorbidity and in-hospital medication. Comorbidity was graded using the Charlson Comorbidity Index (CCI) and divided into 10 major categories based on related systems. Other common underlying diseases in ICUs were analyzed separately. Laboratory indices, including leukocyte count, serum albumin level, serum creatinine level and blood glucose level, were measured and recorded on admission. Antibiotics and PPIs were the most commonly used medications. For CDI patients, medication history was recorded from admission until the onset of CDI. For C. difficile negative (CDN) patients, data were collected from admission to 2 weeks post-EN, which were the median days from the start of EN to the onset of CDI.

To investigate the gut microbiota features, we also recruited 12 healthy individuals from four communities in Shanghai, who did not have any gastrointestinal diseases or use of antibiotics in the past month. All fecal samples were screened for C. difficile and stored at − 80°C for subsequent DNA extraction.

The present study was approved by the Ethics Committee of Ruijin Hospital, Shanghai, China.

C. difficile detection

Stool samples were analyzed for toxin A/B by enzyme-linked fluorescence assay (ELFA) with a VIDAS automatic analyzer (Biomerieux, Marcy-l'Etoile, France). C. difficile isolates were cultured on Clostridium difficile agar base (Oxoid, Basingstoke, UK). Typical colonies were identified based on their odor, appearance and morphology after Gram staining, and confirmed using gluD gene detection by polymerase chain reaction (PCR). Purified C. difficile isolates were characterized by detection of toxinA and toxinB genes.

DNA extraction, 16S rRNA gene sequencing and data processing

Fecal genomic DNA was extracted from each stool specimen with TIANamp Stool DNA Kit (Tiangen Biotech, Beijing, China). After quality verification, DNA was submitted to Majorbio Bio-Pharm Technology Co. Ltd. (Shanghai, China) for 16S rRNA gene amplification and sequencing. The hypervariable region V3–V4 of the bacterial 16S rRNA gene was amplified with primer pairs 338F (5'-ACTCCTACGGGAGGCAGCAG-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3'). Purified amplicons were sequenced on an Illumina MiSeq platform (Illumina, San Diego, CA, USA). Operational taxonomic units (OTUs) with 97% similarity cutoff were clustered using UPARSE (version 7.1) and chimeric sequences were identified and removed. The taxonomy of each OTU representative sequence was analyzed by RDP Classifier against the 16S rRNA database (Silva SSU132) using a confidence threshold of 0.7. All processes were performed on a platform (www.i-sanger.com) provided by Majorbio Bio-Pharm Technology Co. Ltd.

Real-time PCR

Quantitative PCR was performed using the TB Green qPCR Kit (Takara, Tokyo, Japan) and LightCycler 480 Real-Time PCR system (Roche, Shanghai, China). Relative abundance of each bacterium was calculated by the ΔCt method and normalized to total bacteria (16S rRNA). The primer sequences are listed in Table S1.

Statistical Analyses

The results are expressed as medians and quartiles for continuous variables and as frequencies and percentages for categorical variables. The Wilcoxon rank-sum tests were used to examine differences in those data not normally distributed, including duration of hospitalization, leukocyte count, serum creatinine level and blood glucose level. Student's t tests were used to compare normally distributed continuous variables, including age, CCI score and serum albumin. All categorical data were compared by χ^2 test or Fisher's exact test. The statistically significant variables from the above analysis were then included in a multivariate logistic regression model to evaluate the potential risk factors relevant to CDI

and presented as odds ratios (ORs) with 95% confidence intervals (CIs). These analyses were performed with SPSS version 24.0.

The alpha diversity (Chao and Shannon indexes) of the microbiome was calculated at the OTU level on the Majorbio BioTech platform and compared among groups using Student's t test or paired t test. Principal coordinates analysis (PCoA) of Bray–Curtis distance metric was conducted to evaluate the variability in OTUs among groups, and the differences were tested through Adonis analysis. Linear discriminant analysis effect size (LEfSe) was evaluated from phylum to genus and the linear discriminant analysis (LDA) score was set at > 4.0. The predominant phyla or genera were also compared among groups using the Wilcoxon rank-sum test or Wilcoxon signed-rank test. Correlations between genus or species relative abundance were calculated using Spearman's analysis. The t tests and Spearman's correlation tests were conducted in GraphPad Prism 5 and the others were analyzed using the Majorbio BioTech platform.

Differences were considered significant at $P < 0.05$.

Results

Patient population and C. difficile detection

A total of 480 adult patients were admitted to the ICU from July 2018 to December 2019 (Fig. 1). Of these, 168 had received EN for at least 1 week and were recruited to the present study. The patients had average age of 50.5 ± 16.0 (mean ± SD) years, and 31% (52/168) were elderly (> 60 years old). All patients had received antibiotic treatment and 160 (95.2%) had also received PPIs.

We analyzed 695 fecal samples, of which 30 from 23 patients were positive for C. difficile (culture or ELFA) (Table S1). Eighteen patients developed diarrhea and were identified to have CDI, while five were defined as C. difficile colonization (CDC). Overall, the prevalences of CDI and CDC in ICU patients receiving EN were 10.71% and 2.98%, respectively. The median duration from EN therapy to CDI diagnosis was 12 days (interquartile range, 7–21 days).

Clinical characteristics and risk factors for CDI in ICU patients with EN

We compared demographics, clinical features, and in-hospital medication between the CDI and CDN groups (Table 1). The results showed that CDI patients were significantly older (media 66 vs. 48, p = 0.021) and had a longer ICU stay (media 30 vs. 20, p = 0.001). Besides, CDI patients had a distinctly higher CCI score (media 2.5 vs. 2, p = 0.015), but no significant differences were detected in any comorbidity categorized by systems. For other clinically common diseases, we found that a markedly larger proportion of CDI patients had a history of cerebral infarction (22.2% vs. 3.4%, p = 0.006). Laboratory results showed that CDI patients exhibited lower baseline level of serum albumin (media 30 vs. 32, p = 0.047) but comparable leukocyte count, serum creatine and blood glucose. Compared with CDN patients, CDI patients were likely to receive more carbapenems (83.3% vs 61.4, p = 0.068) but

significantly less metronidazole (27.8% vs. 63.2%, p = 0.037). Additionally, there were no significant differences in PPI use or the number of antibiotics received between the two groups.

Table 1

Characteristics and risk factors of CDI and CDN patients with EN admitted to ICU.

Characteristics	CDI (n = 18)	CDN (n = 145)	P value	Multivariable Analysis	
	N (%)/Media (IQR)	N (%)/Media (IQR)		OR (95% CI)	P value
Demographics					
Female	6 (33.3)	62 (42.8)	0.444	_[a]	-
Age, years	66 (57.75–73.75)	48 (37–64)	0.021*	-	0.173
Duration of hospitalization (days)	30 (27.75–50.75)	20 (14–32)	0.001**	-	0.211
In-hospital mortality	1 (5.6)	17 (11.7)	0.697	-	-
Surgical intervention in previous six months	5 (27.8)	32 (22.1)	0.805	-	-
Clinical features					
CCI[b]	2.5 (1–5)	2 (1–3)	0.015*	-	0.194
Comorbidities by category					
Gastrointestinal disease	0 (0)	18 (12.4)	0.236	-	-
Liver disease	5 (27.8)	52 (35.9)	0.498	-	-
Gall bladder, biliary tract or pancreatic disease	13 (72.2)	110 (75.9)	0.962	-	-
Respiratory disease	3 (16.7)	26 (17.9)	1.000	-	-
Cardiovascular disease	5 (27.8)	55 (37.9)	0.400	-	-
Renal disease	5 (27.8)	18 (12.4)	0.159	-	-
Neurologic disease	0 (0)	5 (3.4)	0.940	-	-
Malignancy	1 (5.6)	7 (4.8)	1.000	-	-

Numerical data are shown as media (Interquartile range), and categorical data are described as frequency (percentage).

a. Not applicable; b. CCI, Charlson comorbidities index

Characteristics	CDI (n = 18)	CDN (n = 145)	P value	Multivariable Analysis	
	N (%)/Media (IQR)	N (%)/Media (IQR)		OR (95% CI)	P value
Hematologic or immunologic disorders	2 (11.1)	20 (13.8)	1.000	-	-
Metabolic disorders	9 (50)	91 (62.8)	0.294	-	-
Clinical common underlying disease					
Diabetes	4 (22.2)	31 (21.4)	1.000	-	-
Fatty liver	4 (22.2)	41 (28.3)	0.588	-	-
Hypertension	2 (11.1)	42 (29)	0.184	-	-
History of cerebral infarction	4 (22.2)	5 (3.4)	0.006**	9.759 (2.140-44.498)	0.003**
Laboratory results					
Leukocyte count (× 10^9 /L)	11.44 (8.32–13.86)	11.63 (8.53–15.31)	0.781	-	-
Serum albumin (g/L)	30 (26-33.5)	32 (28–36)	0.047*	-	0.367
Serum creatinine (μmol/L)	70 (54.8–177)	71 (55–134)	0.470	-	-
Blood glucose(mmol/L)	11.64 (9.71–13.27)	9.57 (6.95–12.98)	0.405	-	-
In-hospital medications					
PPIs	16 (88.9)	138 (95.2)	0.580	-	-
Antibiotics					
3rd and 4th generation cephalosporins	9 (50)	97 (66.9)	0.156	-	-
Carbapenems	15 (83.3)	89 (61.4)	0.068	-	0.181

Numerical data are shown as media (Interquartile range), and categorical data are described as frequency (percentage).

a. Not applicable; b. CCI, Charlson comorbidities index

Characteristics	CDI (n = 18)	CDN (n = 145)	P value	Multivariable Analysis	
	N (%)/Media (IQR)	N (%)/Media (IQR)		OR (95% CI)	P value
Metronidazole	5 (27.8)	78 (63.2)	0.037*	0.287 (0.091–0.902)	0.033*
Vancomycin	6 (33.3)	49 (34)	0.953	-	-
Fluoroquinolones	5 (27.8)	26 (17.9)	0.493	-	-
Linezolid	2 (11.1)	20 (13.8)	1.000	-	-
Aminoglycoside	1 (5.6)	10 (6.9)	1.000	-	-
Tetracycline	2 (11.1)	9 (6.2)	0.776	-	-
Antifungal agents	3 (16.7)	26 (17.9)	1.000	-	-
Antiviral drugs	0 (0)	8 (5.6)	0.654	-	-
NO. of antibiotics received					
1 ~ 2	9 (50)	74 (51)	0.895		
3 ~ 4	7 (38.9)	50 (34.5)		-	-
≥ 5	2 (11.1)	21 (14.5)			
Numerical data are shown as media (Interquartile range), and categorical data are described as frequency (percentage).					
a. Not applicable; b. CCI, Charlson comorbidities index					
*P□0.05; ** P□0.01.					

Finally, to assess the potential risk factors for CDI in patients with EN, we performed a multivariable logistic regression analysis on those variables with apparent differences. After forward selection, history of cerebral infarction seemed to be an independent risk factor associated with CDI among patients with EN (OR, 9.759; 95% CI, 2.140–44.498), while prior therapy with metronidazole could be a protective factor (OR, 0.287; 95% CI, 0.091–0.902; Table 2).

Characteristics of intestinal microbiota in CDI

To clarify the features of gut microbiota of patients receiving EN associated with *C. difficile*, we analyzed the microbial community of feces collected from 13 CDP (12 CDI and 1 CDC) and 16 CDN patients and 12 healthy controls (HCs; Figure 2a). The CDI and CDN groups had comparable demographics, clinical

wo CDI patients were detected *C. difficile*

positive at the start of EN (P108, with a negative culture result, was finally identified using 16S gene sequencing), and the CDI sample from P166 was excluded because of poor fecal DNA quality. Overall, 15 CDI feces were collected in total. Next, we assessed the microbial composition of CDI (n=15, marked as black circles in Figure 2a), CDN (n=16, collected two weeks after EN started from CDN patients as controls), and HC (n=12) stool samples to determine the feature of intestinal microbiota among those patients.

Compared with the HC group, microbial richness and diversity decreased significantly in the CDI and CDN stool samples, as demonstrated by Chao richness and Shannon diversity indices (P<0.001) (Figure 2b). Interestingly, microbial diversity of the CDI group was higher than that of the CDN group (P<0.05, Figure 2b), although no significant differences were found in their microbial richness. The PCoA plot of Bray–Curtis distance (Figure 2c) revealed that CDI, CDN and HC samples could be different subject clusters (Adonis analysis: HC vs. CDI, CDN: R^2=0.2414, P=0.001; CDI vs. CDN: R^2=0.0670, P=0.009). In addition, it is shown that the points are more widely dispersed in cluster CDI and CDN, indicating that the samples of CDI patients and CDN patients had greater interindividual variation in microbiota structure (Figure 2c).

The distribution of dominant bacterial phyla, families and genera in each group is listed in Figure S1. Bacteroidetes and Firmicutes were the predominant bacterial phyla, whereas CDI and CDN samples had a greater proportion of Proteobacteria (CDI, CDN vs. HC; 33.7%, 39.0% vs. 12.4%). At the genus level, CDI and CDN groups consisted of higher proportions of *Klebsiella* and *Enterococcu*s. Then, we performed a logarithmic LDA score cutoff 4.0 to identify important taxonomic differences among the groups (Figure 2d). Compared with HCs, we observed significant decreases in the abundance of Bacteroidaceae (*Bacteroides*), *Prevotella_9*, Lachnospiraceae and Ruminococcaceae (*Faecalibacterium*) in CDI and CDN samples. Concerning the differences between CDI and CDN group, we found that the relative abundance of *Clostridioides,* Ruminococcaceae and *Ruminococcus_gnavus_group* was significantly higher, while *Acinetobacter* and *Fusobacterium* had a lower abundance in CDI samples.

Intestinal microbiota dynamics in CDI patients with EN

The high microbial diversity of CDI samples caught our attention. By comparing the microbiota of CDN patients at the start of EN and 2 weeks later, we observed that the microbial richness (P=0.005) and diversity (P=0.057) were declining during EN (Figure 3a). However, things were different in CDI patients. The overall changes in intestinal microbiota of CDI patients are shown Figures S2 and 3. For CDI patients, the diversity increased significantly after *C. difficile* emerged (P=0.019, Figure 3b), and this trend subsequently disappeared when *C. difficile* was cleared (P=0.027, Figure 3b). These results indicate that the presence of *C. difficile* might cause a transient increase in the diversity of gut microbiota. Moreover, accompanied by the alteration of microbial diversity, the composition of microbiota also changed. For example, in P60, the relative abundance of Lachnospiraceae and Ruminococcaceae increased when *C. difficile* emerged, but decreased when *C. difficile* disappeared, in accordance with the changes in microbial diversity (Figure 3c). Similarly, this consistent trend in changes in microbial diversity and relative abundance of Lachnospiraceae or Ruminococcaceae was found in all *C. difficile* positive (CDP)

patients (Figure S2) except P68, whose microbial diversity was accompanied by the emergence of *Phascolarctobacterium*, a short chain fatty acid (SCFA)-producing genus (Figure S2) [12]. To further investigate the effect of this phenomenon, we focused on those patients who remained *C. difficile* positive for at least 2 weeks. As shown in Figure3d for P156, the diversity of gut microbiome decreased obviously with the increase of *C. difficile* load, and so did the Lachnospiraceae and Ruminococcaceae load.

Role of *Bacteroides* in CDI during EN

Although the ICU patients treated with EN received similar in-hospital treatment, they had different outcomes for CDI. Besides demographics, clinical features and in-hospital medications, we speculated whether the gastrointestinal microbiome at the beginning of EN could also predict CDI. Seven CDP patients (six CDI and one CDC) without *C. difficile* colonization at the time EN started were included in the next comparison with CDN patients.

Although there were no significant differences in microbial richness or diversity at the beginning of EN between CDN and CDP patients, PCoA analysis revealed that their composition of microbiota differed significantly (Adonis analysis, R^2=0.0896, P=0.019) (Figures S3a, 4a). By LEfSe analysis (LDA score cutoff 4.0), we found a series of bacterial taxa with distinct abundances between CDP and CDN patients, including *Bacteroides, Escherichia–Shigella, Serratia*, *Ralstonia* and *Anaerostipes* (Figures S3b, 4b). Since metronidazole, the commonly used antibiotic in clinic, could reduce the amount of *Bacteroides* in the human intestine, we excluded all patients received metronidazole treatment within 3 days prior to the start of EN to avoid the interference of medications[12]. The difference in abundance of *Bacteroides* was still significant between the two groups (P=0.011, Figure S3c). It was suggested that early at the time of EN started, CDP patients already had different structure of gut microbiota, featuring in higher proportion of *Bacteroides*.

The high abundance of *Bacteroides* at initiation of EN sparked our interest. To clarify the role of *Bacteroides* in CDI, we observed that the *Bacteroides* load tended to decrease after acquisition of *C. difficile* (40.64% vs. 23.09% P=0.093), while the abundance of *Bacteroides* remained stable for CDN patients during EN (10.51% vs. 8.53%, P=0.451) (Figure S3d). Then, we conducted a correlation analysis among all feces positive for *C. difficile*. Interestingly, the relative abundance of *Clostridioides* was significantly negatively correlated with that of *Bacteroides* (R= -0.58, P=0.016) but significantly positively correlated with that of *Enterococcus* (R=0.66, P=0.002) (Figure 4c). These correlations were verified using quantitative PCR analysis (Figure 4d), which indicates a possible mutual inhibitory relationship between *Bacteroides* and *C. difficile* in CDI.

Discussion

CDI has emerged as one of the most threatening problems globally throughout various healthcare facilities, especially in ICUs [13]. ICU patients are reported to have a significantly higher prevalence of

approximately 2% for CDI, compared with 0.9% in patients in general wards [14]. In our previous study [15], we found that EN could be an important risk factor for CDI patients admitted to ICUs. However, few studies have been conducted on this potential high-risk population. In the present study, we investigated patients admitted to ICUs receiving EN therapy for at least 1 week. The prevalence of CDI reached 10.71%, which was higher than 0.4–4% estimated in ICU patients in European countries and 4.12% in our previous study of ICU patients in the same institution [13, 15, 16]. The present study focusing on ICU patients receiving EN revealed that the patients with CDI were older and had longer hospital stays, higher CCI score, and lower serum albumin level. Despite the frequent risk factors, we also noticed that history of cerebral infarction was strongly and independently related to CDI, which might be due to old age, low microbial diversity and possible prior long-term exposure to healthcare institutions [17]. Metronidazole is commonly used for CDI, and was still recognized as a protective factor here, consistent with many other studies, which emphasizes its current importance in prevention and treatment in CDI [15, 18, 19]. Overall, these results have enriched the epidemiological data for CDI and increased our attention to ICU patients receiving EN.

EN, always along with prophylactic use of antibiotics and PPIs, are likely to be accompanied by disruption or remodeling of gut microbiota, which plays an essential role in occurrence and development of CDI [9, 20]. Compared with HCs, samples from our CDI and CDN patients showed a significant decrease in microbial richness and diversity, and lower abundance of Bacteroidaceae (Bacteroides), Lachnospiraceae and Ruminococcaceae (Faecalibacterium), which are necessary to maintain intestinal homeostasis [21–24]. During the two weeks of EN, the microbial richness and diversity continued to decline. Besides, Enterococcus species, which are highly associated with nosocomial infection in ICUs, obviously increased in those samples and exhibited a positive correlation with C. difficile [25]. All these results indicate that ICU patients with EN have a fragile gut microbiota and the course of EN treatment makes it even worse. This adverse situation may not only be triggered by heavy use of antibiotics and PPIs, but also by the elemental diets in EN. Although elemental diets contain essential nutrients for patients, they lack fiber and food residues, which can be fermented by colonic microbiota to produce regulators of colonic epithelial proliferation to protect against gut pathogens [9]. Perhaps CDN patients were also susceptible to C. difficile because of their equally bad microbial structure but they had not been exposed to C. difficile spores. Overall, the poor intestinal microbiota of ICU patients receiving EN may facilitate C. difficile expansion and make patients vulnerable to CDI.

Previous studies have often described the microbial characteristics of CDI by comparison with HCs or C. difficile-negative patients with diarrhea [26–29]. However, it is difficult to clarify accurately to what these microbial changes should be attributed, either diarrhea, complex clinical management, or C. difficile itself. Receiving consistent diets and clinical management, patients with EN could be a suitable group in which to observe the microbial features in CDI. By comparison of feces from CDI and CDN patients, we found a surprising increase in the microbial diversity in CDI samples, along with higher abundance of Ruminococcaceae and R. gnavus_group (Lachnospiraceae family). We further analyzed the dynamics of the intestinal microbiota in the whole process of CDI. This revealed that the presence of C. difficile might

nsistent change in the abundance of

Ruminococcaceae and Lachnospiraceae families or other SCFA-producing bacteria. Ruminococcaceae and Lachnospiraceae families are usually recognized as protective microbes against CDI, depending on their ability to produce SCFAs and secondary bile acids [24, 30]. SCFAs, especially butyrate, can enhance colonic defense barriers by secreting antimicrobial peptides, and secondary bile acids can directly restrain C. difficile germination or vegetative growth [31, 32]. Accordingly, although the accurate mechanism for these noteworthy microbial alterations remains unclear, we speculate that it might be a protective reaction to prevent C. difficile overgrowth. The opposite relationship between microbial diversity and C. difficile load observed in several patients with long duration of CDI also supports that hypothesis. Besides, Vincent et al. [33] also described a similar response to C. difficile colonization, proposing an increase of beneficial bacterial taxa in gut including Clostridiales Family XI Incertae Sedis, Clostridium and Eubacterium. In a word, these findings suggest that a potential protective microbial reaction might appear at the emergence of C. difficile, enriching our understanding of host response to CDI.

Given that EN therapy increased the risk of CDI, we attempted to evaluate relevant risk factors for CDI within the intestinal microbiota. We observed a greater proportion of Bacteroides before EN therapy in CDP patients, which suggests that Bacteroides promotes colonization by C. difficile. However, we also detected an inhibitory relationship between Bacteroides and C. difficile, which was demonstrated as their negative correlation in abundance. These controversial results raise an interesting question for us: what is the role of Bacteroides in the development of CDI: a risk factor or defender? Previous investigations also had inconsistent conclusions. Based on mouse models, Li et al. [34] demonstrated that Bacteroides was positively correlated with C. difficile load, while Sangster et al. [35] found the opposite in clinical CDI samples. In fact, Bacteroides species also interact with C. difficile differently. Bacteroides fragilis, Bacteroides ovatus and Bacteroides vulgatus can protect against CDI through production of SCFAs or secondary bile acids [36, 37]. However, Ferreyra et al. [38] and Ng et al. [39] have demonstrated that Bacteroides thetaiotaomicron metabolizes polysaccharide to provide C. difficile with a nutrition source, including sialic acid and succinate, and help it proliferate in the perturbed intestine. Thus, we propose that Bacteroides species might play different roles in CDI at different stages of EN. Maybe early in EN, because of rich colonic polysaccharide, Bacteroides could play a dominant role in providing substrates for C. difficile growth. After a period of elemental diets lacking polysaccharide, the function of Bacteroides in producing SCFAs and secondary bile acids might start to dominate and protect against CDI. The complex interactions among intestinal microbiota could be the main reason for divergent conclusions. To clarify the detailed mechanism of how different species of Bacteroides act during the course of CDI, further research is needed.

The current study is believed to be the first to focus on CDI in ICU patients with EN. After effectively ruling out dietary interventions and clinical management, we took a more specific look at the structure of the intestinal microbiota, which provides new insights into the association between gut pathogens and symbiotic microflora. However, there were several limitations to our work. First, all participants were from a single center, which means that the results may not be applicable to all healthcare institutions. Second, our observations on microbial characteristics and dynamics were limited by the small sample size. The

ies to verify our results. To further understand

the association between C. difficile and intestinal microbiota, we need more experimental results. Investigation based on metabolomics could also be helpful.

Conclusion

Our study assessed the incidence, risk factors and microbial characteristics of CDI in ICU patients with EN. The overall incidence of CDI reached 10.7%. History of cerebral infarction significantly increased the risk of CDI, while treatment with metronidazole was a protective factor. Patients with EN showed a significantly lower microbial richness and diversity along with apparent alterations in the composition of intestinal microbiota. C. difficile can cause a series of intestinal microbial reactions, potentially protective, represented by changes in diversity and some specific bacteria. Patients developing CDI after EN had a noteworthy Bacteroides load early after the start of EN. Conversely, the abundance of Bacteroides was negatively correlated with C. difficile burden, which indicates that Bacteroides might play different roles in CDI formation and development. Our study provides useful epidemiological data for CDI development in patients with EN and enhances our understanding of the interaction between C. difficile and intestinal microbiomes.

Abbreviations

EN: enteral nutrition; ICU: intensive care unit; CDI: *C. difficile* infection; PPI: proton pump inhibitor; CDC: *C. difficile* colonization; CDP: *C. difficile* positive; CDN: *C. difficile* negative; ELFA: enzyme-linked fluorescence assay; OTU: operational taxonomic unit; CCI: Charlson comorbidity index; OR: odds ratios; CI: confidence interval; PCoA: principal coordinates analysis; LEfSe: linear discriminant analysis effect size; LDA: linear discriminant analysis; IQR: interquartile range; SCFA: short chain fatty acid

Declarations

Ethics Approval And Consent To Participate

The Ruijin Hospital Ethics Committee approved the study protocol and verbal informed consent because our work only involved stool samples and all data collected were anonymized. All participants provided verbal consent prior to participation.

Consent For Publication

Not applicable.

Availability Of Data And Materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

Funding

This work was supported by the National Natural Science Foundation of China (grant nos. 81902117, and 81971993), the Shanghai Sailing Program (grant no. 19YF1431300) and Research Project of Shanghai Municipal Health Commission (grant no.20194Y0318). The National Natural Science Foundation of China (grant no. 81902117) and Shanghai Sailing Program (grant no. 19YF1431300) funded in the design of the study, sample collection, related experiments and collection of case records. The National Natural Science Foundation of China (grant no. 81971993) and Research Project of Shanghai Municipal Health Commission (grant no.20194Y0318) funded data analysis, interpretation of data and in writing and revision of the manuscript. The funding body had no role in the design of the study and collection, analysis, and interpretation of data and in writing the manuscript.

Authors' Contributions

DW, DD and YP designed and executed experiments, interpreted data, and prepared the manuscript. EM, CW, TS and GW contributed to the collection of clinical samples, related experiments, and case records. YC, CJ and QN assisted with the statistical analysis. All authors have read and approved the final manuscript.

Acknowledgements

The authors appreciate the contributions of all ICU teams and participants involved in the study. We are grateful to Jiu Chen, Mingbing Yu, Zihang Sheng and Feiyu Qian, students from Faculty of Medical Laboratory Science, Shanghai Jiaotong University School of Medicine, for their participation in sample collection. We thank Cathel Kerr BSc, PhD, from Liwen Bianji, Edanz Editing China (www.liwenbianji.cn/ac), for editing the English text of a draft of this manuscript.

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Figures

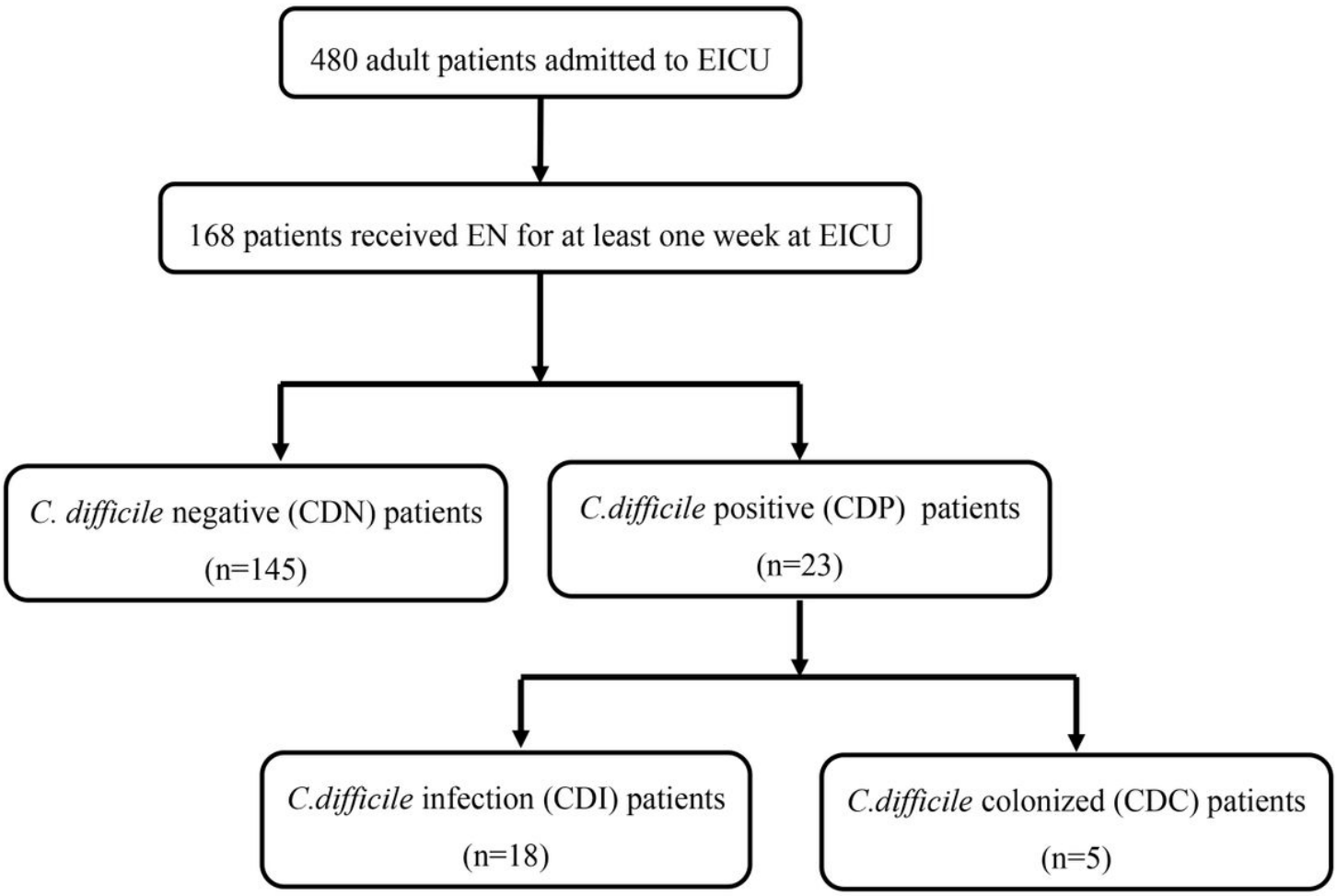


Figure 1

Study flowchart of Clostridioides difficile infection (CDI), C. difficile colonization (CDC) and C. difficile negative (CDN) patients among the ICU patients. Overall, 168 patients were included in the study and were divided into two groups according to whether they were positive for C. difficile. Further grouping was performed according to diarrhea symptoms.

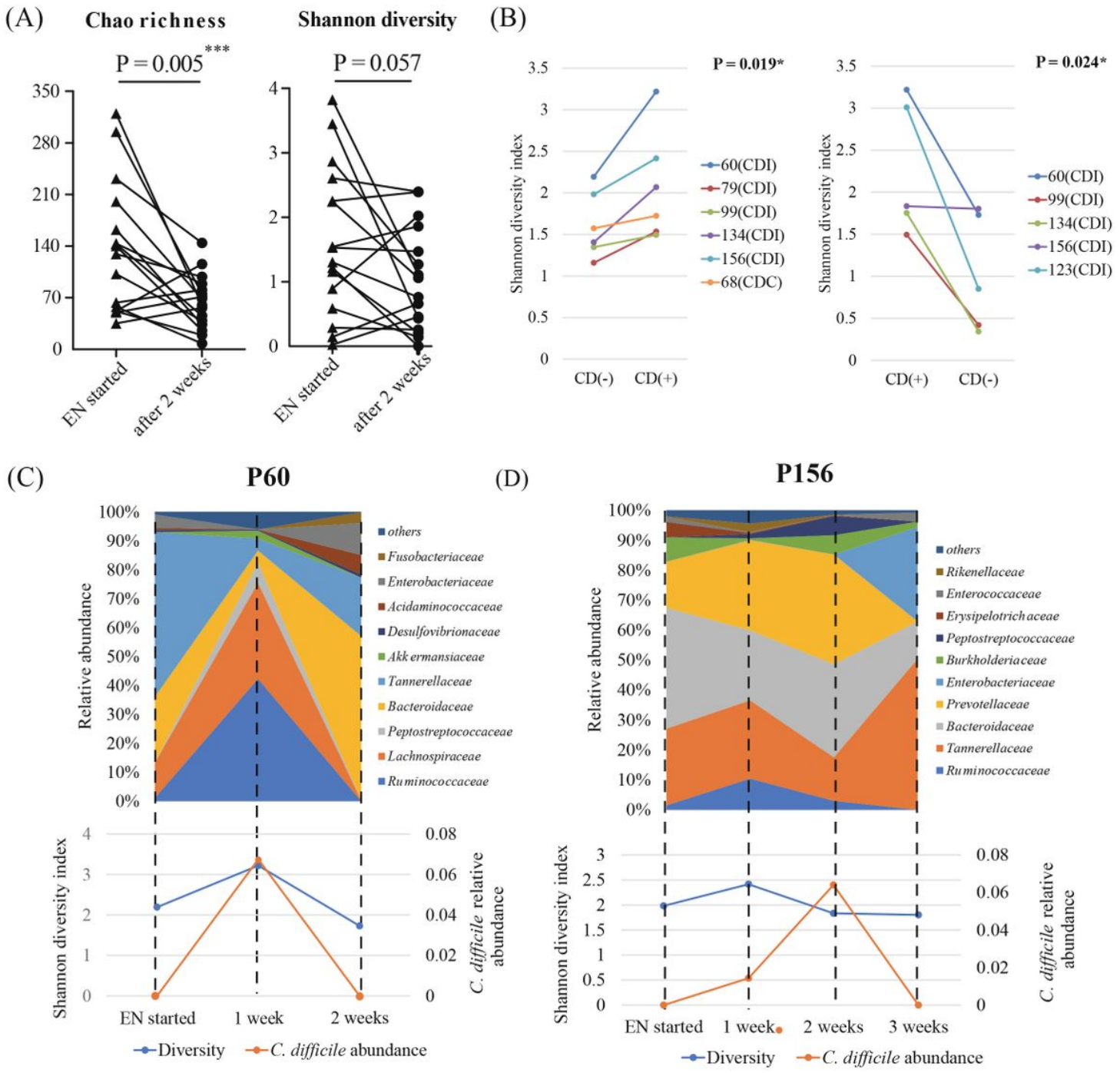
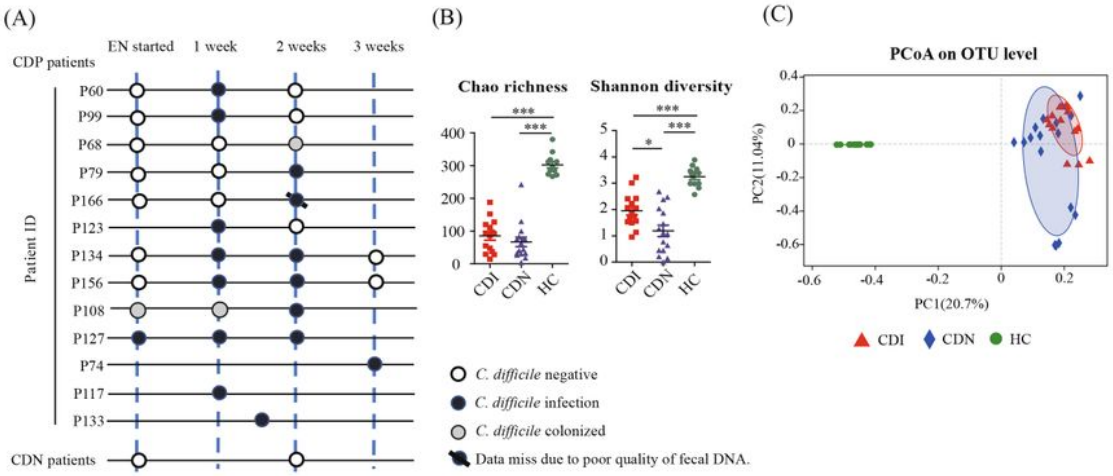
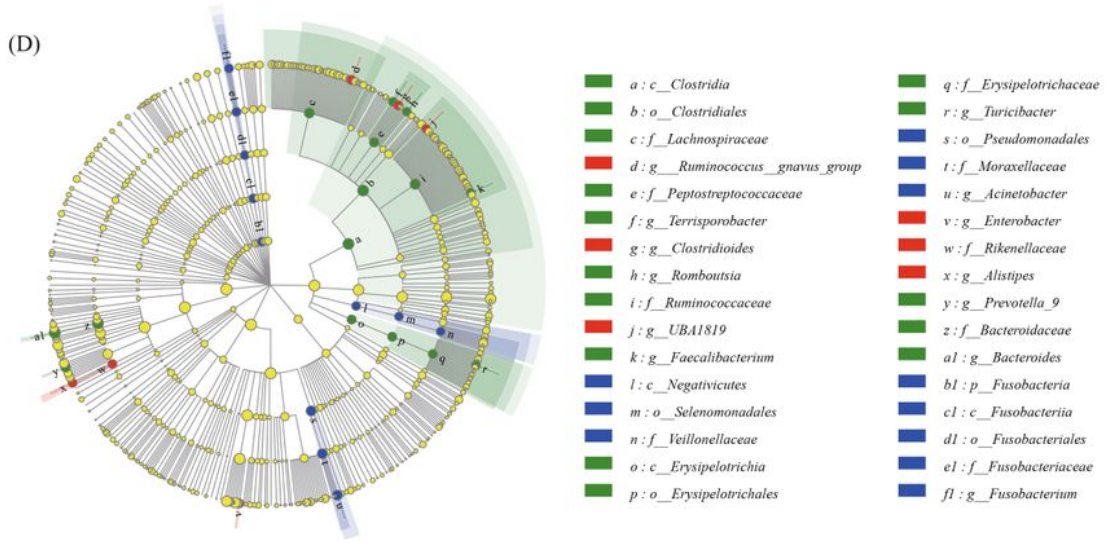


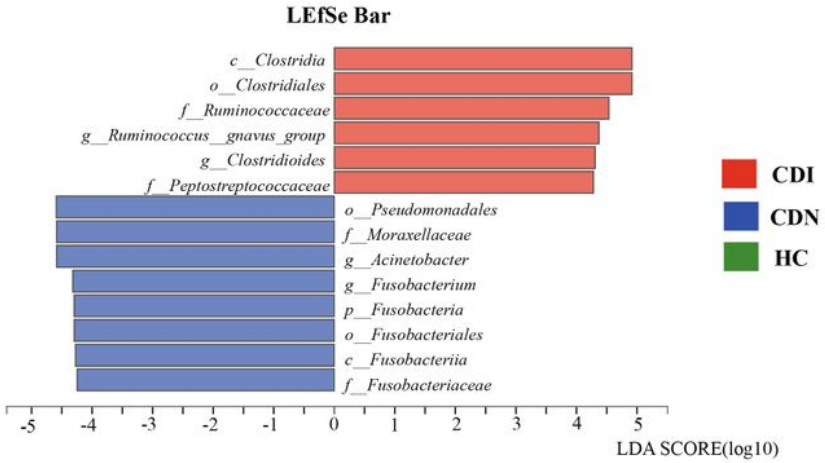
Figure 2

Different distribution of intestinal microbiota in CDI and CDN patients and healthy controls (HCs). (A)Fecal samples from CDP and CDN patients were collected at the indicated times and tested for the presence of C. difficile. White, black and grey circles represent C. difficile negative, C. difficile infection and C. difficle colonization, respectively. (B) Student's t-test shows differences in the indices of microbial richness and diversity between CDI (n=15), CDN (n=16) and HC (n=12) samples. Data represent the mean value and standard error of each group. *P < 0.05; ***P < 0.001. (C) Principal coordinates analysis for CDI, CDN and HC sample groups, with plots based on the Bray–Curtis distance. The horizontal and vertical

axes represent 20.7% and 11.04% of the inter-sample variation, respectively. Each point represents a sample and the colors represent different groups. (D) Linear discriminant analysis effect size was used to identify essential differences in abundance among CDI, CDN and HC groups from phylum to genus. Only taxa with a significant LDA threshold value of >4 are shown. Different-colored regions represent different groups. Circles indicate phylogenetic levels from phylum to genus. The diameter of each circle is proportional to the abundance of the group.







Alterations in the composition of gut microbiota in the presence of C. difficile (A) Alterations in the microbial richness and diversity in CDN patients (n=16) at the start of EN and 2 weeks after receiving EN. (B) Changes in microbial diversity of C. difficile positive patients from C. difficile negative to positive (left) or C. difficile positive to negative (right). Statistical significance in (A) and (B) was determined using paired t-tests. (C) and (D) Intestinal microbiota dynamics in P60 and P156. Changes in microbial composition at the family level are illustrated on the above axis, corresponding to the alterations in the C. difficile load (right) and microbial diversity (left) on the same timeline shown below.

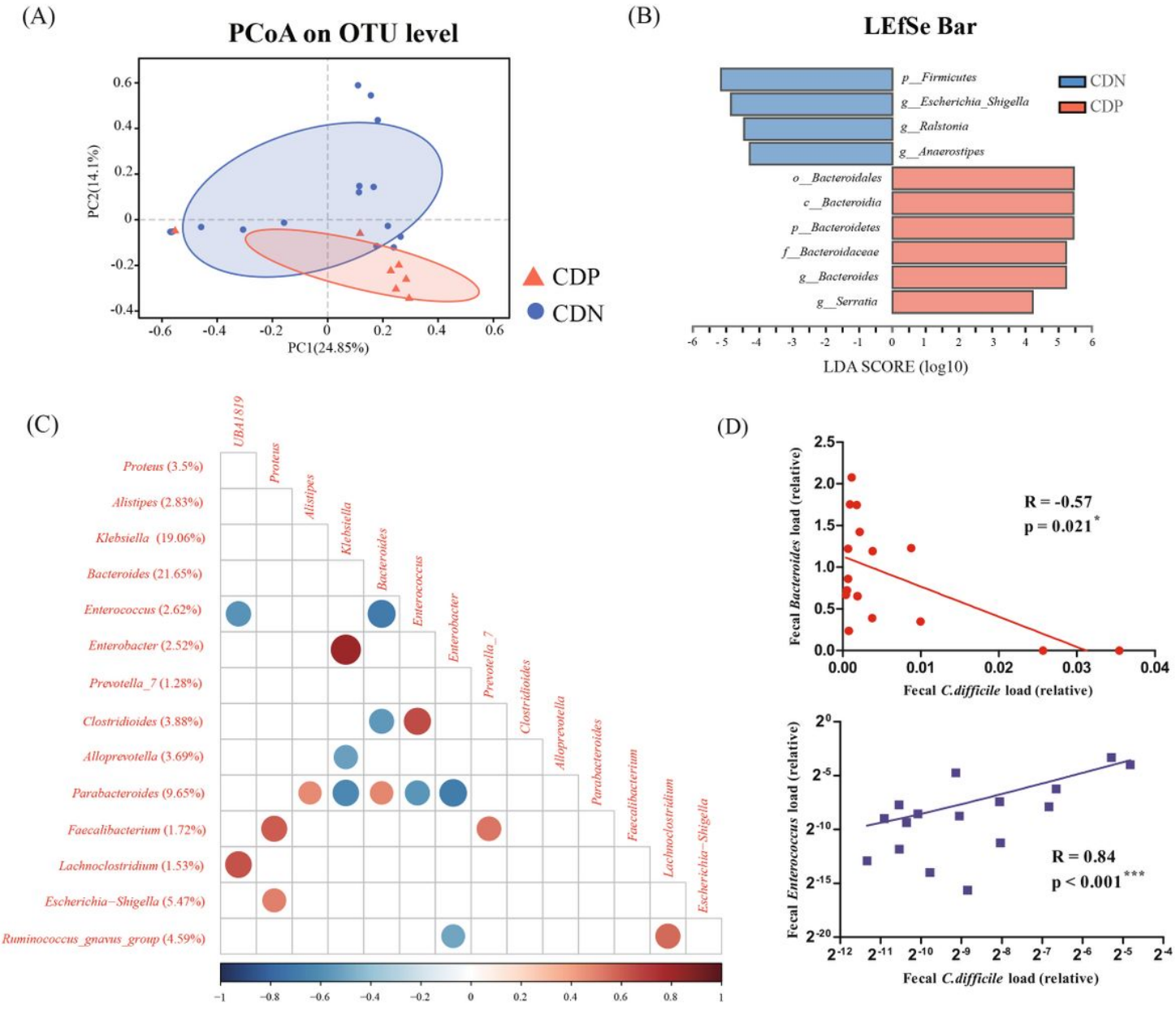
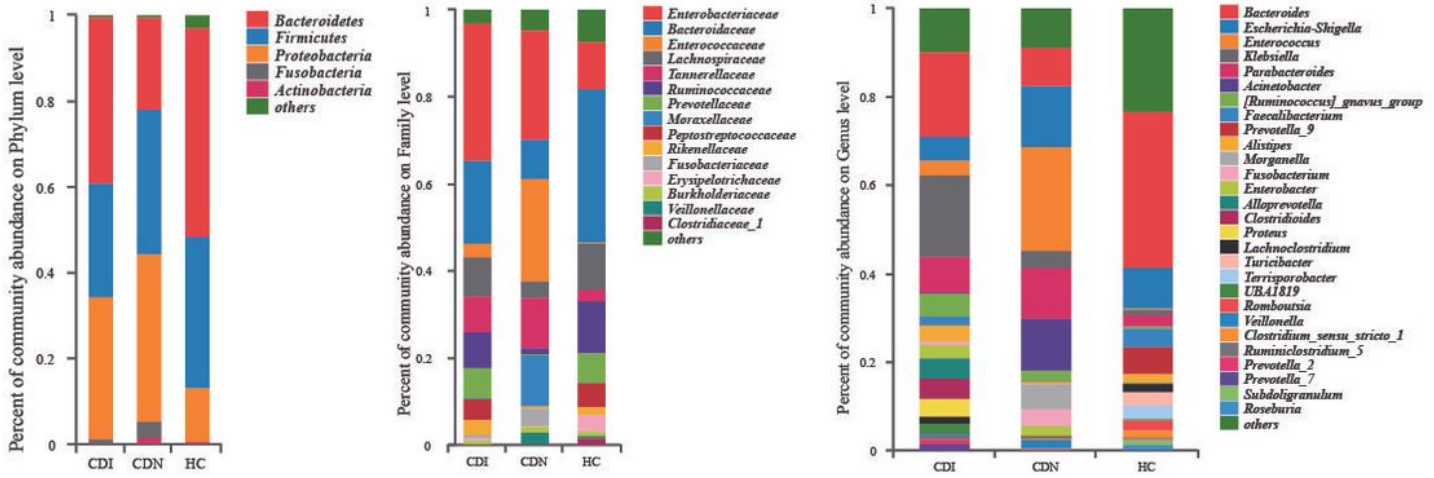


Figure 4

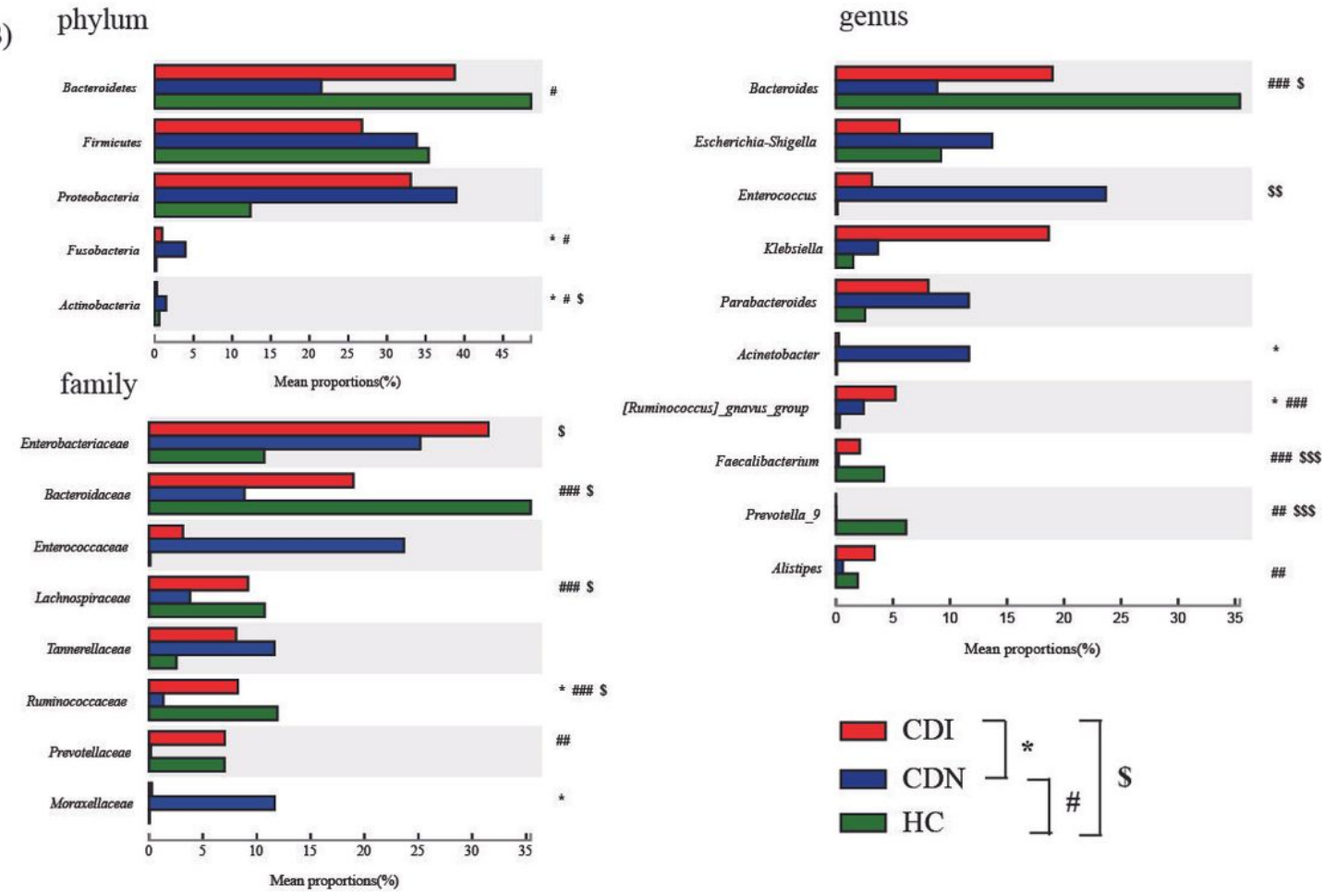
Role of Bacteroides in CDI. (A) and (B) The comparation in the composition of intestinal microbiota between CDP (n=7) and CDN (n=16) patients at the beginning of EN. (A) shows principal coordinates analysis plots based on the Bray-Curtis distance. (B) provides the linear discriminant analysis effect size om phylum to genus level between the two

groups. (C) Correlation matrix of the relative abundance of predominant genera in all C. difficile positive samples (n=18). The average percentage of each genus among all samples is shown to the left of the name of the genus. Only significant correlations are shown ($P < 0.05$). The size and color of the circles reflect the degree and direction of the correlation, respectively. (D) Correlation between the relative abundance of Bacteroides or Enterococcus and C. difficile calculated using qPCR. Correlations were all tested using Spearman's correlation test.

(A)



(B)



Microbial composition in CDI, CDN and HC samples. (A) Average relative proportions of phyla, families and genera in each group. (B) Wilcoxon rank sum test was used to compare relative abundances at the phylum, family, and genus levels among groups. Significant differences of CDI vs. CDN, CDN vs. HC, and CDI vs. HC are illustrated as “*”, “#”, and “$”, respectively. *#$ P < 0.05; ** ##

P < 0.01; *** ###

$ P < 0.001. (PDF 1111KB)

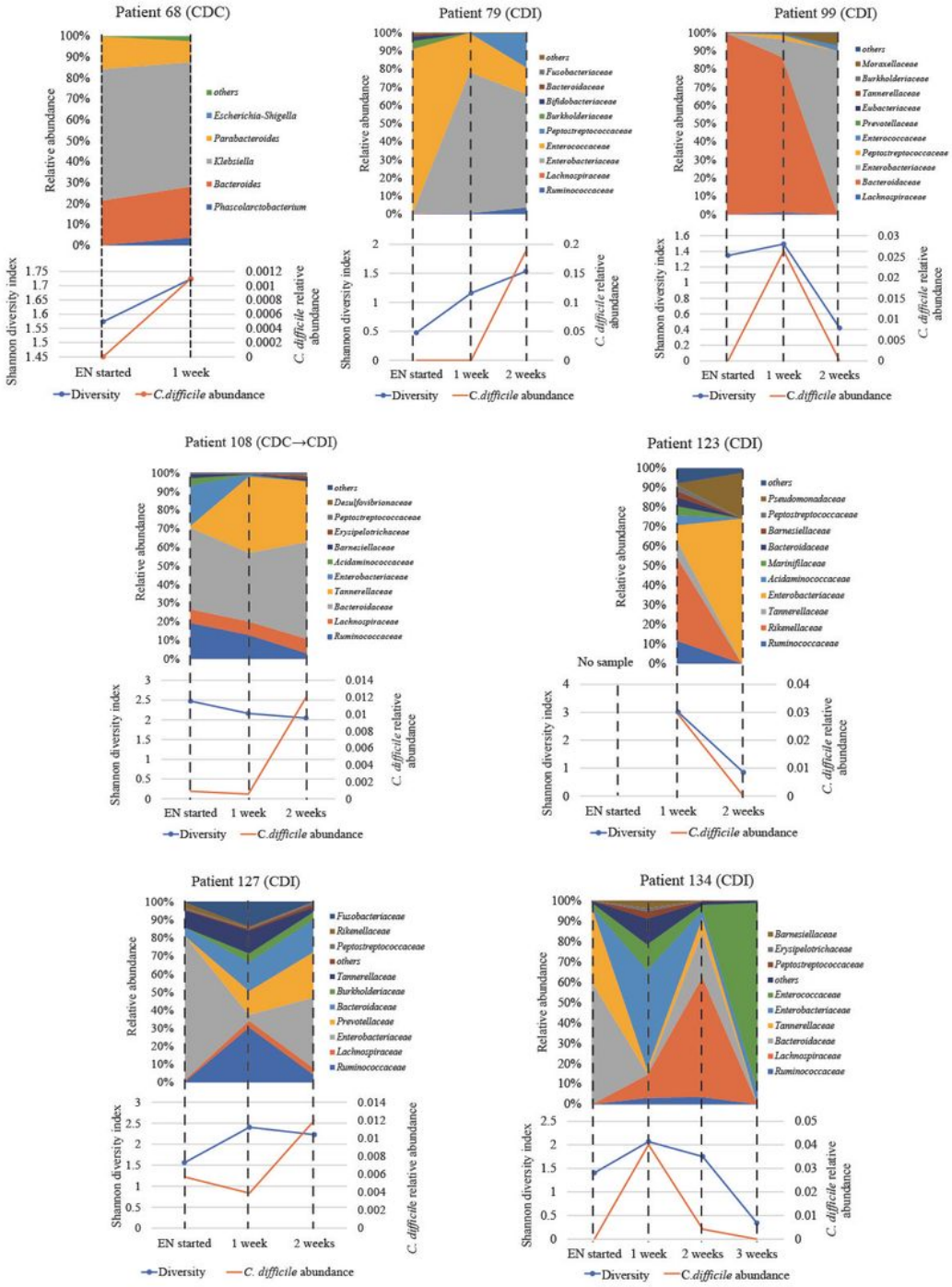


Figure 6

Intestinal microbiota dynamics in C. difficile positive (CDP) patients. For each panel, changes in microbial composition at the family level are illustrated on the above axis, corresponding to the alterations in the C. difficile load (right) and microbial diversity (left) on the same timeline shown below. (PDF 342KB)

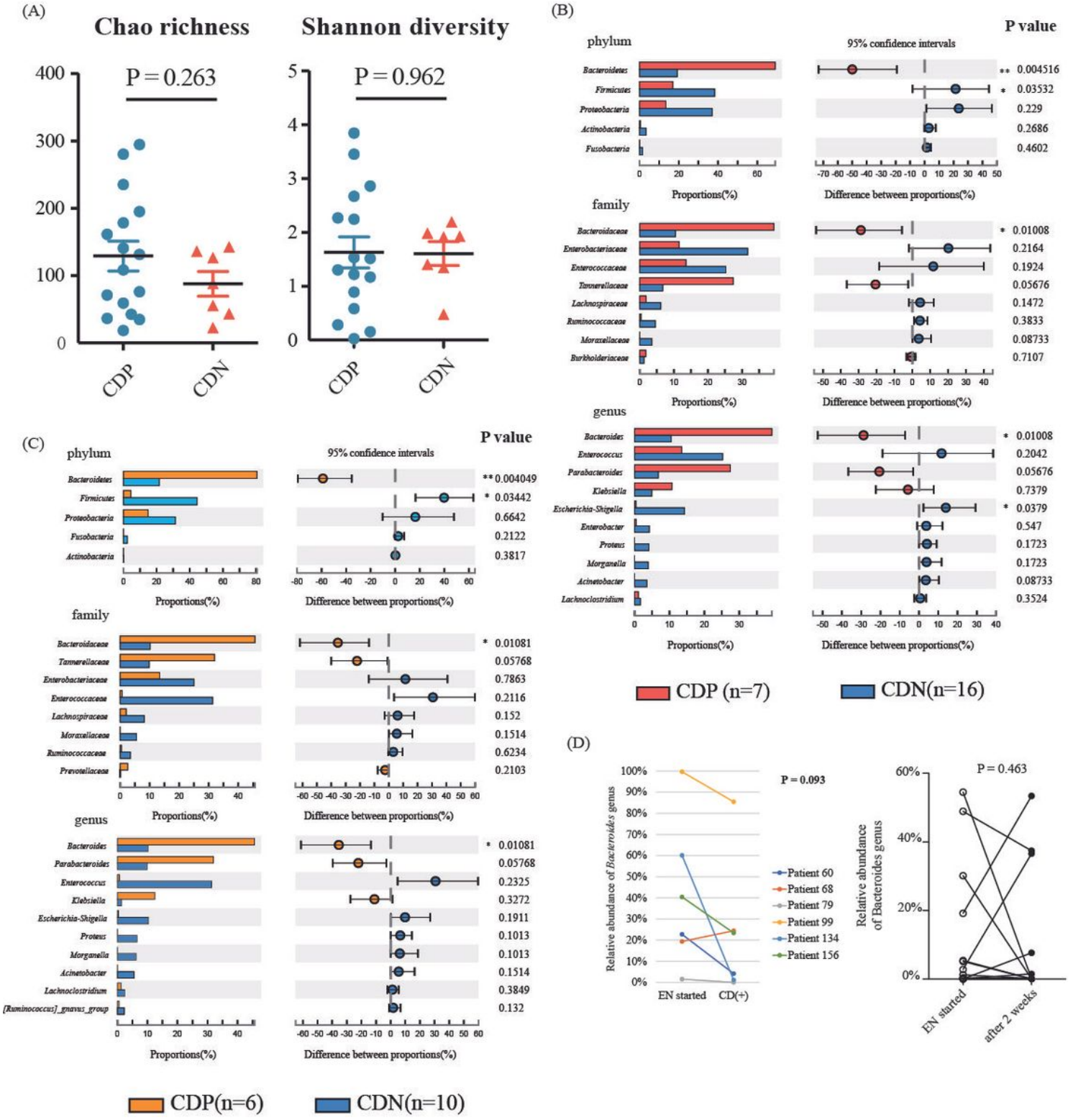


Figure 7

Role of Bacteroides in CDI (A) Differences of microbial richness and diversity between CDP (n=7) and CDN (n=16) patients at the beginning of EN, tested using Student's t-tests. (B) and (C) Wilcoxon rank sum tests were performed to analyze the differences between 7 CDP and 16 CDN patients (B) or those excluded because they were treated with metronidazole within 3 days of the start of EN (C). (D) Relative abundance of Bacteroides genus from the time EN started to the first presence of C. difficile for CDP patients (n=6; left), or from the time EN started to 2 weeks later for CDN patients (n=16; right). The differences in (A) and (B) were tested using Wilcoxon signed-rank test. *P<0.05, **P<0.01. (PDF 1156KB)

Supplementary Files

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