Microbiota diversity following antibiotic treatment of Clostridium difficile infection

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Research

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Abstract

Background: *Clostridium difficile* (*C. difficile*) is a major nosocomial pathogen that infects the human gut and can cause *C. difficile* infection (CDI), a diarrheal disease. A dominant risk factor is antibiotic treatment, which disrupts the normal gut microbiota. The aim of the study was to examine the correlation between antibiotic treatment received prior to CDI onset and patient gut microbiota during the infection.

Methods: Stool samples were collected from patients with CDI, presenting at the Baruch Padeh Medical Center Poriya, Israel. Demographic and clinical information, including previous antibiotic treatments, was collected from patient charts, and CDI severity score was calculated. Bacteria were isolated from stool samples, and gut microbiome was analyzed by sequencing the 16S rRNA gene, using the Illumina MiSeq platform and QIIME2.

Results: In total, 84 patients with CDI were enrolled in the study; all had received antibiotics prior to disease onset. Due to comorbidities, 46 patients (55%) received more than one class of antibiotics. The most common class of antibiotics used was cephalosporins (n=44 cases). The intestinal microbiota of the patients was not uniform. Differences in intestinal microbiome were influenced by the different numbers of antibiotics families that the patients received (p = 0.022)

Conclusions: The number of different antibiotics amount has a major impact on the gut microbiome of CDI patients, particularly on its bacterial richness.

Background

Clostridium difficile (*C. difficile*) is a Gram-positive, obligate anaerobic bacterium that is a member of the Firmicutes phylum. Its highly resistant spores survive on surfaces for long periods, rendering it highly transmissible from person to person. This occurs mainly in hospitalization facilities, categorizing *C. difficile* infection (CDI) as a nosocomial infection [1]. This bacterium can also asymptomatically colonize the gut, potentially leading to a “silent” onward transmission [2]. The major risk factor for CDI is antibiotic administration, which triggers diarrheal diseases, termed antibiotic-associated diarrhea [3]. Although nearly all antimicrobial classes have been associated with CDI, clindamycin, third-generation cephalosporins, fluoroquinolones, and penicillins are most commonly implicated [4].

The gut microbiome plays a central role in CDI. The human body is colonized by a large number of microorganisms, including bacteria, fungi, parasites, and viruses, together termed the human microbiome, whose composition is influenced by several factors, such as diet and host genetics [5]. Opportunistic pathogens are primarily blunted by activation of the immune system [6]. This mode of colonization resistance is altered by antibiotics; bacterial composition, richness, and diversity change (dysbiosis) several days after antibiotic administration, generating a convenient niche for spore germination, proliferation, and toxin production [4]. Indeed, data from human studies have shown that the presence of *C. difficile*, either as a colonizer or as a pathogen, is associated with reduced microbiota diversity. The various antibiotics families may have differential effects on the gut microbiota, thus varying in their

impact on predisposition for CDI. The current study examined the changes in the gut microbiota of patients with *C. difficile* infection on antibiotic treatment.

Results

Demographic and clinical profiles

Overall, 84 CDI patients, of an average age of 72.42 ± 16.74 years, were enrolled in this study (Table 1). Of the 84 patients, 41 (48.81%) were women. Seventeen patients (20.24%) died during hospitalization. More than half of the patients (55.95%) had a nosocomial CDI. Disease severity was calculated for 81 patients, as medical information of three ICU patients was inaccessible; 58 (71.6%) patients suffered from mild disease, 20 (24.7%) from moderate disease, and 3 (3.7%) from severe disease. All patients had received antibiotics prior to disease onset due to other illnesses; 38 patients (45.24%) received one class of antibiotics, 32 patients (38.1%) received two classes, 10 patients (11.9%) received three classes, and 4 patients (4.76%) received 4 classes.

Table 1
Demographic and baseline characteristics of *C. difficile* infection patients (N = 84)

Parameter	
Age (years), mean ± SD	72.42 ± 16.74
Gender, n (%)	41 (48.81)
Male	43 (51.19)
Female	
Infection acquisition, n (%)	47 (55.95)
Nosocomial	37 (44.05)
Community-acquired	
In-hospital mortality, n (%)	67 (79.76)
Alive	17 (20.24)
Died during hospitalization	
Disease severity*, n(%)	58 (71.6)
Mild	20 (24.7)
Moderate	3 (3.7)
Severe	
Number of antibiotic classes received before CDI onset, n(%)	38 (45.24)
1	32 (38.1)
2	10 (11.9)
3	4 (4.76)
4	
*Disease severity was calculated for 81 patients only.	

Antibiotics Received Before Cdi Onset

Twelve classes of antibiotics taken before CDI onset were recorded (Table 2). Antibiotics from the cephalosporin class were most widely used (n = 44), followed by penicillin (n = 32), which was often administered with other antibiotics (n = 24). Sulfa antibiotics were used in only 2 patients and tetracycline in one patient. In 18 cases, one antibiotic from the cephalosporin class was sufficient to trigger disease,

but CDI was often diagnosed after treatment with cephalosporin in combination with other antibiotics (n = 26).

Table 2
Antibiotics classes received by CDI patients*

Antibiotic Class	1 class of antibiotic N = 38	2 classes of antibiotic N = 32	3 classes of antibiotic N = 10	4 classes of antibiotic N = 4	Overall
Aminoglycoside	2	6	0	3	11
Carbapenem	2	4	3	0	9
Cephalosporin	18	17	7	2	44
Chloramphenicol	2	3	0	1	6
Glycopeptide	0	2	4	2	8
Lincosamides	4	1	1	1	7
Macrolide	1	1	4	0	6
Penicillin	8	16	6	2	32
Quinolone	1	6	2	2	11
Tetracycline	0	1	0	1	2
Trim/sulfa	0	1	0	0	1
Other	0	6	3	2	11
*Some patients received more than one antibiotic					

Microbiome Analysis

Microbiome analysis was performed on 67 out of 84 CDI patient samples (79.76%); 17 samples (20.24%) did not pass quality control. Proteobacteria was the dominant phylum in 50% of samples, followed by Bacteroidetes (20%), Firmicutes and Verrucomicrobia phyla (12%, each) (Fig. 1A). At the family level, *Enterobacteriaceae* and *Bacteroidaceae* each dominated 20% of the patient samples and *Verrucomicrobiaceae* dominated 11% of the samples. Other families were present in smaller percentages (Fig. 1B).

Calculation of beta-diversity in CDI patients who received different combinations of antibiotics (Fig. 2A) showed that only patients who had received four classes of antibiotics clustered significantly distant from the other groups ($p < 0.05$). Bacterial richness (alpha-diversity) negatively correlated with the number of antibiotics received (Fig. 2B). Bacterial richness among CDI patients who had received four classes of

antibiotics was the lowest and was significantly different from the other groups ((*$p = 0.03$, **[1]$p=0.007$, **[2]$p=0.005$).).

Discussion

The current study examined the correlation between gut bacterial composition of CDI patients and antibiotic treatment received prior to infection onset. The epidemiological data of the study population was in correlation with the known characteristics of CDI patients, i.e., older age and high mortality rate. Additionally, the percentages of nosocomial and community-acquired cases were similar to earlier reports [17]. Recently, the prevalence of community-acquired infections has increased due to elevated use of antibiotics that were previously only administered in hospitals via intravenous infusion [17].

Most patients were diagnosed with mild disease, while only a few were diagnosed with severe disease. When compared to a study conducted in 2016 in northern Israel, which found that most patients had mild disease, a few had moderate disease, and none were diagnosed with severe disease, the current analysis suggests an increase in the prevalence of moderate-severe CDI [18]. This increase in disease severity can be attributed to an increase in antibiotic resistance or emergence of more virulent strains [1].

The majority of patients received one or two classes of antibiotics prior to CDI onset, corresponding with previous reports demonstrating that one type of antibiotic is sufficient to induce CDI [4]. Cephalosporins and penicillins were the most commonly used antibiotics, two drugs which have previously been shown to significantly increase the risk of CDI as compared to other antibiotics [4, 19–20]. Fluoroquinolone and clindamycin use has also been highly correlated with CDI development, yet, in our study, only a small percentage of patients received these antibiotics.

Examination of intestinal bacterial populations of CDI patients and their correlation with previous antibiotic treatment, showed that there was no phylum- and family-level composition common to all CDI patients, as has been described in other studies [21–23]. In their study comparing the gut microbiome profile of CDI versus non-CDI patients, Manges et al. found an increase in Firmicutes, Proteobacteria and Actinobacteria phyla, as well as a decrease in Bacteroidetes [24]. Antharam et al., surveying the distal gut microbiota of individuals with CDI, found that these patients had significantly less diverse communities, particularly a less diverse Firmicutes population than patients with non-CD diarrhea or healthy controls [21]. In addition, there was depletion of gut commensals such as the *Ruminococcaceae* and *Lachnospiraceae* families and butyrate-producing anaerobic fermenters. This interpatient variability can be explained by the various factors affecting the intestinal bacteria, such as nutrition [25], although we tried to control for these factors during data analysis. More specifically, several parameters (such as age, gender, and disease severity) were tested; none of which had significant effects on bacterial population. In contrast, we found that the antibiotic combinations administered to CDI patients before disease onset correlated with the intestinal microbiota. Patients who had received four classes of antibiotics had more similar microbiomes. In addition, an inverse correlation between bacterial richness and the number of antibiotics received was noted, with significant differences between patients who received four classes of

antibiotics versus those who received one or two classes of antibiotics. These findings can likely be ascribed to the broader range of bacterial species targeted by multi-class antibiotic treatment regimens, which subsequently leads to reduced microbiota richness. A limitation of this study was the lack of a comparison to the gut microbiome of healthy individuals, due to the difficulty in finding healthy elderly controls. Such a comparison could have identified specific gut bacteria absent in CDI patients, that may have the potential to prevent *C. difficile* colonization.

Conclusions

In conclusion, no uniform microbiome profile was observed among the tested CDI patients. Antibiotics were the main cause for disease onset, with all CDI patients in the study having received antibiotics prior to onset. The greatest influence of antibiotic treatment on the gut microbiome was observed in patients who received four different antibiotics classes; these patients demonstrated significantly lower microbial richness and diversity compared to patients who received fewer than four different antibiotics classes.

Methods

Study population

The study population consisted of adults diagnosed with CDI at the Baruch Padeh Medical Center Poriya, Israel. Patients or a legal guardian signed a consent form. Pregnant women and patients suffering from mental illness were not eligible to participate in the study. The study was approved by the Helsinki Committee of the Medical Center, approval No. 0003–15 POR.

Sample collection

C. difficile identification in stool samples was performed at the medical center's clinical microbiology laboratory, as part of the routine CDI screening procedures. A polymerase chain reaction (PCR) test was performed using the GeneXpert *C. difficile* PCR assay (Cepheid, Sunnyvale, CA, USA), to identify toxin B, binary toxin, and *tcdC* deletion.

Severity score calculation

To classify CDI severity, the severity score index (SSI) was calculated according to the "Score indices for *Clostridium difficile* infection severity" [7], which incorporates nine parameters that are associated with increased CDI morbidity and mortality. One point is given for each of the following parameters: altered mental status, abdominal pain or distention, white blood cell (WBC) count below 1500 or above 20,000 cells per cubic meter, hypoalbuminemia (< 2.5 mg/dL albumin, ALB), ascites or colitis (imaging), mean arterial pressure (MAP) < 65 mmHg, tachycardia ≥ 110 beats/min, intensive care unit (ICU) transfer. A score of 0–3 reflects mild disease, 4–6 moderate disease, and ≥ 7 severe disease.

The following demographic and clinical data were collected from patient medical records: age, gender, community- versus hospital-acquired CDI, death during hospitalization, and laboratory test (leukocyte

count, serum albumin, C-reactive protein (CRP)) results.

Gut microbiome

DNA extraction, amplification and sequencing: DNA was extracted from 0.25 ml liquid fecal samples using the Power Soil DNA Isolation Kit (MoBio, Carlsbad, USA), according to the manufacturer's instructions and following a 2-min bead-beating step (Biospec) [8]. DNA was stored at -20 °C until use.

The V4 region of the bacterial 16S rRNA gene was amplified using the 515F and 806R barcoded primers, as per the Earth Microbiome Project protocol [9]. PCR reaction conditions included an initial denaturing step for 3 min at 95 °C, followed by 30 cycles of denaturation (98 °C, 10 sec), annealing (55 °C, 5 sec), and extension (72 °C, 20 sec), with a final elongation at 72 °C (1 min). PCR products were purified using AMPure magnetic beads (Beckman Coulter, Florida, USA) [10], and quantified using the Qubit dsDNA HS Assay (Thermo Fisher, Bartlesville, USA) [11]. Samples were then pooled to equal concentrations (50 ng/µl) and purified again using 2% E-Gel agarose inserted in an E-Gel PowerBase device (Invitrogen, Carlsbad, USA). Then, DNA fragments were purified from the agarose using the NucleoSpin® Gel and PCR Clean-up kit (Macherey-Nagel, Düren, Germany), and sequenced using the Illumina MiSeq platform, at the Genomic Center, Faculty of Medicine, Bar-Ilan University, Israel.

Analysis: Data analysis was performed using QIIME2 [12]. Sequence reads were demultiplexed by per-sample barcodes and Illumina-sequenced amplicon read errors were corrected by the Divisive Amplicon Denoising Algorithm (DADA2) [13]. A phylogenetic tree was generated and taxonomy was classified using the Greengenes reference database at a confidence threshold of 99% [14]. Alpha and beta diversities were calculated based on a feature table containing features observed in at least 40 samples (50%) and on samples containing at least 8000 sequences. Richness was calculated using Faith's Phylogenetic Diversity, a qualitative measure of community richness that incorporates phylogenetic relationships between taxa [15]. Beta diversity was analyzed using principal coordinate analysis (PCoA) based on weighted UniFrac distance matrices [16].

Statistical analysis

In Faith's phylogenetic diversity measure, differences between groups were analyzed by the Kruskal-Wallis (pairwise) test. Differences between weighted UniFrac distances were analyzed by pairwise Permanova test. Statistical significance was defined as $p < 0.05$.

Abbreviations

C. difficile- *Clostridium difficile*

CDI- *C. difficile* infection

PCR- Polymerase chain reaction

SSI- Severity score index

WBC- White blood cell

MAP- Mean arterial pressure

ICU- Intensive care unit

CRP- C-reactive protein

DADA2- Divisive Amplicon Denoising Algorithm

PCoA- principal coordinate analysis

Declarations

Ethics approval and consent to participate

The study was approved by the Helsinki Committee of the Medical Center, approval No. 0003-15 POR.

Consent for publication

Not applicable

Availability of data and materials

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

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

DN, MA, OK and AP designed the study, analyzed, interpreted the data, and wrote the final manuscript. ZH and ON were involved in development of the protocols and contributed to the biobank sample or data

collection. All authors read and approved the final manuscript.

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Not applicable

References

1. Xia J, Gao J, Tang W. Nosocomial infection and its molecular mechanisms of antibiotic resistance. Biosci Trends. 2016;10:14–21.
2. Clabots CR, Johnson S, Olson MM, Peterson LR, Gerding DN. Acquisition of *Clostridium difficile* by hospitalized patients: evidence for colonized new admissions as a source of infection. J Infect Dis. 1992;166:561–7.
3. Högenauer C, Hammer HF, Krejs GJ, Reisinger EC. Mechanisms and management of antibiotic-associated diarrhea. Clin Infect Dis. 1998;27:702–10.
4. Owens RC Jr, Donskey CJ, Gaynes RP, Loo VG, Muto CA. Antimicrobial-associated risk factors for *Clostridium difficile* infection. Clin Infect Dis. 2008;46(S1):19–31.
5. Human Microbiome Project Consortium. Structure, function and diversity of the healthy human microbiome. Nature. 2012;486:207–14.
6. Monaghan TM. New perspectives in *Clostridium difficile* disease pathogenesis. Infect Dis Clin. 2015;29:1–11.
7. Velazquez-Gomez I, Rocha-Rodriguez R, Toro DH, Gutierrez-Nuñez JJ, Gonzalez G, Saavedra S. A severity score index for *Clostridium difficile* infection. Infect Dis Clin Pract. 2008;16:376–8.
8. Santos SS, Nielsen TK, Hansen LH, Winding A. Comparison of three DNA extraction methods for recovery of soil protist DNA. J Microbiol Methods. 2015;115:13–9.
9. Wu L, Wen C, Qin Y, Yin H, Tu Q, Van Nostrand JD, et al. Phasing amplicon sequencing on Illumina Miseq for robust environmental microbial community analysis. BMC Microbiol. 2015;15:125.
10. Meyer M, Kircher M. Illumina sequencing library preparation for highly multiplexed target capture and sequencing. Cold Spring Harb Protoc. 2010;2010:pdb–prot5448.
11. Nakayama Y, Yamaguchi H, Einaga N, Esumi M. Pitfalls of DNA quantification using DNA-binding fluorescent dyes and suggested solutions. PLoS One. 2016;1:e0150528.
12. Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK, et al. QIIME allows analysis of high-throughput community sequencing data. Nat Methods. 2010;7:335–6.
13. 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.
14. DeSantis TZ, Hugenholtz P, Larsen N, Rojas M, Brodie EL, Keller K, et al. Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB. Appl Environ Microbiol. 2006;72:5069–72.

15. Faith DP. Conservation evaluation and phylogenetic diversity. Biol Conserv. 1992;61:1–10.
16. Lozupone C, Knight R. UniFrac: a new phylogenetic method for comparing microbial communities. Appl Environ Microbiol. 2005;71:8228–35.
17. Warriner K, Xu C, Habash M, Sultan S, Weese SJ. Dissemination of *Clostridium difficile* in food and the environment: Significant sources of *C. difficile* community-acquired infection? J Appl Microbiol. 2017;122:542–53.
18. Peretz A, Tkhawkho L, Pastukh N, Brodsky D, Halevi CN, Nitzan O. Correlation between fecal calprotectin levels, disease severity and the hypervirulent ribotype 027 strain in patients with *Clostridium difficile* infection. BMC Infect Dis. 2016;16:309.
19. Debast SB, Vaessen N, Choudry A, Wiegers-Ligtvoet EA, van den Berg RJ, Kuijper EJ. Successful combat of an outbreak due to *Clostridium difficile* PCR ribotype 027 and recognition of specific risk factors. Clin Microbiol Infect. 2009;15:427–34.
20. Goudarzi M, Seyedjavadi SS, Goudarzi H, Mehdizadeh Aghdam E, Nazeri S. *Clostridium difficile* infection: epidemiology, pathogenesis, risk factors, and therapeutic options. Scientifica (Cairo). 2014;2014:916826.
21. Antharam VC, Li EC, Ishmael A, Sharma A, Mai V, Rand KH, et al. Intestinal dysbiosis and depletion of butyrogenic bacteria in *Clostridium difficile* infection and nosocomial diarrhea. J Clin Microbiol. 2013;51:2884–92.
22. Pakpour S, Bhanvadia A, Zhu R, Amarnani A, Gibbons SM, Gurry T, et al. Identifying predictive features of *Clostridium difficile* infection recurrence before, during, and after primary antibiotic treatment. Microbiome. 2017;5:148.
23. Schneider D, Thürmer A, Gollnow K, Lugert R, Gunka K, Grob U, et al. Gut bacterial communities of diarrheic patients with indications of *Clostridioides difficile* infection. Sci Data. 2017;4:170152.
24. Manges AR, Labbe A, Loo VG, Atherton JK, Behr MA, Masson L, et al. Comparative metagenomic study of alterations to the intestinal microbiota and risk of nosocomial Clostridum difficile-associated disease. Infect Dis. 2010;202:1877–84.
25. Baines SD, Wilcox MH. Antimicrobial resistance and reduced susceptibility in Clostridium difficile: potential consequences for induction, treatment, and recurrence of C. difficile infection. Antibiotics. 2015;4:267–98.

Figures

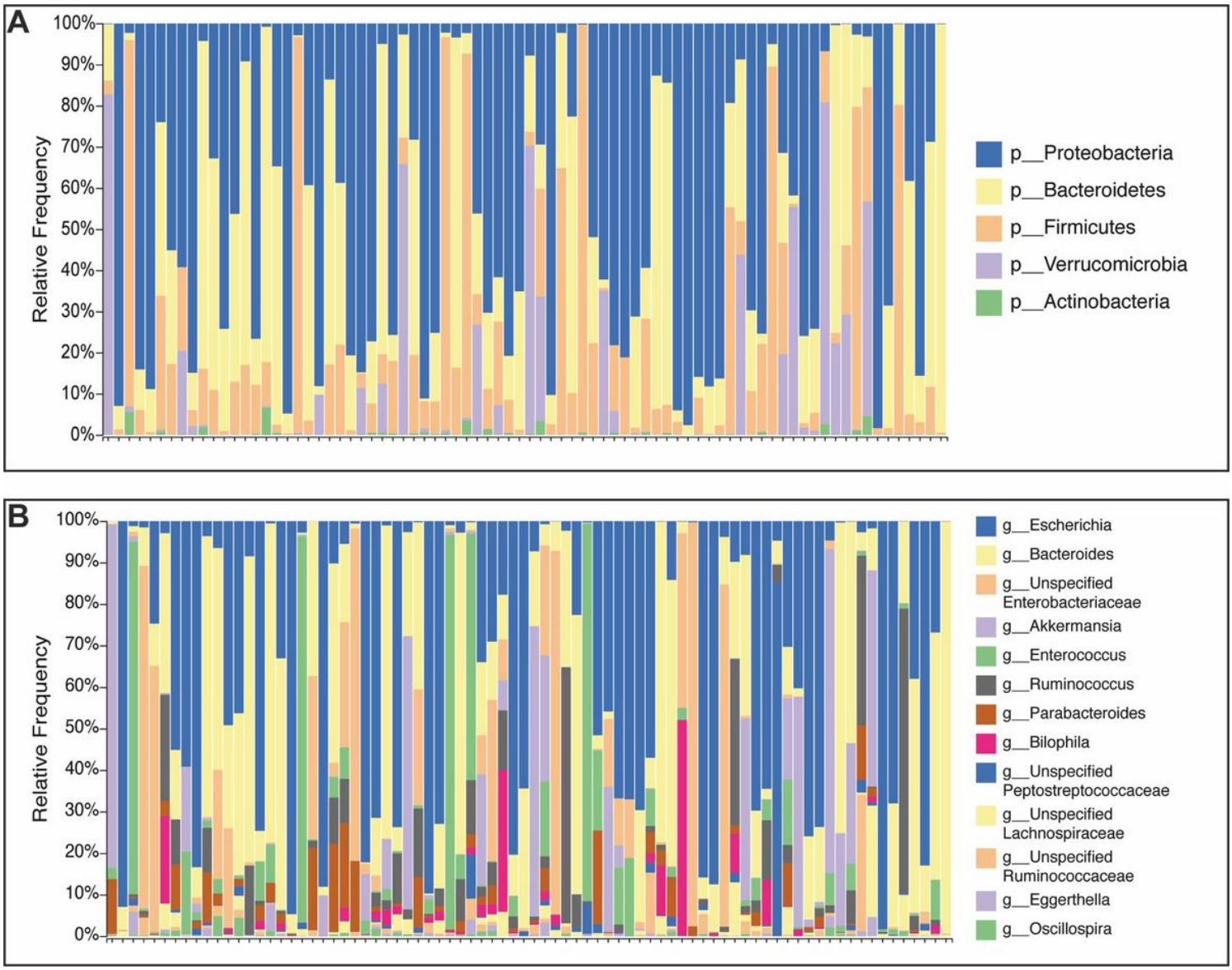

Figure 1

Microbial community structure of C. difficile infection (CDI) patients. The gut microbiota composition of 67 CDI patients. Taxonomy plot at (A) phylum and (B) family levels.

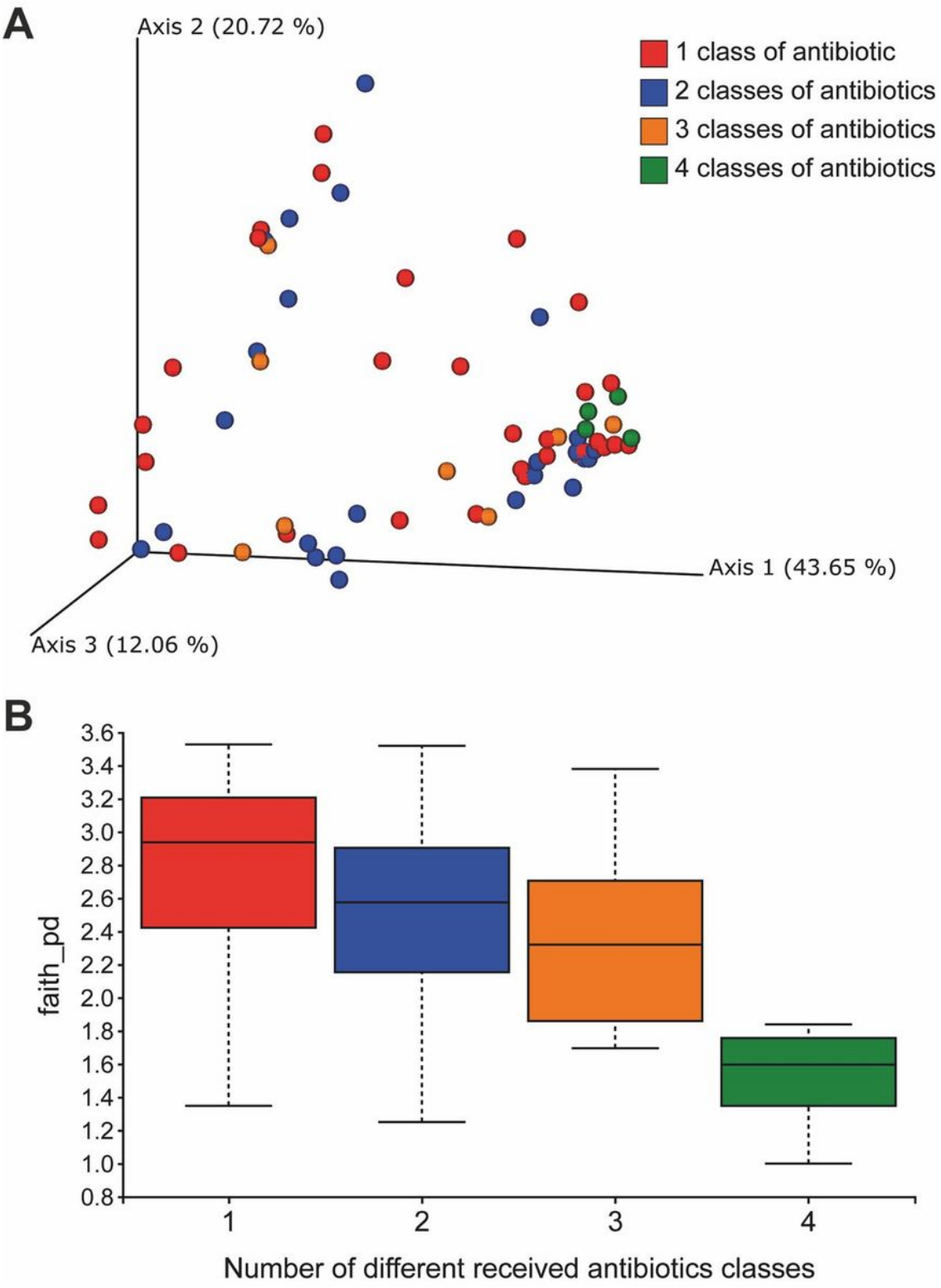

Figure 2

Microbial diversity of C. difficile infection patients, by combinations of antibiotics received prior to CDI onset. CDI patients were divided to four groups according to the number of classes of antibiotics received prior to onset of infection: 1 (n=30), 2 (n=24), 3 (n=9), and 4 (n=4) classes of antibiotics. (A) Beta diversity (between samples) using weighted UniFrac, (B) Alpha diversity (within samples) using Faith's Phylogenetic Diversity (*p = 0.022).