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# Impacts of infection with different toxigenic *Clostridium difficile* strains on faecal microbiota in children

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Increasing evidence suggests that altered intestinal microbial composition and function result in an increased risk of *Clostridium difficile*-associated diarrhoea (CDAD); however, the specific changes of intestinal microbiota in children suffering from CDAD and their associations with *C. difficile* strain toxigenicity are poorly understood. High-throughput pyrosequencing showed that reduced faecal bacterial diversity and dramatic shifts of microbial composition were found in children with CDAD. The Firmicutes/Bacteroidetes ratio was increased significantly in patients with CDAD, which indicated that dysbiosis of faecal microbiota was closely associated with CDAD. *C. difficile* infection resulted in an increase in lactate-producing phylotypes, with a corresponding decrease in butyrate-producing bacteria. The decrease in butyrate and lactate buildup impaired intestinal colonisation resistance, which increased the susceptibility to *C. difficile* colonisation. Strains of *C. difficile* which were positive for both toxin A and toxin B reduced faecal bacterial diversity to a greater degree than strains that were only toxin B-positive, and were associated with unusually abundant *Enterococcus*, which implies that the *C. difficile* toxins have different impacts on the faecal microbiota of children. Greater understanding of the relationships between disruption of the normal faecal microbiota and colonisation with *C. difficile* that produces different toxins might lead to improved treatment.

*Clostridium difficile*-associated diarrhoea (CDAD) is the leading cause of antibiotic-induced diarrhoea and colitis in hospitalised patients. In serving as a source of disease transmission or recurrence, *C. difficile* produces highly resistant and transmissible spores; these can colonize and germinate after antibiotic treatment, thus leading to *C. difficile* infection (CDI)<sup>1</sup>. Generally, infants and children have been considered to be at low risk of CDI<sup>2</sup>; however, recent population-based studies have demonstrated that CDI has become a common cause of diarrhoea in infants and children<sup>3,4</sup>. Rousseau *et al.* provided information that carriage of *C. difficile* by healthy infants represents a potential reservoir of pathogenic strains for susceptible adults<sup>5</sup>. The emergence of the epidemic strains with increased virulence (hypervirulent strains) and more antibiotic resistance, such as 027/BI/NAP1, may have changed the epidemiology in children<sup>6,7</sup>. Because of the virulence, spore-forming ability and persistence of the bacterium, its incidence, recurrence, and all-cause mortality rates have increased markedly in children during the past decade. These changes have increased the economic burden associated with these patients, and CDI has become an increasing public health problem<sup>3,8</sup>.

The human intestinal tract harbours a complex community of microorganisms, which play vital roles in maintaining host-microbe homeostasis and inhibiting pathogen colonisation and overgrowth (i.e., colonisation resistance). These processes are attributable to the existence of a stable and diverse population of resident microorganisms that compete with an invading pathogen directly for niches and nutrients or through production of antibacterial substances<sup>9,10</sup>. However, the equilibrium of the intestinal microbiota is seriously disturbed during the onset of CDAD. This disturbance is characterised by markedly decreased diversity, which breaks down colonisation resistance against *C. difficile* and allows the overgrowth of *C. difficile* derived from environmental spores or endogenous strains<sup>11-14</sup>. A previous study has shown that increased Firmicutes and decreased



Bacteroidetes in the intestinal microbiota are associated with CDI in elderly patients<sup>15</sup>. The mechanisms of CDAD development after antibiotic administration are still unclear. However, the restoration of both the structure and the function of the intestinal microbiota with faecal microbiota transplantation (FMT) has been utilised as an effective therapy for CDAD<sup>16–19</sup>. This may be associated with the reestablishment of colonisation resistance after FMT, which suppresses *C. difficile* to low levels or even eradicates the pathogen completely. All the studies referenced have verified the existence of dysbiosis of the intestinal microbiota in patients with CDAD. Therefore, therapeutic restoration of the altered intestinal microbiota may be an ideal treatment option for patients with CDI.

Toxigenic strains of *C. difficile* usually produce two key virulence determinants, toxin A and toxin B, which can evoke severe colonic inflammation and cause extensive damage to epithelial tissues in infected patients<sup>20</sup>. Early research considered toxin A to be the major virulence factor; however, recent studies have confirmed that toxin B is more potent than toxin A in damaging human intestinal epithelium<sup>21,22</sup>. Previous studies have demonstrated that either toxin A or toxin B alone can cause fulminant disease in experimental animal infections<sup>22,23</sup>, while the presence of both toxins is linked to more severe disease outcomes<sup>24,25</sup>. Clinical diagnosis of CDI requires identification of *C. difficile* toxin A or B in diarrhoeal stool. In contrast to *C. difficile* ribotyping, toxigenic typing with multiplex PCR has shown that most pathogenic strains are toxin A-positive, toxin B-positive (A+B+) strains, or toxin A-negative, toxin B-positive (A-B+) variant strains. Toxin A-positive, toxin B-negative (A+B-) strains are not found naturally in clinical isolates<sup>22,26,27</sup>. The epidemiology, clinical manifestations, therapy and outcome of CDAD are also highly dependent on the toxigenic type of *C. difficile*, which has been found to be an important predisposing factor in the onset of the disease<sup>22</sup>. Early studies found a marked reduction in the microbial diversity of the intestinal tract in elderly patients with CDAD<sup>11–14</sup>; however, the impact of the different toxigenic types of *C. difficile* on the intestinal microbiota in children remains unclear. Given the importance of the *C. difficile* toxigenic typing in clinical practice, we hypothesised that the alterations of the intestinal microbiota in children with CDI were associated with infection with the different toxigenic types of *C. difficile*. Therefore, the focus of the present study was to investigate the alteration of the faecal microbiota in children with CDAD and its associations with different *C. difficile* toxigenic types. To do so, we used massively parallel barcoded 454 pyrosequencing targeting of the 16S rRNA gene V1–V3 hypervariable regions. The results will provide new insights into the microecological divergence of the intestinal microbiota after infection with different toxigenic *C. difficile*.

## Results

### Characteristics of the participants and pyrosequencing data.

Following toxigenic typing of *C. difficile* using multiplex PCR, 37 children with CDAD were enrolled in the study, including 14 children suffering from CDAD with *C. difficile* that produced toxin

A and toxin B (CDAD A+B+) and 23 children with *C. difficile* that produced only toxin B (CDAD A-B+); 43 children were recruited as healthy controls. The ages of both patients and healthy control subjects ranged from 28 to 48 months. Most of the patients with CDAD had been given one or more antibiotics, such as cephalosporins, amoxicillin, azithromycin, sulfonamides and vancomycin, within the 3 months prior to sampling. The frequency of diarrhoea was not significantly different between the CDAD A+B+ and CDAD A-B+ groups ( $p > 0.05$ ).

The pyrosequencing studies provided 952,526 raw sequences with a median read length of 457 base pairs (range from 221 to 540). Following quality trimming and chimera checking, 705,357 high-quality reads remained, accounting for 74.1% of the valid reads with an average of 8,817 reads (range from 6,086 to 23,593) per barcoded sample recovered for downstream analysis. The summary information is shown in Table 1, and detailed characteristics of each sample are shown in Table S1. The total number of unique sequences from the three groups was 11,828, and represented all phylotypes. The values of Good's coverage were nearly 99.0% for all sequences in the three groups, indicating sufficient sequencing depth for the investigation of CDAD-associated faecal microbiota in children.

### Decreased bacterial diversity of the faecal microbiota associated with CDAD.

Both Shannon and Simpson indices demonstrated that the faecal microbiota diversity in children with CDAD was significantly lower than that in healthy controls ( $p < 0.01$ ). Unexpectedly, the bacterial diversity in patients with CDAD A-B+ was significantly higher than that in those with CDAD A+B+ ( $p < 0.05$ , Figure 1A and 1B). Noticeably, the richness indices in patients with CDAD were significantly lower than those in the healthy control subjects ( $p < 0.01$ ). In contrast to the diversity indices, however, there were no significant differences in the richness indices between the two CDAD groups. The rarefaction curves also showed that the species richness of the faecal microbiota in children with CDAD was lower than that in healthy controls, although the shape of the curve revealed that the total richness of the microbial community had not been sampled completely (Figure 1C and S1). Figure 1D shows a long tail in the rank abundance curves, indicating that the majority of operational taxonomic units (OTUs) were present at low abundance in the faecal microbiota from children. To better understand the shared richness among the three groups, a Venn diagram displaying the overlaps between groups was developed. This analysis showed that only 168 of the total richness of 11,828 OTUs were shared among all the samples, while 762 of 4,785 OTUs were shared between the samples in the two CDAD groups (Figure 1E). These data demonstrated that more than one third of the observed OTUs in the healthy controls were undetected in the CDAD groups. To measure the extent of the similarity between microbial communities, beta diversity was calculated using unweighted UniFrac, and principal coordinate analysis was performed. Despite significant inter-individual variation, the faecal microbiota from children with CDAD and healthy controls could be divided into

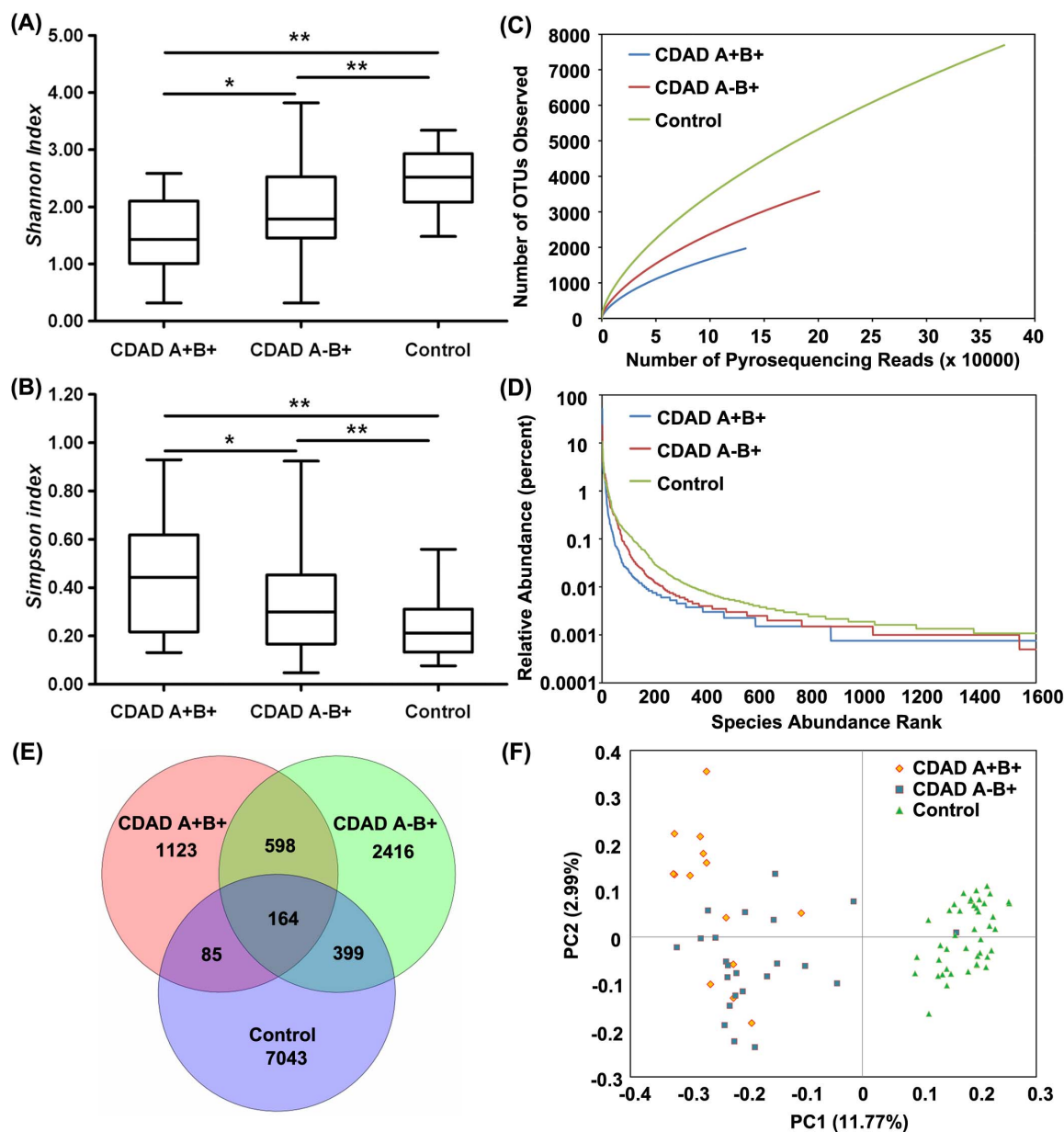
**Table 1** | Comparison of phylotype coverage and diversity estimation of the 16S rRNA gene libraries at 97% similarity from the pyrosequencing analysis

Group	No. of reads	No. of OTUs <sup>1</sup>	Good's(%) <sup>2</sup>	Richness estimator				Diversity index		
				ACE	95%CI	Chao 1	95%CI	Shannon	Simpson	Evenness <sup>3</sup>
CDAD A+B+	132856	1970	99.15%	6463	6136.5–6815.2	4244	3888.4–4666.0	2.585089	0.277023	0.001832
CDAD A-B+	200761	3577	98.98%	10972	10549.6–11419.4	7418	6966.7–7928.4	3.880802	0.072468	0.003858
Control	371740	7691	98.79%	25727	25042.2–26438.2	17191	16405.2–18047.4	4.631818	0.032787	0.003966

<sup>1</sup>The operational taxonomic units (OTUs) were defined at the 97% similarity level.

<sup>2</sup>The coverage percentage (Good's), richness estimators (ACE and Chao1) and diversity indices (Shannon and Simpson) were calculated using Good's method and the mothur program, respectively.

<sup>3</sup>The Shannon index of evenness was calculated with the formula  $E = H/\ln(S)$ , where H is the Shannon diversity index and S is the total number of sequences in that group.

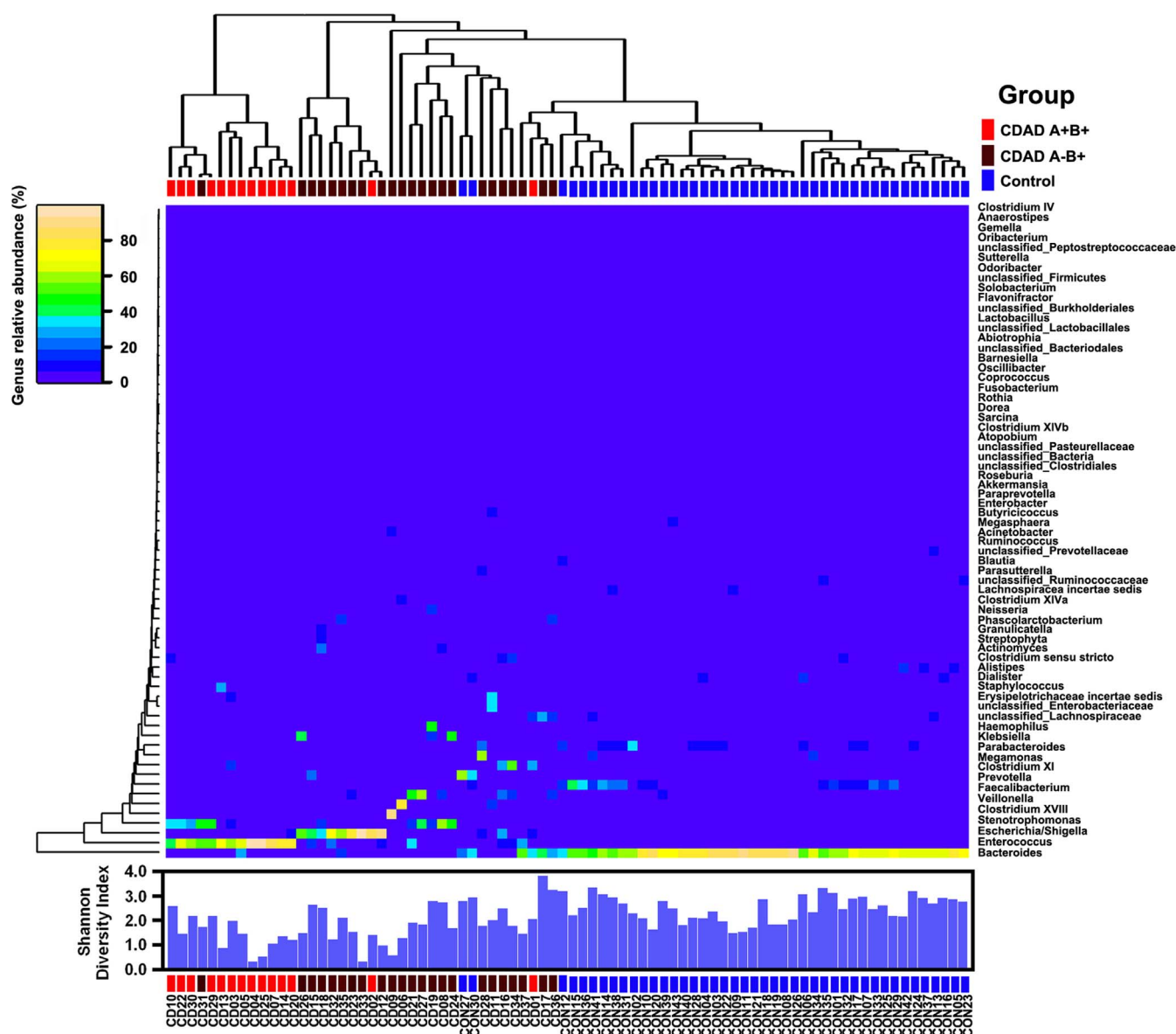


**Figure 1 | Structural comparison of faecal microbiota among healthy controls, CDAD A+B+ and CDAD A-B+ groups.** The Shannon (A) and Simpson (B) indices were used to estimate diversity (i.e., a combined assessment of the number of 97% similar bacterial taxa and their abundance) of the faecal microbiota in children (data shown as mean with SEM). Rarefaction curves were used to estimate richness (at a 97% similarity level) of children's faecal microbiota among the three groups (C). The vertical axis shows the number of OTUs that would be expected to be found after sampling the number of tags or sequences shown on the horizontal axis. Rank abundance curve of bacterial OTUs derived from the three groups (D). Principal coordinate analysis plot of the faecal microbiota based on the unweighted UniFrac metric (E). Venn diagram illustrating overlap of OTUs in faecal microbiota among the three groups of children (F).

two different clusters according to the community composition using unweighted UniFrac metrics (Figure S2), and could be separated clearly by principal coordinates analysis (Figure 1F). However, two CDAD samples clustered with healthy controls. In addition, children with CDAD A+B+ could also be separated from those with CDAD A-B+, although several CDAD A+B+ samples were clustered with the CDAD A-B+ group.

**CDAD-associated alterations in faecal microbiota.** The taxonomy of the faecal microbiota was assessed by a taxon-dependent analysis using the RDP classifier. Nineteen phyla, including three candidate divisions (SR1, TM7 and OD1), were found in the faecal microbiota of all samples, including 10 phyla in the healthy controls, 14 phyla in

the CDAD A+B+ samples and 17 phyla in the CDAD A-B+ samples. In total, sequences from the faecal microbiota could be classified into 342 genera, with 140 genera in healthy controls, 211 genera in CDAD A+B+ samples and 225 genera in CDAD A-B+ samples. Consistent with our rank abundance curves, the majority of the genera were present at low abundance in the faecal microbiota samples with the greater sequencing depth. Of the total number of genera identified in the faecal microbiota, 10 abundant genera (> 1% of the total DNA sequences) were detected in healthy controls, while eight and 16 abundant genera were detected in CDAD A+B+ and CDAD A-B+ samples, respectively. Figure 2 represents a heatmap showing the correlations between *C. difficile* toxin types and the abundances of selected genera. The heatmap showed the genus-



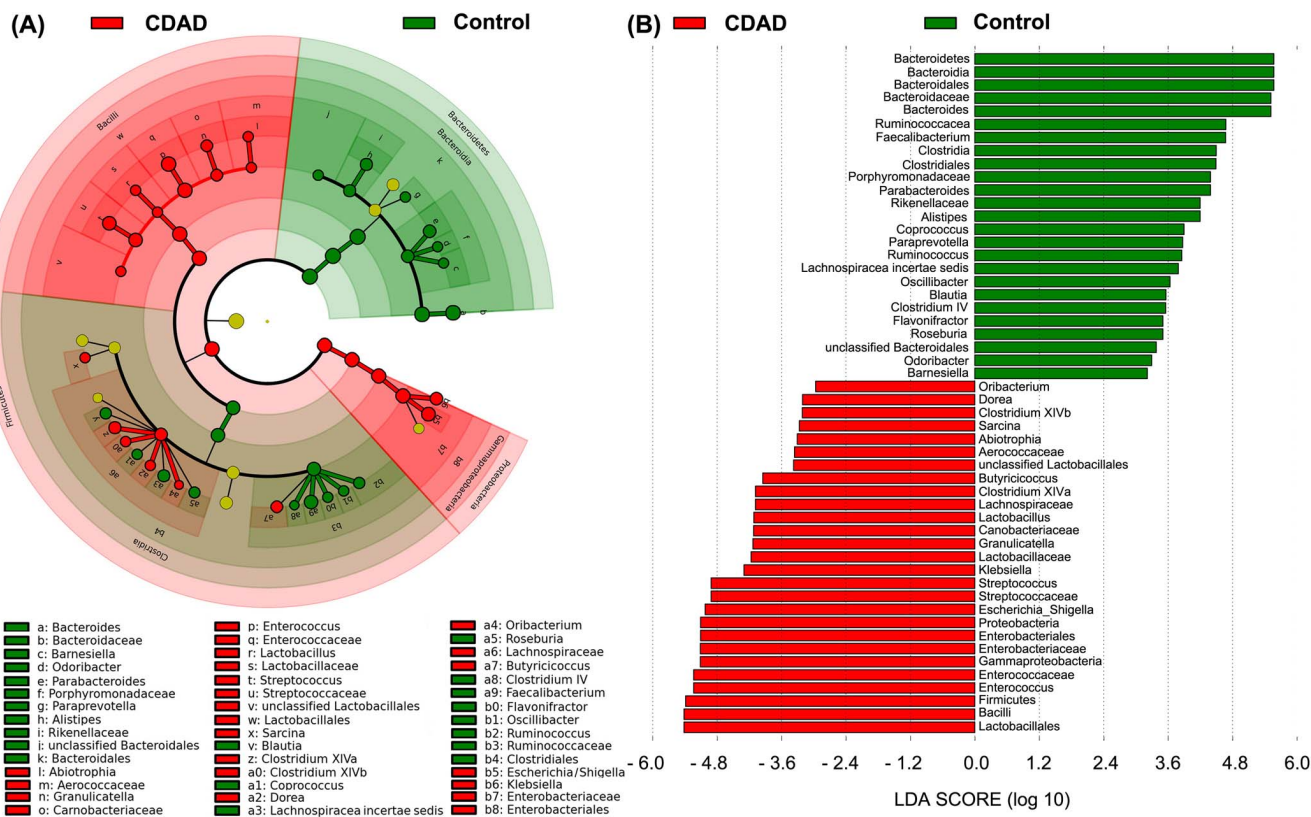
**Figure 2** | Heatmap indicating genus-level changes among healthy controls, CDAD A+B+ and CDAD A-B+ groups. Legends above and below the heatmap represent each participant. The relative abundance of each genus is indicated by a gradient of color from green (low abundance) to yellow (high abundance). Complete linkage clustering of samples was based on the genus composition and abundance of faecal microbiota in children. Distinctive bacterial composition was found in the faecal microbiota of children with or without CDAD, which was also significantly associated with the Shannon index.

level clustering according to frequency within each sample; abundant genera were colour coded yellow, and green colour coding indicated missing genera. Consistent with alpha diversity indices such as the Shannon index and beta diversity metrics, clustering analysis of these genera highlighted the apparent differences in their distributions according to CDI and its toxin types. Clearly, these aberrant compositions of the faecal microbiota were associated with the *C. difficile* toxin types in the CDAD samples.

To identify the specific bacterial taxa associated with CDI, we compared the faecal microbiota in healthy controls and children with CDAD using LEfSe. A cladogram representative of the structure of the faecal microbiota and their predominant bacteria is shown in Figure 3; the greatest differences in taxa between the two communities are displayed. The data indicated that intestinal dysbiosis was extensive in the children with CDAD, and several genera belonging to the three predominant phyla could be used as distinguishing biomarkers. The changes in the faecal microbiota after CDI were also

explored using the Mann-Whitney U test at different taxon levels. At the phylum level, Firmicutes, Bacteroidetes and Proteobacteria were the most predominant phyla in the children's faecal samples. The ratio between Firmicutes and Bacteroidetes was lower in healthy controls ( $0.312 \pm 0.234$ ) and was increased significantly after CDI ( $> 1.000$ ). Despite high inter-individual variability, Firmicutes, Proteobacteria, Actinobacteria and Acidobacteria were significantly less abundant in the faecal microbiota of healthy controls compared to those of patients with CDAD, while Bacteroidetes was significantly more abundant in the faecal microbiota of healthy controls ( $p < 0.05$ , Figure S3A). Compared to healthy controls, the abundant orders Bacteroidales and Clostridiales were decreased in patients with CDAD, while Lactobacillales, Enterobacteriales, Selenomonadales, Actinomycetales and Bacillales were increased ( $p < 0.05$ , Figure S3B). At the family level, Bacteroidaceae, Ruminococcaceae, Lachnospiraceae and Porphyromonadaceae were prevalent in healthy controls, while Enterococcaceae, Enterobacteriaceae,





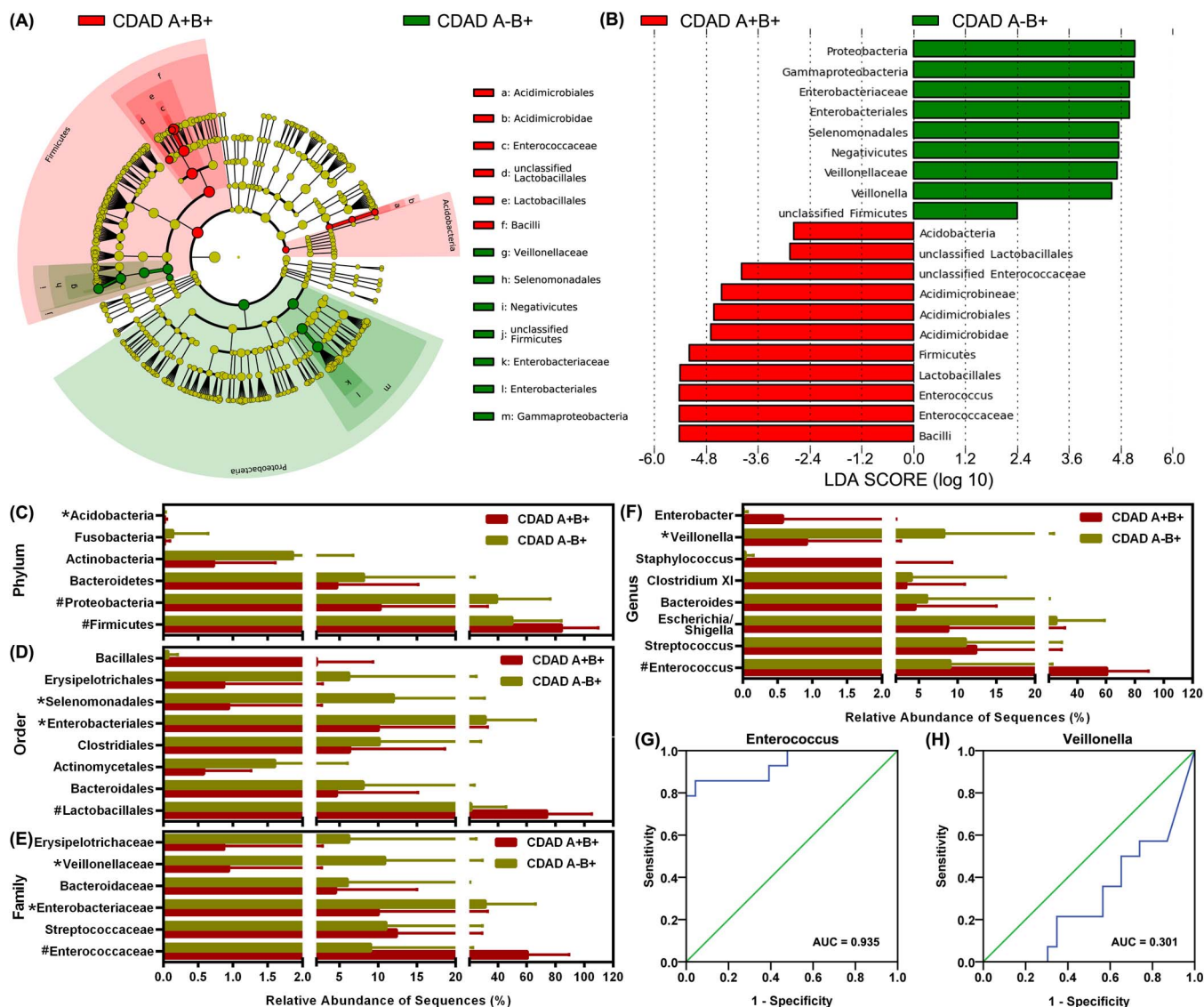
**Figure 3** | LefSe identified the most differentially abundant taxa between healthy controls and patients with CDAD. Taxonomic cladogram obtained from LefSe analysis of 16S sequences (relative abundance  $\geq 0.5\%$ ). (Red) CDAD-enriched taxa; (Green) taxa enriched in healthy controls. The brightness of each dot is proportional to its effect size (A). Healthy control-enriched taxa are indicated with a positive LDA score (green), and taxa enriched in CDAD have a negative score (red). Only taxa meeting an LDA significant threshold  $>2$  are shown (B).

Streptococcaceae, Xanthomonadaceae, Actinomycetaceae, Carnobacteriaceae, Staphylococcaceae, Neisseriaceae, Micrococcineae and Lactobacillaceae were enriched in CDAD subjects (Figure S3C). As shown in the faecal microbiota of adults, butyrate-producing bacteria such as Ruminococcaceae and Lachnospiraceae were also decreased dramatically (for Ruminococcaceae, 10.96% in healthy controls vs. 0.65% in patients with CDAD; for Lachnospiraceae, 4.58% in healthy controls vs. 2.97% in patients with CDAD).

At the genus level, several genera were present at different levels in healthy controls compared to CDAD samples ( $p < 0.05$ , Figure S3D). Among the abundant genera, six (*Enterococcus*, *Streptococcus*, *Escherichia/Shigella*, *Klebsiella*, *Stenotrophomonas* and *Haemophilus*) were increased in CDAD samples, while another six (*Bacteroides*, *Faecalibacterium*, *Parabacteroides*, *Lachnospiraceae incertae sedis*, *Dialister*, and *Alistipes*) were decreased significantly. The abundant genera could be clearly classified into two groups: compared to healthy controls, butyrate- and acetate-producing bacteria were decreased or even eliminated in patients with CDAD, and lactic acid-producing bacteria were increased dramatically. Unexpectedly, *Enterococcus*, *Streptococcus* and *Escherichia/Shigella* were extremely abundant in CDAD samples. Other genera with lower relative abundance (shown in Figure S3D) belonged mainly to butyrate-producing bacterial families, such as Ruminococcaceae and Lachnospiraceae. Further, *Ruminococcus*, *Oscillibacter*, *Clostridium IV*, *Roseburia*, *Coprococcus* and *Blautia* were almost eliminated in patients with CDAD. However, the altered patterns of these butyrate-producing genera were not always the same in CDAD samples. The genera *Clostridium XIVa*, *Clostridium XIVb* and *Butyrivibrio* were enriched in association with CDAD, although they also produced butyrate as a major fermentation prod-

uct. Collectively, these changes in the faecal microbiota revealed the intestinal dysbiosis involved in the development of CDAD.

**Impacts of infection with different toxigenic *C. difficile* strains on faecal microbiota.** Figures 4A and 4B showed the greatest differences in taxa between the two communities, and identified key phylotypes as microbiological markers at different phylogenetic levels. In contrast to the large differences in faecal microbiota between healthy controls and patients with CDAD, only a few microbial signatures in the faecal microbiota were different between the CDAD groups. Specifically, Firmicutes and Acidobacteria were significantly more abundant in the faecal microbiota of patients with CDAD A+B+ than in those with CDAD A-B+, while Proteobacteria was less abundant in CDAD A+B+ samples ( $p < 0.05$ , Figure 4C). The Firmicutes/Bacteroidetes ratio was also significantly different between the two CDAD groups. At the level of Order, Lactobacillales were prevalent in the CDAD A+B+ group (73.92% vs. 21.44% in the CDAD A-B+ group), whereas Selenomonadales and Enterobacteriales were significantly decreased when compared with the CDAD A-B+ group ( $p < 0.05$ , Figure 4D). At the family level, levels of Enterococcaceae, Enterobacteriaceae and Veillonellaceae were significantly different between both CDAD groups. Directly under the order Lactobacillales, Enterococcaceae was unusually abundant in the CDAD A+B+ group ( $p < 0.05$ , Figure 4E). In addition, we found that *Enterococcus* sequences were significantly enriched in children from the CDAD A+B+ group and *Veillonella* sequences were prevalent in the CDAD A-B+ group, as were sequences from the families of Enterococcaceae and Veillonellaceae (Figure 4F). The high abundance of *Enterococcus* sequences, with the best cut-off value  $> 5.35\%$  of the total bacterial sequences, was a feature of



**Figure 4 | Taxonomic differences of faecal microbiota between CDAD A+B+ and CDAD A-B+ groups.** Cladogram representing the features that are discriminative with respect to *C. difficile* toxin types using the LDA model results on the bacterial hierarchy (A). LDA coupled with effect size measurements identifies the most differentially abundant taxa between the CDAD groups (B). Comparison of relative abundance at the bacterial phylum (C), order (D), family (E) and genus (F) levels between CDAD A+B+ and CDAD A-B+ groups; \* indicates  $p < 0.05$ ; # indicates  $p < 0.01$ . Receiver operating characteristic (ROC) curves for *Enterococcus* (G) and *Veillonella* (H) used to predict CDAD A+B+.

children with CDAD A+B+, and could be used as a microbial signature for the differential diagnosis of CDAD A+B+ (the area under the ROC curve was 0.935; Figure 4G). In combination with the previous diversity analysis, our present data showed that infection with different strains of toxigenic *C. difficile* are associated with different compositions of faecal microbiota, regardless of antibiotic administration; this may affect the treatment and prognosis of children suffering from CDAD.

## Discussion

Recently, following the advent of next-generation high-throughput sequencing techniques, many studies have focused on the effect of CDI on the diversity and composition of intestinal microbiota in different populations<sup>12,13,28</sup>. In contrast to traditional microbiological techniques, these approaches provide a relatively comprehensive description of the intestinal microbiota, and large numbers of previously undiscovered bacterial phylotypes have been found. This allows a deeper understanding of the relationships among the intestinal microbiota in patients who have acquired *C. difficile*. Generally,

the human intestinal tract inhabited by a complex community of microorganisms, approximately 10 times more numerous than our own cells, and the currently known number of uncultured phylotypes has been estimated to amount to around 1,800<sup>29</sup>. The Good's coverage of nearly 99.0% found in the present study indicated that the 16S rRNA sequences identified in these groups represented the majority of bacterial sequences present in the samples and was therefore representative of the faecal microbiota of children. With an average of 8,817 reads per sample, a large number of rare taxa present at relatively low abundance could be detected, which influenced the overall bacterial diversity of the faecal microbiota. In health, the intestinal microbiota maintains intestinal homeostasis through a balanced symbiosis with the host, and plays vital roles in human development, physiology, immunity, nutrition and resistance to pathogen invasion. In particular, the diversity and composition of indigenous intestinal microbiota is one of the key determinants of colonisation resistance against invading pathogens<sup>30</sup>. Alterations in intestinal microbiota have been reported in patients with CDAD or recurrent CDAD<sup>12,13,28</sup>, although it is also possible that these changes



preceded the development of CDI, and thus were a cause rather than (or as well as) a consequence. However, previous studies have shown that asymptomatic *C. difficile* carriage in infants and elderly individuals without antibiotic exposure was associated with significant changes in the composition of the intestinal ecosystem<sup>14,28</sup>. The KOALA birth cohort study showed that *C. difficile* colonisation in early infancy altered the composition of the intestinal microbiota, which preceded the development of atopy<sup>31</sup>. It is difficult to determine from correlational studies such as these whether changes in microbial communities are the cause or the consequence of infections, but disturbances of intestinal microbiota are closely associated with CDI. The differences in phylogenetic clustering of the faecal microbiota between healthy controls and patients with CDAD suggested that substantial changes of the faecal microbiota could lead to intestinal dysfunction and diarrhoea.

Many previous studies have shown that treating *C. difficile* with FMT, rather than antibiotics, is a rational, durable, safe, acceptable and highly efficacious treatment option. FMT aims to restore the phylogenetic richness of the recipient's intestinal microbiota without prolonging the perturbation of the normal microbiological composition<sup>16–19,32</sup>. Consistent with previous studies<sup>12,13,32,33</sup>, our study revealed different patterns of intestinal microbiota in patients with CDAD when compared with healthy controls, which were characterised by dramatically reduced bacterial diversity and richness. The importance of increased richness and diversity has been highlighted by the successful use of FMT for treatment of CDAD<sup>32</sup>. Interestingly, given that *C. difficile* toxins are associated with its pathogenicity<sup>21,22</sup>, we also found differences in bacterial diversity between CDAD A+B+ and CDAD A–B+ samples. The bacterial diversity in patients with CDAD A+B+ was significantly lower than in those with CDAD A–B+, which indicates that *C. difficile* toxins also influenced the bacterial diversity of the faecal microbiota differently in both groups. The functions of the *C. difficile* toxins have been studied extensively<sup>21,22</sup>; however, the mechanisms that underlie their different effects on the intestinal microbiota are unknown. An alternative hypothesis for our findings is that *C. difficile* toxins may interact directly or indirectly with specific bacterial taxa, which may give rise to differences in bacterial diversity in the faecal microbiota.

In contrast to young adults and elderly patients, the faecal microbiota from children is dominated by Bacteroidetes, while the Firmicutes/Bacteroidetes ratio undergoes an increase from birth to adulthood and is further altered with advanced age<sup>34,35</sup>. Despite significant inter-individual variation, a large decrease in Bacteroidetes with concomitant relative expansion of Firmicutes and Proteobacteria was observed in patients with CDAD, with a corresponding increase in the Firmicutes/Bacteroidetes ratio. The Firmicutes/Bacteroidetes ratio is considered representative of health status, and may reflect the eubiosis or dysbiosis of the gastrointestinal tract. With regard to an increased Firmicutes/Bacteroidetes ratio in the intestinal microbiota, Bishara *et al.* indicated that obesity was a significant and independent risk factor for CDI in a recent retrospective study<sup>36,37</sup>. In the present study, the increased ratio of Firmicutes/Bacteroidetes could be considered an important marker for intestinal dysbiosis, and used as an indicator to predict susceptibility to CDI. A previous study showed that Bacteroidetes increased markedly after successful FMT, which may be critical to restoration of eubiosis and eradication of *C. difficile*<sup>32</sup>. We also observed a significant difference between CDAD A+B+ and CDAD A–B+ samples, with a higher ratio of Firmicutes/Bacteroidetes in the CDAD A+B+ group. These differences indicate that the combination of *C. difficile* toxins A and B was able to induce a greater decrease in Bacteroidetes than toxin B alone.

A predominance of fermenting bacteria such as Bacteroidales and Clostridiales were found in healthy children, accounting for more than 90% of the intestinal microbiota; this percentage was lower in the patients with CDAD. The presence of both Bacteroidales and

Clostridiales is concordant with a substantial amount of short chain fatty acids (SCFAs) such as acetate and butyric acid, typical end-products of microbial fermentation of carbohydrates that provide beneficial immunomodulatory properties and nutrition to the host<sup>38,39</sup>. SCFAs may improve colonisation resistance and inhibit pathogen growth by lowering the pH and redox potential (eH) in the intestinal lumen<sup>40</sup>. Our pyrosequencing analysis demonstrated that the dominant bacteria shifted from the Orders Bacteroidales and Clostridiales to Lactobacillales and Enterobacteriales. Early evidence has indicated that Lactobacillales occur in gut microbiota in association with obesity<sup>41</sup>, while an increase in Enterobacteriales was also seen in the microbiota of obesity-prone rats<sup>42</sup>. Both capable of surviving in aerobic environments, Lactobacillales and Enterobacteriales may populate a niche at the expense of obligate anaerobes such as Bacteroidales and Clostridiales<sup>43</sup>. With regard to different strains of toxigenic *C. difficile*, Lactobacillales was the dominant order in the CDAD A+B+ group, while other orders such as Bacteroidales, Selenomonadales and Enterobacteriales were more abundant in the CDAD A–B+ group. The higher bacterial taxonomic ranks contained diverse and complex phylotypes, which made it difficult to determine the role of the taxa accurately. However, changes in the faecal microbiota after CDI at these taxonomic levels may help to determine the presence of intestinal dysbiosis.

Consistent with a previous study<sup>13</sup>, the potentially beneficial autochthonous bacteria including acetogens such as Bacteroidaceae (including *Bacteroides*), and butyrate-producing bacteria such as Ruminococcaceae (including *Faecalibacterium*), Lachnospiraceae (including *Faecalibacterium*, *Lachnospiraceae incertae sedis* and the majority of butyrate-producing bacteria) and Porphyromonadaceae (including *Parabacteroides*), decreased significantly after CDI. In contrast, lactic acid-producing bacteria such as Enterococcaceae (including *Enterococcus*), Streptococcaceae (including *Streptococcus*), Staphylococcaceae (including *Staphylococcus*), Actinomycetaceae (including *Actinomyces*) and Lactobacillaceae (including *Lactobacillus*) increased dramatically. The obvious shifts of SCFA-producing bacteria implied that these products of fermentation may be responsible for the development of CDAD, which is linked to epithelial intestinal barrier function<sup>44</sup>. However, the observed effects of butyrate largely depend on its concentration. The decrease or elimination of butyrate and lactic acid buildup will impair the intestinal defense barrier and increase osmotic load in the intestinal lumen, finally leading to diarrhoea. Butyrate, rather than lactic acid, plays a central role in maintaining gut homeostasis<sup>45</sup>. Lactic acid accumulation has been previously associated with disease states such as ulcerative colitis and gut resection, both in humans and in horses<sup>46,47</sup>. This may explain why the evidence for the efficacy of *Lactobacillus* GG (which mainly mediates lactic acid production) in CDAD is less convincing. A previous study also reported that butyrate had the opposite impact on *C. difficile* toxin production<sup>48</sup>. Evidence has shown that some butyrate producers can use exogenous acetate for butyrate production; therefore, a decrease in the population of acetate-producing bacteria in patients with CDAD may lead to a decrease in both acetate and butyrate. *Faecalibacterium*, which is strongly correlated with butyrate production, also exhibits anti-inflammatory effects and counterbalances dysbiosis of the intestinal microbiota<sup>49</sup>. In addition to the role of butyrate, the predominant genus *Bacteroides* also plays a central role in colonisation resistance against *C. difficile*. Tvede *et al.* showed that adoptive transfer of a defined consortium of 10 bacterial species could eradicate *C. difficile*, and correlated the reconstitution of *Bacteroides* species with clinical cure of CDI<sup>50</sup>. Another mechanism that the gut microbiota uses against *C. difficile* is metabolising bile, which is proven to have a role in both spore germination and growth of the vegetative form of the bacterium<sup>11</sup>. Theriot *et al.*, by characterising the gut microbiome and metabolome using a multi-omics approach, have recently identified other specific metabolites, such as the secondary bile acid deoxycholate. These act as key mechanistic





links between alterations of the gut microbiome and *C. difficile* susceptibility<sup>51</sup>. The other group of opportunistic pathogens (*Enterococcus*, *Streptococcus*, *Escherichia/Shigella*, *Klebsiella*, *Stenotrophomonas* and *Haemophilus*) that is enriched in patients with CDAD may interact with *C. difficile* synergistically, and contribute to alterations in intestinal homeostasis. Thus, both structural and functional alterations of the faecal microbiota are correlated significantly with CDI. However, our knowledge of the possible mechanisms by which the intestinal microbiota inhibits *C. difficile* is still rudimentary. Further understanding is necessary for the introduction of novel treatments and preventative strategies for CDI.

The differential changes of acetate- and butyrate-producing bacteria were not found in both CDAD groups, which indicated that both *C. difficile* toxins could affect the SCFA-producing bacteria. In the present study, three families were influenced dramatically by the different *C. difficile* toxins. The relative overgrowth of Enterococcaceae (including *Enterococcus*) and Enterobacteriaceae (including *Escherichia/Shigella*) in the disturbed microbiota could result in endotoxaemia. This may result in increased endotoxin production with increased intestinal permeability, which facilitates *C. difficile* colonisation. A previous study showed that the overgrowth of enterococci was concomitant with antibiotic administration and appeared to be due to direct antibiotic selection<sup>52</sup>. Given the antibiotic exposure in both CDAD groups in the current study, the presence of high amounts of *Enterococcus* implied that the overall disturbance of the faecal microbiota caused by the presence of both *C. difficile* toxins, A and B, was more serious than that caused by toxin B only. This possibility is consistent with the changes in the faecal microbiota diversity. The extremely high relative abundance of *Enterococcus* in the CDAD A+B+ group could serve as a simple and helpful marker to distinguish the two CDAD groups. By contrast, the elevation of Enterobacteriaceae, usually considered to be pathogens, might be due to indirect influence of antibiotic selection but the delayed establishment of stable microbiota<sup>52</sup>. After successful FMT for CDI, the largest increases were observed in *Bacteroides*, *Lactobacillus*, *Dorea*, *Roseburia*, and *Faecalibacterium*, with the corresponding greatest decreases being observed in Enterobacteriaceae<sup>32</sup>. A previous study showed that an increase in Enterobacteriaceae was positively correlated with the inflammatory markers interleukin-6 and interleukin-8<sup>53</sup>. Given that both *C. difficile* toxins A and B act as potent inflammatory enterotoxins in the human intestine, different effects on these inflammatory cytokines may provide a mechanistic explanation for the discrepancies in the amounts of Enterobacteriaceae. Taken together, therefore, *C. difficile* A+B+ strains may produce a greater decrease in bacterial diversity than *C. difficile* CDAD A-B+ strains, while only influencing a few specific phylotypes in the faecal microbiota.

This study has several limitations. First, the findings indicated that toxins A and B acted synergistically to cause tissue damage. However, the impacts of toxins A and B in different *C. difficile* strains on the faecal microbiota remained unclear. To clarify the relative roles of each toxin, and their combination, studies with purified toxins will be required to determine their influence on the diversity and composition of faecal microbiota in animal models *in vivo*, and their effects on specific phylotypes *in vitro*. This would provide clues that may explain our present results. In addition, it is known that *C. difficile* A-B+ strains produce a divergent toxin B; however, it is also true that it is not known whether this divergent toxin influences the microbiota differently. Second, no microbiome data were obtained on the faecal samples after the children had been treated with specific antibodies active against the toxins; this would have strengthened the relationships between changes in faecal microbiota and the *C. difficile* toxins. Third, the impacts of exposure to different antibiotics in children should also be taken into account when analysing the susceptibility to different *C. difficile* strains in future studies.

In summary, our present study demonstrated that decreased faecal bacterial diversity in children was associated with the development of

CDAD. Intestinal dysbiosis, especially a decrease in or elimination of butyrate-producing bacteria and increase in lactic acid-producing bacteria, may contribute to the breakdown of colonisation resistance, resulting in *C. difficile* colonisation. In addition to the impact of antibiotic exposure, infection with *C. difficile* producing different toxins also led to alterations of the faecal microbiota in children. Understanding the relationship between disruption of the normal faecal microbiota and colonisation by *C. difficile* strains that produce different toxins, may also lead to improvements in treatment. Further investigations that focus on the interplay between *C. difficile* toxins and key bacterial phylotypes would be helpful to clarify the mechanisms for the development of CDI.

## Methods

**Subjects' recruitment and sample collection.** The protocols for the present study were approved by the Ethics Committee of Affiliated Beijing Children's Hospital, Capital Medical University (Beijing, China) and the methods were carried out in accordance with the approved guidelines. Informed written consent was obtained from the parents or guardians of all participants prior to enrollment. Children with suspected CDAD who were brought to the Department of Gastroenterology, Affiliated Beijing Children's Hospital for evaluation between December 2011 and April 2013 had their CDAD established by means of clinical history or symptoms and medication history, and confirmed with *C. difficile* culture and identification from fresh feces. For each *C. difficile* isolate, genes encoding toxins were screened by multiplex PCR for *tcdA* and *tcdB* (encoding toxin A and toxin B)<sup>27</sup>. Healthy control subjects were recruited from children who visited the Affiliated Beijing Children's Hospital for routine physical examination. All these control subjects were matched for age with CDAD patients and free of known active bacterial, fungal, or viral infections and intestinal diseases. They had not taken antibiotics, probiotics, prebiotics, or synbiotics in the previous month before faecal samples collected. The fresh feces of these participants were collected in a sterile plastic cup and stored at -80°C after preparation within 15 min for further microbiome analysis.

**DNA extraction, bacterial 16S rRNA gene amplification and pyrosequencing analysis.** Frozen faecal samples were thawed, and bacterial genomic DNA was extracted from 200 mg of feces using QIAamp® DNA Stool Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions, with the additional glass-bead beating steps on a Precellys 24 homogenizer (Bertin Technologies, Montigny, France). The bacterial genomic DNA was amplified in 50-µl triplicates with the 27F (5'-AGAGTTTGA TCCTGGCTCAG-3') and 533R (5'-TTACCGCGCTGTG-TGGCAC-3') primers specific for the V1-V3 hypervariable regions of the 16S rRNA gene according to our previous studies<sup>54</sup>. Each forward primer incorporated FLX Titanium adapters and a sample barcode at the 5' end of the reverse primer to allow all samples to be included in the single 454 FLX sequencing run. After PCR products extracted and quantified, equimolar concentrations were pooled and sequenced on a 454 Life Sciences Genome Sequencer FLX system (Roche, Basel, Switzerland) according to the manufacturer's recommendations.

**Bioinformatics and statistical analysis.** Raw pyrosequencing reads obtained from the sequencer were denoised using Titanium PyroNoise software. The resulting pyrosequencing reads were filtered according to barcode and primer sequences using a combination of tools from Mothur (version 1.25.0; <http://www.mothur.org>) and custom Perl scripts. Preliminary quality control steps included the removal of sequences shorter than 150 nt with homopolymers longer than 8 nucleotides, average quality score < 25, and all reads containing ambiguous base calls or > 2 incorrect primer sequences. Using Mothur implementation of ChimeraSlayer algorithm, chimera sequences arising from the PCR amplification were detected and excluded from the denoised sequences. The high-quality sequences were assigned to samples according to barcodes. The high-quality reads were clustered into operational taxonomic units (OTUs) using Mothur. The OTUs that reached at a 97% nucleotide similarity level were used for alpha diversity (Shannon, Simpson, and Evenness), richness (ACE and Chao1), Good's coverage, Venn diagram, and rarefaction curve analysis using Mothur. A heatmap was generated on the basis of the relative abundance of OTUs using R (version 2.15; The R Project for Statistical Computing, <http://www.R-project.org>). Phylogenetic beta diversity measures such as unweighted UniFrac distance metrics analysis and principal coordinate analysis (PCoA) were performed using OTUs for each sample using the Mothur program<sup>54</sup>.

Taxonomy-based analyses were performed by classifying each sequence using the Naïve Bayesian Classifier program of the Michigan State University Center for Microbial Ecology Ribosomal Database Project (RDP) database (<http://rdp.cme.msu.edu/>) with a 50% bootstrap score. Microbiome features of healthy controls were compared to patients with CDAD with the Mann-Whitney U test using SPSS version 16.0 for Windows (SPSS Inc., Chicago, IL). The characterization of microorganismal features differentiating the faecal microbiota specific to different toxicigenic types was performed using linear discriminant analysis (LDA) effect size (LEfSe) method (<http://huttenhower.sph.harvard.edu/lefse/>) for biomarker discovery, which emphasizes both statistical significance and biological relevance<sup>55</sup>. With a normalized relative abundance matrix, LEfSe uses the Kruskal-Wallis rank sum test to detect





features with significantly different abundances between assigned taxa and performs LDA to estimate the effect size of each feature. A significance alpha of 0.05 and an effect size threshold of 2 were used for all biomarkers discussed in this study. All tests for significance were two-sided, and  $p$  values  $< 0.05$  were considered statistically significant.

**Accession numbers.** The sequence data from this study has been deposited in the GenBank Sequence Read Archive with the accession number SRP044352.

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## Author contributions

Z.X.L., X.L., L.J.L., X.W.X. and C.X. conceived and designed the experiments. Z.X.L., X.L., X.Y.J., Y.W.C., Y.Q.L., L.Y. C.N.Z. and S.G. performed the experiments. Z.X.L., X.L.,



Y.Z.W., L.J.L., X.W.X. and C.X. analyzed the data. Z.X.L., X.L., L.J.L., X.W.X. and C.X. wrote the paper and edited the manuscript. All authors read and approved the final manuscript.

### Additional information

**Supplementary information** accompanies this paper at <http://www.nature.com/scientificreports>

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