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Clostridioides difficile concentration-dependant alterations in gut microbiota of asymptomatic infants

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Abstract

Background Asymptomatic carriage of *Clostridioides difficile* is highly prevalent in early infancy, affecting approximately 40% of infants. This phenomenon offers a unique opportunity to study its impact on the gut microbiota without the confounding effects of disease. In this study, we analysed *C. difficile*-associated gut microbiome alterations in 76 asymptomatic infants, one year after receiving antibiotic treatment during early infancy. The presence and concentration of *C. difficile* were assessed in relation to gut microbiota structure and an extensive set of metadata.

Results Bacterial gut community structure was characterized using 16 S rRNA amplicon sequencing, while *C. difficile* concentration and the presence of the *tcdB* gene were quantified via digital PCR. *C. difficile* was detected in 36.8% of infants, with 10.5% testing positive for the *tcdB* gene. Significant alterations in gut microbiota were observed in relation to *C. difficile* concentration. Specifically, higher *C. difficile* loads were associated with reduced microbial diversity, greater deviations from average community structure, and co-occurrence with the genus *Escherichia*. Conversely, *C. difficile* colonization alone or the presence of the *tcdB* gene did not result in significant gut microbiota alterations. Additionally, no host-specific factors were significantly linked to *C. difficile* prevalence or concentration.

Conclusions Asymptomatic carriage of *C. difficile* in neonates is not associated with significant gut microbiota alterations unless pathogen concentration is considered. Our findings suggest that elevated *C. difficile* proliferation occurs in dysbiotic infant gut microbiota, characterized by reduced alpha diversity and an increase in *Escherichia*.

Keywords Gut microbiota, *Clostridioides difficile*, Infant, Asymptomatic, Dysbiosis

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Background

Prevalence of asymptomatic colonization with *Clostridioides difficile* in infants is estimated at approximately 40% [1], however varies considerably according to geographic location, age, breastfeeding, and underlying medical conditions [2, 3]. Colonization rates with both toxigenic and non-toxigenic strains peak in the first year of life before declining after the age of 2 [1, 4], with considerable disparities in reported prevalences across various studies [5–8]. However, symptomatic *C. difficile* infection (CDI) remains low in infants, ranging from 2.6 to 4 cases per 1000 admissions [9] and 7.24 to 12.80 CDI-related hospitalizations per 10.000 [10], both reported in the United States of America.

In adults, the development of CDI is often preceded by disruption in the gut microbiota leading to decreased colonization resistance [11]. The disruption is commonly attributed to antibiotic therapy, age, and other underlying gastrointestinal complications [12–15]. Consequently, the microbiota associated with CDI typically displays dysbiosis, characterized by lower diversity and reduced relative abundance of Bacteroidota (previously Bacteroidetes) and Bacillota (previously Firmicutes), with subsequent increase in Pseudomonadota (previously Proteobacteria) [16]. Notably, asymptomatic colonization with *C. difficile* has also been linked to adverse effects on gut microbiota composition, suggesting the bacterium's active role in inducing alterations to facilitate its own proliferation [17, 18]. This phenomenon has been further demonstrated in vitro, showing ribotype-specific effect of *C. difficile* on infant's gut microbiota [19] and adult dysbiotic microbiota [20].

Understanding of gut microbiota in correlation to *C. difficile* in infants remains limited to a few studies. The most notable among them is a large study involving longitudinal surveillance of 817 children up to 2 years of age, which found no significant association between *C. difficile* colonization and specific gut microbiota characteristics [3]. Conversely, other studies have reported conflicting results, with some indicating reduced microbiota diversity [21, 22], while others suggest increased diversity in *C. difficile* colonized children [5]. Additionally, the taxa found to be differentially abundant vary considerably across studies, underscoring substantial, unexplained cohort-specific variability.

In this study, we present findings on *C. difficile* colonization and concentration-specific microbiota characteristics in a cohort of 76 infants who received antibiotic therapy during neonatal period. Furthermore, we explore potential risk factors associated with *C. difficile* status by analysing extensive metadata collected at an early age.

Methods

Sample collection

The stool samples used in this study were collected as part of a previously published study [23]. This was a randomized, double-blind, placebo-controlled trial conducted at three Slovenian neonatal units between November 2016 and March 2019. The trial was registered at ClinicalTrials.gov (Identifier: NCT02865564) and received approval from the National Medical Ethics Committee (No. 89/03/15). Detailed methodologies are provided in [23]. Briefly, stool samples used in this study were obtained from 76 infants along with clinical, anthropometric, demographic and feeding data. The inclusion criteria into original study were antibiotic administration for suspected early or late neonatal sepsis for at least five days (ampicillin/flucloxacillin and gentamicin), at the age of less than 21 days, and gestational age of at least 37 weeks. In present study, we included only stool samples collected from these infants at follow-up visit, one year after the completion of antibiotic treatment. None of the infants included in this study were diagnosed with or actively treated for *C. difficile* infection during the study period.

Total DNA isolation

Stool samples were collected in sealed sterile containers and promptly refrigerated for less than 24 h before storage at − 80 °C for subsequent analyses. Initially, suspensions of stool samples weighing between 0.1 and 0.5 g were prepared in Ringer's solution, diluted at a ratio of 1:100. Following centrifugation at 3600 g for 10 min at 10 °C, the resultant pellet underwent lysis and sonication according to the methodology outlined by Matijašić et al. 2014 [24]. DNA extraction was then carried out using the automated Maxwell 16 System protocol (Promega, USA).

C. difficile and *tcdB* quantification

Number of copies of *C. difficile* 16S rRNA gene and toxin *tcdB* gene were determined using digital PCR (dPCR) technology on the QX200 system (Bio-Rad, USA), employing ddPCR EvaGreen Supermix (Bio-Rad, USA) for fluorescence detection with *C. difficile* specific primers Fcdiff (5'-TTGAGCGATTTACTTCGGTAAAGA-3') and Rcdiff (5'-CCATCCTGTACTGGCTCACCT-3') [25] and *tcdB* gene specific primers TcdB_398F (5'- GAAAG TCCAAGTTTACGCTCAAT-3') and TcdB_399R (5'-GCTGCACCTAAACTTACACCA-3') [26]. The dPCR analysis was performed to quantify the absolute number of copies of *C. difficile* per ng of DNA. To achieve this, droplet PCR events were normalized to the input DNA concentration, which was assessed using the Quant-iT™ PicoGreen™ dsDNA Assay (ThermoFisher, USA).

Microbiota 16S amplicon sequencing and statistical analysis

Bacterial community structure was acquired through targeted amplicon sequencing of the V3-V4 variable region of the 16S rRNA gene utilizing the primer pair Bakt_341F (5'-CCTACGGGNGGCWGCAG-3')-Bakt_805R (5'-GACTACHVGGGTATCTAATCC-3') [17]. Sequencing was conducted on the MiSeq Illumina platform (2 × 300 bp, Illumina, USA). Sequence data processing involved quality filtering of reads and construction of Zero-radius Operational Taxonomic Unit (ZOTUs) using the UNOISE3 pipeline implemented in USEARCH v.11.0.667 [27, 28] with default settings except the addition of parameter -fastq-minlen 400 (fastq_filter command). Taxonomy was assigned using the RDP trainset (version 18). The sequencing data generated in this study are available in the NCBI Sequence Read Archive (SRA) under the accession number PRJNA954698.

Statistical analysis and visualization of the microbiome data was performed in R (v4.2.2, R Core Team, 2022) implementing packages 'vegan', 'picante' and 'ggplot'. Alpha diversity was presented as Faith's phylogenetic diversity calculated based on neighbour-joining tree constructed from ZOTU representative sequences and Shannon diversity index. Beta diversity analysis was conducted using Bray-Curtis dissimilarities. Differences in community composition between groups were evaluated with permutation-based test ANOSIM (R package 'vegan').

Results

Our dataset comprises 76 infants who all underwent antibiotic treatment within the first 3 weeks of life. Stool samples were collected one year after treatment completion. In our cohort, we observed a *C. difficile* colonization rate of 36.8% (95% CI: 26.0-47.7%), out of which 28.6% (95% CI: 11.9–45.3%) were toxigenic based on the presence of *tcdB* gene. None of the cases resulted in hospitalization due to *C. difficile* infection. Several cases reported diarrhoea in the previous 6 months (Table 1); however, its association with *C. difficile* was not confirmed (clinical investigations have not been performed).

Subject metadata correlation with *C. difficile* colonization and concentration

C. difficile colonization and concentration was analysed in relation to an extensive set of subject metadata. Risk for *C. difficile* colonization did not significantly correlate with metadata collected, including factors typically associated with CDI in adults such as antibiotic use and hospitalization (Table 1).

C. difficile concentration dependant alterations in gut microbiota composition

C. difficile colonization was in our cohort not associated with specific microbial community structure characteristics. No significant differences were found in either alpha diversity (Shannon diversity, $p = 0.235$; Faith's phylogenetic diversity, $p = 0.245$) or beta diversity (ANOSIM,

Table 1 Correlation between *C. difficile* colonization and concentration with subject-collected metadata. Fisher's exact test was employed for categorical environmental values, and student's t-test was utilized for comparing distributions of continuous environmental values when addressing *C. difficile* colonization status. Wilcoxon test was applied for categorical environmental values, and Pearson's correlation was used for continuous environmental values when analysing *C. difficile* concentration associations. None of the categories in metadata yielded statistically significant correlation with *C. difficile* status beyond the 0.05 significance threshold. Data on maternal antibiotic use was available only for breastfeeding mothers

	***C. difficile* colonization status**		***C. difficile* concentration**
Categorical environmental factors	Percent colonized [%]	Significance (*p* value)	Significance (*p* value)
Gender (Male/Female)	37.0 / 36.6	1.000	0.547
Diarrhoea between 1 and 6 months of age (Yes/No)	42.9 / 39.6	1.000	0.586
Diarrhoea between 6 and- 12 months of age (Yes/No)	50.0 / 37.3	0.495	0.679
Visit to the doctor between 1 and 6 months of age (Yes/No)	39.6 / 35.7	1.000	0.570
Visit to the doctor between 6 and 12 months of age (Yes/No)	40.4 / 30.8	0.753	0.111
Hospitalization between 1 and 6 months of age (Yes/No)	37.5 / 39.0	1.000	0.157
Hospitalization between 6 and 12 months of age (Yes/No)	53.3 / 34.5	0.236	0.075
Antibiotics between 1 and 6 months of age (Yes/No)	39.1 / 38.6	1.000	0.650
Antibiotics between 6 and 12 months of age (Yes/No)	43.8 / 34.2	0.466	0.312
Probiotics between 2 and 12 months of age (Yes/No)	42.1 / 31.6	0.476	0.159
Delivery mode (Caesarean section/vaginal)	28.6 / 37.1	1.000	0.480
Breastfed at 6 months (Yes/No)	50.0 / 50.0	1.000	0.894
Mother - antibiotics between 1 and 6 months of child's age (Yes/No)	33.3 / 33.3	1.000	0.250
Mother - antibiotics between 1 and 6 months of child's age (Yes/No)	33.3 / 33.3	1.000	0.250
Continuous environmental factors	Mean (SD)	Significance (p value)	Significance (p value)
Gestational age [weeks] (Colonized/non-colonized)	39.6 (1.0) / 39.4 (1.2)	0.456	0.638
Weight at birth [g] (Colonized/non-colonized)	3567 (518) / 3536 (500)	0.805	0.757

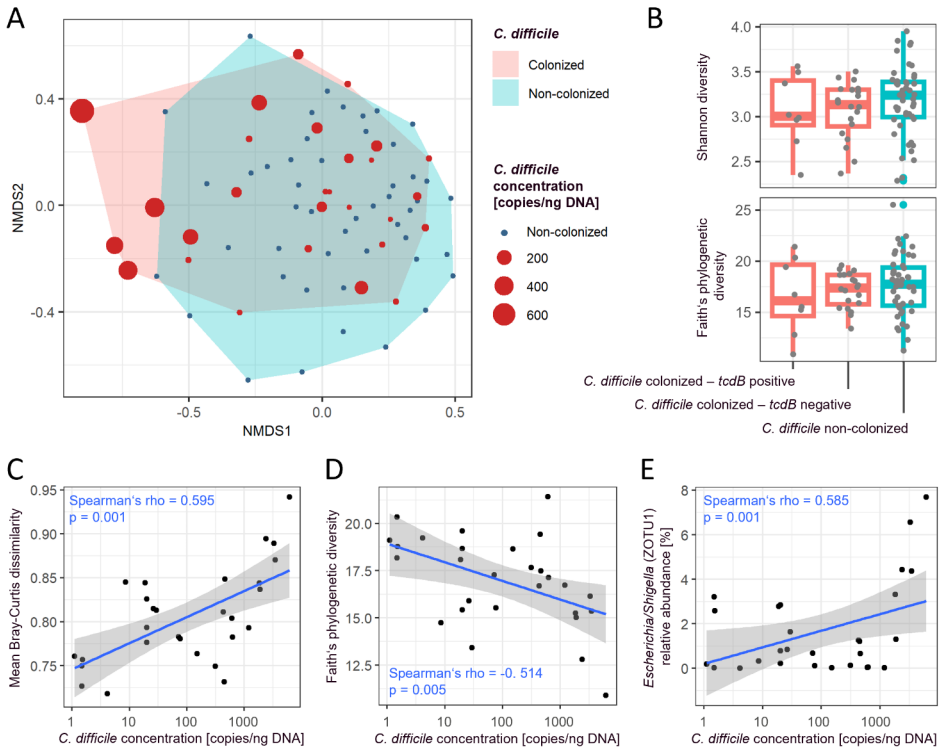

Fig. 1 *C. difficile* associated gut microbial community characteristics. (**A**) *C. difficile* colonization associated characteristics presented with non-metric multidimensional scaling (NMDS) plot illustrating beta diversity using Bray-Curtis dissimilarities, with colours indicating *C. difficile* colonization status and dot size representing *C. difficile* concentration. (**B**) Alpha diversity analysis depicted by Shannon diversity and Faith's phylogenetic diversity. (**C**, **D** and **E**) *C. difficile* concentration (ddPCR; copies per ng DNA) associated changes in (**C**) mean Bray-Curtis dissimilarity, (**D**) Faith's phylogenetic diversity and (**E**) *Escherichia/Shigella* (ZOTU1) relative abundance. All correlations are supported with Spearman's rho and significance

$p = 0.369$) (Fig. 1A and B). Furthermore, among *C. difficile* colonized, no differences between *tcdB* positive and negative samples were found in either alpha (Shannon diversity, $p = 0.980$; Faith's phylogenetic diversity, $p = 0.823$) or beta diversity (ANOSIM, $p = 0.220$) (Fig. 1B). Finally, population level analysis did not yield any differentially abundant taxa between *C. difficile* colonized and non-colonized infants.

On the other hand, we found strong associations between microbiome composition and *C. difficile* concentration. Higher concentration of *C. difficile* was significantly positively correlated with the mean Bray-Curtis dissimilarity from the cohort average (Spearman's rho = 0.595, $p = 0.001$; Fig. 1C), indicating alterations in community composition, found to be predominantly associated with decreased alpha diversity. While concentration-associated decreases in both richness and diversity were observed (observed ZOTUs, $p = 0.016$; Shannon diversity, $p = 0.017$; Shannon evenness, $p = 0.044$; Supplementary Fig. 1), the correlation was most significant with Faith's phylogenetic diversity (Spearman's rho = -0.514, $p = 0.005$, Fig. 1D). The lowest alpha diversity was observed in samples with highest concentrations of *C. difficile*, but group minimum values were comparable to those observed in the group of *C. difficile* non-colonized infants, therefore not indicating any exacerbating effect of *C. difficile* colonization on dysbiosis-like features in gut microbiota. Among detected ZOTUs, the one linked to *C. difficile* (ZOTU171) exhibited strongest positive correlation with increasing mean Bray-Curtis dissimilarity (Pearson's $r = 0.427$, $p < 0.001$). Along with negative correlation observed with *Fusicatenibacter* (ZOTU13) (Pearson's r = -0.470, $p < 0.001$), these two represented the only taxa significantly correlated with mean Bray-Curtis dissimilarity beyond false discovery rate (FDR) < 0.05 (Supplementary Fig. 2). *C. difficile* concentration also positively correlated with the relative abundance of *Escherichia*, with the correlation most evident at the highest concentrations of *C. difficile* (Spearman's rho = 0.585, $p = 0.001$) (Fig. 1E).

Discussion

The predominantly asymptomatic carriage of C. difficile in infants is well documented, yet it still presents certain diagnostic challenges. However, it also offers an opportunity to study *C. difficile* dynamics in the absence of disease. In this study, we report *C. difficile* concentration-associated alterations in the gut microbiome of 76

asymptomatic one year old infants, all of whom received antibiotic therapy during the first three weeks of life.

In our cohort, *C. difficile* was detected in 36.8% of the infants, consistent with the 41% colonisation prevalence reported in a systematic review of 95 studies [1]. Additionally, our prevalence of toxigenic *C. difficile* at 10.5% is comparable to the 14% reported in the same review [1] and the 7.1% reported by Rousseau et al. [6], while significantly lower than the 40% reported by Mani et al. [3]. It is most likely that infants acquire *C. difficile* from the environment, as indicated by its lower prevalence immediately after birth [3]. The potential sources are numerous, as *C. difficile* is commonly found in both hospital and home environments, including pets [7, 29]. In our study, none of the collected environmental factors were associated with *C. difficile* prevalence, including typical adult risk factors such as antibiotic use and hospitalization [30, 31]. Higher *C. difficile* prevalence has also been reported in non-breastfed infants [3, 7, 32], Caesarean deliveries, and infants with diarrhoea [7], none of which was confirmed as statistically significant in our cohort.

No statistically significant differences in microbial community structure were associated solely with either *C. difficile* colonization or toxigenic strain colonization. Previous studies have reported a *C. difficile* colonization-associated decrease in diversity [21, 22], a feature typically seen in adult CDI cases. However, the most comprehensive studies to date, in agreement with ours, did not confirm these findings in infants [3, 6]. In contrast to earlier studies, we did not identify any differentially abundant taxa between *C. difficile*-colonized and non-colonized infants. However, the reported taxa tend to be largely study-specific [3, 6, 21, 22].

We did, however, observe significant alterations in the gut microbiota related to *C. difficile* concentration, particularly lower phylogenetic diversity. Importantly, samples with large deviations from the population average and low microbial abundance were found in both *C. difficile*-colonized and non-colonized groups. This suggests that disrupted microbiota supports better growth of *C. difficile* while we found no indications that the presence of *C. difficile* further exacerbates microbiota disruption. High *C. difficile* concentrations were also positively correlated with increased *Escherichia* relative abundance, a co-occurrence previously described in murine model [33]. To our knowledge, ours is the first study to investigate *C. difficile* concentration-associated alterations in the gut microbiota of infants. Studies in adults have reported different alterations, mainly a negative correlation between *C. difficile* concentration and *Clostridium scindens*, as well as different *Blautia* species [34, 35]. However, adult microbiota differs significantly from that in early childhood, which likely affects *C. difficile* interactions and contributes to the frequent symptomatic disease seen in adults compared to the high prevalence of asymptomatic carriage in infants. Therefore, future studies are required to further validate our observations.

A significant limitation of our study is the small sample size, particularly regarding the statistical analyses performed on the subgroup of *C. difficile*-positive subjects. The observed associations with metadata, influenced by the heterogeneity of the analysed cohort, would greatly benefit from validation in a larger cohort. Furthermore, all children included in this study received antibiotic therapy within the first three weeks after birth, which potentially influenced the development of their microbiota, and the subsequent *C. difficile* colonization associated microbiota characteristics.

Conclusion

Our study of 76 asymptomatic infants found that *C. difficile*-associated alterations in the gut microbiota, primarily lower microbial diversity and greater deviation from population averages, are closely linked to *C. difficile* concentration in the gut. Colonization alone, however, did not significantly alter the bacterial community structure. Furthermore, no host-specific factors from the extensive metadata were significantly correlated with either colonization status or *C. difficile* concentration.

Supplementary information

The online version contains supplementary material available at https://doi.org/10.1186/s13099-025-00687-3.

Supplementary Material 1

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Author contributions
Conception and design: J.L.K., E.B., D.P.P., A.M.; data acquisition: J.L.K., A.M., P.B., A.V., T.O.; analysis and interpretation of data: A.M., J.L.K., T.O., B.B.M., A.M.; drafting the manuscript: A.M., J.L.K., M.R.; critically revising manuscript, approved final version and agreed to be accountable for all aspects of the work: A.M., J.L.K., M.R., P.B., A.V., E.B., T.O., B.B.M., D.P.P.

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Data availability
The sequencing data generated in this study are available in the NCBI Sequence Read Archive (SRA) under the accession number PRJNA954698.

Declarations

Ethics approval and consent to participate
The trial was registered at ClinicalTrials.gov (Identifier: NCT02865564) and received approval from the National Medical Ethics Committee (No. 89/03/15). The study was conducted in accordance with the ethical principles outlined in the Declaration of Helsinki and the legal requirements of the study country. Informed consent was obtained from the parent or legal guardian of each neonate after written and verbal study information was provided.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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