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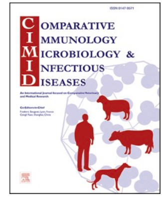

Acquisition site-based remodelling of *Clostridium perfringens*- and *Clostridioides difficile*-related gut microbiota

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ABSTRACT

Introduction: Clostridium perfringens is a gram-positive, anaerobic sporulating bacillus which can infect several hosts, thereby being considered the causative agent of many gut illnesses. Some studies have suggested that *C. perfringens*'s virulence factors may negatively affect gut microbiota homeostasis by decreasing beneficial bacteria; however, studies have failed to evaluate the simultaneous presence of other pathogenic bacteria, such as *C. difficile* (another sporulating bacillus known to play a role in gut microbiota imbalance). Conscious of the lack of compelling data, this work has ascertained how such microorganisms' coexistence can be associated with a variation in gut microbiota composition, compared to that of *C. perfringens* colonisation.

Methods: PCR was thus used for identifying *C. perfringens* and *C. difficile* in 98 samples. Amplicon-based sequencing of 16S- and 18S-rRNA genes' V4 hypervariable region from such samples was used for determining the microbiota's taxonomical composition and diversity.

Results: Small differences were observed in bacterial communities' taxonomic composition and diversity; such imbalance was mainly associated with groups having hospital-acquired diarrhoea.

Conclusion: The alterations reported herein may have been influenced by *C. difficile* and diarrhoea acquisition site, despite *C. perfringens*' ability to cause alterations in microbiota due to its virulence factors. Our findings highlight the need for a holistic view of gut microbiota.

1. Introduction

Multiple pathogens can affect gut microbiota homeostasis due to the production of virulence factors (such as toxins) by detrimentally modifying the gut environment [1]. *Clostridium perfringens* (CPF) is a gram-positive, anaerobic, spore-forming bacteria which is found in varied environments, e.g., soil, food and human and animal gut microbiota [2]. Clinical manifestations are usually associated with this bacterium, i.e. gas gangrene, necrotising enteritis, food poisoning, colitis and other non-specific gastrointestinal alterations [2]. The CPF genome can encode more than 20 toxins, six of which are clinically relevant and useful for toxinotyping: alpha (CPA), beta (CPB), epsilon (ETX), iota (ITX), enterotoxin (CPE) and necrotic enteritis-causing B-like (NetB) toxins [3]. Some CPF strains can carry accessory enzymes, thereby increasing their virulence (considering this species' strong genomic plasticity).

CPF is considered to be the second cause of food poisoning in the USA and Canada; it causes around 5% of outbreaks and 4% of hospitalisations [4]. Few CPF infection studies have been carried out in Colombia; they have revealed 18.3–41.3% infection frequency in patients who have contracted community- or intrahospital-acquired diarrhoea [5]. Such high frequency has been explained by CPF's occurrence as a gut

Abbreviations: CPF, Clostridium perfringens; CDI, Clostridioides difficile Infection; CO, community; HCFO, healthcare facility onset; ASV, amplicon sequence variants; SCFA, short-chain fatty acids.

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microbiota member in healthy individuals [2,6]. Further research is needed to ascertain the effect of CPF colonisation on human gut microbiota, considering that some of this bacterium's strains can acquire genes from plasmids and cause intestinal disease [6].

A few studies have evaluated CPF-related gut microbiota changes [7–9], finding a decrease in beneficial species such as the *Bacteroides fragilis* group, *Bifidobacterium* spp. and *Lactobacillus* spp. However, such studies have not considered the presence of other pathogens, such as *Clostridioides difficile* whose coexistence with CPF can directly and/or indirectly affect gut microbiota composition [10–12], thereby biasing microbiota profiling estimation. *Clostridioides difficile* Infection (CDI)'s prominent role is related to its ability to produce toxins (A, B and binary toxins) and other virulence factors (genetic diversity, sporulation and antibiotic resistance) [13]. Such virulence factors can negatively affect patients' health, causing various clinical manifestations and disrupting intestinal ecosystem homeostasis [11]. Future studies should determine whether bacterial pathogens' coexistence is associated with changes in gut microbiota regarding patients suffering diarrhoea.

This study was thus aimed at describing the gut microbiota composition (bacteria, archaea, and eukaryotes) of patients suffering CPF- and/or CDI-related community- and intrahospital-acquired diarrhoea (considering the lack of Latin American studies in this field). Prokaryote and eukaryote composition was described through amplicon-based sequencing of either the 16S-rRNA or 18S-rRNA V4 hypervariable marker regions, respectively. Bacterial communities had differences regarding taxonomic composition and alpha and beta diversity, mainly influenced by diarrhoea acquisition site, whilst such indexes remained constant for eukaryotic communities.

Interestingly, significant differences were not observed due to the lack of samples having simultaneous CPF and CDI detection. The greatest imbalance occurred within groups having intrahospital-acquired diarrhoea when stratifying the groups by place of diarrhoea acquisition, suggesting this factor's influence on microbiota modification.

2. Materials and methods

2.1. Sample selection and group consolidation

Ninety-eight DNA samples were randomly selected from the Universidad de Rosario's (UR) Microbiology and Biotechnology Research Centre (CIMBIUR) biobank, based on the following quantity standards: > 20 ng/μL, 260/280 > 1.8. A Norgen Biotek Corp (Ontario, Canada) kit was used for extracting DNA from faecal samples, following the manufacturer's instructions; the samples had been PCR screened for CPF targeting the CPA toxin gene [5] as it has been detected in all CPF toxinotypes [3]. The samples were concurrently screened for CDI, as described elsewhere [14].

Briefly, PCR was used for CDI molecular detection, using primers targeting 16S-rRNA and glutamate dehydrogenase (*gdh*) genes. A positive PCR result targeting CPF and CDI was denoted as coexistence. Sample groups were established according to diarrhoea acquisition site, following the Society for Healthcare Epidemiology of America (SHEA) and the Infectious Diseases Society of America (IDSA) criteria [15].

This gave four groups: a) community (CO)-associated diarrhoea positive for CPF '+ ' (CO/+, n = 27), b) CO-associated diarrhoea negative for CPF '-' (CO/- n = 23), c) healthcare facility onset (HCFO)-associated diarrhoea positive for CPF (HCFO/+, n = 7) and d) HCFO-associated diarrhoea negative for CPF (HCFO/-, n = 41). Groups were constructed based on bacterial coexistence: + /+ : CDI and CPF coexistence, + /-: the presence of just CPF, -/+ : the presence of just CDI, -/-: a lack of both types of bacteria.

2.2. Quality assessment and sequencing

A NanoDrop/2000/2000c spectrophotometer (Thermo Fisher Scientific, Massachusetts, USA) was used for verifying DNA quality by agarose gel electrophoresis, along with concentration measurement; a 260:280 ratio at 1.8 and 2.0 and 20 ng/μL minimum concentration were confirmed for each sample.

The NovaSeq PE-250 platform (Illumina Inc) was used for sequencing all samples that met the quality criteria, using a minimum of 100,000 raw reads per sample. The Novogene Corporation Inc. (Sacramento, CA, USA) used 515-F (5′-GTGCCAGCMGCCGCGGTAA-3′) and 806-R primers (5′-GGACTACHVGGGTWTCTAAT-3′) [16] targeting the 16S-rRNA gene's V4 hypervariable region for bacteria and archaea sequencing; by contrast, 528 F (5′-GCGGTAATTCCAGCTCCAA-3′) and 706 R primers (5′-AATCCRAGAATTTCACCTCT-3′) [17] were used for amplifying and sequencing the 18S-rRNA gene's V4 hypervariable region for eukaryotes.

2.3. Taxonomic assignment

FastQC [18] and MultiQC [19] summary tools were used for assessing sequence quality,

considering parameters such as Phred score (minimum Q20) and the presence of adapters. The sequences were then merged, and chimeras eliminated; amplicon sequence variants (ASV) were obtained (defined as sequences varying by at least one nucleotide). The DADA2 pipeline package [20] was used for inferring exact ASVs from high-throughput amplicon sequencing data in R studio [21], using the recommended parameters. The DADA2 formatted SILVA database (version 132) [22] was used for ASV taxonomic assignment of bacteria and Archaea and the Protist Ribosomal Reference database (PR^2) [23] for eukaryotes.

2.4. Alpha and beta diversity analysis

Phyloseq, Vegan, DESeq2, RCy3, FSA, ggplot2 and reshape2 R packages were used for analysing diversity. Alpha diversity was evaluated for determining differences regarding ASV richness and abundance amongst groups by calculating Shannon and inverted Simpson indexes. Beta diversity was analysed by Bray Curtis similarity matrix-based principal coordinates analysis (PCoA) for establishing potential differences concerning sample clustering according to group.

2.5. Statistical analysis

Kruskal-Wallis tests with Benjamini-Hochberg correction for multiple comparisons and post hoc analysis using Dunn's test were used for assessing differences amongst groups regarding alpha diversity and genus abundance. A Man-Whitney test was used for comparing CPF infection status. Permutational multivariate analysis of variance (PERMANOVA) using analysis and partitioning sums of squares using dissimilarities (*adonis*) was used for evaluating differences in centroids in PCoA plots; < 0.05 *p*-values were assumed to be significant for the statistical analysis.

3. Results

3.1. *C. perfringens* infection and microbiota composition

Thirty-four of the 98 samples tested (34.7%) were CPF- and CPA-gene positive, 60 (61.2%) were CDI gene-positive and coexistence was seen in 24 (24.5%) samples. No samples from patients having hospital-acquired diarrhoea contained CPF whilst lacking CDI (HCFO +/-) (Supplementary Table 1). Microbiota composition did not vary significantly amongst groups as similar relative abundance of the phyla Bacillota, Bacteroidota and Pseudomonadota was observed, along with low relative abundance of Actynomicetota, Verrucomicrobia and Fusobacteria phyla (Fig. 1A and B).

Increased relative abundance of the phylum Bacteroidota was only observed in CPF-positive samples ($p = 0.0349$) (Fig. 1A). Increased

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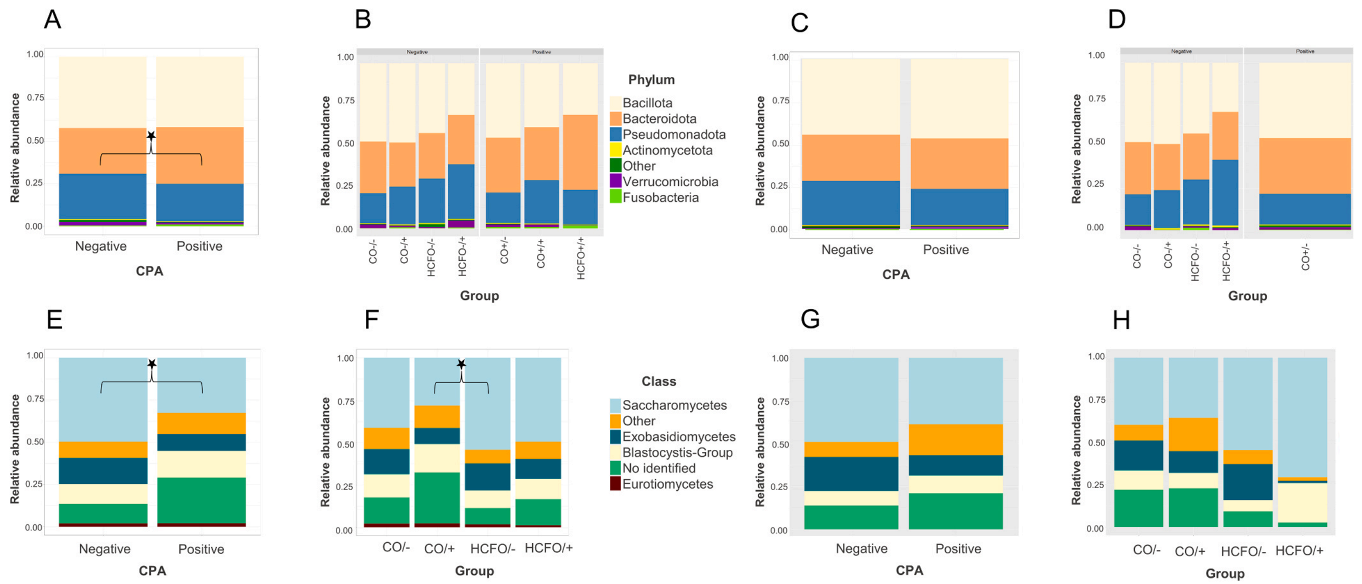

Fig. 1. Taxonomic composition of bacterial and eukaryotic microbiota by *C. perfringens/C. difficile* coexistence status and by group. A) Distribution of bacterial phyla involving *C. perfringens*; B) 16S-rRNA taxonomical composition by group (mixing diarrhoea acquisition site and *C. perfringens* and *C. difficile* detection); C) Bacterial phyla distribution by *C. perfringens* detection status in groups lacking simultaneous *C. perfringens* and *C. difficile* detection; D) Bacterial phyla distribution by group (mixing diarrhoea acquisition site and *C. perfringens* and *C. difficile* detection) in groups lacking simultaneous *C. perfringens* and *C. difficile* detection; E) Eukaryotic class distribution by *C. perfringens* detection status; F) Eukaryotic class distribution by group; G) Eukaryotic class distribution by *C. perfringens* detection status in groups lacking simultaneous *C. perfringens* and *C. difficile* detection; H) Eukaryotic class distribution by group re groups lacking simultaneous *C. perfringens* and *C. difficile* detection. Statistically significant differences are represented by an asterisk.

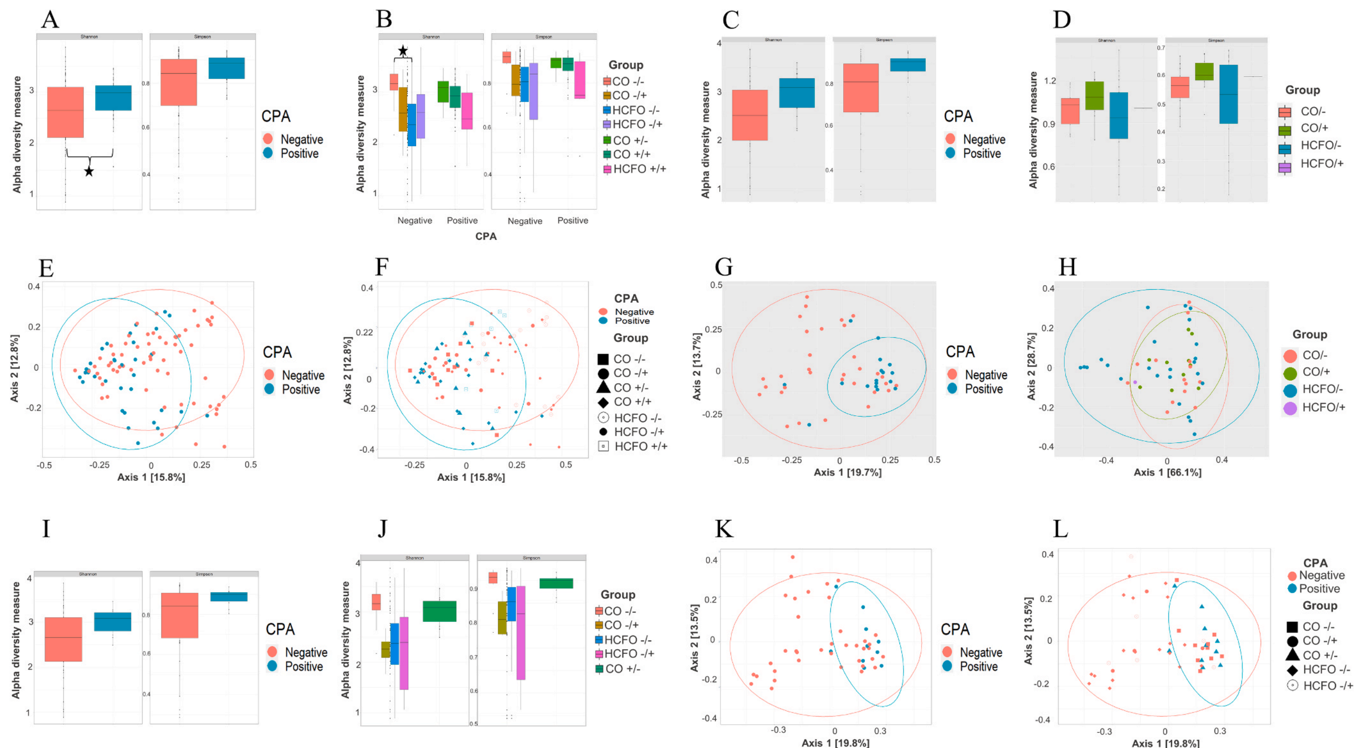

Fig. 2. Alpha and beta diversity measurement differences: statistically significant differences between groups are represented by a star; A) Alpha diversity indexes by bacterial sequence-based *C. perfringens* detection status; B) Alpha diversity indexes by bacterial sequence-based group (mixing diarrhoea acquisition site and *C. perfringens* and *C. difficile* detection); C) Alpha diversity indexes by eukaryotic sequence-based *C. perfringens* detection status; D) Alpha diversity indexes by eukaryotic sequence-based group in groups lacking simultaneous *C. perfringens* and *C. difficile* detection; E) PCoA by bacterial sequence-based *C. perfringens* detection status; F) PCoA by bacterial sequence-based group (mixing diarrhoea acquisition site and *C. perfringens* and *C. difficile* detection); G) PCoA by eukaryotic sequence-based *C. perfringens* detection status; H) PCoA by eukaryotic sequence-based group; I) Alpha diversity indexes by *C. perfringens* detection status-based bacterial sequences in samples lacking simultaneous *C. perfringens* and *C. difficile* detection; J) Alpha diversity indexes by group (mixing diarrhoea acquisition site and *C. perfringens* and *C. difficile* detection) based on bacterial sequences in samples lacking simultaneous *C. perfringens* and *C. difficile* detection; K) PCoA by eukaryotic sequence-based *C. perfringens* detection status; L) PCoA by eukaryotic sequence-based group.

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relative abundance of this phylum was evident in the HCFO + /+ group, although this difference was not observed when stratifying groups by coinfection status (Fig. 1B). No statistically significant differences were observed between phyla when re-analysing samples; this involved excluding groups in which CDI and CPF had been detected (Figs. 1C and 1D).

The groups' eukaryote composition had differential profiles characterised by high relative Saccharomycetes abundance, followed by *Blastocystis*-group and Exobasidiomycetes classes (Figs. 1E and 1F). Decreased relative Saccharomycetes class abundance was observed in positive samples ($p = 0.002474$) when considering CPF presence (Fig. 1E). Statistical analysis revealed a decrease in the CO/+ group versus the HCFO/- group ($p = 8.601011e\text{-}07$) in this class (Fig. 1F); conversely, no changes were observed regarding eukaryotic community abundance amongst groups when CDI-positive samples were eliminated from analysis (Figs. 1G and 1H).

3.2. Differences in alpha and beta diversity

Moderate bacterial diversity values were observed regarding CPF and CDI infection samples (Shannon $p = 0.006$, Simpson $p = 0.008$); higher bacterial diversity was observed in the CO -/- group compared to the HCFO -/- group (Shannon $p = 0.04$) (Fig. 2A and B). A lack of difference regarding diversity was observed when groups were analysed without CDI- and CPF-positive samples (Figs. 2C and 2D).

Bacterial PCoA highlighted spatial sample clustering by CPA result; the *adonis* test gave differences regarding centroid positions regarding infection status (PERMANOVA F = 3.2526, $p = 0.001$) along with great dispersion, especially of HCFO groups (Figs. 2E and 2F). Spatial clustering remained when analysing data without CPF- and CDI-positive samples (PERMANOVA F = 1.9555, $p = 0.031$) (Figs. 2G and 2H). Eukaryote diversity was moderate and did not vary regarding infection status or group (Figs. 2I and 2J); spatial clustering was not observed (Figs. 2K and 2L).

4. Discussion

Forero et al., reported up to 33.3% frequency for simultaneous CPF and CDI infection in Colombia [5]; such coexistence's effect on microbiota had not been explored previously. Although much research has shown CDI's direct and indirect effect on gut microbiota, i.e. common pathogens increase whereas beneficial bacteria become depleted [10,11,24], other factors must be taken into account when analysing microbiota from patients suffering CPF- and/or CDI-related community- and/or intrahospital-acquired diarrhoea. The pathogen's possible acquisition site must be analysed in depth since its presence alone does not necessarily trigger drastic changes in host microbiota [11].

Bacteroidota's increased relative abundance observed in CPF-positive samples (Fig. 1A) conflicted with previous research reporting a decrease in specific genera belonging to this phylum in Bacteroidota-positive samples [7,12]. However, such increase was largely due to the hospital-acquired diarrhoea group, along with simultaneous CDI and CPF (HCFO +/+) (Fig. 1B), where a considerable increase in *Bacteroides*' relative abundance was observed (despite few samples: n = 7) (data not shown).

The beneficial role played by many members of this genus [25,26] could suggest that patients in this group preserve a balance within their microbiota (even when two bacteria are associated with diarrheal symptoms). Such balance is promoted by their carbohydrate use machinery which could influence butyrate production in other microorganisms [27] (butyrate being a significant metabolite for gut microbiota homeostasis maintenance).

This poses a challenge for studying microbiota since the probable pathogen acquisition site adds to the list of factors that could influence results. This became evident when eliminating CO + /+ and HCFO + /+ groups from analysis, as statistically significant differences could no longer be observed (Figs. 1C and 1D). It must be mentioned that information regarding patients' health status and sociodemographic characteristics was lacking as this could have directly affected the results [11,28,29].

CDI and CPF interaction is another variable contributing to modifications within microbiota due to damage to the epithelium from toxins and/or other virulence factors [3,10]. Both bacterial species' potential for producing toxins was not evaluated as it is difficult to determine toxigenic profiles from DNA extracted directly from samples due to limitations regarding molecular test sensitivity (i.e. few copies of these genes during initial infection phases) or the potential presence of more than one toxinotype, thereby giving unreliable results. This highlights the need for reliable in vitro culture procedures for obtaining isolates from these species for genomic characterisation for determining virulence factors' true effect within gut microbiota's complex relationships.

The infection status and group differences observed for the class Saccharomycetes (Fig. 1E - 1F) contrasted with previous reports concerning inflammatory disease (i.e. irritable bowel syndrome (IBD); an increase in such fungi by as-yet-unknown mechanisms was shown [30]. Such differences were not observed when eliminating CDI- and CPF-positive samples, suggesting a complex interaction between bacteria and eukaryotes meriting further research.

Moderate alpha diversity values (mainly associated with positive samples - Fig. 2A), suggested complex relationships between microbiota members and CPF and CDI, despite the diarrhoea status of all patients included in this study. Eliminating samples having both CPF and CDI caused the lack of difference amongst groups, thereby highlighting the fact that microbiota modifications did not result from a single group, but rather the product of many components' interaction [10,24,28].

The groups of patients having diarrhoea associated with an in-hospital setting could have influenced the differences, since stratifying the results showed that the HCFO -/-, HCFO -/+ and HCFO + /+ groups had the greatest data dispersion diversity (i.e. a downward trend - Fig. 2B). It was evident that decreased diversity could have resulted from the bacillus' interactions with other intestinal ecosystem members in CPF-negative groups having CDI; this could have been promoted by factors such as nutrient competition, toxins and/or a decrease in short-chain fatty acids (SCFA) [31].

This has been the first report regarding bacterial and eukaryotic microbiota associated with CPF and CDI coexistence. Composition and diversity index variations suggested that such microorganisms' coexistence and pathogen acquisition sites could have affected gut microbiota, and that CDI could have further affected it by modulating ecosystem balance and contributing to an increase in its diversity.

Our study has limitations concerning a lack of clinical and demographic factors associated with the target patients from whom the samples were taken and a lack of information concerning CPF–positive samples' toxinotyping which could have affected the results.

Further research is required for evaluating CPF's effect and that of the toxinotypes and their load regarding gut microbial ecology. Collecting and evaluating clinical and sociodemographic data is also recommended for determining the effect of other factors on CPF and CDI role regarding gut microbiota. This study has highlighted the importance of analysing gut microbiota in a broad context for determining the effect of the relationships between microorganisms and intestinal homeostasis, rather than concentrating on their influence on a single element concerning this ecosystem. (Table 1).

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Table 1
C. perfringens and *C. difficile* found in the samples.

Group		CPA positive	CPA negative	Total
CO		27	23	50
HCFO		7	41	48
Total		34	64	98
Coinfection				
Status		**CPA positive**	**CPA negative**	**Total**
Cdiff positive		24	36	60
Cdiff Negative		10	28	38
Total		34	64	98
Coinfection by group				
Status		**CPA positive**	**CPA negative**	**Total**
Cdiff/+	CO	17	13	30
	HCFO	7	23	30
Cdiff/-	CO	10	10	20
	HCFO	0	18	18
Total		34	64	98

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Ethical approval statement

The study was considered low risk according to Colombian Ministry of Health resolution 8430/1993. Samples were coded according to Colombian ethical guidelines and the Declaration of Helsinki to avoid patient identification. This project was approved by the Universidad del Rosario's (UR) Research Ethics Committee (approval 339). Written informed consent was obtained for using the samples in this research, as authorised by the UR ethics committee.

CRediT authorship contribution statement

GH, JDR, and MM: conceptualisation and methodology; GH and AC: investigation and data curation; GH and MM: software, validation, and formal analysis; MAP, JDR and MM: funding acquisition, review and editing the final manuscript. All authors have read and approved the final manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.cimid.2023.102074.

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