



Metagenome-assembled genomes (MAGs) suggest an acetate-driven protective role in gut microbiota disrupted by *Clostridioides difficile*

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ABSTRACT

Clostridioides difficile may have a negative impact on gut microbiota composition in terms of diversity and abundance, thereby triggering functional changes supported by the differential presence of genes involved in significant metabolic pathways, such as short-chain fatty acids (SCFA). This work has evaluated shotgun metagenomics data regarding 48 samples from four groups classified according to diarrhea acquisition site (community- and healthcare facility-onset) and positive or negative *Clostridioides difficile* infection (CDI) result. The metagenomic-assembled genomes (MAGs) obtained from each sample were taxonomically assigned for preliminary comparative analysis concerning differences in composition among groups. The predicted genes involved in metabolism, transport, and signaling remained constant in microbiota members; characteristic patterns were observed in MAGs and genes involved in SCFA butyrate and acetate metabolic pathways for each study group. A decrease in genera and species, as well as relative MAG abundance with the presence of the acetate metabolism-related gene, was evident in the HCFO/- group. Increased antibiotic resistance markers (ARM) were observed in MAGs along with the genes involved in acetate metabolism. The results highlight the need to explore the role of acetate in greater depth as a potential protector of the imbalances produced by CDI, as occurs in other inflammatory intestinal diseases.

1. Introduction

Clostridioides difficile infection (CDI) is considered a public health problem in developed countries due to its high morbidity-mortality and high health system costs (Balsells et al., 2019; Desai et al., 2016; Heister et al., 2019). CDI is caused by a sporulated anaerobic bacillus, resistant to multiple antibiotic regimens. It results in recurrent infections that lead to pseudomembranous colitis, sepsis, and even death (Abt et al.,

2016; Ma et al., 2021). *C. difficile* can proliferate due to the action of antibiotics and other drugs; multiple interactions among intestinal microbiota members become altered, resulting in the worsening of symptoms and even in the proliferation of other opportunistic pathogens due to the imbalance caused by the decrease in commensal bacteria (Abbas and Zackular, 2020; Herrera, Paredes-Sabja, et al., 2021; Herrera, Vega, et al., 2021). This highlights the importance of this intestinal microorganism in terms of disease development and recurrence and the

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elements that could contribute to protection against it (Abt et al., 2016; Herrera, Paredes-Sabja, et al., 2021).

Shotgun metagenomics unveiling the gut ecosystem has led to reconstructing the taxonomic composition of the studied environments, exploring functional diversity, and comparing metagenomic-assembled genomes (MAGs) (Quince et al., 2017). This has facilitated the description of new microorganism species (Parks et al., 2017), epidemiological surveillance of antibiotic resistance markers (e.g., in wastewater) (G. Chen et al., 2022; Singleton et al., 2021), and the in-depth description of various species' microbiome composition and potential functioning (Crook et al., 2021; Thongsripong et al., 2021; Zeng et al., 2022).

Few CDI studies have shown commensal carbohydrate-degrading clostridial interaction with *C. difficile*; this inhibits bacillus growth, decreasing associated symptoms (Fishbein et al., 2022). Other studies have proposed some short-chain fatty acids (SCFA) roles, butyrate being the most studied due to its protective role against CDI; it activates an immune response by neutrophil recruitment and modulating chemotaxis (i.e., neutrophil migration) (Fachi et al., 2020).

Other research has demonstrated butyrate's modulation of macrophage and dendritic cell (DC) proinflammatory activity as well as controlling pathogenic bacteria overgrowth by increasing peroxisome proliferator-activated receptor- γ (PPAR- γ), which increases beta-oxidation in colonocytes, thereby decreasing available luminal oxygen (Akhtar et al., 2022; Antharam et al., 2013; J. Chen and Vitetta, 2020; L. Zhang et al., 2021). Recent studies have highlighted the active involvement of acetate in intestinal processes, particularly in neutrophil-mediated responses against *C. difficile* (Fachi et al., 2020) and antiviral responses (Niu et al., 2023). Moreover, emerging evidence suggests that acetate plays a vital role in the gut-brain axis, promoting metabolic fitness in the brain's immune system (Erny et al., 2021). This indicates that SCFAs play a crucial role in maintaining gut microbiome homeostasis by creating a beneficial environment with low levels of inflammation. Consequently, this reduces the impact of microorganisms with pathogenic potential, such as *C. difficile*. Therefore, it is essential to determine which microorganisms can contribute to the production of SCFAs and thus contribute to the homeostasis of the gut microbiota. Approaches such as shotgun metagenomics can be used for taxonomic and functional profiling and the reconstruction of MAGs to better understand their contribution to this process.

Few studies have used shotgun metagenomics for investigating CDI in Latin America; our team's study has highlighted taxonomic alterations and differentially abundant species in each study group, along with a decrease in the genes involved in butyrate metabolism, mainly in the groups suffering hospital-acquired diarrhea (Herrera et al., 2022). However, intestinal microbiota's metabolic and functional profiles in Latin American CDI patients remain unknown.

The study's second stage was thus aimed at evaluating SCFAs' taxonomic and functional profile, emphasizing MAGs recovered from samples taken from patients with and without CDI in two hospitals in Bogota, Colombia. Given the significant role of SCFAs in maintaining gut microbiota homeostasis, it is crucial to assess the diversity and relative abundance of MAGs carrying genes involved in SCFAs metabolism, specifically acetate and butyrate, in community- and intrahospital-onset groups (CO and IH). This hypothesis stems from our previous observations of notable modifications in gut microbiome composition, both taxonomically and functionally.

We utilized shotgun metagenomics data to assemble MAGs, which were analyzed based on their taxonomic, functional, and antibiotic resistance profiles. Subsequently, we compared the MAGs among different groups based on the presence of genes related to SCFA metabolism. An increase in MAGs' relative abundance with the presence of genes involved in acetate metabolism in the groups having less microbiota imbalance suggested an essential role for this SCFA in intestinal homeostasis that should be addressed in future research.

2. Materials and methods

2.1. DNA selection and shotgun metagenomic sequencing

Forty-eight out of 98 DNA samples from a study by Muñoz et al. (2018), stored in the Universidad del Rosario's Microbiology and Biotechnology Research Centre's cryobank, were selected for the present research. Samples were randomly chosen in compliance with technical requirements (DNA concentration, purity, and volume) and had been previously classified according to the Society for Healthcare Epidemiology of America and Infectious Diseases Society of America guidelines (Cohen et al., 2010) and the presence/absence of *C. difficile* as described in Muñoz et al. (2018) in four populations: community-onset CDI positive (CO/+, n=13), community-onset CDI negative (CO/-, n=14), healthcare facility-acquired CDI positive (HCFO/+, n=13) and healthcare facility-acquired CDI negative (HCFO/-, n=8). Sequencing was conducted by the external facility Novogene (USA, Sacramento, CA), generating reads of 150 base pairs in length under the following conditions: PE150, Q30>80%, and 4 Gb of raw data per sample.

2.2. Data quality and filtering

FastQC (v. 0.11.9) (Andrews, 2010) and MultiQC (v. 1.6) (Ewels et al., 2016) were used for assessing sequence quality; the Trimmomatic tool (v. 0.38) (Bolger et al., 2014) was used for filtering and cutting sequences (Q score <20, length <150 bp). The Bowtie2 tool (v. 2.4.4) (Langmead and Salzberg, 2012) was used for deleting human host reads using the reference genome reported by the National Center for Biotechnology Information (NCBI) (GRCh38, accession number PRJNA31257).

2.3. Metagenomic assembled genomes (MAGs)

The SPAdes assembler (v.3.15.4) with the meta parameter (35) toolkit/pipeline was used to assemble reads from the metagenomic data set, using 1500 nt as the contig size. Bowtie2 and SAMtools (v. 1.17) sequencing alignment tools were used to map the decontaminated reads, using the MAGs as reference (Li et al., 2009).

Maxbin (v. 2.2.7) (Wu et al., 2016), Metabat2 (v. 2.15) (Kang et al., 2019), and Concoct (v. 1.1.0) (Alneberg et al., 2013) were used for binning the assemblies. The CheckM tool (v. 1.2.2) (Parks et al., 2015) was used to check bin quality, and the DAS Tool (v. 1.7.0) (Sieber, 2017) was used to refine the bins. Only high-quality drafts were selected for downstream analysis, defined by Bowers et al.'s parameters (Bowers et al., 2017) (completion >90%, contamination <5%) (Figure S1).

2.4. MAGs taxonomical assignment

The Genome Taxonomy Database Toolkit (GTDB-Tk was used for taxonomic classification (Chaumeil et al., 2020); the PhyloPhlan (v. 3.0.2) integrated pipeline (Segata et al., 2013) was used for phylogenetic reconstruction using the 400 universal PhyloPhlan markers with the following options: -diversity medium -fast -min_num_markers 100, using the phylophlan database as reference, displayed in interactive tree of life - iTol (v. 6) (Letunic and Bork, 2019).

2.5. Functional annotation and MAG analysis

EggNOG-mapper functional annotation tool (v. 2.1.9) (Cantalapiedra et al., 2021) was used from high-quality MAG drafts, identifying clusters of orthologous groups (COGs). These COGs were categorized based on their functional categories, including cellular processes and signaling, information storage and processing, metabolism, and poorly characterized functions. The COGs were then plotted for each previously defined working group. Furthermore, Prokka (Seemann, 2014) was utilized to annotate the MAGs functionally. Acetate kinase (ackA),

butyrate kinase (buk), butyryl-CoA: acetate CoA-transferase (but), methyl malonyl-CoA decarboxylase (mm), lactose-CoA dehydratase (lcdA), and CoA-dependent propionaldehyde dehydrogenase (pduP) were the specific enzymes involved in SCFA metabolism identified.

Each MAG's relative abundance was determined by calculating the total number of reads mapped to MAG based on each sample's total number of reads (Hua et al.) (Hua et al., 2019). Abricate (v. 1.0.1) (Seemann, 2018) was used to search for antibiotic resistance markers (ARM) and virulence factors by comparing it with the Comprehensive Antibiotic Resistance Database (CARD) (updated in July 2022) (Alcock et al., 2020) and the virulence factor database (updated in July 2022) (L. Chen et al., 2005).

2.6. Statistical analysis

A bubble plot demonstrated MAG quality dispersion using the statistical software. Mann-Whitney U and Kruskal-Wallis tests with Bonferroni correction and Dunn's test were used for multiple comparisons in R studio software (R Core Team 2021. R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria., n.d.) for post hoc analysis, taking < 0.05 *p*-values as statistically significant.

3. Results

3.1. Data quality assessment

After filtering out host sequences, an average of 15.9 million reads per sample remained from the initial 16.4 million reads, each with a Phred score >33 . The data quality has been previously described (Herrera et al., 2022).

3.2. MAGs quality

The workflow regarding bin assembly and dereplication gave 437 drafts of high-quality MAGs to be assembled from the samples; 130 (29.7%) were recovered from CO/- group samples, 144 (33.0%) from the CO/+ group, 60 (13.7%) from the HCFO/- group and 103 (23.6%) from the HCFO/+ group. Table S1 summarizes the quality of the recovered MAG statistics. MAG sizes ranged from 1.4 to 7.0 megabases, 97 kb N50 average (3.7 kb to 1.5 Mb). The % GC ranged from 24.9% to 65%; 6 of the 437 MAGs had 100% completeness and 0% contamination (Figure S1A).

3.3. MAG taxonomic assignment and functional annotation

Taxonomically, high-quality draft MAGs belonged to 11 phyla, Bacillota ($n=222$), Bacteroidota ($n=109$), and Pseudomonadota ($n=61$) being the most abundant (Fig. 1A); the Clostridia class was the most abundant ($n=181$). *Prevotella* (23), *Alistipes* (20), and *Escherichia* (19) genera were most abundant. A slight increase in MAGs belonging to the Verrucomicrobiota and Actinomycetota phyla was observed in the HCFO/- group (Fig. 1B), although no statistically significant differences were observed. It was found that most genes were involved in metabolism, followed by information processing and storage. No differences were observed between groups when analyzing the MAGs according to COG categories (Fig. 1C).

Enzymes buk and ackA were found in 229 MAGs (90 for both enzymes, four only for buk, and 135 only for ackA) when ascertaining MAGs in SCFA metabolism-encoding genes; genes encoding both enzymes were found in 5 phyla, Bacteroidota and Bacillota being the most abundant (Figure S1B).

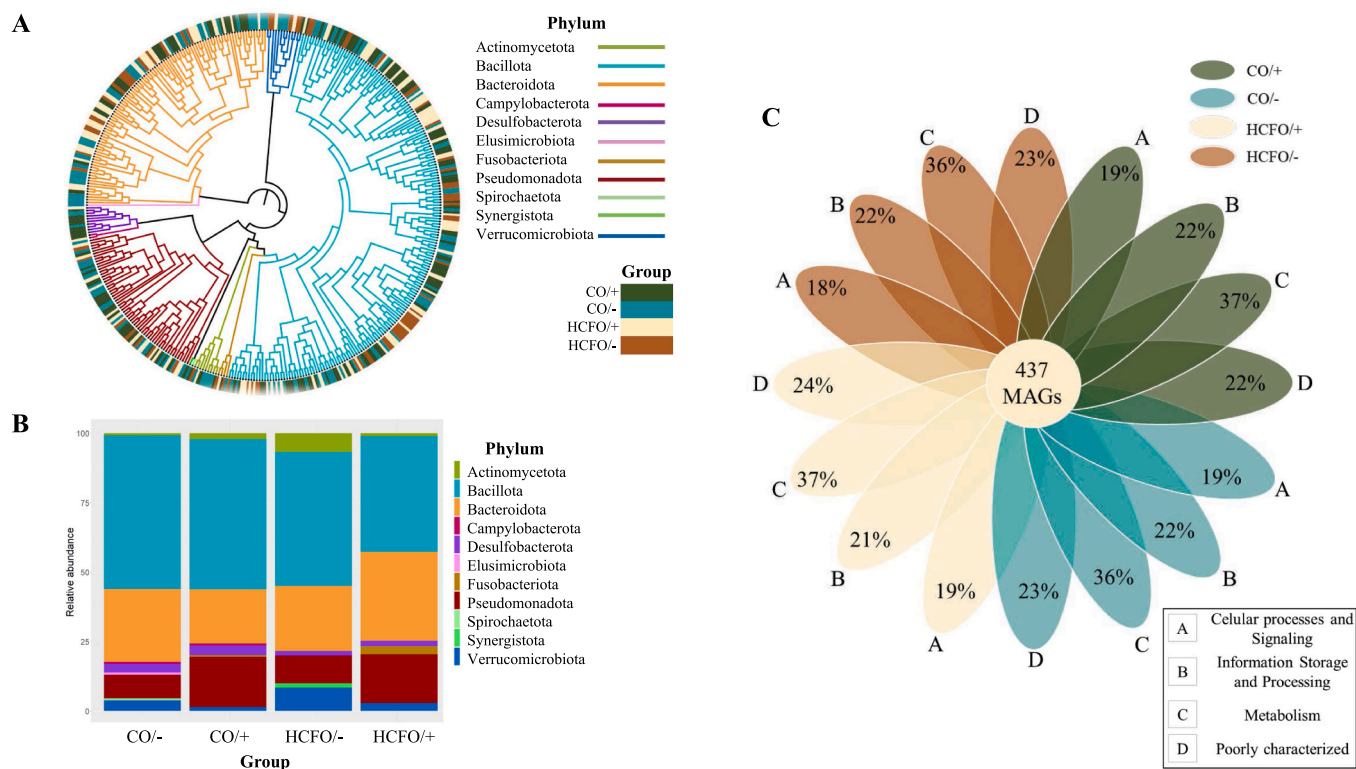


Fig. 1. MAG's taxonomic and functional profiles. A) Phylogenetic tree of the MAGs assembled from the samples. Branch colors denote the Phylum to which they belong, while outer ring colors identify the one from which the MAG was assembled. B) MAG taxonomic composition by group. C) Functional profiling of MAGs according to the function of each identified COG.

3.4. Differential profile of acetate producers among groups

MAG analysis of *ackA* and *buk* enzyme presence gave each study group a differential microorganism profile capable of metabolizing SCFA (Fig. 2A). The CO/- group had 25 species capable of metabolizing acetate and 10 for butyrate, which was not found in other groups (differentially present species); a more significant percentage of MAGs identified as *Butyrivibrio crossotus* and *Alistipes finegoldii* was observed.

The CO/+ group was characterized by 21 unique species having *ackA* and 11 with *buk*, the genus *Prevotella* predominating. The intrahospital-associated groups had profiles, where from the samples of the HCFO/- group, 16 differentially present species metabolizing acetate and five metabolizing butyrate were recovered, while the HCFO/+ group had 25 differentially present species with enzymes for acetate metabolism and 7 for butyrate, mainly characterized by the presence of the genera *Bacteroides*, *Enterococcus* and *Alistipes* (Fig. 2A and B). Statistically significant differences were found when analyzing the number of genera having enzymes for metabolizing SCFA in each group, specifically for genera having *ackA* in the CO/- vs. HCFO/- ($p=0.0046$), CO/+ vs. HCFO/- ($p=0.028$) and HCFO/- vs. HCFO/+ ($p=0.034$) groups (Fig. 2C). No differences were observed between groups regarding butyrate.

3.5. MAG relative abundance, resistance, and virulence profile

MAGs' relative abundance varied markedly, with the CO/+ and CO/- groups having the highest relative abundance and the HCFO/- group having low relative abundance re most MAGs (Fig. 3A). *Faecalibacterium prausnitzii* (CO/+ vs. HCFO/- $p=0.036$; HCFO/- vs. HCFO/+ $p=0.045$) and *Pseudomonas helleri* (CO/- vs. HCFO/-, $p=0.031$; CO/+ vs. HCFO/+, $p=0.033$) had statistically significant differences. There were no statistically significant differences between ARMs and virulence factors (Figure S1C).

However, when analyzing ARMs regarding the class of antibiotics against which they conferred resistance, differences were found concerning aminocoumarin (acetate vs. both, $p=0.022$; acetate vs. none, $p=0.032$), aminoglycoside (acetate vs. both, $p=0.027$; acetate vs. none, $p=0.035$), phosphomycin (acetate vs. none, $p=0.018$) and peptide (acetate vs. none, $p=0.015$) (Fig. 3B). The same analysis discriminating by study group revealed differences in the abundance of ARMs related to acridine dye (CO/- vs. HCFO/+, $p=0.022$; CO/+ vs. HCFO/+, $p=0.030$; HCFO/- vs. HCFO/+, $p=0.02$), diamminopyrimidine (CO/- vs. HCFO/+, $p=0.007$; CO/+ vs. HCFO/+, $p=0.010$; HCFO/- vs. HCFO/+, $p=0.011$) and lincosamide (CO/- vs. HCFO/+, $p=0.003$; CO/+ vs. HCFO/+, $p=0.010$; HCFO/- vs. HCFO/+, $p=0.012$) (Fig. 3B).

4. Discussion

Shotgun metagenomics enabled the exploration of differences between taxonomic groups and functional changes that might have occurred in the microbiota of the individuals from which the samples were collected. The taxonomic composition of the MAGs recovered from diarrheal patient samples exhibited profiles like those previously described through read-based analysis on the same samples (Herrera et al., 2022). This analysis revealed increased microorganisms belonging to the Phylum Pseudomonadota in both CDI-positive samples and in patients with diarrhea associated with the in-hospital setting (HCFO/+ and HCFO/-) (Fig. 1A and B). Multiple factors may contribute to the increased abundance of Pseudomonadota members in HCFO groups. These factors include intestinal imbalances, interactions during CDI, and the acquisition location of the diarrhea. Notably, in-hospital environments can harbor highly virulent pathogens, which may also contribute to the rise in CDI cases (Abt et al., 2016; Edwardson and Cairns, 2019; Herrera, Paredes-Sabja, et al., 2021).

Despite MAG taxonomic composition differences, COG analysis did not reveal marked variations regarding macro processes amongst groups

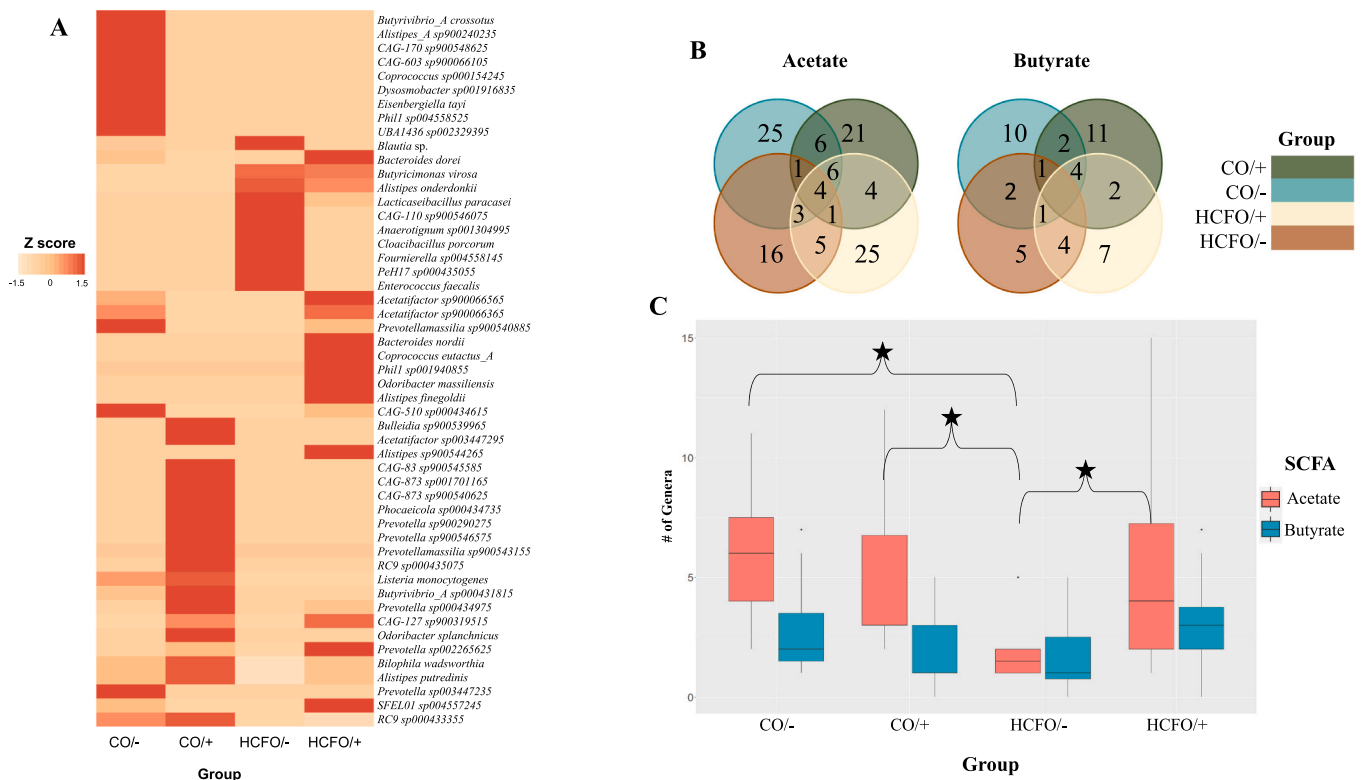


Fig. 2. Differences in the profiles of bacteria able to metabolize MAGs amongst study groups. A) Relative MAG abundance in which genes encoding *ackA* and *buk* enzymes were recovered for each group. B) Amount of unique and shared species among groups having *ackA* (acetate) or *buk* (butyrate) encoding genes. C) Boxplot with the number of genera with *ackA* and *buk* enzymes for each group.

Previous studies regarding these samples have shown taxonomic alterations in the HCFO/- group characterized by a reduction in beneficial bacteria's relative abundance (Herrera et al., 2022; Herrera, Vega, et al., 2021); this could have decreased SCFA metabolism, in turn negatively affecting immunoglobulin A (IgA) production, which would have facilitated pathogenic bacteria proliferation and decreased beneficial bacteria maintenance capability (Deleu et al., 2021; Gutzeit et al., 2014; Markowiak-Kopeć and Śliżewska, 2020; W. Wu et al., 2017). However, since SCFA levels could not be quantified in this study's samples, the impact of MAG reduction on the enzymes involved in their metabolism could not be determined (requiring further research adopting a multi-omics approach).

The large amount of genera and species, along with high relative MAG abundance with *ackA* and *bukA* observed in the CDI groups (CO/+ and HCFO/+) (Figs. 2 and 3) contrasted with what was expected, since this pathogen is associated with altered microbiota characterized by decreased SCFA metabolism-associated beneficial bacteria (Abt et al., 2016; Antharam et al., 2013; Fishbein et al., 2022; Gregory et al., 2021). However, despite the varied toxigenic profiles in *C. difficile* isolates recovered from samples, (Muñoz et al., 2018, 2019) our research revealed slightly altered microbiota in the target patients, characterized by beneficial bacteria's substantial diversity and relative abundance (Herrera et al., 2022; Herrera, Vega, et al., 2021). This might have explained the increase of MAGs potentially affecting SCFA metabolism, as acetate production-related metabolic pathways are widely distributed among classes of bacteria (Deleu et al., 2021).

SCFA's ability to inhibit *C. difficile* growth has been demonstrated. It has also been shown that SCFA can inhibit this potentially pathogenic bacteria's growth (Herrera, Paredes-Sabja, et al., 2021; Kondepudi et al., 2012; McDonald et al., 2018). Some studies have shown that these metabolites may exacerbate its toxin production (Hryckowian et al., 2018; Karlsson et al., 2000), which could be related to the varied toxigenic profiles mentioned above. However, further studies are required to establish the relationship between these elements.

Despite the increase in AMRs observed in MAGs with enzymes for acetate metabolism (Fig. 3B), further studies are required to determine whether such markers confer antibiotic resistance. Different factors such as abundance, ability to express ARMs, and propensity to be transferred must be evaluated to establish whether they pose a patient risk (Zhang, Zhang, Wang, et al., 2022).

C. difficile's survival mechanisms should be determined in microbiota, which has the potential for SCFA metabolism, primarily acetate, as this fatty acid is closely related to a neutrophil-mediated response against the bacillus (Fachi et al., 2020). Dual transcriptomic studies are needed to demonstrate *C. difficile*'s metabolic profiles, the members of the microbiota, and the host, along with interactome studies focused on establishing relationships between the bacillus and the host. Multicenter studies are required to evaluate the genomic, transcriptomic, and metabolic variations that may occur compared to healthy patients' profiles to establish specific therapies and even the usefulness of previous metabolomic studies (Dawkins et al., 2022; Fletcher et al., 2018; Robinson et al., 2019).

The study was limited because clinical data that could help explain the results were unavailable for the patients from whom the samples were taken. Additionally, there was no control group without diarrhea, which would have enabled more robust comparisons. Therefore, developing future studies that include more samples and incorporate epidemiological and clinical metadata is pivotal. This will facilitate the identification of variables involved in the changes identified in this study.

This also emphasized that the presence of genes alone does not demonstrate a microorganism's ability to perform a given function. It should be noted that the clinical data regarding the patients from whom the samples were taken could not be obtained. Likewise, there was no control group without diarrhea. It is crucial to note that some SCFA-producing bacteria could be overlooked due to the strict quality

standards applied to this data. Therefore, this approach should be supplemented by measuring SCFA levels in serum and stool samples to better understand these metabolites in conditions such as CDI and other inflammatory bowel diseases. Likewise, research involving multiple omics is required for a holistic understanding of the process occurring during CDI.

5. Conclusion

This has been the first study reporting MAG assembly from CDI samples in Colombia. A reduction in bacteria able to metabolize SCFA appears to affect intestinal microbiota's taxonomic composition and diversity, mainly in patients with diarrhea in an in-hospital setting. Acetate could play a pivotal role in the microbiota during CDI, reducing the impact of *C. difficile*. Further research is needed to ascertain the influence of acetate and other SCFAs during CDI and their potential as a therapy for intestinal inflammatory diseases.

Ethical approval

The current project was conducted with the approval of the Universidad del Rosario's Research Ethics Committee (approval number 339). According to Colombian Ministry of Health Resolution 8430/1993, this study was considered low risk.

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CRediT authorship contribution statement

Giovanni Herrera: Writing – original draft, Visualization, Software, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Sergio Castañeda:** Visualization, Formal analysis. **Marina Muñoz:** Writing – review & editing, Visualization, Validation, Supervision, Software, Methodology, Funding acquisition, Formal analysis, Conceptualization. **Juan Camilo Arboleda:** Software, Methodology, Conceptualization. **Juan E. Pérez-Jaramillo:** Software, Methodology. **Manuel Alfonso Patarroyo:** Writing – review & editing, Funding acquisition. **Juan David Ramírez:** Writing – review & editing, Validation, Supervision, Investigation, Funding acquisition, Conceptualization.

Declaration of Competing Interest

none

Data availability

Data will be made available on request.

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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.micres.2024.127739](https://doi.org/10.1016/j.micres.2024.127739).

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