DETERMINACIÓN DEL MICROBIOMA INTESTINAL EN PACIENTES CON INFECCIÓN POR *Clostridioides difficile* ADQUIRIDA EN UNIDAD DE CUIDADOS INTENSIVOS Y COMUNIDAD

GIOVANNY ANDRÉS HERRERA OSSA

Documento de tesis presentado como requisito para optar al título de Doctor en Ciencias Biomédicas y Biológicas y Doctor en Salud y Desarrollo en los Trópicos

DOCTORADO EN CIENCIAS BIOMÉDICAS Y BIOLÓGICAS

UNIVERSIDAD DEL ROSARIO

BOGOTÁ D.C., COLOMBIA

DOCTORADO EN SALUD Y DESARROLLO EN LOS TRÓPICOS

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SALAMANCA, ESPAÑA

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PROGRAMA DE DOCTORADO EN SALUD Y DESARROLLO EN LOS TRÓPICOS

ESCUELA DE DOCTORADO "STUDII SALAMANTINI"

UNIVERSIDAD DE SALAMANCA

Los directores de la Tesis Doctoral titulada *"**Determinación del microbioma intestinal en pacientes con infección por Clostridioides difficile adquirida en unidad de cuidados intensivos y comunidad**"*, elaborada por el doctorando Giovanny Andrés Herrera Ossa, autorizan la presentación de esta Tesis en la modalidad de compendio de artículos.

en Salamanca, a 30 de marzo de 2023

Fdo. Dr. Juan D. Ramírez Fdo. Dra. Marina Muñoz Fdo. Dr. Julio López Abán

PROGRAMA DE DOCTORADO EN SALUD Y DESARROLLO EN LOS TRÓPICOS

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Dr. Juan David Ramírez, Profesor Asociado de la Facultad de Ciencias Naturales de la Universidad del Rosario; **Dra. Claudia Marina Muñoz,** Directora Técnica del Centro de Investigaciones en Microbiología y Biotecnología-UR (CIMBIUR) de la Facultad de Ciencias Naturales de la Universidad del Rosario; y **Dr. Julio López Abán,** Profesor Titular de Parasitología de la Universidad de Salamanca

INFORMAN:

Que la Tesis Doctoral realizada bajo nuestra dirección por D. Giovanny Andrés Herrera Ossa, con el título: *"**Determinación del microbioma intestinal en pacientes con infección por Clostridioides difficile adquirida en unidad de cuidados intensivos y comunidad**"*, reúne los requisitos de calidad, originalidad y presentación exigibles a una investigación científica, y está en condiciones de ser sometida a la valoración del Tribunal encargado de juzgarla en la modalidad de Tesis por compendio de publicaciones para optar al GRADO DE DOCTOR por la Universidad de Salamanca.

Para que así conste a los efectos oportunos, firman la presente en Salamanca, a 30 de marzo de 2023

Fdo. Dr. Juan D. Ramírez Fdo. Dra. Marina Muñoz Fdo. Dr. Julio López Abán

La presente Tesis Doctoral titulada *"**Determinación del microbioma intestinal en pacientes con infección por Clostridioides difficile adquirida en unidad de cuidados intensivos y comunidad**"*, realizada por D. Giovanny Andrés Herrera Ossa, bajo la dirección del Dr. Juan David Ramírez, codirección de la Dra. Claudia Marina Muñoz y Tutoría del Dr. Julio López Abán, corresponde a un compendio de artículos originales publicados en revistas científicas de prestigio internacional e indexadas en el Science Citation Reports, cuyos datos bibliométricos se detallan en el Anexo I. A continuación, se describen todos los artículos producidos como resultados de la presente tesis, su título, afiliaciones y autores, junto a la referencia completa de la revista científica donde fueron publicados

Artículo primero:

Título: Occurrence of *Blastocystis* in patients with *Clostridioides difficile* infection
Autores: Laura Vega[1], Giovanny Herrera[1], Marina Muñoz[1], Manuel Alfonso Patarroyo[2,3], Juan David Ramírez[1]
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Revista: Pathogens
Referencia completa:
Vega, L., Herrera, G., Muñoz, M., Patarroyo, M. A., & Ramírez, J. D. (2020). Occurrence of *Blastocystis* in patients with *Clostridioides difficile* infection. Pathogens, 9(4), 283.

Artículo segundo:

Título: Gut microbiota profiles in diarrheic patients with co-occurrence of *Clostridioides difficile* and *Blastocystis*
Autores: Laura Vega[1], Giovanny Herrera[1], Marina Muñoz[1], Manuel Alfonso Patarroyo[2,3], Jenny G. Maloney[4], Monica Santín[4], Juan David Ramírez[1]
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Revista: PLoS One

Referencia completa:

Vega L, Herrera G, Muñoz M, Patarroyo MA, Maloney JG, et al. (2021) Gut microbiota profiles in diarrheic patients with co-occurrence of *Clostridioides difficile* and *Blastocystis*. PLOS ONE 16(3): e0248185. https://doi.org/10.1371/journal.pone.0248185

Artículo Tercero:

Título: Gut microbiota composition in health-care facility-and community-onset diarrheic patients with *Clostridioides difficile* infection

Autores: Giovanny Herrera[1], Laura Vega[1], Manuel Alfonso Patarroyo[2,3,4], Juan David Ramírez[1] & Marina Muñoz[1]

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Revista: Scientific Reports

Referencia completa:

Herrera, G., Vega, L., Patarroyo, M.A. et al. Gut microbiota composition in health-care facility-and community-onset diarrheic patients with *Clostridioides difficile* infection. Sci Rep 11, 10849 (2021). https://doi.org/10.1038/s41598-021-90380-7

Artículo cuarto:

Título: Updating changes in human gut microbial communities associated with *Clostridioides difficile* infection

Autores: Giovanny Herrera[a], Daniel Paredes-Sabja[b,c], Manuel Alfonso Patarroyo[d,e,f], Juan David Ramírez[a,h], Marina Muñoz[a]

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Revista: Microbiology Spectrum

Referencia completa: Herrera, G., Paredes-Sabja, D., Patarroyo, M. A., Ramírez, J. D., & Muñoz, M. (2021). Updating changes in human gut microbial communities associated with *Clostridioides difficile* infection. Gut Microbes, 13(1), 1966277.

Artículo quinto:

Título: Microbial inter-domain interactions delineate the disruptive intestinal homeostasis in *Clostridioides difficile* infection

Autores: Giovanny Herrera[a], Juan Camilo Arboleda[b,c,d], Juan E. Pérez-Jaramillo[c,d], Manuel Alfonso Patarroyo[e,f,g], Juan David Ramírez[a,h], Marina Muñoz[a]

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Revista: Microbiology Spectrum

Referencia completa: Herrera, G., Arboleda, J. C., Pérez-Jaramillo, J. E., Patarroyo, M. A., Ramírez, J. D., & Muñoz, M. (2022). Microbial Interdomain Interactions Delineate the Disruptive Intestinal Homeostasis in *Clostridioides difficile* Infection. *Microbiology Spectrum*, *10*(5), e00502-22.

Artículo sexto:

Título: Interrogating the gut microbiota from several human-biting tick species in Northwestern Spain

Autores: Giovanny Herrera[1], María Carmen Vieira Lista[2], Luisa Páez-Triana[1], Antonio Muro[2], Julio López-Abán[2], Marina Muñoz[1], Juan David Ramírez[1,3]

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Revista: Journal of Medical Entomology

Referencia completa: Herrera G, Vieira Lista MC, Páez-Triana L, Muro A, López-Abán J, Muñoz M, Ramírez JD. Interrogating the gut microbiota from several human-biting tick species in Northwestern Spain. J Med Entomol (Sometido)

Artículo séptimo:

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

Autores: Giovanny Herrera[a], Juan Camilo Arboleda[b,c,d], Juan E. Pérez-Jaramillo[c,d], Manuel Alfonso Patarroyo[e,f,g], Juan David Ramírez[a,h], Marina Muñoz[a]

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Revista:

Referencia completa: Herrera, G., Castañeda, S., Arboleda, J. C., Pérez-Jaramillo, J. E., Patarroyo, M. A., Ramírez, J. D., Muñoz M. Metagenome-assembled genomes (MAGs) suggest an Acetate-driven protective role in gut microbiota disrupted by *Clostridioides difficile.* (Sometido)

A mis padres Diana y Giovanny

Por la vida, el apoyo y el amor.

... y a vos, por cumplir tu sueño de niño

"No hay nada repartido de modo más equitativo
en el mundo que la razón: todo el mundo
está convencido de tener suficiente."
René Descartes

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ABREVIATURAS

CD: *Clostridioides difficile*

CdtLoc: *Clostridioides difficile* transferase locus

CLIPP: Common luminal intestinal parasitic protists

Ctn: Transposones de tipo conjugativo

ICD: infección por *Clostridioides difficile*

ITS: espaciador de transcrito interno

Kb: Kilobases

MAG: Metagenome – assembled genome

Mb: Megabases

SCP: Secuencias codificantes de proteínas

UCI: Unidad de cuidados intensivos

TABLA DE CONTENIDOS

LISTA DE FIGURAS

RESUMEN

Clostridioides difficile (CD), es considerada como la principal causa de diarrea asociada al uso de antibióticos, la cual se asocia a una desregulación de la microbiota intestinal del hospedero, que afecta a los diferentes componentes de la microbiota, siendo el bacteriano el más ampliamente estudioado. Sin embargo, otros grupos de organismos, cómo virus y eucariotas, se han descrito como miembros fundamentales dentro del ecosistema intestinal, e incluso se destaca la función de loci clínicamente importantes, especialmente aquellos asociados con resistencia a antibióticos. A pesar de la gran relevancia de los miembros de la microbiota intestinal sobre la homeóstasis de dicho ecosistema, poco se conoce sobre su papel en el ámbito de la ICD, así como la influencia del lugar de adquisición de la diarrea sobre la composición (diversidad y abundancia) del microbioma, en especial en el contexto de las enfermedades inflamatorias intestinales.

Por estas razones, este estudio se dirigió a determinar la composición del microbioma intestinal de pacientes con diarrea asociada a la ICD, adquirida en Unidad de Cuidados Intensivos (UCI) y en comunidad, a través de la implementación de técnicas de secuenciación de alto rendimiento. Para esto, se seleccionaron 98 muestras de ADN provenientes de pacientes con diarrea adquirida en comunidad y a nivel intrahospitalario, tanto positivos como negativos para ICD*,* las cuales fueron sometidas a secuenciación de marcador único ARNr-16S y -18S. Posteriormente, se seleccionaron 48 muestras de este grupo inicial y fueron sometidas a secuenciación metagenómica.

Inicialmente, se observaron cambios en la composición de la microbiota asociada a los grupos de pacientes con diarrea adquirida a nivel intrahospitalario (HCFO/+, HCFO/-) caracterizada por la disminución de microorganismos benéficos*,* sugiriendo la importancia del lugar de adquisición de la infección sobre la modulación del ecosistema intestinal. Así mismo, se observaron interacciones entre los diferentes miembros de la microbiota intestinal. La secuenciación metagenómica permitió evidenciar un grupo de 51 especies diferencialmente abundantes entre los grupos, con una reducción en los genes implicados en el metabolismo del butirato en los grupos de pacientes con diarrea adquirida a nivel intrahospitalario. Finalmente, se observaron microorganismos productores de acetato y butirato característicos para cada uno de los grupos, con diferencias marcadas tanto en sus abundancias como en sus perfiles de resistencia. Se destaca, además, el papel de los microorganismos asociados al metabolismo del butirato sobre el ecosistema intestinal en el ámbito de la ICD.

Por su parte, el estudio de la microbiota de las garrapatas se ha convertido en una herramienta de gran utilidad en la vigilancia y control de las enfermedades transmitidas por garrapatas, las cuales se encuentran ampliamente distribuidas en el continente europeo, siendo un problema de salud pública, con especial énfasis en España, donde regiones como Castilla y León han presentado unas tendencias al aumento en las poblaciones de garrapatas así como en los reportes de picaduras. Por lo anterior, empleando el esquema de análisis para secuenciación profunda de marcador único generado en los apartados anteriores se realizó la descripción de 29 ejemplares de 5 especies de garrapatas duras recolectadas en dicha región encontrando diferencias en la composición taxonómica así como en las correlaciones entre los miembros de su microbiota, siendo este el primer estudio piloto realizado en dicho territorio

ABSTRACT

Clostridioides difficile (CD) is the main cause of antibiotic-associated diarrhea, associated with dysregulation of the host intestinal microbiota. This dysregulation affects the different components of the microbiota, with the bacterial component being the most widely studied. However, other groups of organisms, such as viruses and eukaryotes, have also been described as fundamental members of the intestinal ecosystem. Additionally, the function of clinically important loci, especially those associated with antibiotic resistance, has been highlighted. Despite the great relevance of the members of the intestinal microbiota in maintaining the homeostasis of the intestinal ecosystem, little is known about their role in the field of CDI. Furthermore, the influence of the acquisition site of diarrhea on the composition (diversity and abundance) of the microbiome, especially in the context of inflammatory bowel diseases, is poorly understood.

Therefore, this study aims to determine the intestinal microbiome composition in patients with diarrhea associated with CDI, acquired in the Intensive Care Unit (ICU) and in the community, by implementing high-throughput sequencing techniques. To achieve this, 98 DNA samples from patients with community-acquired and hospital-acquired diarrhea, both positive and negative for CDI, were selected and subjected to 16S rRNA and 18S rRNA single marker sequencing. Subsequently, 48 samples were chosen from this initial pool and subjected to metagenomic sequencing.

Initially, we observed changes in the microbiota composition of the groups with hospital-acquired diarrhea (HCFO/+, HCFO/-) characterized by a decrease in beneficial microorganisms, suggesting the importance of the place of acquisition of the infection in the modulation of the intestinal ecosystem. Similarly, we observed interactions between the different members of the intestinal microbiota. The metagenomic sequencing revealed 51 differentially abundant species between the study groups, with a reduction of the genes involved in the butyrate metabolism in the groups of patients with hospital-acquired diarrhea. Finally, we observed distinctive acetate and butyrate-producing microorganisms for each group, with marked differences in their abundances and resistance profiles. The role of microorganisms associated with butyrate metabolism on the intestinal ecosystem in the CDI scenario is also emphasized.

On the other hand, the study of tick microbiota has become a useful tool in the surveillance and control of tick-borne diseases, which are widely distributed in the European continent and pose a significant public health problem. This is particularly true in Spain, where regions such as Castilla y León have reported increasing trends in tick populations and bites. Therefore, using the single marker deep sequencing analysis scheme generated in the previous sections, we described the microbiota of 29 specimens of 5 species of hard ticks collected in that region. We found differences in the taxonomic composition as well as in the correlations between the members of their microbiota. This pilot study is the first of its kind carried out in that territory.

1. MARCO TEÓRICO

1.1.Generalidades de *Clostridioides difficile*

Clostridioides difficile (CD) (anteriormente conocido como *Clostridium difficile*) (1) es un bacilo Gram positivo, esporulado, anaerobio estricto, que en cultivo forma colonias circulares a irregulares (2). CD puede colonizar el intestino de humanos y varios animales por contacto directo vía oro-fecal de las esporas de la bacteria presentes en heces de un paciente infectado (3). CD suele encontrarse en individuos adultos inmunocompetentes como parte de la microbiota intestinal sin causar sintomatología, sin embargo, en individuos con alteración de esta microbiota, principalmente en el caso de pacientes sometidos a terapia antibiótica (4), se favorece la germinación de las esporas de CD, la generación de formas vegetativas móviles productoras de múltiples adhesinas (requisitos para la penetración de la bacteria en las células gastrointestinales) y la progresión a la primera fase de la infección dentro del tracto gastrointestinal (denominada colonización), en donde se producen las toxinas clostridiales, las cuales causan daño en el epitelio intestinal y favorecen el desarrollo de una respuesta inmune inflamatoria (5–7).

1.2.Infección por *Clostridioides difficile* (ICD*)*

Entre los síntomas y signos característicos se encuentran la diarrea acuosa, dolor abdominal, fiebre, anorexia, náuseas, malestar, según el cuadro clínico puede haber presencia de leucocitosis y sangre en las heces. Los casos de colitis moderada-grave se caracterizan por la presencia de dolor, distensión abdominal y, en algunos casos, hemorragia digestiva oculta (2,8). Algunos pacientes pueden sufrir daño en el ciego y el colon, además de leucocitosis. En algunos casos severos, como la colitis pseudomembranosa grave, megacolon tóxico e íleo paralitico, los síntomas sistémicos pueden generarse en ausencia de diarrea (7,8).

A nivel epidemiológico, CD puede ser identificado en las heces de al menos el 3% de los individuos adultos saludables de la población general y en cerca del 10 al 25% de las diarreas asociadas a uso de antibióticos, en el 50 al 75% de los pacientes que desarrollan colitis posteriores al tratamiento con antibiótico, y en el 90 al 100% de los casos de colitis pseudomembranosa (9–11). En general, entre un 16 y un 35% de los pacientes hospitalizados puede ser portadores, con tasas proporcionales a la duración de la hospitalización y al uso de antibióticos (12). Si bien, algunos pacientes pueden desarrollar colitis en ausencia de ICD, el porcentaje global de este escenario es muy reducido (13).

La ICD se ha reportado como una de las cinco infecciones más importantes en Unidades de Cuidados Intensivos (UCI) (14,15). Los casos más graves se presentan en 1 a 3% de los pacientes infectados, quienes pueden desarrollar colitis fulminante, con íleo, megacolon tóxico e incluso perforación (16).

1.3.Factores de virulencia de CD

La virulencia de CD se ha relacionado con los siguientes mecanismos: Diversidad genética, Producción de toxinas, Resistencia a antibióticos, y Capacidad de esporulación/germinación. A continuación, se describe cada uno de estos mecanismos.

1.3.1. Diversidad genética

El primer reporte de genoma completo realizado en 2006, a partir de una cepa hiper-epidémica y multidrogo-resistente (630-RT012), permitió identificar un cromosoma circular de entre 4,1 y 4,3 megabases (Mb), con 3.776 Secuencias Codificantes para Proteínas (SCP) y un contenido de GC de alrededor del 29%; además de un plásmido de casi 8 kilobases (Kb) con 11 SCP (17). Estudios posteriores han conducido a la reanotación del genoma de la cepa 630 y a la secuenciación de genoma completo de otras cepas (18–20). En general los hallazgos muestran una arquitectura genómica altamente dinámica, que involucra un alto contenido de elementos genéticos móviles (~11% para el caso de la cepa 630), dentro de los que se incluyen bacteriófagos, secuencias de inserción, elementos tipo CRISPR (por las siglas en inglés de Clustered Regularly Interspersed Short Palindromic Repeats), islas genómicas y elementos transponibles o de tipo conjugativo, entre otros; estos últimos acompañados de un amplio rango de genes accesorios (21). Los elementos mayoritarios en el genoma de CD corresponden a transposones de tipo conjugativo (CTns, por las siglas en inglés Conjugative Transposons), definido como un tipo de elemento genético móvil que tiene la capacidad de escindirse de su sitio blanco en el genoma y transferirse entre células bacterianas por un proceso que requiere contacto célula-célula (22). Las principales familias de CTns son Tn916 y Tn1549, debido a su alta frecuencia en bacterias, por estar relacionados con resistencia a antibióticos (generalmente tetraciclina) y por ser los más ampliamente caracterizados (22,23). Es precisamente este intercambio de elementos genéticos móviles (de ocurrencia frecuente) el que contribuye a la plasticidad del genoma de CD (24).

Las características propias del genoma le han permitido a CD adaptarse a condiciones adversas y coexistir con su hospedero durante un largo periodo de tiempo. En este sentido, la plasticidad de su genoma, dada por la presencia de los elementos genéticos móviles, ha sido asociada a un largo periodo de adaptación de la especie patógena a su hospedero, contrario a lo que ocurre en especies con genomas más estables, como *Clostridium botulinum*, que indica una asociación más corta con el hospedero cuando se compara con CD. La segunda de las características genómicas de interés para la supervivencia, es el tamaño del genoma, mayor al de otras bacterias, que se ha asociado con la adaptación a múltiples ambientes adversos, como ocurre en *Pseudomonas aeruginosa*; de acuerdo con esto, se ha descrito que CD posee un genoma >42% más grande que otras especies Clostridiales y la mayoría de Firmicutes (25).

Recientemente, se han caracterizado siete CTns a lo largo del genoma de la cepa 630, siendo el CTn3 quien transporta un gen de resistencia a tetraciclina, los CTn1, CTn3, CTn6 y CTn7 relacionados con la familia Tn916, de acuerdo con la secuencia de su módulo de conjugación, y los elementos CTn2, CTn4 y CTn5 pertenecientes a la familia 1549, basado en la similitud

de secuencia. Así mismo, los módulos accesorios de los CTns codifican para transportadores ABC no caracterizados aún (24). Todos estos elementos anteriormente mencionados se detallan en la Figura 1.

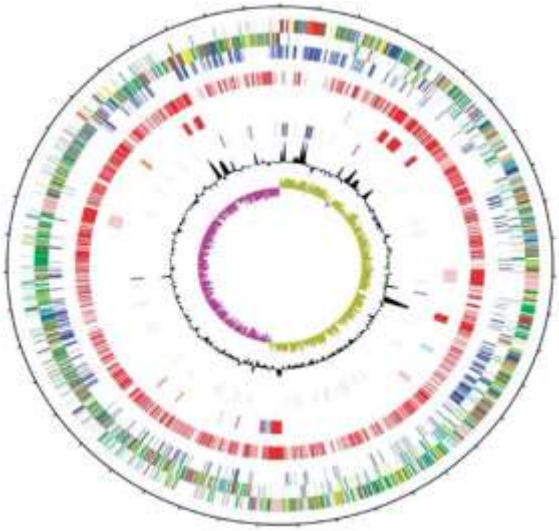

Figura 1. Ilustración circular del cromosoma de la cepa 630 de CD. Los dos círculos externos evidencian 3776 secuencias codificantes de proteínas (SCP) en ambos sentidos (a favor y en contra de las manecillas del reloj). El tercer círculo muestra las SCP compartidas con otros individuos de la misma familia. En Rojo se muestran los elementos genéticos móviles (Transposones, profagos e islas genómicas). Más al interior se evidencian los genes codificantes para ARN y finalmente los círculos internos muestran el contenido Guanina/Citosina. Adaptado de Knight y colaboradores (25)

1.3.2. Producción de toxinas

La patogenicidad de CD se ha relacionado también con la acción de dos de las principales toxinas mayores codificadas por algunos genes de esta bacteria: Toxina A y Toxina B (codificadas por los genes *tcdA* y *tcdB*, respectivamente), las cuales pertenecen la familia de toxinas clostridiales con acción glucosiltransferasa (6,26). La actividad de estas toxinas radica en la modificación irreversible, a través de glicosilación, de GTPasas de la superfamilia Rho/Ras. Dado que estas GTPasas son proteínas celulares involucradas en muchos procesos regulatorios de la célula hospedera, su inactivación permanente causa disrupción en muchas vías de señalización esenciales, dentro de las que resultan críticas: la regulación transcripcional, la apoptosis, la integridad del citoesqueleto y la función de barrera de las células del epitelio colónico, entre otras (27). En la Figura 2 se presenta un esquema del Locus de patogenicidad (PaLoc) y sus componentes.

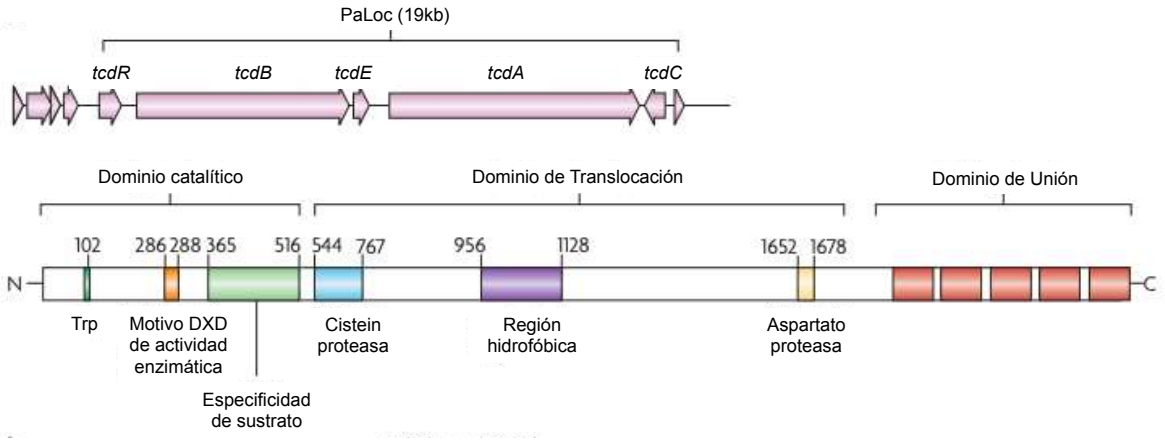

Figura 2. Esquema del Locus de Patogenicidad (PaLoc). En la parte superior se observan los cinco genes que componen el PaLoc. En la parte inferior se detalla la composición de *tcdB*. Adaptado de Rupnik y colaboradores (28).

A nivel clínico, se ha identificado la presencia de la toxina A en individuos que muestran secreción de líquidos y proceso inflamatorio hemorrágico, y de la toxina B, en individuos con alteraciones en el citoesqueleto, ratificando su participación en la estimulación de la muerte celular (29). Las cepas que producen la toxina B se han visto asociadas a las presentaciones más severas de la enfermedad, y las no productoras de las dos toxinas, usualmente son cepas no patógenas. Estas toxinas son codificadas por genes ubicados en una región del cromosoma de aproximadamente 20 Kb, que constituye el PaLoc (30), que podría tener origen por un evento de transposición, mediado por elementos tipo CTn (23).

Adicional a los genes codificantes para las dos grandes toxinas clostridiales, tres genes accesorios son codificados en el PaLoc, estos son: *tcdR, tcdC* y *tcdE*. Se ha identificado que *tcdR* codifica para un factor sigma alternativo, que juega un papel crítico para la expresión de *tcdA* y *tcdB*. En el caso de *tcdC*, los estudios sugieren que codifica para un factor anti-sigma, que secuestra TcdR y evita la interacción de éste con la ARN polimerasa, por lo que se ha propuesto a TcdC como regulador negativo de la transcripción. *tcdE*, el último gen incluido en el PaLoc, codifica para una proteína similar a una clase de proteínas de bacteriófago conocidas como holinas, por lo que se ha descrito que podría estar involucrada en procesos de exporte de las toxinas de CD, a través de un mecanismo aún no caracterizado (31).

Se ha identificado que la variación en la organización de los genes ubicados en el PaLoc, se relaciona con el grado de virulencia de CD, siendo el caso más evidente el de la cepa hipervirulenta ribotipo 027 (RT027/BI/NAP1), que presenta una deleción de un par de bases en la posición 117 del gen *tcdC*, lo cual resalta la necesidad de llevar a cabo una caracterización toxigénica que permita identificar la circulación de cepas con importancia a nivel clínico y epidemiológico (32). Diferentes estudios encaminados a esta caracterización toxigénica, han permitido identificar que el PaLoc presenta diferentes polimorfismos, entre los que se incluyen las repeticiones directas y secuencias de inserción (responsables de la alta

frecuencia de deleciones, las duplicaciones y/o amplificaciones), que conducen al alto nivel de variación y diversidad evolutiva, siendo la transferencia horizontal de genes y los eventos de recombinación, los principales mecanismos relacionados con esta diversidad (18).

Se ha identificado que el PaLoc está ausente en las no toxigénicas, donde éste es reemplazado por una región no codificante altamente conservada de 115/75 pares de bases (pb), conocida como sitio de integración. En el caso de las cepas toxigénicas, se han encontrado diferentes perfiles de deleción de fragmentos en el regulador negativo *tcdC*, siendo los más frecuentes: -18pb / -39 pb / -54 pb (18).

Adicional a las toxinas TcdA y TcdB, un limitado número de aislamientos (~23%), tienen la capacidad de producir la toxina binaria, que tiene actividad ADP-ribosil transferasa. Esta toxina binaria es codificada por una región cromosómica denominada Locus CDT (CdtLoc), ubicada corriente abajo del PaLoc, que contiene los genes *cdtA* y *cdtB*, codificantes para sus dos componentes: CdtA, que corresponde al componente enzimático y CdtB, componente de unión (33), y para su regulador *cdtR* (34). Si bien la función de la toxina binaria no ha sido totalmente caracterizada, se ha propuesto que podría tener un papel en la adherencia de CD, actuando de forma sinérgica con otros factores como proteínas de superficie (31). Esta organización se muestra en la figura 3.

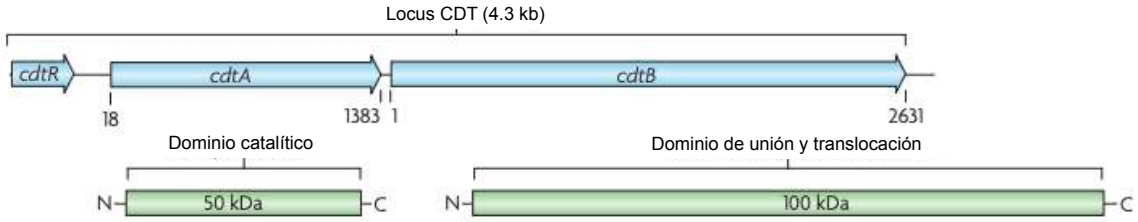

Figura 3. Representación del Locus CDT (cdtLoc). Se observa los genes que codifican para las toxinas CdtA y CdtB. En la parte inferior se detallan ambas proteínas que se encuentran separadas formando el dominio catalítico y el de unión y translocación. Adaptado de Rupnik y colaboradores (28).

1.3.3. Resistencia a antibióticos

A pesar de la necesidad del uso de antibióticos en el manejo clínico de la ICD, se ha identificado que este tratamiento contribuye al avance de la historia natural de la enfermedad, debido a su habilidad para alterar la microbiota intestinal y por tanto estimular la proliferación de CD. De acuerdo a esto, los incrementos observados en las tasas de incidencia y de recurrencia, podrían estar atribuidos a la reducción en la efectividad del tratamiento causada por la emergencia de cepas resistentes a diferentes antibióticos, que representan un serio problema a nivel mundial (35).

La resistencia a antibióticos ha sido atribuida a la diversa estructura poblacional de CD y a la plasticidad del genoma, asociada a los elementos genéticos móviles (descritos en la sección de Diversidad Genética), siendo este tipo de marcador molecular (asociados a resistencia) los que se movilizan con mayor frecuencia en CD. En la mayoría de los casos es la presencia/ausencia de estos marcadores moleculares la que confiere la resistencia a antibióticos, como es el caso de los genes *erm*(B) (que confieren resistencia a eritromicina y clindamicina) (36,37) y los genes *tet*(M) y *tet*(W) (relacionados con resistencia a tetraciclina) (38,39). Sin embargo, en algunos casos el mecanismo que conduce a la resistencia es la presencia de mutaciones en ciertos genes, como es el caso de los genes *gyrA* y *gyrB,* cuyas mutaciones se han asociado con resistencia a fluoroquinolonas (40). Adicionalmente, se ha relacionado la organización genética de estos mecanismos moleculares de resistencia a antibióticos con el aumento de la virulencia de CD, siendo determinante el número de copias de los genes o el tipo de elemento genético en el que son movilizados (41,42).

1.3.4. Esporulación/germinación

Los mecanismos por los cuales se produce la esporulación en CD aún siguen siendo estudiados, sin embargo, las comparaciones con los modelos de esporulación en otros microorganismos han permitido proponer que el bacilo sensa constantemente las condiciones ambientales, y este quorum sensing es el que le permite la detección de ausencia de nutrientes y otras características ambientales que desencadenan un conjunto de señales producidas por estrés que derivan en la formación de la espora (7,43). El proceso de esporulación contiene cuatro estadios morfogénicos: en un primer estadio, se forma un compartimiento pequeño separado por un septo en una célula madre (célula que da paso a la espora), posterior a esto, la célula madre interioriza el compartimiento recién formado, dando paso a una pequeña espora, al exterior de la cual en un tercer momento se comienza a formar la corteza y la cubierta. Finalmente, la célula madre se lisa y libera la espora madura. La espora contiene el ADN genómico, el ARN mensajero, ribosomas, proteína y es rico en un compuesto rico en ácido 2, 6 piridinico-dicarboxílico (APD). La membrana externa está compuesta de una pared que contiene peptidoglicano, una corteza especializada que contiene este mismo compuesto y una membrana con capas proteicas (5,43).

El proceso de germinación de la espora está mediado por señales recibidas del entorno que le indican a la bacteria que se encuentra en un entorno favorable para su desarrollo. Elementos como los ácidos biliares primarios y algunos aminoácidos que se encuentran en la parte baja del tracto gastrointestinal, donde no hay presencia de oxígeno. Todos estos elementos en conjunto, activan un conjunto de enzimas líticas que degradan las estructuras externas de la espora y permiten que la forma vegetativa emerja al exterior (5). Vale la pena destacar que este mecanismo de supervivencia del bacilo es de gran importancia en la dispersión de la infección.

1.4.Tratamiento de la ICD

Luego de la confirmación de ICD, se debe iniciar tratamiento antibiótico, en el cual se contempla convencionalmente el uso de vancomicina o metronidazol, para el tratamiento del primer episodio de ICD (44). Sin embargo, se ha reportado que el uso de vancomicina conduce con mayor eficiencia al éxito clínico. Para 2012, la FDA aprobó el uso de Fidaxomicina para el tratamiento de ICD, que muestra una baja actividad contra la microbiota intestinal normal y además, su actividad contra CD es similar a la reportada para la

vancomicina. Esquemas con múltiples antibióticos han sido implementados para el tratamiento de ICD severas, de acuerdo a los lineamientos de tratamiento establecidos por cada país (45). La más reciente guía de práctica clínica solo recomienda el trasplante de materia fecal (TMF) en pacientes con múltiples recurrencias de la ICD en los cuales han fallado los esquemas terapéuticos apropiados y posterior a una correcta tamización de la muestra del donante siguiendo las recomendaciones de la FDA (44).

1.5.Factores de riesgo del hospedero

Reportes disponibles en la literatura han permitido identificar ciertos factores asociados al hospedero, relacionados con el aumento de la diarrea por CD, siendo algunos de estos: la edad avanzada, la inmunodepresión, un antecedente de cirugía gastrointestinal y, como factor fundamental, el tratamiento antibiótico (17).

CD afecta con mayor frecuencia a pacientes con factores de riesgo como adultos mayores de 65 años, pacientes que presentan co-morbilidad (con algunas patologías renales y hepáticas), pacientes con inmunosupresión (VIH), pacientes con cáncer (tratamiento con quimio-radioterapia), con enfermedades gastrointestinales como las enfermedades inflamatorias intestinales y pacientes con hospitalización previa (4,46). Uno de los grupos de riesgo más importantes son los pacientes en Unidad de Cuidados Intensivos (UCI), donde se reconoce como una de las infecciones más frecuentes (46).

El factor de riesgo más importante para el avance en la historia natural de la ICD es el desequilibrio de la microbiota, que puede ser causada en la mayoría de los casos por la exposición a diferentes antibióticos, siendo los de mayor impacto aquellos de amplio uso en la práctica clínica, como: penicilinas, cefalosporinas de segunda y tercera generación, clindamicina y fluoroquinolonas; procedimientos gastrointestinales, la presencia de tubos nasofaríngeos, enemas y el consumo de antiácidos puede llevar facilitar la colonización y el proceso infeccioso. Por otro lado, los neonatos portan mayor cantidad de cepas toxigénicas debido a la microbiota inmadura que poseen, sin embargo, son asintomáticos por la misma razón, ya que los enterocitos del colón al ser inmaduros, no presentan receptores para las toxinas de CD, por este motivo no generan su efecto tóxico (47).

Si bien la ICD también puede aparecer en ausencia de tratamiento previo con antibióticos, este tipo de eventos ha sido identificado en pacientes sometidos a quimioterapia o en tratamiento con inmunosupresores, en quienes la eficiencia del sistema inmune no es suficiente para evitar el éxito del proceso de infección por CD (48). El uso de inhibidores de la bomba de protones (IBP) se ha descrito también como posible factor de riesgo. Así mismo, la infección por CD puede ocasionar brotes en pacientes con enfermedad inflamatoria intestinal (48).

2. Microbioma intestinal

El microbioma del intestino humano es un complejo sistema de múltiples microorganismos, sus productos génicos y funciones fisiológicas correspondientes, que pueden ser encontrados en el tracto gastrointestinal humano (49). Dentro de esta denominación se incluyen bacterias, arqueas, virus y organismos eucariotas, que mantienen una interacción constante entre ellas y con el hospedero (50). Se ha descrito que alrededor de 100 billones de células microbianas habitan el intestino humano, las cuales en conjunto son las encargadas de producir numerosas

enzimas necesarias para llevar a cabo procesos digestivos (51). De esta manera, la mayoría de los microorganismos en un intestino son inocuos o benéficos para el hospedero, protegiéndolo contra la colonización de enteropatógenos, produciendo metabolitos necesarios para la absorción de nutrientes por parte del hospedero y/o promoviendo la funcionalidad normal del sistema inmunológico. El desequilibrio de la microbiota intestinal está asociado con muchas enfermedades, como la obesidad, la malnutrición, las enfermedades inflamatorias intestinales y el desarrollo de cáncer de colon (52–54). Así mismo, la microbiota intestinal ayuda a la fermentación de carbohidratos y la síntesis de las vitaminas mediante la reducción de la permeabilidad intestinal y el aumento del mecanismo de defensa epitelial para formar una barrera mucosa (55). El sistema inmune de la mucosa intestinal constituye el componente inmune más grande en los vertebrados, funcionando de cerca con el microbioma intestinal. El equilibrio del sistema inmune de la mucosa intestinal juega un papel clave en la homeostasis y defensa del huésped (51).

El estudio del microbioma intestinal ha atravesado por múltiples cambios tanto en las técnicas de recolección de datos como en las herramientas utilizadas para su posterior análisis (56,57). El empleo de medios y técnicas tradicionales de cultivo para intentar descifrar todos los componentes de la microbiota representaba una aproximación muy limitada teniendo en cuenta que se estima que menos del 2% de las bacterias ambientales son cultivables y que muchos microorganismos son desconocidos tanto en sus características físicas como metabólicas, dificultando aún más su identificación por estos métodos (58). Con el desarrollo de las técnicas de secuenciación de nueva generación y el advenimiento de las ómicas los estudios de microbiomas aumentaron exponencialmente dando paso a datos cada vez más complejos sobre los integrantes de este ecosistema y su homeostasis.

El secuenciación profunda de marcador único es uno de los métodos más utilizados a nivel mundial para los estudios de microbiomas para la identificación de sus componentes (59). Este método se basa en la amplificación y posterior secuenciación de regiones altamente conservadas entre todos los individuos pero que presentan variaciones que permiten la identificación y diferenciación de los miembros de una comunidad microbiana (60). Los genes más ampliamente utilizados como marcadores genéticos en los estudios de microbiomas varían en función del componente que se desea indagar, siendo considerado el ARNr-16S el estándar de oro para el estudio de las bacterias (61) con la utilización de primers dirigidos a diferentes regiones variables a lo largo de dicho gen, como se detalla en la sección de bacterioma del presente documento. Para el caso de los eucariotas, el marcador ARNr 18s se constituye en el más empleado para los estudios de microbiomas junto con el espaciador del transcrito interno (ITS por sus siglas en inglés) específicamente para hongos (62). La rapidez, facilidad y reproducibilidad obtenidas mediante el empleo de esta técnica la ha convertido en una herramienta fundamental y casi que un primer paso en el estudio de los ecosistemas microbianos tanto intestinales como de diversas fuentes, llevando al descubrimiento de múltiples comunidades de microorganismos habitando ambientes en los que anteriormente no se tenía evidencia de su presencia (60). Cabe resaltar que esta técnica presenta dentro de sus dificultades el análisis de los errores de secuenciación, las cuales podrían pasar inadvertidas de no ser por la utilización de herramientas bioinformáticas orientadas a su detección con el fin de brindar análisis cada vez más confiables (60).

A pesar de los múltiples beneficios evidenciados en la utilización del secuenciación profunda de marcador único, en ocasiones esta metodología evidencia falencias que no permiten una comprensión completa de todos los elementos que componen la microbiota intestinal. Como alternativa a estos vacíos, surge la aproximación metagenómica, la cual consiste en la amplificación aleatoria y posterior secuenciación de todo el contenido genético presente en una muestra (60,63). La metagenómica sistematiza la composición genética de todos los genes de las comunidades microbianas y, por lo tanto, proporciona una mejor resolución taxonómica e información genómica en comparación con las técnicas de marcador único, facilitando además el posterior análisis y predicción funcional de los genes circulantes (64,65) lo que brinda una información mucho más amplia como se observa en la figura 4. Dentro de esta técnica se resalta que su costo dificulta en ocasiones el análisis en masa de las poblaciones de estudio, por lo que la mayoría de los estudios que emplean esta metodología, la aplican sobre subgrupos pequeños de las poblaciones estudiadas inicialmente con secuenciación profunda de marcador único. Así mismo, los requerimientos a nivel computacional para el análisis bioinformático de los datos generados en los estudios de metagenómica representan una barrera, ya que dentro de estos se deben tener en cuenta aspectos como el control de calidad, ensamblaje, predicción de genes, clasificación taxonómica y funcional, la cual demanda la utilización de servidores computacionales de gran poder con los que no se cuentan en algunos países en desarrollo limitando su aplicación (60). Existen igualmente otras metodologías que se agrupan junto con la metagenómica, denominadas las metaómicas, (meta transcriptómica, metaproteómica y metabolómica) las cuales están orientadas al estudio de componentes específicos del microbioma, cuyo funcionamiento es similar al descrito en la metagenómica, con la variante del tipo de componente a analizar en cada uno de ellos como se detalla en la figura 4.

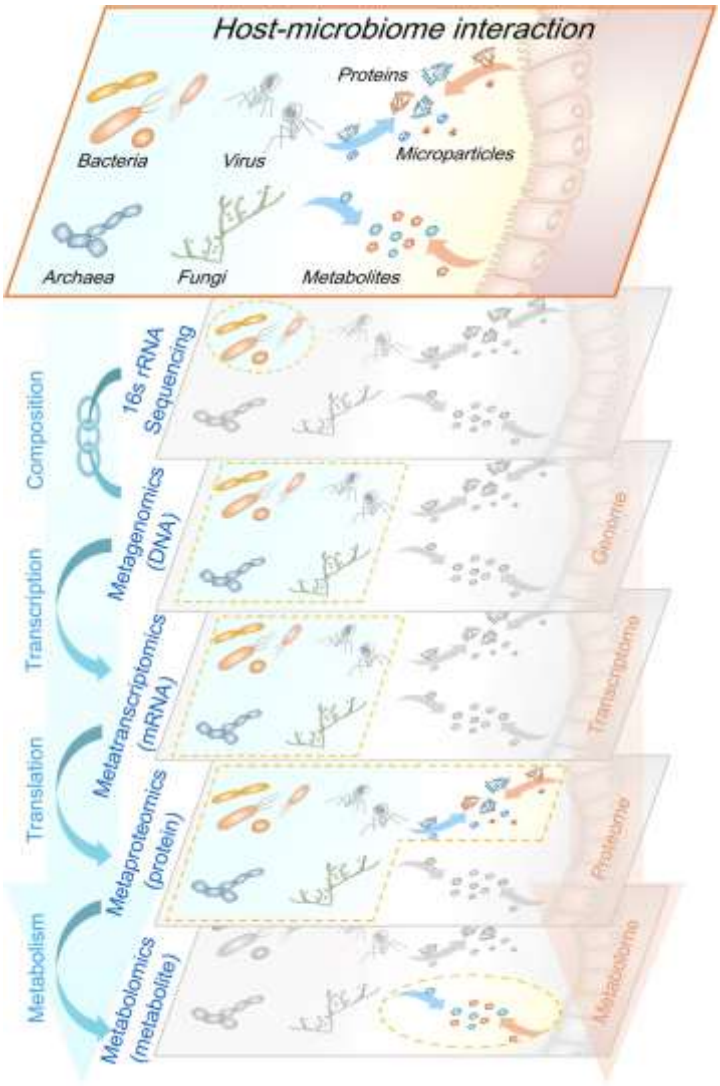

Figura 4. Esquema de las diferentes aproximaciones para el estudio de microbiomas. Se detalla en cada una de las casillas el componente del microbioma al cual va dirigida cada metodología. Tomado de Zhang y colaboradores (108)

2.1.Microbioma bacteriano

Los métodos de investigación para explorar las comunidades bacterianas de la microbiota se han dirigido a la implementación de estudios dependientes de cultivo y la secuenciación de genes usados como marcadores funcionales (como es el caso de la región codificante para subunidad pequeña del ARN ribosomal *SSU-ARNr* o el gen *nifH*) (66). Aunque la implementación de estrategias de cultivo ha permitido dilucidar algunos de los miembros de esas comunidades en diferentes ambientes y superficies (67), a través de una aproximación relativamente sencilla y de bajo costo, se presenta la limitación de que existe una vasta diversidad microbiana que no puede ser cultivable. Queda claro entonces, que los estudios basados en cultivo se limitan a evaluar la presencia de un pequeño grupo de microorganismos, por lo que la secuenciación del gen ARNr-16S usando plataformas de

secuenciación de alto rendimiento actualmente se posiciona como la estrategia más utilizada para describir la composición, abundancia y estructura poblacional de la mayoría de las especies microbianas (66,67).

La mayoría de los estudios utilizan las regiones hipervariables V5 / V6, V3 / V4 o V4, que producen la mejor precisión para la identificación de las comunidades, siendo la región V4 la más comúnmente utilizada, debido a que también puede encontrarse en arqueas (68,69). En la figura 5 se detalla la diversidad (medida en términos del índice de Shannon) de cada una de las regiones de este marcador, el cual representa uno de los aspectos más críticos dentro de los estudios de microbiomas, ya que la selección de una región altamente variable no permitiría la identificación de los microorganismos con un par de primers, mientras que regiones altamente conservadas impediría una correcta discriminación de los miembros de la microbiota, siendo las regiones reportadas como más eficientes para el estudio de microbiomas las que exhiben un índice de Shannon entre 0,4 y 0,6.

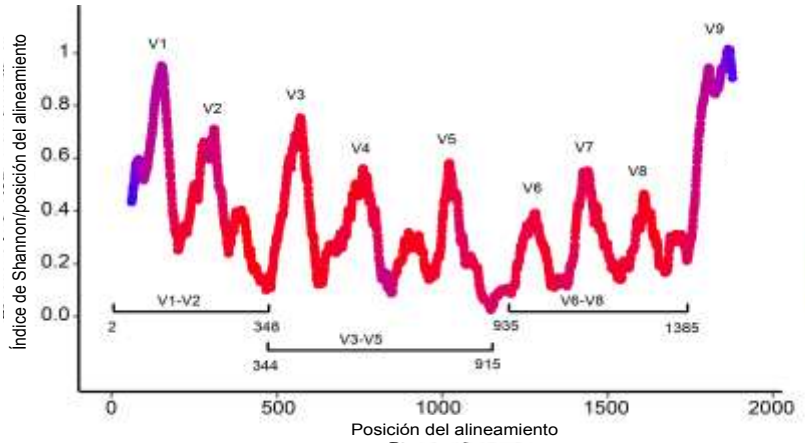

Figura 5. Esquema de las 9 regiones hipervariables del gen del ARNr-16S . Se observan regiones con una alta variabilidad lo que impide que las bacterias puedan ser detectadas al usarse estos marcadores, mientras que regiones altamente conservadas no permiten diferenciar entre las diferentes especies. Adaptado de Seedorf y colaboradores (70)

Se ha descrito que la diversidad microbiana que se encuentra en el colon de un adulto saludable contiene aproximadamente 200 especies bacterianas comunes en el intestino humano, siendo las especies pertenecientes a las Filos Firmicutes y Bacteroidetes las más comunes, abarcando más del 70-75% del total de especies. Los siguientes Filos. di más comunes son Actinobacteria, Proteobacteria y Verrucomicrobia. Los géneros más predominantes son *Bacteroides, Clostridium, Faecalibacterium, Eubacterium, Ruminococcus, Peptidococcus, Peptidostreptococcus* y *Bifidobacterium* (71). Interesantemente, las bacterias más estudiadas en el tracto gastrointestinal: *Lactobacillus* y *Escherichia* están presentes en mucho menor grado.

La diversidad y composición de la microbiota son altamente dinámicas, dependiendo del estado físico del huésped, genotipo y fenotipo inmune, pero también de los factores ambientales como la dieta, el uso de antibióticos y los comportamientos de estilo de vida. Estos factores ambientales pueden afectar adversamente el ecosistema intestinal (disbiosis),

que frecuentemente se asocia con una susceptibilidad a infecciones, así como enfermedades no transmisibles como obesidad, síndromes metabólicos (por ejemplo, diabetes y enfermedades cardiovasculares), alergia y otras enfermedades inflamatorias (72). La evidencia emergente de estudios más recientes también demuestra la existencia de una vía de comunicación bidireccional que une el intestino y la microbiota con el cerebro, lo que sugiere que estos microorganismos pueden desempeñar un papel en los trastornos neurológicos, así como la percepción del huésped, el comportamiento y la respuesta emocional (73,74).

Uno de los factores asociados a las variaciones en la microbiota es el uso de antibióticos, ya que además de la disminución de la diversidad y abundancia microbiana durante su uso, genera efectos a largo plazo que impiden la recuperación de una microbiota idéntica al estado previo al tratamiento, o que requieren de extensos periodos posteriores al tratamiento para inducir un estado de equilibrio (75). Las alteraciones en la composición de la comunidad microbiana como resultado del uso de antibióticos son importantes en el contexto de las infecciones agudas y recurrentes por CD, que se han relacionado fuertemente con los cambios en la microbiota intestinal debido a los efectos diferenciales. La hipótesis de esta interacción se dirige a que el tratamiento antibiótico (o alguna otra perturbación) altera significativamente la estructura de la comunidad microbiana en el intestino (76), impactando el equilibrio de ácidos biliares primarios y secundarios que favorece la colonización del bacilo (5). Un segundo efecto de la antibioticoterapia es el incremento de la concentración de carbohidratos en la mucosa intestinal que puede promover la expansión de CD (5,6). El resultado de esta perturbación de la microbiota se representa en la figura 6.

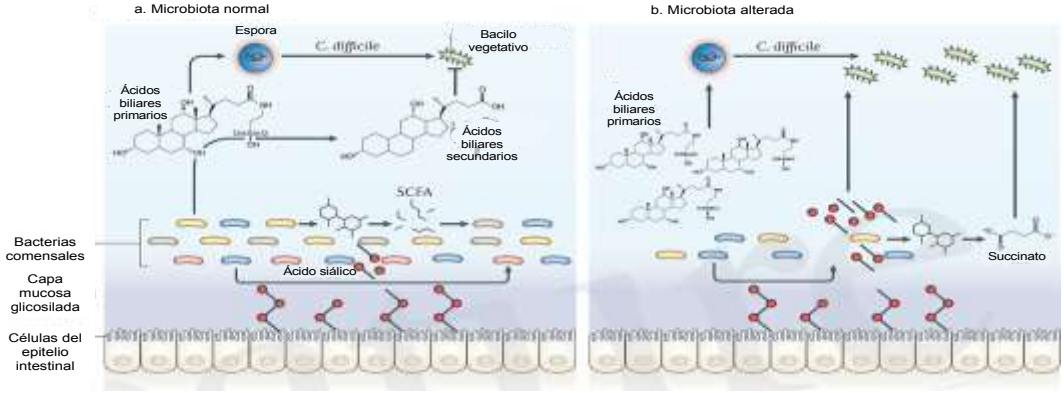

Figura 6. Esquema comparativo del desarrollo de CD en el contexto de una microbiota normal y una microbiota alterada. Se observa que la producción de ácidos grasos de cadena corta y ácidos biliares secundarios por parte de bacterias comensales limitan la aparición de las formas vegetativas, mientras que en la microbiota alterada el exceso de succinato y la ausencia de los anteriores metabolitos es aprovechada por el bacilo para su multiplicación. Adaptado de Abt y colaboradores (5).

Los primeros estudios dirigidos a relacionar la influencia de la microbiota para la prevención de la ICD se realizaron en la década de los 80's en los que mediante cultivos demostraron la inhibición que tenían algunas bacterias de los géneros *Streptococcus, Lactobacillus* y *Bacteroides* sobre el crecimiento de CD llegando a proponer el efecto competitivo sobre los monosacáridos liberados de la mucina mucina (77–79). Así mismo, durante esta década se

desarrollaron los primeros intentos de terapia de trasplante de microorganismos para el restablecimiento de la microbiota con resultados promisorios (80). En la década de los 90's surge evidencia sobre la microbiota intestinal como barrera contra la ICD y el impacto que tienen los antibióticos y otros medicamentos sobre su equilibrio, favoreciendo la germinación de las esporas del microorganismo (81,82). Con el advenimiento de nuevas técnicas moleculares comenzaron a detallarse las poblaciones bacterianas que se encontraban presentes en pacientes con ICD. Para el año 2002 con la ayuda de un sistema de identificación microbiana basada en ácidos grasos celulares se determinó un aumento en la diversidad de especies de *Lactobacillus* y *Clostridium* con una marcada disminución de *Bacteroides, Prevotella* y *Bifidobacterium* (83). Estos cambios en la composición de la microbiota entre pacientes sanos y con ICD llevaron al desarrollo de estudios posteriores en diferentes partes del mundo comparando el microbioma de los diferentes pacientes incluidos en los estudios con el fin de establecer las principales características de la microbiota asociada a la ICD.

En el año 2008 Chang y colaboradores realizaron un estudio realizado en Estados Unidos en 3 grupos de pacientes mayores de 75 años (3 controles sanos, 4 pacientes con ICD y 4 pacientes con ICD recurrente) evidenciaron una disminución de la diversidad alfa en aquellos pacientes con ICD recurrente en comparación con pacientes sanos (84). Los resultados de esta investigación se detallan en la figura 7.

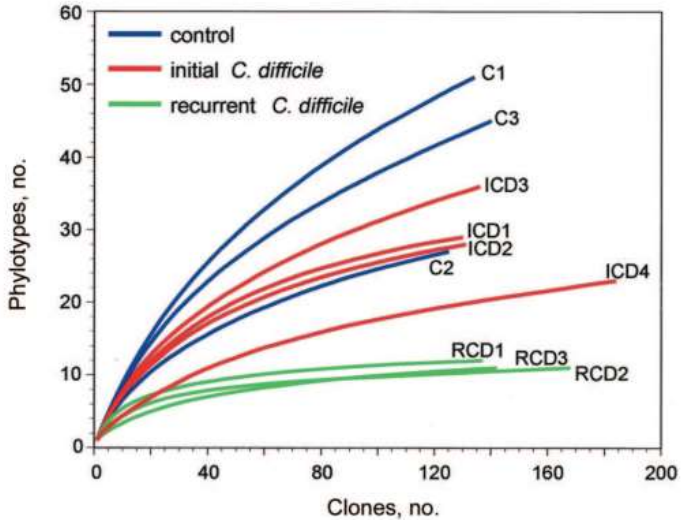

Figura 7. Disminución en la diversidad alfa en pacientes con ICD recurrente descrita por Chang y colaboradores (84)

Precisamente los cambios presentados en estos pacientes, así como los resultados positivos de la bacterioterapia en los años 80, motivaron el desarrollo de estudios orientados al trasplante fecal de un paciente sano a uno con ICD y su impacto sobre la restauración de la microbiota. Uno de estos estudios fue desarrollado en el año 2010 donde uno de los pacientes recibió el trasplante a partir de heces de su esposa sana, lo que llevó a un dramático cambio en la composición de la microbiota del paciente receptor, la cual 14 días posteriores al trasplante evidenciaron una similitud con la del donador, llevando a la resolución de los síntomas (85) como se evidencian en la figura 8.

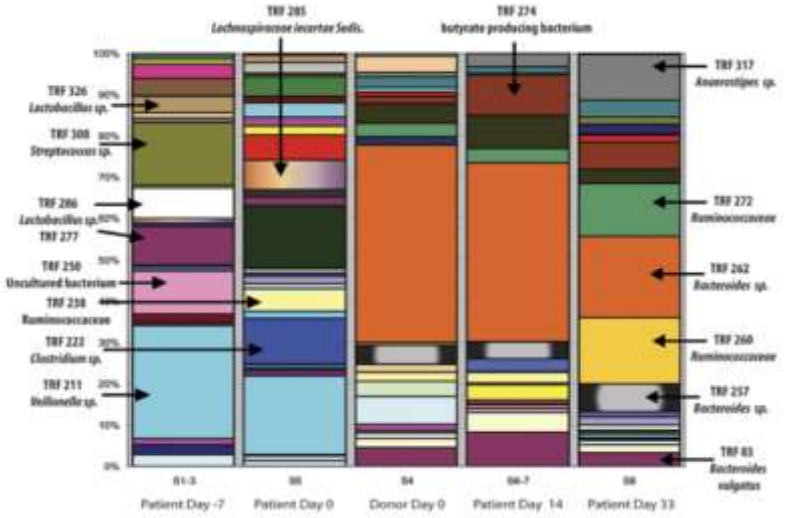

Figura 8. Cambios en la composición de la microbiota de un paciente posterior a trasplante fecal. Se evidencian los cambios en la microbiota del paciente la cual se asemeja a la del donante 14 días después del trasplante. Tomado de Khoruts et al (85).

En este mismo año se desarrolló un estudio de metagenómica en 75 pacientes (25 casos y 50 controles anidados en una cohorte de 599 pacientes) en Canadá, evidenció un aumento de Firmicutes, Proteobacteria y Actinobacteria y una disminución de Bacteroidetes en pacientes con ICD (86). Dos años más tarde, Rea y colaboradores realizaron un estudio en el que seleccionaron 22 de los sujetos de estudio con cultivo positivo y negativo para CD, cuyas muestras fueron sometidas a pirosecuenciación encontrando una marcada diferencia entre los pacientes con presencia de CD ribotipo 027 respecto a los pacientes con ICD por una cepa diferente (87). Llama poderosamente la atención que no se encontraron diferencias marcadas entre los pacientes positivos y negativos para la ICD. En el año 2013 se realiza un estudio en la Florida, con 115 pacientes divididos en 3 grupos: Pacientes sanos (n=40), pacientes con ICD (n=39) y pacientes con diarrea nosocomial negativos para ICD (n=36), encontrando una marcada disminución en la diversidad principalmente en el phylum de los Firmicutes en los grupos de pacientes con diarrea tanto por ICD como por otras causas, la cual estuvo acompañada de una marcada depleción de microorganismos productores de butirato pertenecientes a las familias Ruminococcaceae y Lachnospiraceae como se observa en la figura 9 (88).

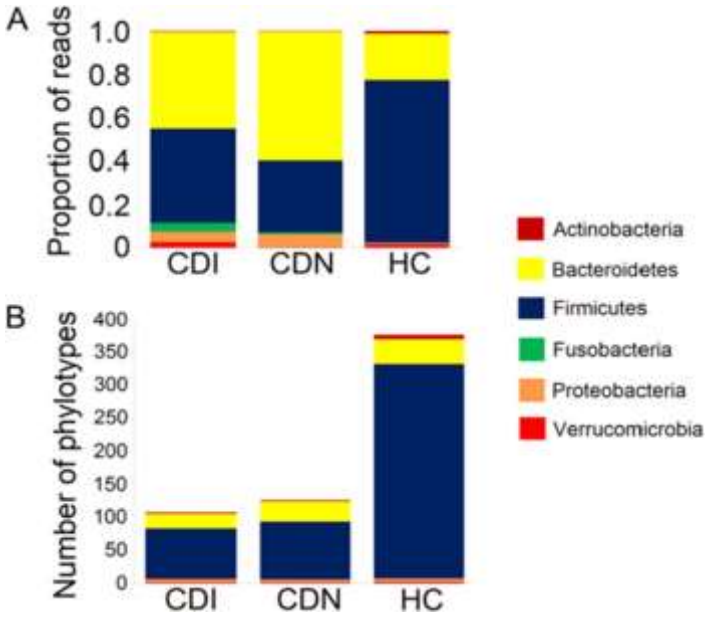

Figura 9. Disminución en la diversidad de la microbiota en pacientes con diarrea nosocomial producida por la ICD (CDI) como por otras causas (CDN) tanto en el número de lecturas (A) como en el número de haplotipos (B) (HC= Controles sanos) Tomado de Antharam y colaboradores (88).

Para el año 2014, Buffie y colaboradores resaltan en un estudio realizado en ratones que la ICD está relacionada con la disminución de unos grupos específicos de microorganismos, entre ellos *Clostridium scindens* el cual es un deshidroxilante de ácidos biliares que actúa como protector por la producción de metabolitos derivados de los ácidos biliares primarios, convirtiéndose en un candidato a probiótico para el tratamiento de la ICD (89). Estos resultados se resaltan en la figura 10.

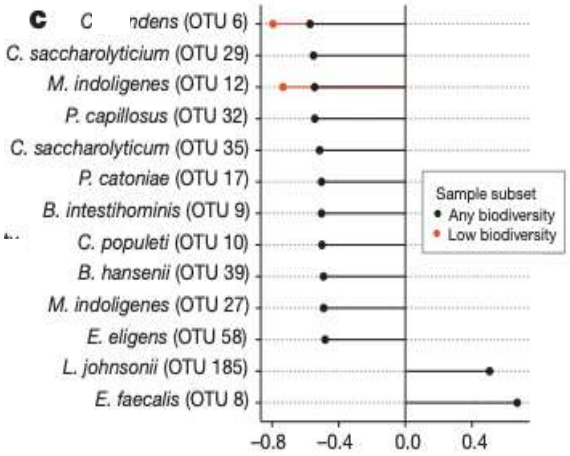

Figura 10. Correlación de Spearman entre las OTU y la susceptibilidad a la ICD. Se evidencia que para *C. scindens* y *M. indoligenes* existe una correlación inversa fuerte entre su diversidad y la susceptibilidad a la ICD. Tomado de Buffie y colaboradores (89).

Posteriormente, entre los años 2015 y 2016 nuevos estudios realizados en población europea comienzan a ahondar en los grupos de microorganismos que se reducen en un escenario de ICD evidenciando una disminución en los Bacteroidetes y Firmicutes y un aumento de las Proteobacterias (90–92). Los resultados de estos estudios se detallan en la figura 11.

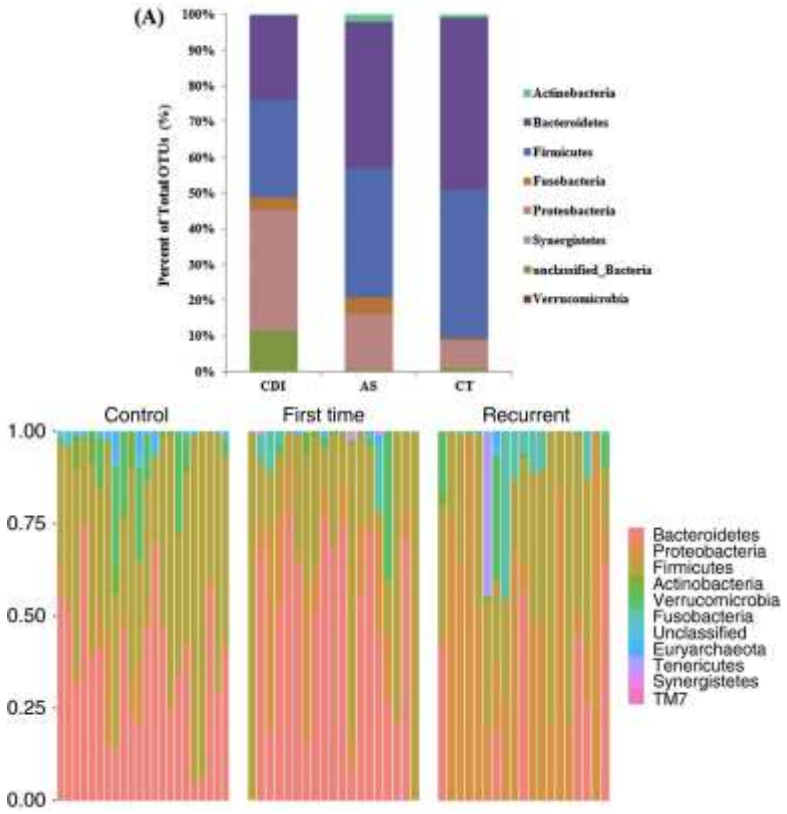

Figura 11. Resultados de diferentes estudios evidenciando diferencias entre pacientes sanos y pacientes con ICD, principalmente en los phyla Firmicutes Bacteroidetes y Proteobacterias (90–92)

Sangster y colaboradores (93) señalan un aumento de *Akkermansia* en pacientes con ICD a la vez que evidencian las principales diferencias en las familias bacterianas intestinales entre pacientes sanos y pacientes con ICD como se detalla en la figura 12.

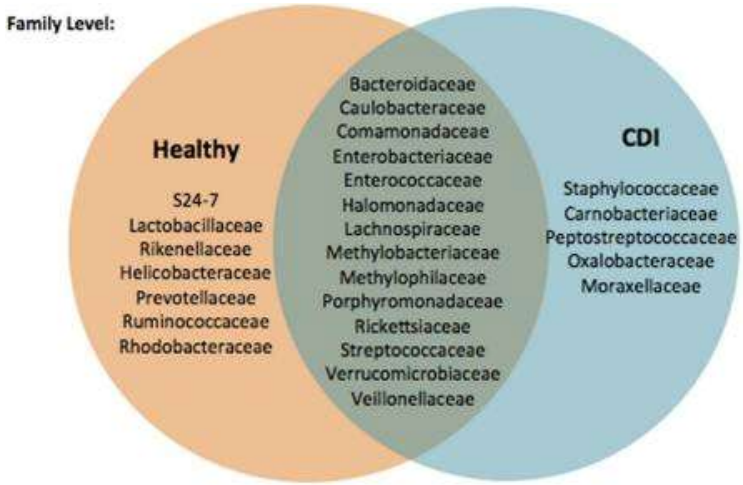

Figura 12. Diagrama propuesto por Sangster y colaboradores en el que se muestran las familias bacterianas que se encuentran diferencialmente en personas sanas y con ICD (93).

Los estudios realizados posteriormente han mostrado de manera reiterada una disminución de la diversidad alfa principalmente en las familias Ruminococcaceae, Lachnospiraceae y Peptoestreptococcaceae y un aumento de las Proteobacterias (94–97). De igual manera han resaltado la recomposición de la microbiota intestinal posterior a un tratamiento de Trasplante de materia fecal como lo resume Lagier (98) y se observa en la figura 13.

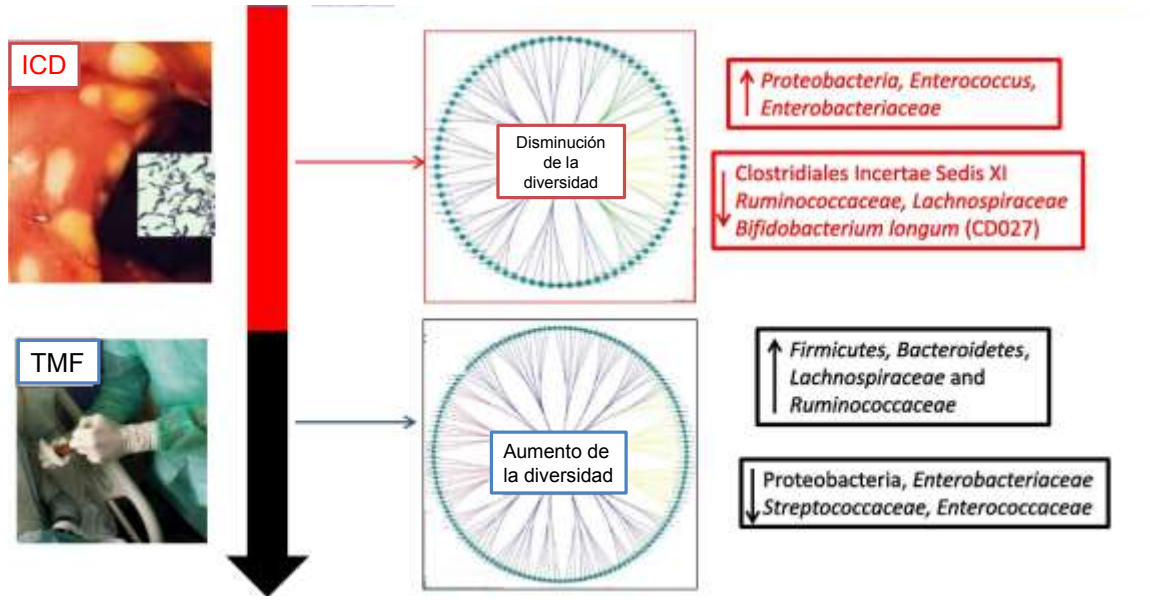

Figura 13. Esquema general de los hallazgos en la microbiota durante la ICD así como el impacto que tiene el trasplante de materia fecal (TMF). Adaptado de Lagier (98).

Finalmente, un estudio realizado en el año 2019 por Amrane y colaboradores mostró mediante la combinación de culturómica y metagenómica un consenso de microorganismos considerados benéficos que pueden ser considerados potenciales candidatos para el trasplante

a pacientes con ICD los cuales se encontraban disminuidos o ausentes en comparación con pacientes sanos (56). El producto de dicho consenso se muestra en la figura 14.

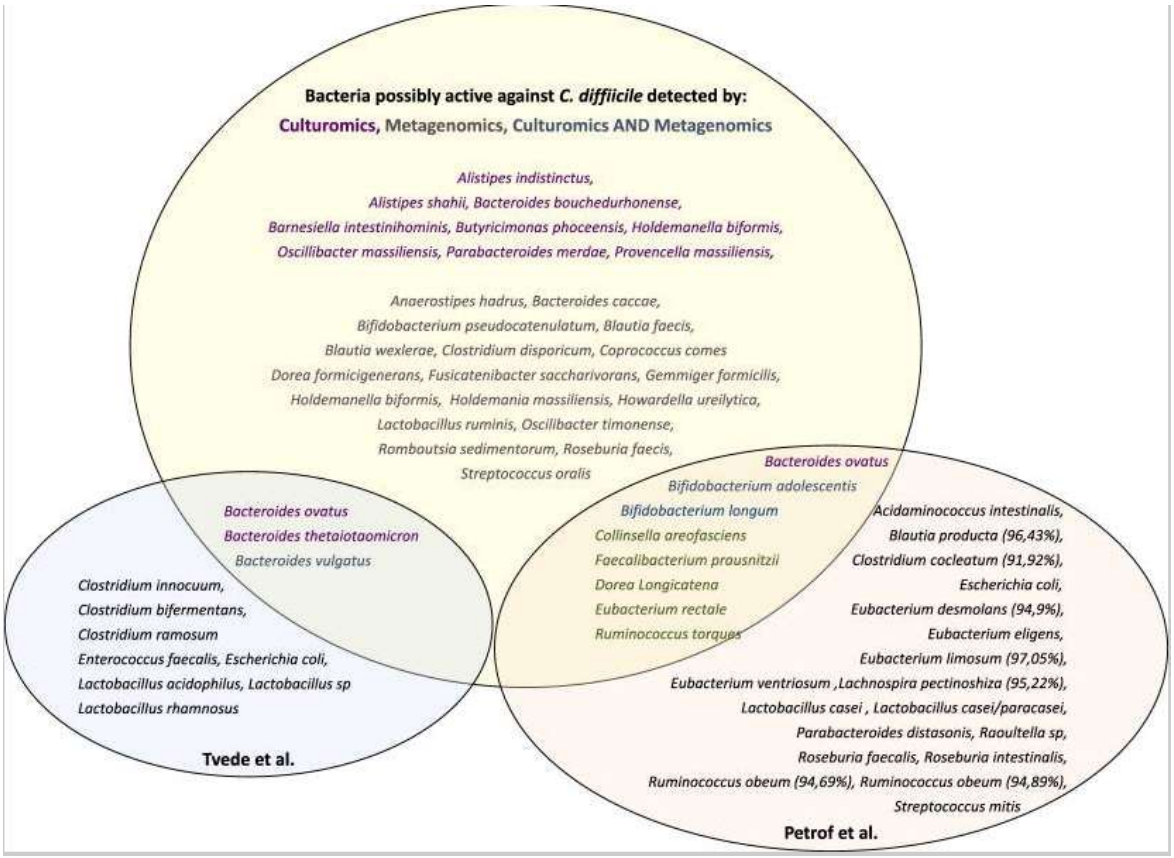

Figura 14. Bacterias con potencial acción protectora contra la ICD. Se muestran las bacterias descritas por diferentes autores y el consenso de algunas de ellas entre los estudios realziados. Tomado de Amrane y colaboradores (56).

2.2.Microbioma eucariota

Los microorganismos eucariotas que residen en el intestino de muchas especies de vertebrados, también muestran un efecto sobre los eventos de salud/enfermedad que puede presentar el hospedero, sin embargo la caracterización de este componente del microbioma tiene un progreso mucho más rezagado en comparación con el grado de avance en la descripción a nivel de bacterias (99). Algunos organismos multicelulares (como helmintos) y otros unicelulares (como amebas y ciertos protozoos), han sido identificados como miembros del microbioma intestinal. Muchos de estos taxones han sido investigados durante décadas desde un punto de vista parasitológico, usando microscopía y enfoques moleculares dirigidos, por lo que se considera que la diversidad de la microbiota eucariótica en el intestino humano aún no ha sido sistemáticamente investigada desde una perspectiva comunitaria (100).

Un estudio realizado en el año 2014 comparó la composición del microbioma eucariota en diversas fuentes como el intestino humano de pacientes sanos, la piel y el aire, encontrando

una gran diversidad en las muestras obtenidas de heces en comparación con las demás fuentes evaluadas (100). En este estudio se resalta la presencia de Stramenopilas, parabasálidos y de hongos como se observa en la figura 15

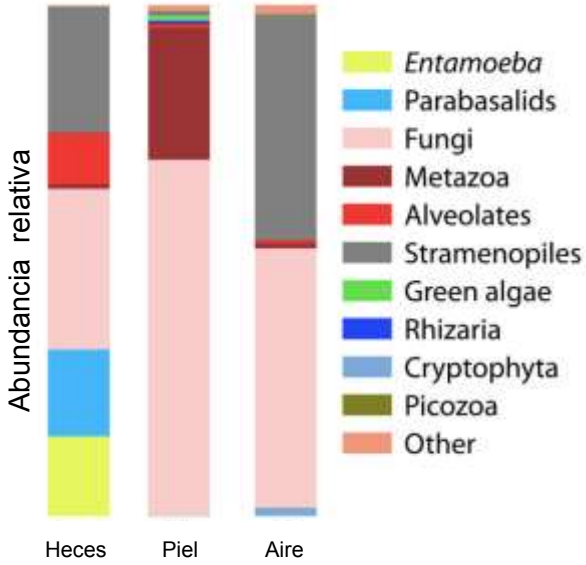

Figura 15. Composición del microbioma eucariota en diversas fuentes. Se evidencia una gran diversidad en la composición del microbioma eucariota obtenido a partir de heces con respecto a los de piel y aire. Adaptado de Parfrey y colaboradores (100).

A pesar de la relación que existe entre algunos microorganismos eucariotas y el desarrollo de enfermedades intestinales en los humanos, los recientes avances han permitido comprender que no todos los eucariotas que se encuentran habitando el tracto intestinal deben ser considerados parásitos, ya que muchos de ellos contribuyen al aumento de la diversidad bacteriana e interactúan con el sistema inmune para evitar la colonización del intestino por parte de agentes patógenos (101). Aunque se ha documentado ampliamente el papel patogénico de especies de eucariotas como *Ascaris lumbricoides, Entamoeba histolytica, Cryptosporidium* spp. y *Strongyloides stercoralis* la evidencia reciente sugiere que otros microorganismos eucariotas que habitan comúnmente el intestino como *Blastocystis* y *Dientamoeba* podrían desempeñar papeles ecológicos importantes en la homeóstasis intestinal (100–103). Para el caso de *Blastocystis,* el debate pareciera interminable en cuanto a la determinación de su rol, ya que estudios evidencian su presencia tanto en pacientes con desórdenes intestinales como en pacientes sanos (104,105). A pesar de no tener claridad sobre su papel patogénico o comensal, la presencia de este eucariota se ha asociado con el aumento de la riqueza de las especies bacterianas y una disminución del índice de masa corporal (106,107). Este microorganismo, junto con *D. fragilis, Giardia lamblia, Entamoeba* spp., *Iodamoeba* y *Endolimax* son considerados como Parásitos protozoos comunes del lumen intestinal (CLIPP por sus siglas en inglés) (108).

Los CLIPPs cobran una especial relevancia debido a la alta frecuencia con que suelen encontrarse en el tracto intestinal humano incluso en países desarrollados, donde en pacientes asintomáticos han llegado a evidenciarse altas prevalencias de algunos de ellos (108). En

términos del ecosistema microbiano, se ha asociado la presencia de CLIPPs con un aumento en la diversidad y abundancia de bacterias e incluso con patrones específicos de la microbiota, lo que sugiere una interacción directa entre estos organismos y los demás constituyentes de dicho ecosistema intestinal (109).

El producto de estas interacciones entre el microbioma eucariota y el microbioma bacteriano pueden ser variables. Un claro ejemplo de ello sucede en ratones con presencia de *Tritrichomonas musculis*, un parabasálido el cual protege a los individuos de la colonización de otros patógenos a la vez que mediante la activación del inflamasoma puede llegar a producir síndrome de colon irritable e incluso cáncer colorrectal (110). Otros ejemplos documentado han sido con la presencia de *Giardia, Toxoplasma* y *Cryptosporidium,* eucariotas que han sido ligado a la disbiosis por disminución de ciertas poblaciones bacterianas benéficas que terminan por agravar la sintomatología asociada a la infección con esto protozoos (111,112). De igual manera, el subtipo 7 de *Blastocystis* mostró un impacto negativo sobre bacterias benéficas como *Bifidobacterium* y *Lactobacillus,* lo que podría llevar a una disbiosis de la microbiota facilitando la aparición de patologías intestinales (113).

Por su parte, los helmintos que generalmente son vistos como patógenos, han mostrado una contribución al aumento de la diversidad de la microbiota intestinal, que tiende a desaparecer posterior a una terapia para removerlos (114), como se observa en la figura 16. Estas relaciones, aunque no se han esclarecido, parecen ser cada vez más notorias, llevando a pensar que hospederos y parásitos no existen de una manera aislada, sino que es el resultado de un proceso de coevolución que ha permitido la coexistencia de un sinnúmero de microorganismos en un nicho de una forma balanceada en la que todos los integrantes se benefician, incluyendo el mismo hospedero (101). Se trata entonces de una relación de doble vía, en la que las interacciones de unos y otros repercute de manera positiva o negativa sobre los demás miembros de la microbiota (114).

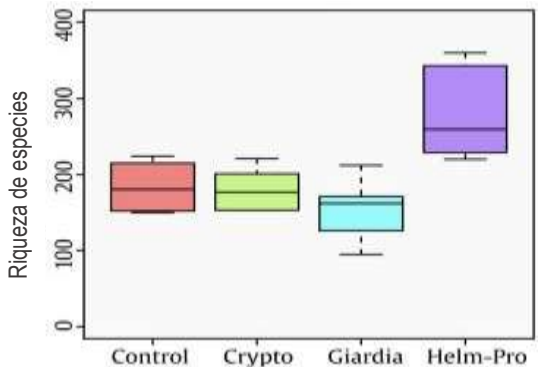

Figura 16. Esquema del impacto de diferentes eucariotas sobre la riqueza de especies en pacientes pediátricos. Los pacientes con presencia de helmintos y protozoos evidenciaron una mayor riqueza de especies. Tomado de Toro-Londoño y colaboradores (115).

Finalmente, la composición del micobioma intestinal (hongos componentes de la microbiota) ha sido uno de los temas con mayor rezago en el estudio de los microbiomas, resaltándose el estudio realizado en una cohorte de pacientes sanos, donde se evidencia que los principales componentes del micobioma son *Saccharomyces, Malassezia* y *Candida* (116). El estudio de

estos elementos de la microbiota reviste gran importancia en el ámbito de la ICD ya que se ha evidenciado el efecto protector de hongos como *Saccharomyces boulardi,* uno de los probióticos más comunes aislados de las frutas, contra la colitis causada por CD, mediada por la producción de inmunoglobulina A (117,118). Estudios observacionales recientes han mostrado que en el contexto de la ICD se observa un incremento de la razón entre Ascomycota/Basidiomycota, así como incremento en la frecuencia relativa de hongos como *Cladosporium, Aspergillus* y *Candida;* mientras que otros estudios han profundizado en algunas de estas relaciones, encontrando que, en ratones, la colonización previa con *C. albicans* reduce la susceptibilidad a la ICD, mientras que, en humanos, la presencia de este hongo reduce la efectividad del trasplante de microbiota fecal en pacientes con ICD (119,120). Los diferentes estudios realizados con especial enfoque en este importante componente de la microbiota intestinal han permitido dilucidar las complejas relaciones que se pueden establecer entre hongos y bacterias en el proceso salud-enfermedad, como se observa en la figura 17.

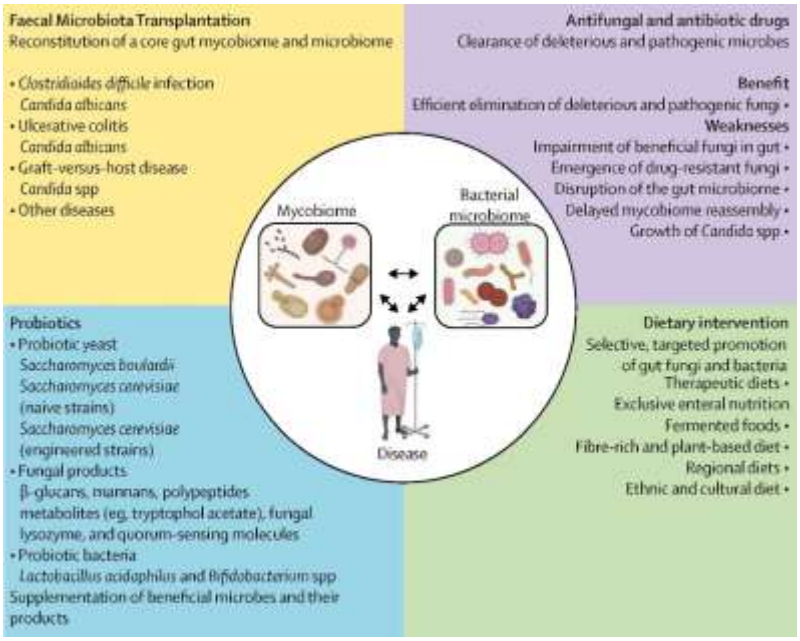

Figura 17. Principales interacciones entre hongos y bacterias de la microbiota intestinal relacionados con el proceso salud-enfermedad. Adaptado de Zhang y colaboradores (120).

2.3.Microbioma viral

Aunque la mayoría de estudios de microbioma se han enfocado en el componente bacteriano de la microbiota en estados de salud y enfermedad, durante años recientes los virus (incluyendo virus de ADN y ARN) presentes a nivel intestinal vienen cobrando cada vez más importancia, por su contribución a la ecología microbiana, por lo que se viene planteando la necesidad de comparar su diversidad, abundancia y función a través de diferentes tipos de células (121).

Una limitación de la secuenciación del microbioma viral es la implementación de métodos adecuados para la purificación de las partículas virales a partir de la muestra de heces, siendo los factores limitantes el pequeño tamaño del genoma (comparado con el ADN del hospedero e incluso del componente bacteriano de la microbiota), lo cual sumado al pequeño porcentaje que representa dentro de las comunidades microbianas, podría haber subestimado su participación en estos ecosistemas (122). Estos factores han conducido a la estandarización de métodos de procesamiento de la muestra que permitan evaluar la verdadera representatividad de estas partículas y llevar a cabo su caracterización (123).

A pesar de estas limitaciones, algunos estudios han permitido avanzar en la descripción del microbioma viral intestinal, identificándose que algunos grupos representativos que incluyen virus de ADN, como los llamados virus gigantes (> 300 kb), entre los que se encuentran las familias: Mimiviridae, Mamaviridae, Marseilleviridae, Poxviridae, Iridoviridae, Ascoviridae, Phycodnaviridae, Asfaviridae y los bacteriófagos (con representación mayoritaria de Anelloviruses), siendo estos últimos relacionados con parasitismo en bacterias. Dentro de estos virus se encuentran algunos que poseen ADN de una sola hebra (familias Microviridae e Inoviridae) y otros con ADN de doble hebra (Myoviridae, Siphoviridae, Podoviridae, Tectiviridae, Leviviridae, Inoviridae) (124,125) los cuales se destacan por representar la mayor abundancia dentro de la microbiota. Otro grupo importante de virus son los de ARN, entre los que se incluyen: Rotavirus, Astrovirus, Calicivirus, Norovirus, Virus de la Hepatitis E, Coronavirus y Torovirus, Adenovirus (serotipos 40 y 41) (126), los cuales generalmente se encuentran en bajas cantidades. Debido a su potencial impacto sobre la modulación de las comunidades microbianas y el consecuente efecto sobre la salud del hospedero, es que se hace necesario realizar la descripción de la dinámica de este tipo de partículas (127). En la microbiota también pueden encontrarse en bajas proporciones virus de eucariotas, siendo *Anelloviridae*, *Geminiviridae*, *Herpesviridae* los virus de ADN más frecuentes, mientras que *Caliciviridae*, *Picornaviridae*, *Reoviridae* se destacan como los virus ARN de eucariotas más abundantes (128,129)

En el contexto de la ICD han sido pocos los estudios que se han enfocado en profundizar la relación entre el componente viral de la microbiota y CD. La primera caracterización del microbioma viral intestinal en pacientes con ICD se realizó en el año 2018 en Asia, en los que se evidenció una disbiosis en el microbioma viral caracterizada principalmente por una disminución de los virus pertenecientes a la familia Microviridae (130). Estos resultados se detallan en la figura 18.

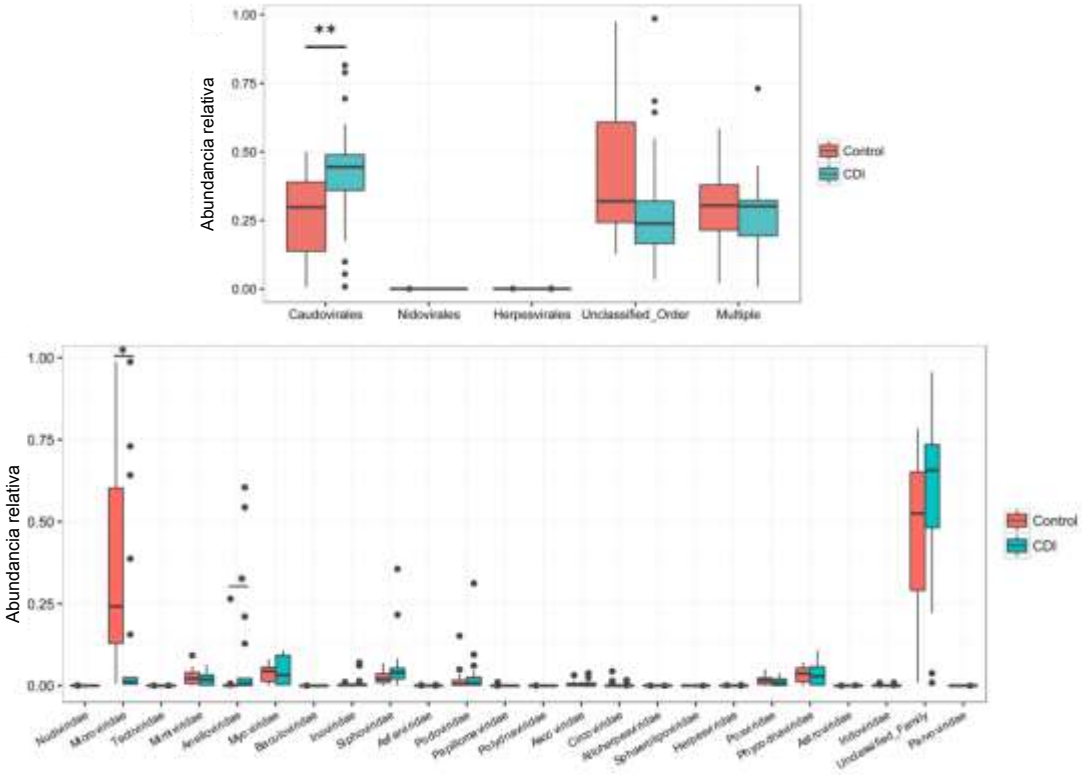

Figura 18. Caracterización del microbioma viral intestinal en pacientes con ICD en comparación con controles. En el panel superior se observa la abundancia relativa de los diferentes virus a nivel de orden. En el panel inferior se observa la abundancia relativa a nivel de familias virales. Tomado de Zuo y colaboradores (130).

Posteriormente, los estudios estuvieron orientados a determinar las modificaciones y el impacto sobre el microbioma viral posterior a un trasplante de materia fecal, donde se ha evidenciado que a pesar de las modificaciones que sufre la microbiota posterior al tratamiento de trasplante, se establece un core viral intestinal que dista del perfil del donador, con una tendencia a la ausencia de fagos en las personas sin alteraciones, sugiriendo que este componente contribuye al establecimiento a largo plazo de la microbiota proveniente del donante (131–133). Estos resultados se evidencian en la figura 19. Un estudio piloto reciente realizado en 5 pacientes con ICD evidenció mejoras en la consistencia de las heces y síntomas gastrointestinales posterior a la administración de un filtrado estéril, sugiriendo el papel de los fagos en el restablecimiento de la microbiota en el contexto de esta enfermedad inflamatoria intestinal (134). Por su parte, otra investigación evidenció que en pacientes con ICD recurrente se observa una disminución de los fagos crAssphage, uno de los fagos más abundantes y comúnmente encontrados en las heces de humanos en el mundo (135).

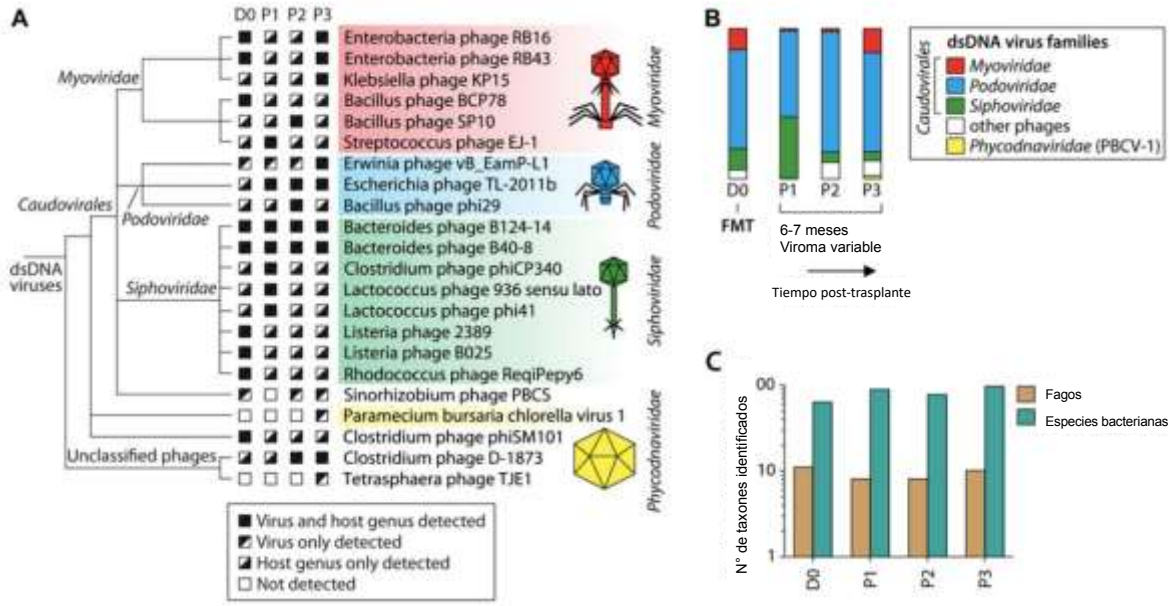

Figura 19. Cambios en el microbioma viral posterior al trasplante de materia fecal. A) Filogenia de los virus de ADN doble cadena encontrados en muestras de heces de donador y pacientes. B) Abundancia relativa de las familias virales encontradas. C) Comparativo de la abundancia relativa de fagos y especies bacterianas en las muestras encontradas. Adaptado de Broecker y colaboradores (132)

Llama poderosamente la atención en estas investigaciones, que, aunque los fagos son un marcador de inflamación, su abundancia no varía entre los donadores y los receptores, por lo que se requieren estudios que profundicen en el papel desempeñado por estos componentes de la microbiota. Revisiones recientes han mostrado el papel fundamental que desempeña el microbioma viral en la modulación de la microbiota intestinal, del sistema inmune y en el desarrollo de enfermedades inflamatorias por interacciones con las células de la lámina propia y los microorganismos comensales del intestino. También se ha evidenciado el papel benéfico que tiene en el metabolismo, regulación de la motilidad y mantenimiento de la barrera intestinal contra patógenos (136–138). A pesar de estas evidencias, este ha sido uno de los temas con menor número de publicaciones en el estudio de microbiota por lo que su campo es aún bastante desconocido. Así mismo, la utilización de herramientas de metagenómica que permite explorar tanto componentes del microbioma bacteriano como de los demás elementos de la microbiota, se ha enfocado principalmente en la caracterización de los primeros (56,90), dejando de lado la información concerniente tanto al microbioma viral como al microbioma eucariota dificultando aún más la comprensión holística de la interacción entre los diferentes componentes del microbioma.

Todo lo anterior evidencia que el microbioma no se limita únicamente al estudio del microbioma bacteriano, sino que cada uno de los componentes, tanto bacterias, virus y eucariotas como los elementos genéticos circulantes tienen un impacto directo sobre el ecosistema intestinal, influyendo de manera positiva o negativa sobre este, facilitando o impidiendo el desarrollo de infecciones como la ICD. Esto se constituye en uno de los elementos más importantes para el desarrollo de investigaciones como la presente tesis, donde se evaluaron los diferentes elementos que componen el microbioma intestinal, no

como elementos individuales, sino dentro de su interacción como parte de un ecosistema que se encuentra en un equilibrio, con el fin de tratar de dilucidar algunos de los factores relacionados con esta enfermedad, para contribuir al mejoramiento de la prevención, diagnóstico y tratamiento de la ICD.

3. HIPÓTESIS DE TRABAJO Y OBJETIVOS

3.1.Hipótesis de trabajo

Clostridioides difficile ha sido asociado con una microbiota intestinal alterada, debido a la disminución de microorganismos productores de ácidos grasos de cadena corta, principalmente butirato, los cuales se ven reducidos por acción de factores externos como los antibióticos y otros medicamentos. Sin embargo, los cambios que ocurren en la microbiota intestinal tanto previamente como a lo largo de la enfermedad dan cuenta de las complejas interacciones que suceden entre los diferentes miembros del ecosistema intestinal las cuales participan activamente tanto en el proceso de infección como en el establecimiento y desarrollo de la enfermedad, por lo que se sugiere que dichos cambios no están restringidos únicamente a la presencia de *C. difficile.* Así mismo, a pesar de que las alteraciones en las comunidades bacterianas intestinales de personas con ICD han sido consistentemente evidenciadas en diferentes investigaciones, los tratamientos dirigidos hacia el restablecimiento de la homeostasis intestinal han obtenido resultados variables, sugiriendo que existen otros elementos de la microbiota intestinal que juegan un papel preponderante en el desarrollo de interacciones y en el establecimiento de una microbiota equilibrada. Así mismo, se sugiere que el lugar de adquisición de la diarrea, así como los factores de virulencia y marcadores de resistencia pueden contribuir al empeoramiento de las condiciones generales de los pacientes.

Nuestra hipótesis de trabajo se fundamenta en la comprensión de las relaciones entre los diferentes elementos de la microbiota intestinal asociada a la ICD*,* así como en el desarrollo de esquemas de análisis que permitan la comprensión de diferentes fenómenos en otros modelos biológicos.

3.2.Objetivo general

Determinar la composición del microbioma intestinal de pacientes con diarrea asociada a la infección por *Clostridioides difficile* (ICD), adquirida en Unidad de Cuidados Intensivos (UCI) y en comunidad a través de la implementación de técnicas de secuenciación de alto rendimiento.

3.3.Objetivos específicos

3.3.1. Describir la composición taxonómica (procariotas y eucariotas) de la microbiota intestinal de pacientes con diarrea asociada a la ICD adquirida en UCI y comunidad mediante secuenciación profunda de los marcadores ARNr-16S y ARNr-18S.

3.3.2. Identificar otros componentes del microbioma intestinal (virus de ADN y conjunto de genes circulantes) aplicando metagenómica a muestras de diarrea asociada a la ICD en los grupos de estudio.

3.3.3. Determinar posibles asociaciones entre la fuente de adquisición de la ICD en UCI o comunidad y la composición del microbioma intestinal de las poblaciones de estudio.

4. INTRODUCCIÓN A LOS CAPÍTULOS

Entre los avances logrados en el estudio de la microbiota intestinal se destaca el entendimiento de la compleja interacción con el sistema inmune del huésped y el metabolismo, destacándose una gran participación en la producción de compuestos esenciales como ácidos biliares secundarios y ácidos grasos de cadena corta (5,72,139,140), los cuales desempeñan un papel fundamental en el desarrollo de la ICD. Así mismo se ha destacado el papel de la microbiota intestinal y su asociación con el desarrollo de enfermedades como obesidad, diabetes tipo 2 (52,141–143), enfermedad de hígado graso (144–146) y enfermedades inflamatorias intestinales (53,147,148), convirtiéndose en un tema central entre la comunidad científica. En este sentido, vale la pena mencionar los avances obtenidos en términos de los esquemas de tratamientos alternativos para la ICD, como el trasplante de materia fecal, el cual ha sido ampliamente difundido con variada evidencia de su efectividad en el proceso restaurativo de la homeostasis intestinal (95,149,150).

Uno de los factores que ha influido en la variabilidad de los tratamientos alternativos dirigidos al restablecimiento del equilibrio intestinal ha sido el limitado conocimiento acerca del papel de otros constituyentes del microbioma diferentes a las bacterias, lo cual no ha permitido dilucidar completamente los factores claves del éxito de este tipo de intervenciones, así como tampoco los riesgos que estos podrían conllevar. Esto podría estar dado por el enfoque de la mayoría de las investigaciones que se realizan en el ámbito de la microbiota intestinal, dirigido principalmente a las poblaciones bacterianas, sin profundizar en los demás miembros de la microbiota (5,6,96,151). De igual manera, se ha dejado de lado el entendimiento de las interacciones que se podrían desarrollar al interior de este ecosistema. Si bien es cierto que las bacterias son el principal constituyente de la microbiota intestinal, existen organismos eucariotas y virus que juegan un papel determinante en el mantenimiento de la homeostasis intestinal, por lo que el estudio del microbioma eucariótico y el microbioma viral representa un desafío para la completa descripción de la composición del microbioma intestinal.

Es precisamente gracias a este escenario que el análisis metagenómico cobra gran relevancia, ya que se ha convertido en una gran opción que permite no solo profundizar en los diferentes miembros del ecosistema intestinal, sino también en los metabolitos y genes asociados, los cuales revisten gran relevancia a nivel clínico debido a que algunos inducen alteraciones en procesos inmunes y metabólicos del hospedero y otros ocasionan resistencia a antibióticos, lo que podría reducir la eficacia de las estrategias terapéuticas actualmente disponibles para diversas patologías e incrementar el impacto clínico de muchas infecciones como la ICD (152). Lo anterior evidencia la necesidad de incluir este aspecto en los estudios de microbiomas, con el fin de brindar una mayor comprensión de las interacciones de los diversos componentes del ecosistema intestinal en relación con la ICD.

Otro elemento fundamental es la carencia en cuanto a los estudios existentes en la región dirigidos a la ICD, siendo la gran mayoría enfocados en la epidemiología, en los cuales se ha destacado el gran subregistro que existe en el continente referente a la infección, lo cual dificulta el abordaje de esta patología en los países latinoamericanos. Así mismo, en Colombia no se han realizado estudios comparativos sobre la composición de la microbiota en pacientes con sintomatología diarreica con y sin ICD, por lo que el impacto de este microorganismo sobre el ecosistema intestinal en el país y en la región permanece desconocido. Por estas razones, se identificó la necesidad de desarrollar estudios que permitan determinar la composición de la microbiota intestinal, factores de virulencia y marcadores de resistencia asociados, por lo que esta tesis se planteó el abordaje del objetivo general dividido en los siguientes 3 capítulos:

Capítulo 1. *"*Identificación de comunidades de bacterias y eucariotas asociadas a la ICD en pacientes con diarrea adquirida en comunidad y a nivel intrahospitalario"

Capítulo 2. *"*Identificación de otros componentes del microbioma intestinal (virus de ADN y conjunto de genes circulantes) mediante metagenómica"

Capítulo 3. *"*Identificación de microbiota asociadas a garrapatas duras que se alimentan de humanos en España"

CAPÍTULO 1. "Identificación de comunidades de bacterias y eucariotas asociadas a la ICD en pacientes con diarrea adquirida en comunidad y a nivel intrahospitalario"

Con el fin de desarrollar los objetivos propuestos en la presente tesis, el primer capítulo se enfocó en determinar las diferencias en la composición, diversidad y abundancia de las bacterias y eucariotas de la microbiota intestinal de pacientes con diarrea asociada a la ICD, adquirida en comunidad y a nivel intrahospitalario mediante secuenciación profunda de marcador único. Se analizaron 98 muestras correspondientes a 4 grupos designados de acuerdo con el lugar de adquisición de la diarrea y el estatus de ICD (HCFO/+, HCFO/-, CO/+, CO/-). Se realizó análisis de los datos secuenciados por Illumina de los marcadores ARNr-16S y 18S, desarrollando un esquema de análisis rápido y preciso para este tipo de muestras, permitiendo la descripción de las comunidades de bacterias y eucariotas de los pacientes. Se evidenció una alta frecuencia de coinfección *C. difficile – Blastocystis* spp., así como un aumento de bacterias benéficas productoras de butirato en los pacientes con infección por *Blastocystis.* También se observó un aumento de los miembros del phylum Firmicutes en los grupos con diarrea adquirida en comunidad, mientras que en los grupos con presencia de *C. difficile* se observó un aumento de los miembros del phylum Proteobacteria. No se encontraron diferencias en las Archaea ni en los eucariotas entre los grupos estudiados. Así mismo, la revisión de la literatura permitió la elaboración de un artículo que reúne los diferentes aspectos de la fisiopatología y de la microbiota asociada a la ICD.

Artículo 1. Vega, L., Herrera, G., Muñoz, M., Patarroyo, M. A., & Ramírez, J. D. (2020). Occurrence of *Blastocystis* in patients with *Clostridioides difficile* infection. *Pathogens*, *9*(4), 283.

pathogens

MDPI

Communication

Occurrence of *Blastocystis* in Patients with *Clostridioides difficile* Infection

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Abstract: *Clostridiodes difficile* comprises a public-health threat that has been understudied in Colombia. Hypervirulent strains of *C. difficile* harbor multiple toxins, can be easily spread, and can have their onset of disease within healthcare facilities (HCFO) and the community (CO). Studies have shown that a disrupted microbiota (e.g., dysbiosis) may allow *C. difficile* infection (CDI). It has been suggested that dysbiosis prevents colonization by the anaerobic eukaryote *Blastocystis*, possibly due to an increase in luminal oxygen tension. No study has found co-occurrence of CDI and *Blastocystis*. Therefore, we aimed to determine the frequencies of *C. difficile* and *Blastocystis* infection/colonization in 220 diarrheal fecal samples. Molecular detection by PCR for both microorganisms was performed, with descriptive analyses of four variables (CDI detection, determination of *C. difficile* toxigenic profiles, *Blastocystis* detection, and patient site of onset). We demonstrate a significant association between the presence of *Blastocystis* and CDI, with coinfection found in 61 patients, and show a high frequency of CDI among both HCFO and CO groups. Our results of coinfection frequencies could support hypotheses that suggest *Blastocystis* can adapt to dysbiosis and oxidative stress. Further, the presence of toxigenic *C. difficile* occurring outside healthcare facilities shown here raises the alarm for community wide spread.

Keywords: *C. difficile*; *Blastocystis*; dysbiosis; toxigenic *C. difficile*; community onset; healthcare facility onset

1. Introduction

Studies focused on the composition of the intestinal microbiota have indicated that some of its members may have a modulating effect on the diversity of other populations of microorganisms [1]. This is the case of *Clostridioides difficile*, which may be a negative modulator of the microbiota [2], which means that it can decrease the diversity of other groups of bacteria in the gut. For example, some authors have pointed out that *C. difficile* infection (CDI) is related to the decrease in beneficial bacteria while increasing the abundance of bacteria belonging to the Enterobacteriaceae family or other groups of bacterial pathogens [3]. Furthermore, some strains of *C. difficile* have importance in public health due to their virulence factors: toxins and mobile genetic elements (MGEs) [4]. Toxins A and B are encoded by the pathogenicity locus (*PaLoc*), which comprises five genes (*tcdA*, *tcdB*, *tcdR*, *tcdC*, and *tcdE*), while binary toxins (CdtA and CdtB) are encoded by *CdtLoc*. Likewise, MGEs of *C. difficile*

are associated with resistance to some antibiotics, and sometimes, they can be transferred from other species of bacteria to strains of *C. difficile*. Hypervirulent strains are also important in public health since they can be easily spread and have aggressive clinical features [4].

Blastocystis is an anaerobic eukaryote that can be found in a wide range of hosts (humans, birds, primates, reptiles, etc.). This eukaryote exhibits a high genetic diversity and, thus, is classified in at least 17 subtypes, of which the distribution varies geographically [5,6]. *Blastocystis* may have a modulatory effect upon some members of the microbiota. However, due to its high genetic diversity, the variation of its subtypes distribution and the different results obtained when studying its modulatory effect on the microbiota has led to the difficulty of considering this eukaryote as a commensal of the gut microbiota or as a pathogen of the same. As a result of these controversies, the effect of *Blastocystis* colonization in the host health is still under study [7].

The microbiota-modulating effect of *Blastocystis* is still under study because microbiome studies in *Blastocystis* show variable results; some of them show that *Blastocystis* is associated with a healthy microbiota, while in others, it may be linked to dysbiosis and gastrointestinal diseases [7]. For instance, the in vivo and in vitro study carried by Yason et al. demonstrated that the presence of *Blastocystis* subtype 7 reduces the populations of beneficial bacteria in the gut microbiota while increasing populations of enterobacteria [8]. In addition, Nourrisson et al. showed that the abundance of beneficial bacteria, such as *Bifidobacterium* are reduced in patients with colonization by *Blastocystis* and inflammatory bowel disease (IBD) [9]. On the contrary, other studies have shown that the presence of *Blastocystis* in gut microbiota increases the abundance of some groups of beneficial bacteria (e.g., Ruminococcaceae, Lachnospiraceae, Clostridiaceae, etc.) [10–12].

Dysbiosis can be defined as changes in the communities of the microbiota that lead to a state of disease [13,14]. For instance, dysbiosis may be a common scenario in cases of diarrhea associated with the extensive use of antibiotics, which in turn allows *C. difficile* infection in the intestine [15]. However, it has been suggested that dysbiosis scenarios can prevent colonization by *Blastocystis* given that there is an increase in luminal oxygen concentrations postulated in dysbiosis [16]. Nonetheless, *Blastocystis* may have some mechanisms that allow it to respond to the oxidative stress of dysbiosis. Currently, no studies have shown the incidence of *C. difficile* and *Blastocystis* coinfection worldwide. Therefore, this work focused on determining the frequencies of *C. difficile* and *Blastocystis* infection/colonization in 220 fecal samples of Colombian patients with diarrhea, belonging to community-onset (CO) origin and healthcare facility-onset (HCFO) origin.

2. Results and Discussion

2.1. Descriptive Analyses of the Population and the Four Outcome Groups

Descriptive analysis showed that 37.3% (n = 82) of the total population (n = 220) had *Blastocystis*. Markers of *C. difficile* were found in 65.4% (n = 144) of the total population, where 87.5% (n = 126) of these patients had *C. difficile* toxins. Also, most of the patients with *Blastocystis* or *C. difficile* or with some *C. difficile* toxin belonged to CO (Figure 1A,B). In the case of CO (n = 138), we found 89 patients with CDI, where 82 of these patients had a positive toxigenic *C. difficile* (Cd_tox); also, 40 patients belonging to this onset group presented three variables at the same time (CDI, positive Cd_tox, and presence of *Blastocystis*). Additionally within CO, 37 patients were negative for both study microorganisms (*Blastocystis* and *C. difficile*) (Figure 1A). Within HCFO (n = 82), we found 55 patients with CDI, where 44 had a positive toxigenic *C. difficile* (Cd_tox); only 17 patients belonging to this onset group presented the three variables mentioned above. Only 18 patients within HCFO were negative for both microorganisms (Figure 1B).

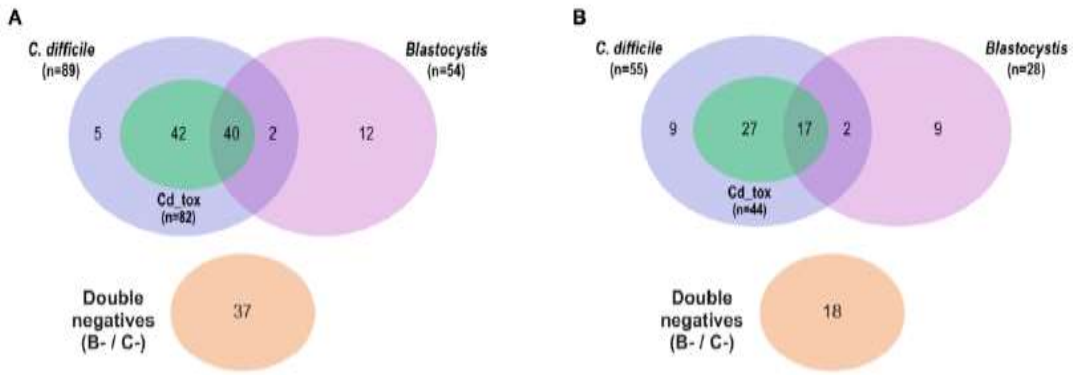

Figure 1. Infection frequencies by *Blastocystis* and *C. difficile* within onset groups: (**A**) Number of community-onset (CO) patients with *C. difficile* infection (CDI), positive toxigenic *C. difficile* (Cd_tox), and presence of *Blastocystis* and patients negative for both micoorganisms. (**B**) Number of healthcare facility-onset (HCFO) patients with *C. difficile* infection (CDI), positive toxigenic *C. difficile* (Cd_tox), and presence of *Blastocystis* and patients negative for both micoorganisms.

The sizes of each of our 4 outcome groups were as follows: 61 patients belonged to B+/C+, 55 patients belonged to B−/C−, 21 patients belonged to B+/C−, and 83 patients belonged to the group B−/C+. In Table 1 the distribution of CDI (toxins positive or negative) and *Blastocystis* among onset groups is detailed, where only a quarter of each population is free of the two microorganisms, and it is evident that most of the patients with CDI are toxin positive for both onset groups. Although for the group B+/C+ there is an increase of 8.3% in toxigenic *C. difficile* within CO in comparison to HCFO, the frequencies of toxigenic *C. difficile* are nearly identical among CO and HCFO of the group B−C+ (30.4% and 32.9%). Also, the results point out an excess of toxigenic *C. difficile* among all positive groups that belong to CO in contrast to HCFO (59.4% vs. 53.6%). A noticeable fact that jumps out is the excess of non-toxigenic *C. difficile* among HCFO, where most of these strains belong to the B−/C+ group (Table 1). In summary, it is important to highlight that, in all of the mentioned outcome groups, the distribution of cases based on CDI and/or *Blastocystis* status is similar among CO and HCFO.

Table 1. Distrbution of CDI (toxins positive or negative) and *Blastocystis* among community-onset (CO) or healthcare facility-onset (HCFO).

	B+/C+ [1]		**B−/C+** [1]		**B−/C−**	**B+/C−**
	Cd_tox Postitive	**Cd_tox Negative**	**Cd_tox Postitive**	**Cd_tox Negative**	–	–
CO [2] $n = 138$	29.0%	1.4%	30.4%	3.6%	26.8%	8.7%
HCFO [2] $n = 82$	20.7%	2.4%	32.9%	11.0%	22.0%	11.0%

[1] These groups present an additional category, positive or negative toxigenic *C. difficile*, because these outcome groups are the only ones positive for CDI; [2] percentages were calculated considering the total samples for each onset type.

The high rates of CDI both in CO and HCFO are in concordance with the studies that have reported similar results for the region. The study performed by Forero et al. (2019) in Colombia showed a high frequency of *C. difficile* infection in both onset groups, where 64.5% of CO patients and 67.1% of HCFO patients had CDI [17]. However, there are only a few studies in Colombia about the prevalence of CDI considering the onset groups mentioned herein, especially studies focusing on CDI within community onset. In the case of Latin America, an underestimation of CDI has been done, maybe because of the low standardization of methods for *C. difficile* detection and because of the lack of

supplies in many laboratories [18]. Despite this fact, many studies performed in some countries such as Argentina, Brazil, Puerto Rico, and Chile have focused on the determination of CDI prevalence, principally within healthcare centers. Even though these studies focus on the healthcare center level, some of them highlight the importance of community-acquired *C. difficile* [19].

2.2. Frequencies of Coinfection Suggest a Possible Adaptation of Blastocystis to Oxidative Stress

The outcome group of greatest interest is the one positive for both microorganisms (B+/C+) since it has been suggested that, in the scenarios of dybiosis, there should be no colonization by *Blastocystis* because high concentrations of oxygen could affect *Blastocystis* survival [16]. Nevertheless, the results exposed here show the presence of *Blastocystis* and *C. difficile* in 61 patients out of 220. Also, a statistically significant association was obtained between the presence of *Blastocystis* and CDI ($p = 0.032$, Odds Ratio = 1.92). The present study is merely descriptive, and its limitations do not allow making conjectures about mechanisms that *Blastocystis* might be implementing to adapt to this environment that is not conducive to its survival, since these factors were not evaluated in this study. However, our results could support some hypothesis that have been proposed by other authors.

A previous study proposed a mechanism in which *Blastocystis* reduces the induced oxidative stress using its alternative oxidase system (AOX) [20], which may allow *Blastocystis* colonization to be successful even in environments where it is believed that its survival could be affected. Some studies not conducted in Latin America have found a low prevalence of *Blastocystis* in scenarios where there are alterations in the microbiota (such as those that may occur in inflammatory disease or Crohn's disease) when compared with a healthy population [3,21]. In the case of Colombia, cases of coinfection of *Blastocystis* with other intestinal protists have been reported, and there has been suggested zoonotic transmission of some subtypes of *Blastocystis* [22,23].

Nevertheless, the results showed herein could suggest that *Blastocystis* may have the ability to adapt to other than ideal conditions (anaerobiosis). One hypothesis suggested by Laforest-Lapointe and Arrieta is that *Blastocystis* may have the ability to predate over some bacterial taxa within the disrupted microbiota [24], thereby promoting its survival under high oxygen concentrations. However, further studies are needed to provide additional information about the possible ecological interaction between *Blastocystis* and other members of the microbiota. Also, future approaches are required to elucidate the mechanisms that *Blastocystis* can use to adapt to a scenario of dysbiosis.

The infection frequencies shown in this work could suggest a possible adaptation of *Blastocystis* to a scenario of dysbiosis when there is also an infection by *C. difficile* and that had not been reported in Colombia or Latin America. Even though we found an association between CDI and *Blastocystis*, it is possible that there is an intermediate bacterial taxa in the microbiome mediating this association. In order to understand more deeply the role of *Blastocystis* in this scenario of dysbiosis, circulating subtypes must be identified within patient samples. Likewise, a descriptive analysis of the composition of each patient's microbiota would help to completely elucidate the modulating role of this eukaryote on the members of the bacterial microbiome, particularly in those cases of coinfection with *C. difficile*.

2.3. Excess of Non-Toxigenic C. difficile among HCFO Patients

Surprisingly, toxigenic *C. difficile* was found equally among CO and HCFO, despite the expectation that it would be more common in the HCFO group. The latter group is expected to have more of the principal risk factor for CDI, alterations of the gut microbiota due to the extensive use of antibiotics and other medical treatments, as well as increased exposure to other patients with *C. difficile* due to prolonged hospitalization of the patients [25]. Indeed, an excess of non-toxigenic *C. difficile* was observed among HCFO patients in contrast to CO patients, especially those belonging to the B−/C+ group (Table 1). The previous result suggests that non-toxigenic *C. difficile* can be prevalent within healthcare facilities, where they could be clonally spread since these strains may be transformed into toxigenic strains by acquiring toxin genes, thus serving as potential pathogens.

On the other hand, a slight increment in the rate of toxigenic *C. difficile* among the CO group was identified in contrast with the HCFO group; however, this difference was not statistically significant. Even though CO has lower exposure to risk factors for CDI, in the last years, there has been an increase of CDI within CO patients [26], including the presence of some hypervirulent strains of *C. difficile* [27]. Our results support what was reported by Gupta and Khanna, where a study conducted in Cleveland found that more than half of asymptomatic residents were carriers of a toxigenic strain of *C. difficile* [28]. Additionally, in Colombia, Muñoz et al. reported a patient whose CDI had been acquired within the CO after antibiotic use that carried toxin-encoding genes and loci associated with antibiotic resistance [27]. In general, these findings support the hypothesis that CO patients can contribute to the transmission of *C. difficile* toxins [2,28].

Since CDI studies commonly use samples belonging to healthcare centers, the prevalence of CDI within CO patients and strain profiles of *C. difficile* within this onset group are less studied [27]. Some studies proposed that CDI in CO patients could arise due to antibiotic exposure and the use of other outpatient medications [29]. In this study, clinical data of the patients was not available, including prior antibiotic use or recent hospitalizations, which might have clarified how CDI within the CO group could have arisen as an emerging transmission scenario. Studies have highlighted the ability of some *C. difficile* CO strains to acquire virulence factors (e.g., toxin-encoding genes) [30] via horizontal gene transfer to the non-toxigenic strains [31]. This high level of genome plasticity is supported by previous studies of our group, where strains circulating in CO were characterized finding rearrangements in these genes [27,30]. Finally, this high frequency of CDI could be explained by an increased clonal spread, in which some limited sequence types (STs) have been detected within the community. However, there is a lack of studies focused on molecular or genomic epidemiology that can help to elucidate population structure and transmission dynamics of *C. difficile* in Colombia [30,32]. Future studies should consider the implications of CDI in CO.

3. Materials and Methods

Only patients with diarrhea (the main manifestation of CDI) were included in this study, since it would allow a greater probability of identifying patients with alterations in intestinal homeostasis, including *C. difficile* infection. Thus, 220 previous stool samples were employed, and the procedures of collection and subsequent DNA extraction of these samples were conducted as reported in Muñoz et al. (2018) [32]. Briefly, CDI was determined by different approaches including in vitro culture and molecular methods: conventional PCR targeting *16S-rRNA* and *gdh* and quantitative PCR targeting *16S-rRNA*. Afterward, toxigenic *C. difficile* of samples positive for any CDI molecular detection were determined by conventional PCR targeting six molecular markers located within *PaLoc* and *CdtLoc*. The amplification of lok1/lok3 markers flanquing the *PaLoc* was performed to determine those *C. difficile* strains without *PaLoc* [32].

The purpose of targeting more than one molecular marker in the previous study is because of its usefulness for increasing the sensibility and specificity in the schemes employed in the molecular epidemiology of pathogens, especially when the microorganism has a highly dynamic genome (e.g., *C. difficile*). The high genetic diversity of the markers can lead to an underestimation of their frequency when only one test is used [32]. Although we employed the same population as in Muñoz et al. (2018), the present study considered different selection criteria. Hence, the selection criteria used to determine positive samples (for CDI or Cd_tox) was when at least one positive result in any of the traditional molecular tests applied to DNA of the sample was obtained. Thus, in the case of toxigenic *C. difficile*, the presence of genes that encode for any of the toxins (toxins A or B, or binary toxin) determined a positive toxigenic *C. difficile*, since some studies show that binary toxins have cytopathic effects in vitro and exacerbate clinical outcomes in *C. difficile* patients [33,34]. Therefore, all toxigenic CDI samples had at least 2 molecular markers positive (*16S-rRNA* or *gdh* gene and at least 1 of the toxin genes).

The origin or onset groups of the patients (community-onset (CO) or healthcare facility-onset (HCFO)) was determined considering the guidelines of the Clinical Practice Guidelines for CDI in

Adults [35]. The mentioned guideline defines three categories for patient classification, where two of them contemplate the community classification: community-onset, healthcare facility-associated disease (CO-HCFA), and community-associated CDI (CA-CDI) [35]. Despite this classification, for the present study, the categories that contemplate the community onset (CO-HCFA and CA-CDI) were grouped as one (CO). This grouping of community onset was performed given the lack of clinical data of CO patients, especially information about prior hospitalizations [32]. Hence, CO was defined when the patient attended the emergency room for different reasons and presented an episode of diarrhea during the 48 h following admission to a medical center. For the CO subjects, 85% of these patients attended the emergency room while 15% were referred for outpatient consult. On the other hand, an HCFO patient was defined when the episode of diarrhea developed after the third day following admission. HCFO also included patients from Intensive Care Unity (ICU), which constitutes a population with major risks for developing complications associated with CDI [32].

Likewise, *Blastocystis* presence in 220 stool samples was determined using a conventional PCR as reported elsewhere [36]. A database was constructed to couple the information corresponding to CDI, successful determination of toxigenic *C. difficile* (Cd_tox), presence of *Blastocystis*, and the patients' onset group (CO or HCFO). Furthermore, the database allowed us to define 4 outcome groups: positive for *Blastocystis* and *C. difficile* (B+/C+), only positive for *C. difficile* (B−/C+), only positive for *Blastocystis* (B+/C−), and negative for both microorganisms (B−/C−). We constructed Venn diagrams to better depict the number of samples in the described groups, including data from Cd_tox that was also plotted. Descriptive analyses were performed to determine the frequencies of coinfection events in terms of percentage. Finally, X^2 tests were performed to identify the associations between the four variables of interest (CDI, presence of *Blastocystis*, Cd_tox, and patients' onset group). For the cases in which a significant association between two variables was obtained, an odds ratio (OR) was calculated considering only the CDI positive samples (n = 144) and taking HCFO as the onset group with increased risk (IC = 95%). The clinical importance of using only CDI positive samples is to know which percentage of these samples carry toxin genes and which of them are non-toxigenic. The statistical analyses were performed in STATA 12.0 (StataCorp LLC, College Station, TX, USA). A significance value $p < 0.05$ was fixed for all hypothesis tests.

Author Contributions: M.M. and J.D.R. designed the study and contributed to the definition of the experimental approach. M.M. and L.V. performed the data acquisition and the respective laboratory protocols. M.M. and G.H. performed statistical analyses of the study. M.A.P. and J.D.R. revised and edited the manuscript. All authors read and approved the final version of the manuscript.

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RESEARCH ARTICLE

Gut microbiota profiles in diarrheic patients with co-occurrence of *Clostridioides difficile* and *Blastocystis*

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Abstract

Blastocystis and *Clostridioides difficile* co-occurrence is considered a rare event since the colonization by *Blastocystis* is prevented under a decrease in beneficial bacteria in the microbiota when there is *C. difficile* infection (CDI). This scenario has been reported once, but no information on the gut microbiota profiling is available. The present study is motivated by knowing which members of the microbiota can be found in this rare scenario and how this co-occurrence may impact the abundance of other bacteria, eukaryotes or archaea present in the gut microbiota. This study aimed to describe the bacterial and eukaryotic communities using amplicon-based sequencing of the 16S- and 18S-rRNA regions of three patient groups: (1) *Blastocystis* and *C. difficile* infection (B+/C+, n = 31), (2) *C. difficile* infection only (B−/C+, n = 44), and (3) without *Blastocystis* or *C. difficile* (B−/C−, n = 40). *Blastocystis* was subtyped using amplicon-based sequencing of the 18S-rRNA gene, revealing circulation of subtypes ST1 (43.4%), ST3 (35.85%) and ST5 (20.75%) among the study population. We found that B+/C+ patients had a higher abundance of some beneficial bacteria (such as butyrate producers or bacteria with anti-inflammatory properties) compared with non-*Blastocystis*-colonized patients, which may suggest a shift towards an increase in beneficial bacteria when *Blastocystis* colonizes patients with CDI. Regarding eukaryotic communities, statistical differences in the abundance of some eukaryotic genera between the study groups were not observed. Thus, this study provides preliminary descriptive information of a potential microbiota profiling of differential presence by *Blastocystis* and *C. difficile*.

Introduction

The gut microbiota is defined as the assembly or microorganisms from different domains (bacteria, archaea, eukaryotes and viruses) that can have a well-defined habitat in the intestine, where their microbial structural elements and the surrounding environmental conditions are

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considered (microbiome) [1]. Several exogenous and endogenous factors that can generate variations in the composition of the microbiome have been described and this has led to the intestinal microbiota being viewed as a potential indicator of host health [2, 3]. Because multiple factors can alter the composition of the microbiome, it can be difficult to establish a direct cause and effect. To date, most studies on the microbiota have focused on the analysis of different groups of bacteria and thus our understanding of the roles and interactions of eukaryotes, archaea and viruses within the microbiome remains limited [2, 4, 5].

In 2016, Gorvitovskaia *et al.* proposed that certain bacterial taxa can be used as biomarkers since they correlated with diet or a state of disease [6]. Also, in the case of eukaryotes a study proposed a set of protists that can be commonly found in the microbiota: Common luminal intestinal parasitic protists (CLIPPs). CLIPPs are protists (*Blastocystis*, *Giardia*, *Cryptosporidium*, *Dientamoeba*, *Endolimax*) that can be found colonizing gut microbiota, and they appear to be more common than previously thought. It has been suggested that some CLIPPs may have pathogenic potential, and also that they can interact with other members of the gut microbiota, influencing the host's health [7].

The possible modulating role of some members of the microbiota has been suggested, including the pathogenic bacterium *Clostridioides difficile* and the protist *Blastocystis* [7, 8]. *C. difficile* is an anaerobic, spore-forming bacterium, whose virulence is mediated by toxin production and some antimicrobial resistance genes [9, 10]. Some authors have pointed out that *C. difficile* infection (CDI) is related to a decrease in some groups of beneficial bacteria producing butyrate, which is accompanied by an increase in some groups of pathogenic bacteria belonging to the *Enterobacteriaceae* family [11]. Therefore, this bacterium is usually considered a negative modulator of the intestinal microbiota.

Blastocystis is an anaerobic eukaryote that exhibits high genetic diversity and is classified into 26 subtypes, whose geographical distribution and host preference are widely heterogeneous [12, 13]. In humans, the presence of ST1 to ST9 [13] and ST12 [14, 15] has been reported, while some other subtypes are commonly found in animals (such as ungulate mammals, rats and birds) [16]. Studies on the modulating role of *Blastocystis* in the microbiota have shown variable results. Some reports have linked *Blastocystis* colonization with a healthy microbiota [17], while other studies have found associations with microbiota disorders and some gastrointestinal diseases [18, 19].

Dysbiosis can be defined as changes in the communities of the microbiota that lead to a state of disease [20]. One of the consequences of CDI is a decrease in beneficial bacteria that produce butyrate, and in turn this low concentration of butyrate increase luminal oxygen concentrations, as does the abundance of pathogenic bacteria. This increase in the oxygen concentration in the intestinal lumen can prevent the colonization of *Blastocystis* because of its anaerobic nature, and therefore, finding this protist in scenarios of CDI infection is not common [7, 21]. However, *Blastocystis* may possess a mechanism to support oxidative stress in the intestine, which would allow it to continue to colonize under scenarios where its survival may otherwise be affected [22, 23]. To date, the co-occurrence of *Blastocystis* and *C. difficile* has been reported in a single study worldwide [24], which is interesting given that this scenario should not occur due to the alterations that CDI generates on bacterial communities and that can prevent the colonization of *Blastocystis*, as explained above.

Nonetheless, there have been no studies on gut microbiota profiling during this co-occurrence scenario. What draws our attention is this controversy in which the simultaneous colonization by *Blastocystis* and *C. difficile* can occur. This particularity prompted us to explore which members of the microbiota could be found in this scenario, as well as detecting changes by comparing with the members of the microbiota found in patients that only have CDI or even in patients without colonization by both microorganisms. The results obtained from this

analysis would allow us to understand how *Blastocystis* may be exploiting a niche that had not been previously described and even to unveil the possible implications that this co-occurrence has on other members of the microbiota. Likewise, it would help to address questions about whether the profile of the microbiota under the co-occurrence of the mentioned microorganisms resembles that of a CDI, or if colonization by *Blastocystis* can change this profile because of the interactions that this protist may have with communities of bacteria and other eukaryotes.

Considering the lack of information about co-occurrence of *Blastocystis* and *C. difficile*, the present study aimed to describe the composition of the gut microbiota for three groups of patients with diarrhea and a differing *Blastocystis* and *C. difficile* colonization/infection status: 1. *Blastocystis* and *C. difficile* colonization/infection-positive patients (B+/C+), 2. *Blastocystis* colonization-negative and *C. difficile* infection-positive patients (B−/C+), and 3. colonization/infection-free patients (B−/C−). Our results are the first to describe the bacterial and eukaryotic communities during the co-occurrence and differential presence of *Blastocystis* and *C. difficile* in patients with diarrhea, which helps to understand this peculiar scenario by acknowledging the microbial communities, becoming a starting point to decipher their possible interactions within the microbiota.

Materials and methods

Ethical statement

This study was approved by the Universidad del Rosario's Research Ethics' Committee (CEI-UR). This research was considered low risk due to Colombian Ministry of Health resolution 008430/1993 criteria stating that experimental interventions cannot be made regarding research subjects. Data concerning patient identification was treated confidentially, in line with Colombian legal and ethical guidelines and according to that expressed by the latest version of the Declaration of Helsinki (World Medical Association). Hospital written informed consent was obtained from patients.

Sample selection and fecal DNA amplification for *Blastocystis* detection and subtyping

The DNA extracted from 115 fecal samples, collected by Muñoz *et al.* (2018) during a previous study that aimed characterizing *C. difficile* infection (CDI) in patients with diarrhea in Colombia [25], was used in the present study. The patients were selected based on symptoms of diarrhea because this is the main manifestation of CDI, but the study did not consider further clinical and sociodemographic information about the patients. DNA extraction from fecal samples was performed with the Stool DNA Isolation Kit (Norgen Biotek Corporation, Ontario, Canada), and the molecular detection of *C. difficile* was performed using two conventional PCR protocols targeting the 16S-rRNA and *gdh* genes, and a quantitative PCR protocol directed at 16S-rRNA. Samples were considered positive for CDI when at least one positive result was obtained with any of the aforementioned molecular tests [25]. It is important to highlight that the 115 samples were also selected based on sample volume (μL) and DNA concentration (ng/μL) optimal for the sequencing process.

For the detection of *Blastocystis* in fecal samples, the hypervariable V4 region of 18S-rRNA was amplified by conventional PCR using the primers F5 (5′-GGTCCGGTGAACACTTTGGATTT-3′) and F2 (5′- CCTACGGAAACCTTGTTACGACTTCA-3′) [26] and the following PCR cycling conditions: initial denaturation at 95˚ C for 10 minutes, followed by 40 cycles of 95˚ C for 15 seconds, 58˚ C for 1 minute and 72˚ C for 10 minutes, and a final extension at 72˚

C for 10 minutes. The presence of *Blastocystis* DNA was determined by observing a band of 119 base pairs (bp) following agarose gel electrophoresis.

The samples for which *Blastocystis* DNA was detected were subjected to the subtyping protocol described by Maloney *et al.* (2019) [12]. The samples were submitted to PCR for the amplification of SSU rRNA gene using one pair of modified primers that contained the Illumina overhang adapter sequences: ILMN_Blast505_532F and ILMN_Blast998_1017R. Sequencing libraries were prepared using a dual indexing strategy. A final pooled library concentration of 8 pM with 20% PhiX control was sequenced on an Illumina MiSeq using 600 cycle v3 chemistry (Illumina, San Diego, CA, USA). The resulting paired end reads were analyzed with an in-house pipeline that used the BBTools package v38.22, VSEARCH v2.8.0 [27], and BLAST+ 2.7.1. The sequences were filtered, trimmed and merged using the mentioned software. After removing singletons, clustering and the assignment of centroid sequences to Operational Taxonomic Units (OTU) was performed for each sample at 98% identity threshold. The OTUs were filtered, so those with a minimum of 100 sequences were retained and blasted against a reference database consisting of *Blastocystis* sequences. All OTUs were assigned a *Blastocystis* subtype based on the best match from the BLAST results using the consensus subtype terminology [28]. Further, the relative abundance of subtypes in each sample was calculated considering the number of reads obtained for each operational taxonomic unit (OTU), with respect to the total reads obtained per sample.

Blastocystis detection data were combined with *C. difficile* infection (CDI) data [25], and this allowed to establish three study groups: 1. colonization/infection (B+/C+, n = 31), 2. CDI only (B−/C+, n = 44), and 3. colonization/infection-free group (B−/C−, n = 40). Overall, the frequency of CDI among the 115 samples was 65.22% (n = 75), whereas the frequency of *Blastocystis* was 26.96% (n = 31). The group showing colonization/infection by both organisms (B+/C+) represented 26.96% (n = 31) of the total population, the colonization/infection-free group for which the study microorganisms were not detected (B−/C−) represented 34.78% (n = 40) of the total population, and the B−/C+ group represented about 40% of the total population (n = 44) (S1 Table).

Illumina sequencing and bioinformatic analyses to determine the bacterial and eukaryotic intestinal communities

Initially, the V4 hypervariable regions of the 16S-rRNA (bacterial communities) and 18S-rRNA (eukaryotic communities) gene fragments were amplified in the 115 samples, using the 341F/806R [29] and 528F/706R [30] high resolution primers, respectively. The sizes of the fragments amplified by the aforementioned primers were 466 bp and 179 bp, respectively. The amplification described above, and the sequencing of the samples was performed by an independent entity (Novogene, Bioinformatics Technology Co., Ltd, Beijing, China). Subsequently, the construction of DNA libraries of microbial amplicons was performed using end repairing, the addition of A to tails and ligation of the index adapter. These libraries were subjected to the sequencing process on a paired-end Illumina platform (Illumina Novaseq PE250) to generate 250 bp paired-end raw reads, and assuming a minimum expected depth of 100 thousand reads per sample. Once paired-end sequences were obtained, QIIME software (version 2019.7) [31] was used to remove barcodes and primers from each pair of these demultiplexed sequences.

The taxonomic allocation of the sequences was performed using R DADA2 package (R Core Team, Vienna, Austria) [32], implementing the recommended pipeline for microbiome analysis (https://benjjneb.github.io/dada2/tutorial.html). Initially, individual reads were filtered considering a Phred score equal to or greater than 30 to minimize erroneous reads.

Subsequently, forward and reverse reads were merged, and central sample inference algorithm of the reads was used to infer Amplicon Sequence Variant (ASV). Once the ASV sequence table was constructed, possible chimeras were removed from the sequences. Finally, the taxonomic allocation used the SILVA v132.16s database [33] for bacterial sequences; and the Protist Ribosomal Reference database (PR2) [34] was used to assign eukaryotic sequences. The latter contains reference sequences for protists, metazoans, embryophytes, and fungi. For the taxonomic assignments, a minimum confidence bootstrap of 50 was considered, according to the functions provided by the DADA2 package.

Additionally, when a large percentage of sequences were classified as "Unidentified", such as the case of the 18S-rRNA sequences, a posterior identification of these sequences was conducted using BLASTn (https://blast.ncbi.nlm.nih.gov/Blast.cgi?LINK_LOC=blasthome&PAGE_TYPE=BlastSearch&PROGRAM=blastn). For this process, we generated a reference database filtering all 18S-rRNA sequences available in NCBI, specifically using the following search algorithm: 18S-rRNA [All Fields] AND (biomol_genomic[PROP] AND refseq[filter]). Also, we generated a multifasta with sequences of interest, to compare them with the constructed reference database, considering an e-value smaller than 10 and a percentage of identity of sequences greater than 95%. The resulting table contained the best assignation for the sequence (one result per sequence considering the parameters of e-value and percentage of identity), sequence accession number, the name of the organism, the e-value, and the sequence identity percentage. Once the identification was complete, this information was cross-checked with respective relative abundance obtained from DADA2 analysis.

Descriptive and statistical analyses of the bacterial and eukaryotic communities

Descriptive analyses of the three groups of patients were performed to determine the infection and colonization/infection frequencies of the studied microorganisms in terms of percentages. Additionally, the relative frequencies of the identified subtypes of *Blastocystis* within the positive samples were determined.

First, we performed alfa diversity analyses for the ASVs corresponding to bacterial and eukaryotic communities of the three study groups. The Abundance-based Coverage Estimator (ACE) was calculated for richness estimation of the mentioned communities, and Shannon-Weaver and Simpson indexes were calculated to estimate the diversity of the communities within the three groups. Statistical differences of richness and diversity between the three groups were evaluated with a Kruskal-Wallis and a post hoc Dunn test considering Benjamini-Hochberg's procedure (False Discovery Rate (FDR)) ($p<0.05$). A principal coordinate analysis (PCoA), with Bray-Curtis calculated distances, was performed for the beta diversity analyses of the ASVs corresponding to bacterial and eukaryotic communities of the three groups. Permutational analysis of variance using distance matrices (adonis) was used to evaluate statistical differences of the sample clustering depending on the study group. Alpha and beta diversity analyses were carried using R phyloseq package [35].

Further, the proportion of ASVs obtained with the DADA2 analysis was calculated considering the total number of reads per sample. Initially, we represented and described bacterial phyla with relative abundance greater than 1%, thus phyla with a lower relative abundance were categorized as "Others". This procedure was also performed for eukaryotic classes, but instead we represented eukaryotic classes with relative abundance greater than 3%. At the family level, we represented and described bacterial families with a relative abundance greater than 10%, and eukaryotic families with a relative abundance greater than 3%. The families with a lower relative abundance were categorized as "Others". Significant differences in the

abundance of bacterial and eukaryotic families between the groups were evaluated with a Kruskal-Wallis and a post hoc Dunn test using Benjamini-Hochberg's procedure ($p<0.05$).

To obtain a further description of the *Blastocystis* and *C. difficile* co-occurrence group (B+/C+), we created two subgroups considering the samples with colonization by a single *Blastocystis* subtype (n = 15) and with mixed subtype infections (n = 16). The description and representation of bacterial phyla of these two subgroups were performed using the phyla with a relative abundance higher than 1%, thus phyla with a lower relative abundance were categorized as "Others". This procedure was also performed for eukaryotic classes, but instead we represented eukaryotic classes with relative abundance greater than 3%. Significant differences in the abundance of bacterial phyla and eukaryotic classes were evaluated using non-parametric Mann-Whitney test ($p<0.05$). On the other hand, only bacterial families of the two subgroups with a relative abundance higher than 5% were represented, and those families with a lower relative abundance were classified as "Others". This procedure was also performed for eukaryotic families, but instead we represented eukaryotic classes with relative abundance greater than 1%. Significant differences in the abundance of these bacterial and eukaryotic families were evaluated using non-parametric Mann-Whitney test ($p<0.05$).

The DESeq2 package [36] was employed to assess significant differences in the abundance of bacterial and eukaryotic genera between the three study groups. A phyloseq object was used as a starting point, and then it was converted to a DESeqDataSet. After, a Wald test was applied to the dataset, and the differences in the abundance of the genera were detected by performing pairwise comparisons of the study groups. The differences were considered significant if the p-value cut-off was <0.01 (adjusted by Benjamini-Hochberg correction). Additionally, we evaluated the differences in the relative abundance of 21 bacterial genera (*Acinetobacter*, *Akkermansia*, *Alistipes*, *Bacteroides*, *Bifidobacterium*, *Bilophila*, *Dorea*, *Enterococcus*, *Escherichia/Shigella*, *Eubacterium*, *Faecalibacterium*, *Fusobacterium*, *Klebsiella*, *Methanobrevibacter*, *Parabacteroides*, *Prevotella*, *Lachnospira*, *Lachnospiraceae* groups, *Ruminococcus*, *Ruminococcaceae* groups and *Roseburia*) that could be considered as potential biomarkers of the microbiota [6, 37] using Kruskal-Wallis ($p<0.05$) or ANOVA test ($p<0.05$), according to normal distribution of the data. The *Lachnospiraceae* groups and *Ruminococcaceae* groups corresponded to undefined genera belonging to the mentioned families, and that were clustered into these categories. It is important to highlight that the selection of the potential bacterial biomarkers was performed considering what the literature has reported about the role of these bacteria, either as beneficial (e.g., butyrate producer bacteria, or bacteria with anti-inflammatory or immunomodulatory properties that help to maintain the balance of the microbiota) or as a potential pathogen.

A similar approach to the bacterial biomarkers was followed for the eukaryotic genera, where we identified all the possible of the genera of common luminal intestinal parasitic protists (CLIPPs) occurring within the groups. These protists (*Blastocystis*, *Giardia*, *Cryptosporidium*, *Dientamoeba*, *Endolimax*) were selected, since studies have reported that some CLIPPs may have pathogenic potential, and also that they can interact with other members of the gut microbiota, influencing the host's health [7, 38]. Also, the most abundant genera of fungi and helminths were selected for this analysis. Thus, significant differences in the relative abundance of the CLIPPs between the three groups were evaluated with a Kruskal-Wallis and a post hoc Dunn test implementing Benjamini-Hochberg's procedure ($p<0.05$).

Statistical differences in the relative abundance of the bacterial biomarkers, according to the *Blastocystis* subtype colonization (single infections and coexistence of different subtypes) were assessed. For this analysis, an ANOVA was performed using Tukey HSD post hoc tests ($p<0.05$). This same procedure was conducted with the CLIPP genera, fungi, and helminth genera. Thus, statistical differences of the mentioned eukaryotic genera, considering

Blastocystis subtypes colonization were assessed with a Kruskal-Wallis test and a post hoc Dunn test with p-value correction using the Benjamini-Hochberg's procedure ($p<0.05$).

Finally, correlation networks between the families of the most abundant bacterial phyla (Firmicutes, Bacteroidetes, and Proteobacteria) and eukaryotic divisions (Ascomycota and Basidiomycota) were performed using non-parametric Spearman correlation test. The p-values were corrected using Benjamini-Hochberg's procedure (False Discovery Rate (FDR)), and only correlations with a correlation coefficient (ρ) above 0.6 and lower than -0.6 were considered. Afterwards, correlation networks were built using igraph, ggraph, and RCy3 R packages, and finally represented in Cytoscape 3.8.0. On the other hand, the correlation analyses were computed for the abundance of bacterial and eukaryotic genera of the most abundant families (Lachnospiraceae, Enterobacteriaceae, Ruminococcaceae, Saccharomycetales, and Exobasidiomycetales). For this analysis corr and psych packages of R were used, and the correlations between genera were performed using non-parametric Spearman correlation test, with p-values corrected with Benjamini-Hochberg's procedure. Descriptive analyses, statistical analyses and generation of the corresponding figures were all performed using the R v.3.6.2 software package, along with Vegan v.2.5–6, phyloseq, corr, FSA, psych, igraph, ggraph, RCy3, dplyr, reshape2, tidyverse, and ggplot2 (R Core Team, Vienna, Austria).

Results

Study groups and relative frequencies of *Blastocystis* subtypes

For the present study, a total of 115 samples [25] belonging to patients with diarrhea (the main manifestation of CDI) were subjected to a *Blastocystis* screening. Only 31 patients out of the total presented this protist. Thus, the available data of the samples allowed the creation of the three mentioned groups: B+/C+ (26.96%, n = 31), B−/C+ (38.26%, n = 44) and B−/C− (34.78%, n = 40) (S1 Table). For the 31 samples in which *Blastocystis* was detected (B+/C+), ST1 (43.4%, n = 23) and ST3 (35.85%, n = 19) were identified as the most frequent subtypes, and ST5 (20.75%, n = 11) was identified as the third most frequent subtype. These subtypes were identified either as single infections (n = 15) or mixed infections (n = 16), where ST5 was only present in mixed infections (Fig 1 and S2 Table). For the 16 samples that presented mixed infections, up to three subtypes coexisted in the same sample (S3 Table), and the highest coexistence frequencies were for ST1/ST3/ST5 and ST1/ST3 (S2 Table).

Description of the bacterial and eukaryotic communities of each study groups

The Abundance-based Coverage Estimator (ACE) showed a higher richness of the bacterial ASVs, compared with the richness of the eukaryotic ASVs of the three groups. The mean values obtained both for Shannon and Simpson indexes would allow to conjecture that, in general, the diversity of bacterial (Shannon index = 2.86 and Simpson index = 0.83) and eukaryotic communities (Shannon index = 1.71 and Simpson index = 0.65) was low in the three groups. The above, considering the assumption of the Simpson index in which the diversity decreases abundance, the closer the index value is to 1. Statistical analyses showed no significant differences in the richness and diversity of bacterial and eukaryotic communities of the three study groups ($p>0.05$, Kruskal-Wallis) (Fig 2A and 2B). On the other hand, principal coordinate analysis with Bray-Curtis calculated distances of the bacterial ASVs showed no differential clustering of the three groups (adonis, $r^2 = 0.024$, $p>0.05$) (S1A Fig). Likewise, the principal coordinate analysis of the eukaryotic ASVs showed no differential clustering of the three groups (adonis, $r^2 = 0.008$, $p>0.05$) (S1B Fig).

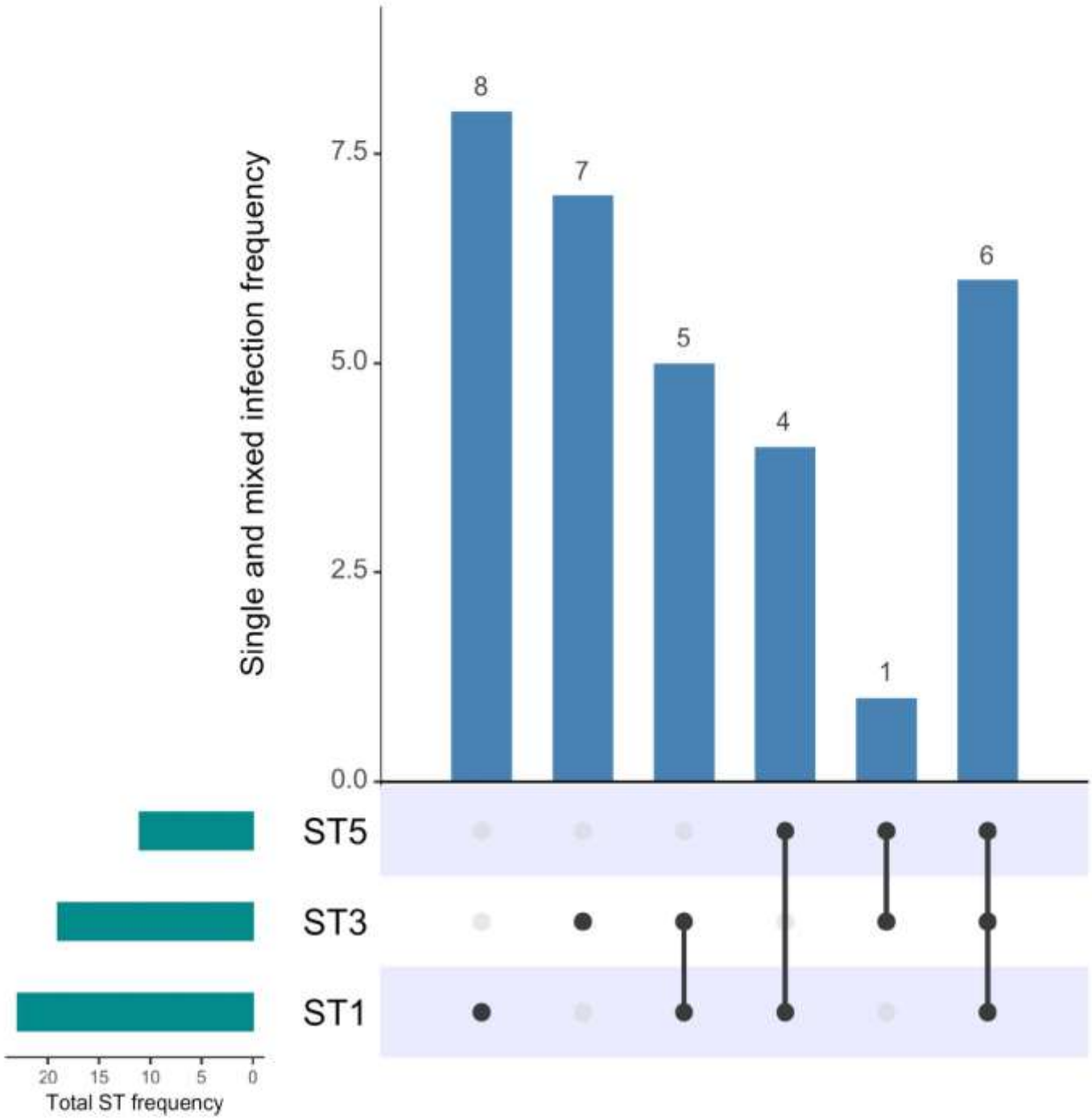

Fig 1. Frequency of *Blastocystis* subtypes in the 31 fecal samples (B+/C+). The absolute frequency of each ST is displayed in the lateral bar plot (green). The panel with the dots represents the single or mixed infections, which frequency is displayed in the main bar plot (blue).

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The sequencing of the 16S-rRNA V4 gene fragment of the 115 fecal samples showed that the most abundant phylum was Firmicutes and represented approximately 30% of reads for the three groups. The most abundant phyla followed by Firmicutes were Bacteroidetes and Proteobacteria, being Bacteroidetes the second most abundant in the B+/C+ and B-/C- groups,

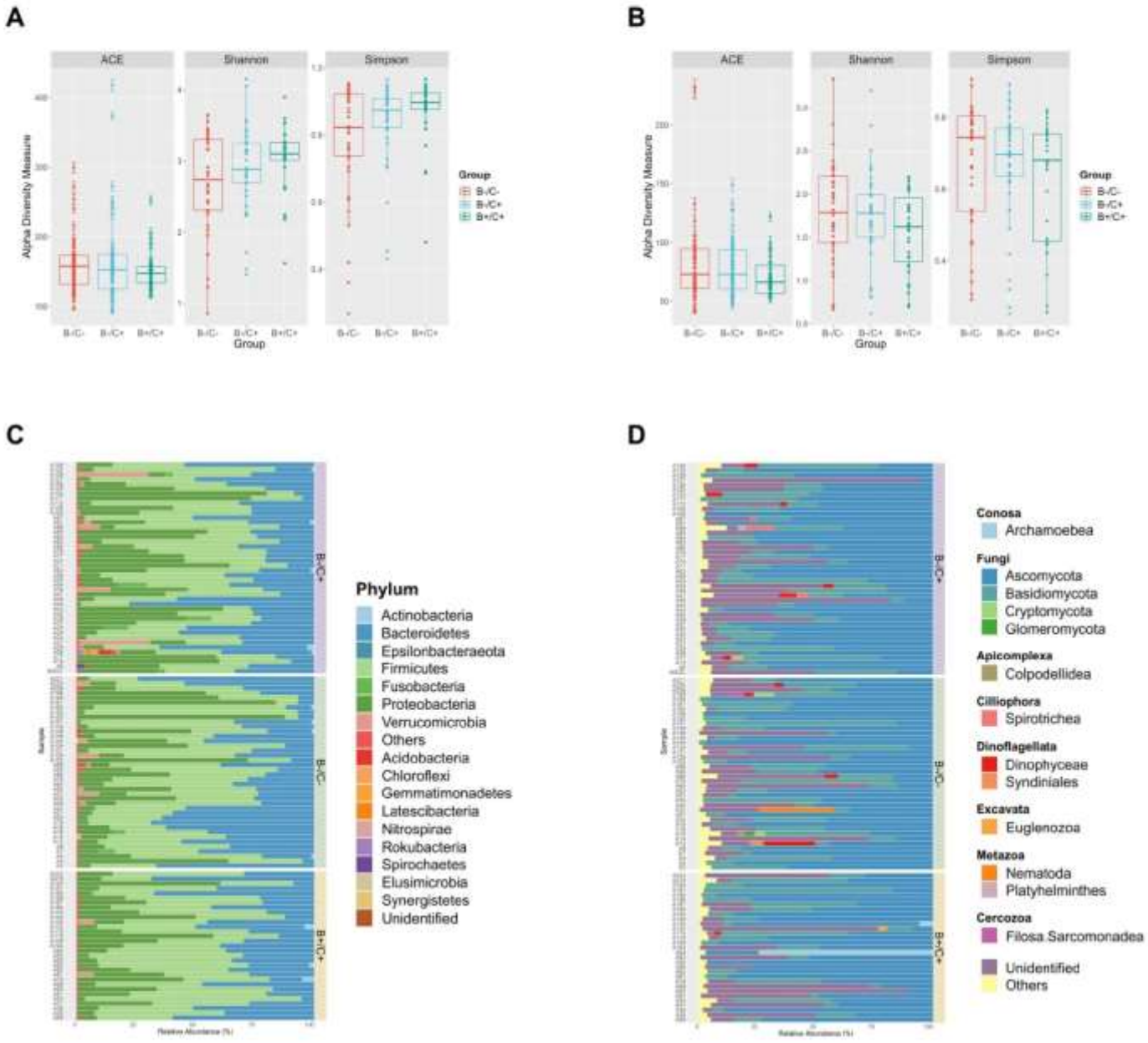

Fig 2. Alpha diversity measures and description of the bacterial phyla and eukaryotic divisions in the three groups. **(A)** Richness and abundance measures for the ASVs corresponding to bacterial communities of the three study groups and **(B)** Richness and abundance measures for the ASVs corresponding to eukaryotic communities of the three study groups. No significant differences were found in the richness and abundance of the bacterial and eukaryotic communities of the study groups ($p<0.05$). **(C)** Relative abundance of bacterial phyla identified for each of the three study groups, where the predominant phyla within each group were Firmicutes, Bacteroidetes and Proteobacteria. **(D)** Relative abundance of the eukaryotic classes identified for each of the three study groups, where the predominant classes within each group were Ascomycota and Basidiomycota.

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and Proteobacteria the second most abundant in the B-/C+ group (Fig 2C). The sequencing of the 18S-rRNA gene fragment allowed for the identification of different groups of fungi, protists, and other eukaryotes. For the eukaryotic community analysis, the ASVs belonging to metazoans (except helminths) and embryophytes were removed to focus the analysis on the fungal, protozoan and intestinal helminth groups because some organisms belonging to these groups are important in the hosts' health [39]. Thus, these results showed that fungi was the

most abundant phylum in the three groups and represented more than 50% of the reads, being Ascomycota (55%) and Basidiomycota (20%) the two most abundant classes of eukaryotes in the three groups (Fig 2D). The third most abundant category in the three groups represented on average 19% of the total reads and corresponded to those ASVs that were not successfully identified ("Unidentified") both by the DADA2 taxonomic allocation process and by BLASTn further analysis. However, in BLASTn analysis 843 ASVs were identified successfully, and corresponded mainly to fungi.

At the bacterial family level, the most abundant families in the B-/C- group were: Enterobacteriaceae, Lachnospiraceae, and Bacteroideaceae, respectively (S2A Fig). Whereas, for the group B-/C+ the most abundant families were: Enterobacteriaceae, Lachnospiraceae and Ruminococcaceae, respectively. Finally, the most abundant families in the B+/C+ group were: Ruminococcaceae, Lachnospiraceae, and Bacteroideaceae, respectively. The descriptive analysis showed that only the Lachnospiraceae family was common in the three groups as one of the most abundant. No significant differences were found in the relative abundance of these families between the study groups ($p>0.05$, [Kruskal-Wallis]).

Despite their low abundance, we detailed the archaeal families identified in the study groups since its presence has been correlated with the host's health status, and the abundance of some archaea may be related to diet or correlated with the presence of other members of the microbiota [40–42]. Overall, the most abundant archaeal families were *Methanobacteriaceae* (22.92%), followed by *Methanomethylophilaceae* (14.37%) and *Nitrosotaleaceae* (14.31%). Table 1 displays the mean relative abundance of the archaeal families in each one of the study groups. No significant differences were found in the relative abundance of these families between the study groups ($p>0.05$, [Kruskal-Wallis]), which may be due to the low relative abundance of these archaeal families in each of the groups.

Regarding eukaryotic families, the descriptive analysis showed that the most abundant eukaryotic family in the three groups was Saccharomycetales, which accounted for more than 50% of the reads on average. Additionally, the third most abundant family in the three groups, followed by the "Unidentified" family category corresponded to Exobasidiomycetales, which represented on average 15% of the reads (S2B Fig). However, no significant differences were found in the relative abundance of these families between the study groups ($p>0.05$, [Kruskal-Wallis]).

Table 1. Mean relative abundances of archaeal families identified in the study groups.

Family	B+/C+ (Mean ± SD)	B-/C+ (Mean ± SD)	B-/C- (Mean ± SD)
Calditrichaceae	2.0e-4 ± 1.1e-3	3.9e-3 ± 2.1e-2	1.2e-3 ± 3.6e-3
Entotheonellaceae	1.0e-4 ± 5.5e-4	3.3e-4 ± 2.2e-3	3.3e-5 ± 2.1e-4
Methanobacteriaceae	0.0e0 ± 0.0e0	5.9e-3 ± 1.9e-2	7.1e-3 ± 3.3e-2
Methanocellaceae	0.0e0 ± 0.0e0	5.0e-4 ± 3.3e-3	0.0e0 ± 0.0e0
Methanofastidiosaceae	0.0e0 ± 0.0e0	1.2e-3 ± 7.6e-3	6.3e-5 ± 3.9e-4
Methanomassiliicoccaceae	0.0e0 ± 0.0e0	0.0e0 ± 0.0e0	2.0e-4 ± 1.3e-3
Methanomethylophilaceae	1.6e-3 ± 8.6e-3	2.0e-3 ± 7.9e-3	5.1e-3 ± 2.5e-2
Methanoregulaceae	0.0e0 ± 0.0e0	2.5e-3 ± 1.6e-2	1.1e-4 ± 6.9e-4
Methanosaetaceae	0.0e0 ± 0.0e0	5.2e-3 ± 3.1e-2	1.1e-3 ± 5.7e-3
Methanosarcinaceae	0.0e0 ± 0.0e0	3.8e-4 ± 2.5e-3	0.0e0 ± 0.0e0
Methanospirillaceae	0.0e0 ± 0.0e0	1.0e-3 ± 6.7e-3	9.4e-5 ± 5.9e-4
Nitrosopumilaceae	0.0e0 ± 0.0e0	6.5e-3 ± 4.3e-2	4.3e-4 ± 1.9e-3
Nitrososphaeraceae	0.0e0 ± 0.0e0	1.3e-3 ± 8.7e-3	0.0e0 ± 0.0e0
Nitrosotaleaceae	0.0e0 ± 0.0e0	7.6e-3 ± 5.0e-2	3.7e-5 ± 2.3e-4

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A description of the bacterial and eukaryotic communities within the B+/C+ group was also performed, considering two subgroups: samples with colonization by a single *Blastocystis* subtype and mixed subtype infections. The relative abundance of the bacterial phyla and the eukaryotic classes found in these two subgroups are displayed in the S3A and S3B Fig.

At the family level, the most abundant bacterial families within the samples with mixed subtype infections were Bacteroidaceae, Ruminococcaceae, and Lachnospiraceae, respectively. Whereas, within the samples with colonization by a single subtype, the most abundant families were Ruminococcaceae, Lachnospiraceae, and Prevotellaceae, respectively (S3C Fig). Statistical analyzes showed significant differences abundance of the family Prevotellaceae, where it displays a higher abundance within samples with colonization by a single subtype compared to the samples with mixed subtype infections ($p = 0.031$, [Mann-Whitney]) (S4 Fig). Additionally, statistical differences were found for the Pseudomonadaceae family, where this family showed a higher relative abundance within the samples with mixed subtype infection compared to those with colonization by a single subtype ($p = 0.033$, [Mann-Whitney]) (S4 Fig). Regarding eukaryotic families, it was observed that Saccharomycetales and Exobasidiomycetales were the most abundant families in both of the subgroups, respectively (S3D Fig). However, no significant differences were observed in the relative abundances of these families between the subgroups ($p>0.05$, [Mann-Whitney]).

Analysis of bacterial biomarkers, fungi, nematodes and Common Luminal Intestinal Parasitic Protists (CLIPPs) of the study groups

Initially, the DESeq analysis showed that the abundance of 5 bacterial genera and two bacterial ASVs unclassified at the genus level were significantly diminished in the B+/C+ group compared to the B-/C+ and B-/C- groups (DESeq2, Benjamini-Hochberg correction p-value <0.01). These differences were due to the low abundance of these genera and ASVs in the B+/C+ group, principally when compared with the B-/C- group, where most of the differences were found (S4 Table). Additionally, the comparison between the groups B-/C+ and B-/C- did not show any significant difference in the abundance of bacterial genera, and therefore it was not displayed in the S4 Table (DESeq2, Benjamini-Hochberg correction p-value >0.01).

For further analyses of the possible changes in the bacterial communities of the three study groups, we evaluated the differences in the relative abundances of specific genera of bacteria, and of some genera of eukaryotes that can play important roles within the microbiota. Regarding bacterial communities, 21 bacterial genera acknowledged as potential biomarkers of the microbiota were selected [6, 37] (Fig 3A). The statistical analyses showed significant differences only in the relative abundance of *Faecalibacterium*, *Dorea*, and in groups of undefined genera belonging to the family Lachnospiraceae (*Lachnospiraceae* groups) among the three study groups. Thus, the mentioned genera exhibited a higher relative abundance within the B+/C+ group compared to the B-/C+ group ($p<0.05$, [Kruskal-Wallis]). Also, the relative abundance of the *Faecalibacterium* genus and *Lachnospiraceae* groups was significantly higher within the B+/C+ group compared to B-/C- group ($p<0.01$, [Kruskal-Wallis]) (Fig 3B).

In the case of the eukaryotic communities of the three groups, the DESeq analysis showed that three fungi genera were significantly diminished in the B+/C+ group compared to the B-/C- group (DESeq2, Benajmini-Hochberg correction p-value <0.01) (S4 Table). The only significant difference found between the B-/C+ and B-/C- groups corresponded to the diminished abundance of the genus *Coniochaeta* in the B-/C+ group (fold change = -26.51, p-value = 1.03e-21). On the other hand, only three genera considered as CLIPPs were identified, namely *Entamoeba*, *Endolimax*, and *Cryptosporidium* [38]. Additionally, the three most abundant genera of fungi (*Candida*, *Saccharomyces*, and *Malassezia*) and the most abundant genus

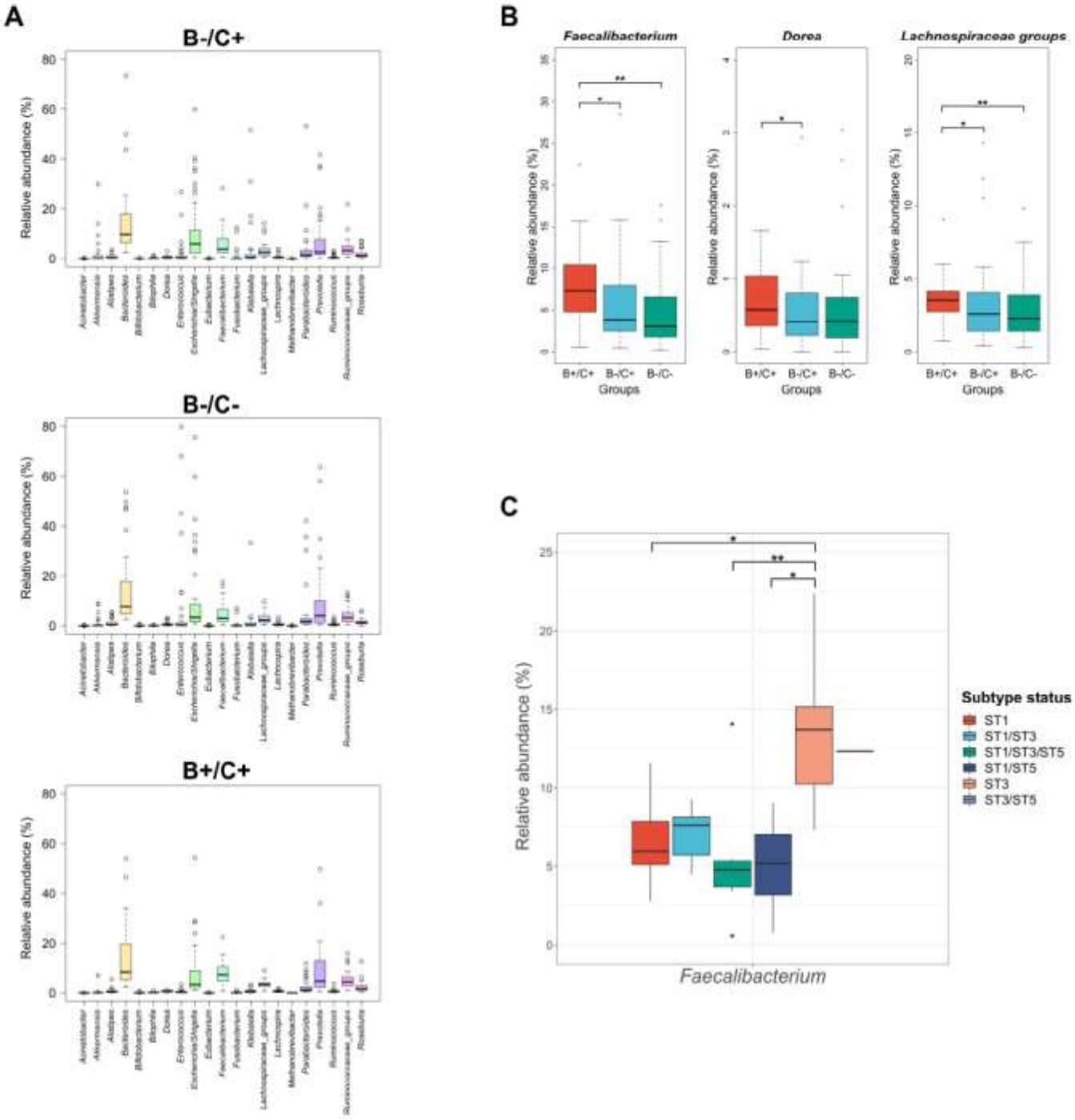

Fig 3. Description and evaluation of the statistical differences in the abundance of bacterial biomarkers among the three study groups. **(A)** Description of the relative abundance of 21 genera of bacteria considered as possible biomarkers of the intestinal microbiota among the three study groups. **(B)** Three genera of bacterial biomarkers presented significant differences in their relative abundance among some of the study groups, where all of these genera display a higher abundance in the B+/C+ group. **(C)** Changes in the relative abundance of *Faecalibacterium* considering the colonization of a determined *Blastocystis* subtype, showing that this genus has a higher abundance in ST3 colonized patients. Significant differences between the study groups were evaluated using Kruskal-Wallis test and ANOVA (*, $p<0.05$; **, $p<0.01$).

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Table 2. Mean relative abundance of the seven genera of eukaryotes (CLIPPs, nematodes and fungi) selected for the three study groups.

Genus	B+/C+ (Mean ± DS)	B−/C+ (Mean ± DS)	B−/C− (Mean ± DS)
Candida	27.62 ± 24.83	24.48 ± 22.73	21.41 ± 20.31
Malassezia	15.67 ± 16.56	16.92 ± 14.49	17.64 ± 17.20
Saccharomyces	21.04 ± 17.33	21.06 ± 18.23	23.26 ± 20.51
Entamoeba	2.65 ± 13.14	4.05e-2 ± 0.11	8.88e-2 ± 0.36
Endolimax	1.53e-3 ± 8.35e-3	3.53e-4 ± 2.31e-3	7.0e-5 ± 4.37e-4
Cryptosporidium	1.25e-2 ± 3.02e-2	4.10e-3 ± 2.01e-2	1.31e-2 ± 4.03e-2
Strongyloides	2.43e-2 ± 0.12	2.0e-2 ± 0.13	0.85 ± 5.08

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of nematodes (*Strongyloides*) were selected. Table 2 shows the value of the mean abundance of the seven genera mentioned above, together with their respective standard deviations (SD). The statistical analyses showed no significant differences in the abundance of these genera between the study groups ($p>0.05$, [Kruskal-Wallis]).

Additionally, within the samples of the B+/C+ group, the possible changes in the abundance of the 21 bacterial biomarkers and the seven selected eukaryotic genera were evaluated, considering single infections and coexistence of *Blastocystis* subtypes (S5 and S6 Figs). In the case of bacterial biomarkers, the statistical analyses showed only significant differences in the relative abundance of *Faecalibacterium*, considering *Blastocystis* subtypes. The relative abundance of this genus was significantly higher in the samples with colonization by ST3 than in the samples with ST1 ($p = 0.017$, [ANOVA]), ST1/ST3/ST5 coexistence ($p = 0.011$, [ANOVA]), and ST1/ST5 coexistence ($p = 0.018$, [ANOVA]) (Fig 3C). On the other hand, the statistical analyses showed no significant differences in the relative abundances of the seven eukaryotic genera, considering single infections or coexistence of *Blastocystis* subtypes [$p>0.05$, [Kruskal-Wallis]).

Correlations between bacterial and eukaryotic communities are different among study groups

To evaluate possible interactions between bacterial and eukaryotic communities of the three study groups, we performed a correlation network of the families belonging to the most abundant bacterial phyla (Firmicutes, Bacteroidetes and Proteobacteria) and the most abundant eukaryotic divisions (Ascomycota and Basidiomycota). The correlation network was performed based on the Spearman correlation test, where Fig 4 shows only significant correlations ($p<0.05$ after FDR correction) with a correlation coefficient (ρ) lower than -0.6 and higher than 0.6 (Fig 4, top). Despite that, most of the correlations were positive and mainly between families of the Proteobacteria phylum, the networks of the groups were notably different from each other.

Only B+/C+ and B-/C- groups displayed moderate negative correlations between some of the bacterial and eukaryotic families (Fig 4, top left and top right). The network of the B-/C+ group was markedly different from the networks of the other two groups since this network had the highest number of strong correlations, mainly between the families of Proteobacteria and Bacteroidetes phyla (Fig 4, top center). Additionally, it is interesting to observe a slight clustering in the networks of each group, where different interactions from the ones presented in the main network were observed. For instance, instead of representing interactions mostly between families of Proteobacteria, these clusters exhibited interactions between Firmicutes, Bacteroidetes, and Basidiomycota.

In the B+/C+ group was notable that the families of the Basidiomycota division correlated mostly with each other, while families of Ascomycota correlated with Proteobacteria and

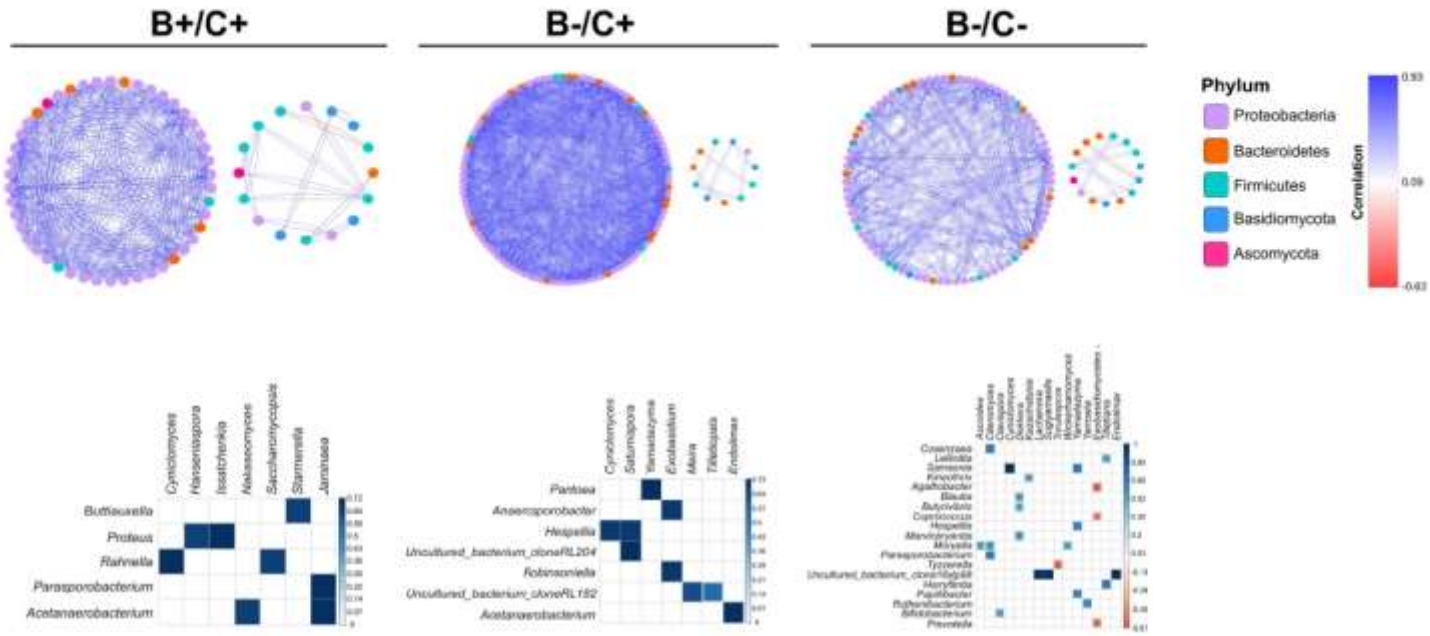

Fig 4. Intra-domain and inter-domain correlations in the three study groups. Correlation networks of the B+/C+ (top left), B-/C+ (top center), and B-/C- (top right) groups. The nodes represent the bacterial and eukaryotic families, and its color represents the phylum to which it belongs. Blue edges and red edges indicate positive and negative correlations, respectively. The networks only represent significant correlations ($p<0.05$, after FDR correction) with ρ above 0.6 and lower than -0.6, and that were computed with the Spearman correlation test. This figure also displays correlation plots of the genera belonging to the most abundant bacterial and eukaryotic families of the B+/C+ (bottom left), B-/C+ (bottom center), and B-/C- (bottom right) groups. Blue and red squares represent positive and negative correlations, respectively. Only significant correlations, computed with the Spearman correlation test, ($p<0.05$, after FDR correction) were represented in the mentioned plots.

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Firmicutes (Fig 4, top left). Conversely, in the B-/C- group, Basidiomycota families correlated with each other as well as with families of Proteobacteria phylum (Fig 4, top right). In general, it can be observed that there are not many correlations between bacterial families and families of the Ascomycota division; in fact, in the group B-/C+ no correlation involving this division of fungi was evidenced (Fig 4, top center).

On the other hand, we computed correlations on the genera belonging to the most abundant families of bacteria (Lachnospiraceae, Ruminococcaceae, and Enterobacteriaceae) and eukaryotes (Saccharomycetales and Exobasidiomycetales) of the three study groups. Also, the 21 bacterial genera considered as biomarkers, and the seven genera of eukaryotes selected for previous analyses were added to the correlation analysis (Fig 4, bottom). The results showed a higher number of statistically significant correlations ($\rho>0.5$, $p<0.05$ after FDR correction) between genera of bacteria and eukaryotes in the B-/C- group (number of correlations: 24) (Fig 4, bottom right), compared to B-/C+ (number of correlations: 9) (Fig 4, bottom center) and B+/C+ groups (number of correlations: 8) (Fig 4, bottom left).

Within the B+/C+ group, most of the significant correlations were displayed between fungi genera belonging to the Saccharomycetales family and bacteria belonging to the Enterobacteriaceae family (Fig 4, bottom left). The B-/C+ group presented mostly correlations between the fungi genera of the two evaluated families and genera of the Lachnospiraceae family (Fig 4, bottom center). Finally, in the B-/C- group, it was observed that various genera of the three bacterial families were correlated mainly with genera of the Saccharomycetales family. Also, this group was the only that displayed moderate inverse correlations ($\rho = -0.6$, $p<0.01$ after FDR correction); for instance, these correlations were observed between *Exobasidiomycetes* and Lachnospiraceae genera (*Agathobacter* and *Coprococcus*), as well as between *Exobasidiomyctes* and *Prevotella* (Fig 4, bottom right).

Discussion

It is currently known that the pathogenic bacterium *C. difficile* usually causes diarrhea associated with the use of antibiotics, which is related to a decrease in the abundance of beneficial bacteria in the intestinal microbiota [11]. However, *Blastocystis* is a protist whose role as a pathogen or commensal within the intestinal microbiota has not been defined, and therefore it is not completely known how it can modulate and interact with the communities of bacteria and other intestinal eukaryotes [17–19]. The coexistence of *Blastocystis* and *C. difficile* has only been reported once [24], and therefore it is interesting to know the bacterial and eukaryotic communities that can be found under this scenario, given that colonization by *Blastocystis* is not usually reported in scenarios of decrease of beneficial bacteria as under CDI [7]. Additionally, few studies have focused on the description of the intestinal eukaryotic communities in certain states of the host's health. Therefore, this study aimed to describe for the first time the communities of intestinal bacteria and eukaryotes within three specific groups of patients (B+/C+, B-/C+ and B-/C-) who presented diarrhea.

First, it is important to highlight the limitations of the present study. Metadata about the patients that would have allowed for other factors that influence the composition of the microbiota (e.g., diet, age, sex, body mass index, prior medical treatments) was not available. Because all of the patients belonging to the three groups presented with diarrhea, there was no group without any physiological imbalance in the microbiota that would allow for broader comparisons. Furthermore, the data of the present study did not allow the creation of a group with colonization by *Blastocystis* only (B+/C−), that would also have allowed for more robust comparisons of the bacterial and eukaryotic communities between groups. Future studies are therefore needed to confirm our preliminary findings from microbiota profiling.

Herein we show a colonization/infection frequency of 26.96% by *Blastocystis* and *C. difficile*. The results obtained from the subtyping of *Blastocystis* were consistent with those reported in other studies, where it is described that ST1 and ST3 are some of the most abundant subtypes in the human intestine worldwide, which includes Colombia as shown in our study [14, 43]. A striking finding in our study was the detection of ST5, which was the first report of this subtype in Colombia and, interestingly was found only in mixed subtype infections (Fig 1). The finding of mixed subtype infections supports the advantage of next generation sequencing in the subtyping of *Blastocystis* because this technique offers high resolution and sensitivity [12]. Therefore, it is likely that ST5 had not been previously reported in Colombia because studies in the country had only employed Sanger sequencing for subtyping [14, 43–45]; a method that has less sensitivity in the detection of multiple *Blastocystis* subtypes compared to next generation sequencing [12].

Although there were no significant differences in the richness and diversity of the ASVs corresponding to the bacterial communities of the three study groups, the Simpson index and ACE could suggest an overall low bacterial diversity in the three groups (Fig 2A and S1A Fig). This potential low bacterial diversity could be related to the effects of CDI in the microbiota, where there is a decrease in bacterial abundance, especially of beneficial ones [46]. As in the bacterial communities, the analyzes of alpha and beta diversity of the ASVs corresponding to the eukaryotic communities did not show significant differences between the study groups (Fig 2B and S1B Fig). Nonetheless, ACE and Simpson index may suggest an overall low eukaryotic diversity in the three groups. Few studies have addressed the changes of eukaryotic communities in CDI infection, but it has been suggested that imbalances in the microbiota can significantly alter fungal communities causing fungal dysbiosis [47, 48]. As a perspective, this should be explored in the patients presented here.

On the other hand, our results showed that the relative abundance of archaea families is low within the three study groups. The low abundance of methanogenic archaea (Table 1) indicates that the microbiota of the patients belonging to the three groups may display a functional imbalance. The uptake of hydrogen from some methanogenic archaea can drive the processes of primary and secondary fermenters within the microbiota. Moreover, a direct correlation between methane production and constipation has been reported, which also supports the fact that the patients belonging to the three study groups presented diarrhea [41, 49, 50]. For the B +/C+ group, a higher relative abundance of archaea was expected because some studies have reported significant associations between *Blastocystis* colonization and the abundance of some archaea [51, 52]. Because of the above, it could be hypothesized that the low relative abundance of archaea in the three study groups may be mainly driven by the low abundance of bacteria (Fig 2A). This, considering the syntrophic relationship that has been described between these organisms, because archaea can optimize the metabolic pathways of fermenting bacteria [53]. Future studies are required to assess this hypothesis and observe how the syntrophic relation between bacteria and archaea could be changing in this scenario. However, fermentation may not be completely restricted due to the presence of butyrate-producing bacteria in the three study groups, although it could be occurring at a very low rate. Therefore, archaea could be competing in an environment with limited resources (H_2). Still, future studies are needed to elucidate the ecological relationships between archaea, whether if they are competing or coexisting in a limited resource environment [54]. Also, as a perspective, it would be interesting to acknowledge if a possible predominance of a determined methanogenesis type (hydrogenotrophic, methylotrophic, and acetotrophic) [40] is occurring in the scenario of the three groups.

Regarding eukaryotic communities of the study groups, descriptive analyses showed that the three groups presented a high abundance of fungi, especially of genera belonging to the Saccharomycetales family. To date, there is no clear information about how *Blastocystis* can interact or modify fungal communities of the microbiota when an intestinal imbalance is present. One of the few studies showed that colonization by *Blastocystis* increased yeast abundance [55]. However, in the case of our study groups, the lack of statistically significant results makes it difficult to establish possible interactions between *Blastocystis* and some fungi genera.

The description of the bacterial phyla within the subgroups with colonization by a subtype and with mixed infection was very similar to the three study groups (B+/C+, B-/C+ and B-/C-), where the Firmicutes, Bacteroidetes, and Proteobacteria phyla were predominant (S3A Fig). The same case was observed for the eukaryotic classes, where Ascomycota and Basidiomycota were the most abundant classes in the two subgroups (S3B Fig). It is interesting to observe that within this scenario of *Blastocystis* and *C. difficile* co-occurrence, the samples with mixed subtype infections show a greater abundance of a family of the phylum Proteobacteria (S4 Fig), specifically Pseudomonadaceae. Whereas when colonization by a single subtype of *Blastocystis* occurs, there is a greater abundance of a potentially beneficial family, namely Prevotellaceae (S4 Fig).

The DESeq analysis applied to the bacterial genera showed a diminished abundance of *Akkermansia* in the B+/C+ group (S4 Table), which is in accordance with that described by other studies where the presence of *Blastocystis* was related to a low abundance of *Akkermansia* [52, 56]. Also, significant differences were observed in the abundances of beneficial bacteria genera (*Faecalibacterium*, *Dorea* and groups of undefined genera belonging to the family Lachnospiraceae) between the three study groups (Fig 3B). Some studies have suggested that colonization by a determined *Blastocystis* subtype can be related with the decrease or increase of some bacterial genera [52, 57]. The study by Gabrielli et al., (2020) reported a higher abundance of some genera of beneficial bacteria such as *Prevotella* and *Ruminococcus* in patients

colonized by *Blastocystis* ST3 [58]. In the case of the results of our study, it is interesting to observe that the abundance of the *Faecalibacterium* genus was significantly higher in the samples of patients who presented colonization by ST3 (Fig 3C). The significantly higher abundance of these three genera within the B+/C+ group could again suggest a predominance of beneficial bacterial genera when compared to the other two groups.

It could even be hypothesized that the presence of *Blastocystis* in cases of CDI can cause a change in some groups of bacteria, increasing the abundance of beneficial bacteria (e.g., butyrate producers). The high abundance of these genera (*Faecalibacterium*, *Dorea*, and *Lachnospiraceae* groups) in the B+/C+ group can be explained by the hypothesis of the predatory role of *Blastocystis*, where an increase in the richness of some beneficial bacterial groups is the result of *Blastocystis* predation over some other groups of bacteria [59].

The analyses of the relative abundance of the genera of fungi, nematodes and CLIPPs did not show to be significant between the study groups (Table 2). However, the DESeq analysis showed a significantly low abundance of the genera *Antrodia* and *Clavispora* in the B+/C + group compared to the other two study groups (S4 Table). The information about the possible role of these fungi in the gut microbiota is limited. Still, *Antrodia* has been described as a genus that harbors some species with potential anti-inflammatory, hepato-protective and immunomodulatory properties [60, 61]. Regarding *Clavispora*, it is possible to hypothesize that this genus may be linked to some disease states since it has been found enriched in samples of patients with Crohn's disease [62].

The detection of only a few CLIPPs in the samples may be explained by the fact that microorganisms such as *Giardia* and *Dientamoeba*, do not possess hydrogenosomes that allow them to survive under the aerobic environment of a disturbed microbiota [63, 64]. Future studies should evaluate the effect of these genera of parasites (*Giardia* and *Dientamoeba*) on the bacterial communities of our three groups to obtain a broader understanding of the interactions between these taxonomic units within the microbiome. Because most microbiota studies focus on the bacterial communities, information on the effect of a disturbed gut because of CDI [65], or the presence of *Blastocystis* on fungal and other eukaryotic communities is limited [66]. Thus, our results tried to contribute toward an understanding of the eukaryotic communities, specifically within a specific group of patients that harbor a physiological imbalance in their microbiota.

Overall, the correlation networks showed a predominance of the interaction between Proteobacteria families, as well as a low interaction between bacterial families and fungal families (Fig 4, top). Additionally, a slight clustering in the three correlation networks could suggest a shortage of cooperation in the whole system [67], considering the lack of connection between families of the cluster with the main network, and the low or absence of interaction with fungal families. However, future studies are needed to elucidate the complex polymicrobial interactions of the microbiota [68] in these specific scenarios. Also, it would be interesting to address the possible implications of these interactions upon the pre-existing condition (intestinal imbalance) of the three types of patients.

Most of the correlations between the abundance of specific bacterial and eukaryotic genera showed to be positive correlations (Fig 4, bottom); this could suggest that an antagonistic interaction between these organisms may not be present. Nonetheless, establishing definite roles for the fungi communities of the microbiota in a scenario of intestinal imbalance is difficult due to the limitations in the information of fungal communities and their interactions in the microbiota. Few studies have suggested that some of these yeasts could have a probiotic effect, and even some yeast species have been studied in the prevention of many bacterial pathogen colonization, among them *C. difficile* [69–71]. Conversely, studies have suggested that commensal fungi can undergo a shift towards pathogen organisms, that can even exacerbate a

pre-existing disease [72]. This shift could be influenced by multiple predisposing factors such as chemical, nutritional or physiological alterations [73]. Future approaches implementing models can help to unravel the possible role of these fungi communities within the microbiota of the three study groups, observing whether if they have a beneficial or pathogenic role.

Despite the limitations of the present study, herein we report the first description of bacterial and eukaryotic communities of three specific groups of patients exhibiting diarrhea, highlighting the scenario of *Blastocystis* and *C. difficile* co-occurrence. In addition, our results may suggest that the presence of *Blastocystis* in patients with CDI (B+/C+) can generate a possible shift towards more beneficial bacterial groups. Therefore, the results of our study may support the hypothesis that *Blastocystis*, as a commensal prototype of the microbiome, may favor the proliferation of potentially beneficial bacteria, such as butyrate producers or bacteria with anti-inflammatory properties (i.e., *Faecalibacterium*, *Dorea* and *Lachnospiraceae* groups). Finally, our results hope to encourage future microbiota studies to analyze eukaryotic communities thoroughly, since their interaction with bacteria and other eukaryotes would give more information about the functioning of this complex environment under specific scenarios.

Supporting information

S1 Table. Frequencies of the three study groups that comprise the overall population (n = 115).
(PDF)

S2 Table. Relative frequency of *Blastocystis* subtypes in single infections and mixed subtype infections within the 31 *Blastocystis* positive samples.
(PDF)

S3 Table. Relative abundance of *Blastocystis* subtypes within each positive sample.
(PDF)

S4 Table. Bacterial and eukaryotic genera found to be significantly different by DESeq analysis between the three study groups.
(PDF)

S1 Fig. Beta diversity of the bacterial and eukaryotic ASVs of the three study groups. (A) Principal coordinate analysis (PCoA) of the bacterial ASVs. **(B)** Principal coordinate analysis (PCoA) of the eukaryotic ASVs of the three study groups. The percentage of variation explained by the two dimensions of the PCoA is displayed on the axis.
(TIF)

S2 Fig. Heatmap of the relative abundance of the most abundant bacterial and eukaryotic families within the study groups. (A) Relative abundance of the 24 most abundant bacterial families of the three study groups. **(B)** Relative abundance of the 21 most abundant eukaryotic families of the three study groups.
(TIF)

S3 Fig. Description of some bacterial and eukaryotic taxonomic groups present in the samples with mixed subtype infections and with colonization by a single subtype. (A) Bar plot of the relative abundance of the bacterial phyla identified in the two subgroups, where Firmicutes, Bacteroidetes, and Proteobacteria are the most abundant. **(B)** Bar plot of the relative abundance of the eukaryotic classes identified in the two subgroups, where Ascomycota and Basidiomycota where the most abundant. **(C)** Heatmap of the relative abundance of the 30 most abundant bacterial families identified in the two subgroups. **(D)** Heatmap of the relative

abundance of the 15 most abundant eukaryotic families identified in the two subgroups.
(TIF)

S4 Fig. Differential relative abundance of Prevotellaceae and Pseudomonadaceae families between the mixed subtype subgroup and the subgroup with colonization by a single *Blastocystis* subtype. Significant differences between the study groups were evaluated using non-parametric Mann-Whitney test (*, $p < 0.05$).
(TIF)

S5 Fig. Relative abundance of the bacterial biomarkers considering colonization by a single subtype of *Blastocystis* or by coexistence of different subtypes (subtype status). For the boxplot the genera: *Acinetobacter*, *Akkermansia*, *Bifidobacterium*, *Bilophila*, *Eubacterium*, *Fusobacterium* and *Methanobrevibacter* were not displayed since their relative abundance was low.
(TIF)

S6 Fig. Relative abundance of the seven eukaryotic genera (CLIPPs, fungi genera, and nematode genus) considering colonization by a single subtype of *Blastocystis* or by coexistence of different subtypes (subtype status).
(TIF)

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Gut microbiota composition in health-care facility-and community-onset diarrheic patients with *Clostridioides difficile* infection

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The role of gut microbiota in the establishment and development of *Clostridioides difficile* infection (CDI) has been widely discussed. Studies showed the impact of CDI on bacterial communities and the importance of some genera and species in recovering from and preventing infection. However, most studies have overlooked important components of the intestinal ecosystem, such as eukaryotes and archaea. We investigated the bacterial, archaea, and eukaryotic intestinal microbiota of patients with health-care-facility- or community-onset (HCFO and CO, respectively) diarrhea who were positive or negative for CDI. The CDI-positive groups (CO/+, HCFO/+) showed an increase in microorganisms belonging to Bacteroidetes, Firmicutes, Proteobacteria, Ascomycota, and Opalinata compared with the CDI-negative groups (CO/−, HCFO/−). Patients with intrahospital-acquired diarrhea (HCFO/+, HCFO/−) showed a marked decrease in bacteria beneficial to the intestine, and there was evidence of increased Archaea and *Candida* and *Malassezia* species compared with the CO groups (CO/+, CO/−). Characteristic microbiota biomarkers were established for each group. Finally, correlations between bacteria and eukaryotes indicated interactions among the different kingdoms making up the intestinal ecosystem. We showed the impact of CDI on microbiota and how it varies with where the infection is acquired, being intrahospital-acquired diarrhea one of the most influential factors in the modulation of bacterial, archaea, and eukaryotic populations. We also highlight interactions between the different kingdoms of the intestinal ecosystem, which need to be evaluated to improve our understanding of CDI pathophysiology.

Health-care-associated infections (HCAIs) are a high-impact issue worldwide, as they favor the development of diseases that put the patient's life at risk and are associated with high expenditure rates within health systems[1]. One HCAI with the greatest global impact is *Clostridioides difficile* Infection (CDI), considered to be the causative agent of diarrhea associated with the use of antibiotics[2–4]. This microorganism can cause a range of problems from asymptomatic infections, dehydration, and diarrhea to severe digestive tract complications, such as toxic megacolon, pseudomembranous colitis, and sepsis, and even death[2,3,5]. The problems associated with CDI have worsened as a result of increased incidence and mortality, mainly in patients of the intensive care unit (ICU), where it is reported as being one of the five infections with the greatest impact worldwide[4,5].

Various studies have shown that the influence of CDI on the intestinal microbiota is characterized by a disruption and alteration of its homeostasis, leading to various consequences including diarrhea[6–9]. Despite the absence of a definition for a core microbiome among individuals with CDI, because of the interindividual variability that may exist, clear differences have been found between groups of people with positive or negative CDI status. Among the main alterations suffered by people with CDI is a decrease in particular bacterial populations, such

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as *Bacteroides*, *Lachnospiraceae*, and *Ruminococcaceae*, which is accompanied on many occasions by an increase in bacteria of the phylum Proteobacteria, as well as a decrease in microbiota bacterial diversity[9–11]. Similarly, various groups of bacteria with the ability to inhibit the growth of *C. difficile*, both in vivo and in vitro, have been described as being associated with CDI, including *C. scindens*, *B. adolescents*, and some members of the *Lachnospiraceae* family[9,11,12].

The role of eukaryotes in the development of CDI has been addressed in only a few investigations in which the involvement of fungi was emphasized[13,14]. Among the main findings was an increase in the relative abundance of fungi of the genus *Penicillium* in patients with CDI compared with patients without CDI[15], as well as a relationship between *C. albicans* and failures in fecal transplant treatment[16]. Recent studies have shown the co-occurrence of *C. difficile* and *Blastocystis*[14]. However, the impact of this eukaryote on intestinal microbiota in relation to CDI has not been clarified.

A description of the composition and abundance of bacterial species, both in healthy individuals and those suffering from disorders related to CDI, has promoted the understanding of various aspects of the pathophysiology of this disease[9,17]. However, the roles played by other organisms that are part of the microbiota, such as archaea and eukaryotes, has not been addressed in recent studies. Therefore, the impacts of both the gut microbiota and the established interactions between the different members of the microbiota remain poorly understood[18]. Furthermore, most of these studies have been conducted in Europe and North America, and we are lacking a description of the microbiota of patients with CDI in South America and, particularly, Colombia. Therefore, this study proposed to determine the intestinal microbiome (including bacteria, archaea, and eukarya) of patients with diarrhea acquired at the intrahospital or community level under either positive or negative CDI statuses. A marked decrease in the relative abundance of bacteria, such as *Dorea*, *Faecalibacterium*, *Lachnospira*, and *Prevotella*, was evidenced in the groups positive for CDI, and there was an increase in fungi of the genus *Candida* in CDI-positive patients with diarrhea acquired in hospital. Inverse correlations were observed between some groups of bacteria and eukaryotes. Finally, the associations among bacterial and eukaryotic families and genera with CDI were investigated.

Results

Compositional differences between groups. The 98 samples used in this study came from diarrheal patients treated in two fourth-level hospitals in Bogotá, which serve an urban population with different clinical, sociodemographic, and economic characteristics. The diversity of specialties served by the hospital guarantees a high variability in the patients included in the study. Of the 48 patients in total included in the HCFO/− and HCFO/+ groups, data were collected for 32 of them. The HCFO/− group comprised eight women and six men aged between 18 and 81 years ($\bar{x}$ = 66.2, SD = 20.4). In the HCFO/+ group, there were 10 women and eight men aged between 26 and 92 years ($\bar{x}$ = 64.5, SD = 14.7). No sociodemographic data were obtained for the CO cases. When reviewing the quality of the sequencing, an average of 350,000 reads were obtained per sample, with a minimum of 200,000 and a maximum of 400,000 reads, which was adequate to determine the diversity in each of the samples considering the rarefaction analysis indicated a minimum of 60,000 reads were needed to reveal diversity (Figure S1). We also found no sequences with ambiguous assignment in any position, and more than 99.9% of the reads had a phred score of more than 30, thus we decided that no sequences needed to be removed before analysis.

During the taxonomic assignment, a total of 75,126 amplicon sequencing variants (ASVs) were found for the 16S-rRNA marker, corresponding to 49 phyla and 659 genera (74,594 ASVs (99.29%) for bacteria and 532 ASVs (0.71%) corresponding to Archaea); while 11,265 ASVs were found for the 18S-rRNA marker, corresponding to 54 classes and 623 genera (3396 ASVs (30.14%) for Fungi). Initially, samples were analyzed based on their CDI status (positive/negative). Our analysis showed 90% of bacteria in the samples belonged to the phyla Bacteroidetes, Firmicutes, or Proteobacteria, while approximately 9% belonged to the phyla Acidobacteria, Actinobacteria, Fusobacteria, Spirochaetes, or Verrucomicrobia (Fig. 1A). In general, the bacterial community composition of the CDI-negative group showed a predominance of Firmicutes and Bacteroidetes, while the pattern of CDI-positive patients was characterized by a decrease of Firmicutes and a small increase in the relative abundance of Proteobacteria and Verrucomicrobia compared to the CDI-negative group (Fig. 1A,E). With the eukaryotes, similar patterns were seen in both groups, with a predominance of organisms belonging to the Fungi and Metazoa kingdoms. However, we observed a significant increase in the relative abundance of microorganisms belonging to the phylum Opalozoa in the CDI-positive patients ($p = 0.04613$) (Fig. 1B,G).

The analysis of the composition of the intestinal bacteria within the four study groups (HCFO/+, CO/+, HCFO/−, and CO/−) showed a decrease in the relative abundance of Bacteroidetes in the HCFO/− group with respect to the CO/− group. Similarly, there was a significant decrease in Firmicutes in the HCFO/+ group with respect to the CO/+ and CO/− groups (p values; CO/− vs HCFO/+ = 0.00773, CO/+ vs HCFO/+ = 0.028) (Fig. 1C,F). The distribution of the Archaea genera within the study groups was deepened, showing more than 10 different genera, among which *Methanobrevibacter* and *Methanosaeta* stood out as the most abundant (Figure S2). The HCFO/+ group was characterized by a higher abundance of most archaea, except *Methanobrevibacter*, as the HCFO/− group presented a marked increase in the relative abundance of this genus with respect to the other groups evaluated. Finally, the compositions of the eukaryotes were more uniform throughout the evaluated groups, with no differences between the organisms belonging to the observed classes (Fig. 1D,H).

Alpha and beta diversity with no differences between groups. When we analyzed the diversity indices for both bacteria and eukaryotes among patients with positive and negative CDI results, we observed Shannon and Simpson indices indicative of low diversity, with no significant differences between patients (Figure S3A and S3B). A similar pattern was evidenced when we analyzed the diversity indices among the four

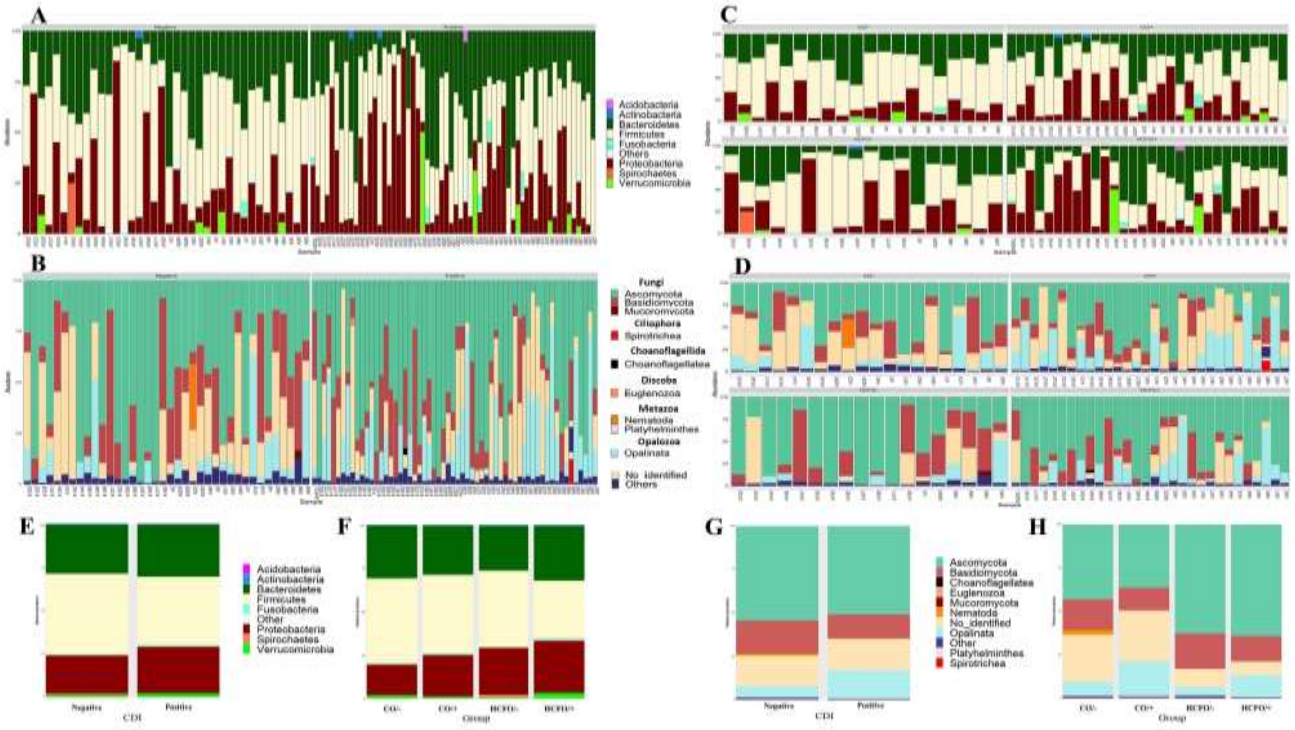

Figure 1. Microbial composition of diarrheic patients' gut microbiota by CDI status and by group (HCFO/+, HCFO/–, CO/+, CO/–). (**A**) Bar plots showing the 9 major bacterial phyla by CDI status. (**B**) Bar plots of major eukaryotic groups by CDI status. (**C**) Bar plots of major bacterial phyla by group. (**D**) Bar plots of major eukaryotes by group. (**E**) Distribution of each bacterial phyla by CDI status. (**F**) Distribution of each bacterial phyla by group. (**G**) Distribution of eukaryotes by CDI status. (**H**) Distribution of eukaryotes by group. Figure created on R studio with ggplot package[64,69].

study groups: no significant differences were found among the groups, which all had relatively low diversities (Figure S3C and S3D). Finally, in the case of beta diversity, we found no characteristic patterns that would allow for clear spatial groupings between the members of the different groups (Figure S3E and S3F).

Robust differences between the genera commonly found in the gut. In the present research, 18 of the bacterial genera and eight of the most common eukaryotic genera from the human intestinal tract[19,20] were compared among the groups. Based on these genera, heatmaps were constructed, in which differences in the relative abundances were investigated (Fig. 2A,B). From the evaluation of the genera, significant differences ($p < 0.05$) in abundances were revealed for 10 bacterial genera, including *Dorea*, *Faecalibacterium*, *Lachnospira*, and *Prevotella*, and one eukaryotic genus, *Candida* (Fig. 2C). The remaining eight bacterial and seven eukaryotic genera did not show significant differences in abundance between the studied groups (Figure S4). Despite this, some eukaryote genera displayed striking patterns. Among these, the increase in the relative abundance of *Blastocystis* in the CO/+ and HCFO/+ groups with respect to the CDI-negative groups was prominent. Furthermore, the HCFO/– group showed an increase in the relative abundance of *Saccharomyces* and *Malassezia* genera with respect to the other groups (Figure S2).

When we analyzed the differential distribution of *Candida, Saccharomyces*, and *Malassezia* spp.; different configurations were observed for each genus in each of the study groups. In the case of *Candida*, an increase was observed in the HCFO/– and HCFO/+ groups, where more than 30 different species were associated to several ASVs (>99% similarity), with *C. albicans* and *C. glabrata* as the most abundant (Figure S2B). In the case of *Saccharomyces*, only the presence of ASVs corresponding to *S. cerevisiae* was evidenced as having a comparable distribution among the different groups (Figure S2C). Finally, ASVs relating to nine *Malassezia* species were identified, with the most abundant ASVs corresponding to *M. restricta*, followed by *M. globosa* and *M. furfur*, which had obvious increased abundances in the intrahospital-acquired diarrhea groups (Figure S2D).

Correlogram between bacteria and eukaryotes. To identify the possible interactions occurring between the intestinal bacteria and eukaryote genera, correlograms were generated using the reads from each of these microorganism groups. In general, an inverse correlation was observed in all study groups between some genera of fungi and bacteria of the phylum Firmicutes (Fig. 3). Strikingly, the CDI-negative groups presented the highest number of significant correlations (Figs. 3A,C). Similarly, in the HCFO/+ group, there were many inverse correlations between bacteria of the genus *Dorea* and various fungal genera (Fig. 3D).

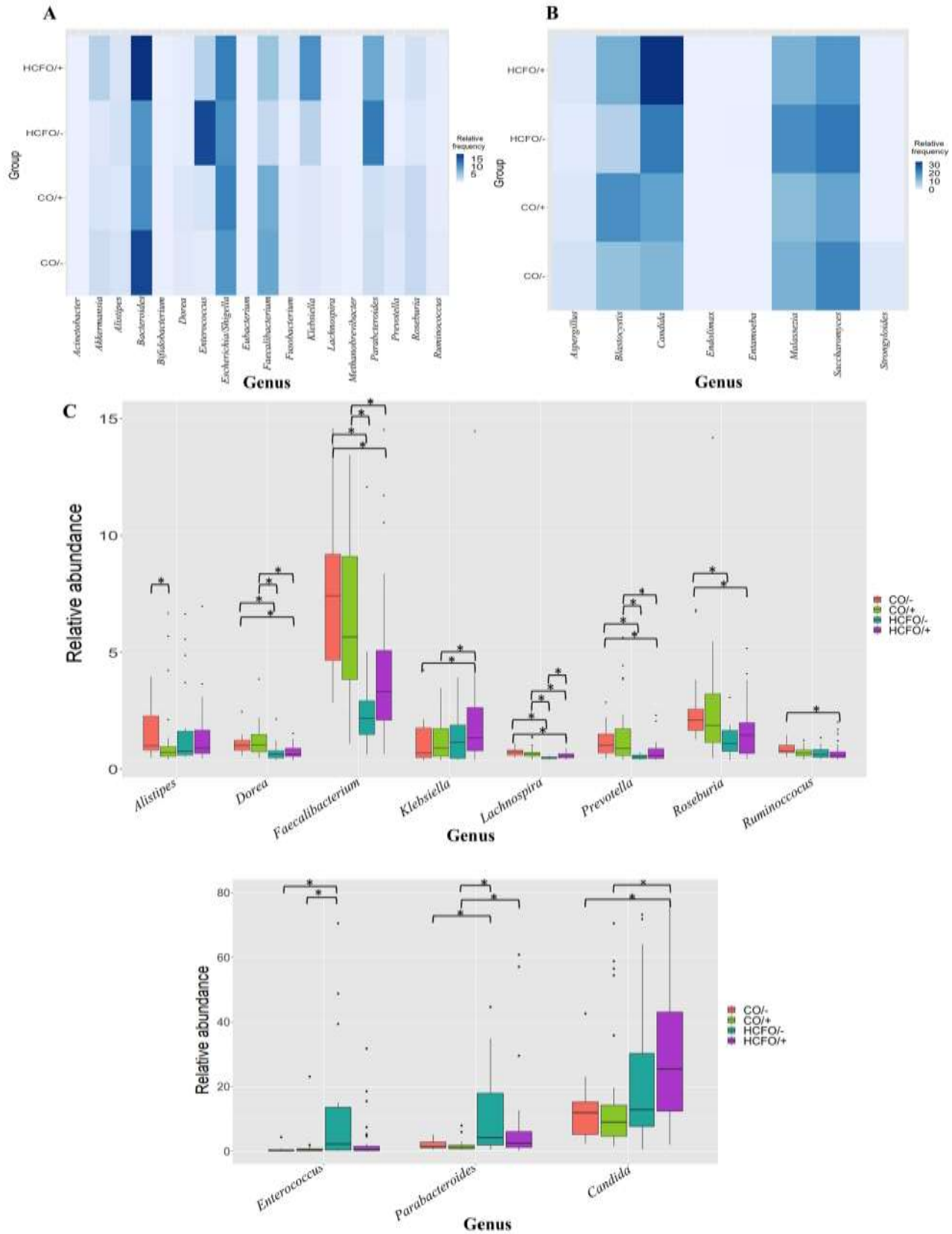

Figure 2. Changes in bacteria and eukaryotes commonly found in the gut microbiota. (**A**) Heatmap of bacterial genera by group. (**B**) Heatmap of eukaryotic genera by group. (**C**) Boxplot showing the differences between groups by relative abundances of each genus. Statistical differences (Kruskall–Wallis test; Post-hoc: Dunn test with Benjamini–Hochberg correction and a confidence level of 95%) ($p < 0.05$) are indicated by an asterisk mark (*). Figure created on R studio with ggplot package[64,69].

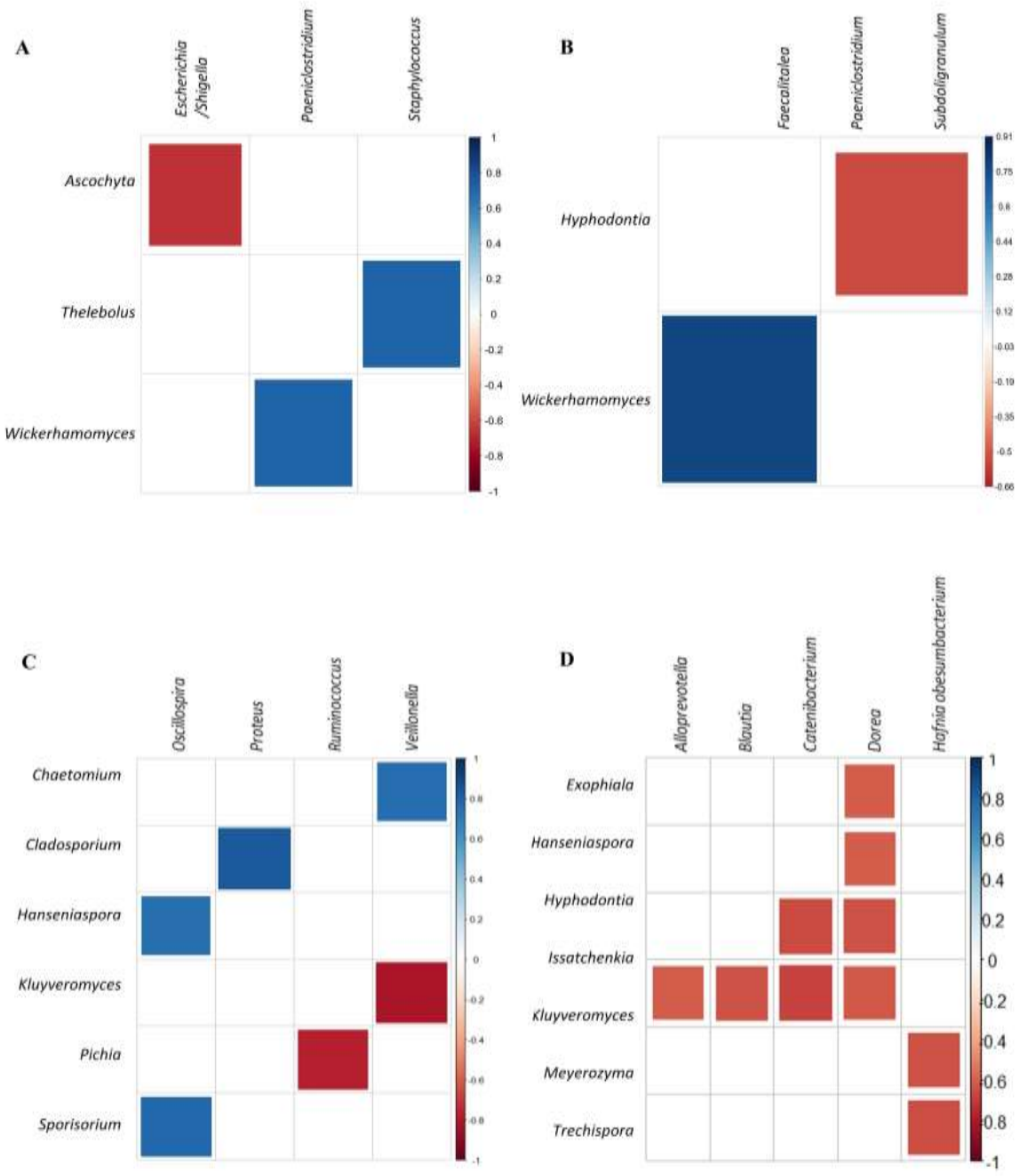

Figure 3. Possible interactions between kingdoms. Correlogram plots between bacteria and eukaryotes. ASVs corresponding to the most abundant phyla (Bacteroidetes, Firmicutes, Proteobacteria, Ascomycota and Basidiomycota) were compared. Outliers were deleted to only compare the ASVs corresponding to the most abundant genera (Spearman's rho correlation method with Benjamini–Hochberg correction). Only were considered strong correlations ($-0.7 < \rho > 0.7$; p value < 0.05). (**A**) CO/− group. (**B**) CO/+ group. (**C**) HCFO/− group. (**D**) HCFO/+ group. Figure created on R studio with psych package[64,72].

Identification of potential biomarkers. Finally, LEfSe analyzes were performed to determine the characteristic microbiological profiles of the four groups by considering the most abundant taxa in each of the study populations to be potential biomarkers (linear discriminant analysis [LDA] score > 4, $p < 0.05$, non-strict analysis). In the CO/− group, a differential predominance of bacteria belonging to three families of the phylum Firmicutes was found compared with the other groups: Ruminococcaceae, Lachnospiraceae, and Clostridiaceae had LDA scores > 4, with the Ruminococcaceae being the most characteristic within this group, followed by bacteria belonging to the genus *Faecalibacterium*. However, the patients of the CO/+ group showed a greater abundance of bacteria belonging to the genera *Alloprevotella* and *Fusicatenibacter*, which showed an LDA score > 3, and represented the greatest difference in this group compared with other groups (Fig. 4A,B).

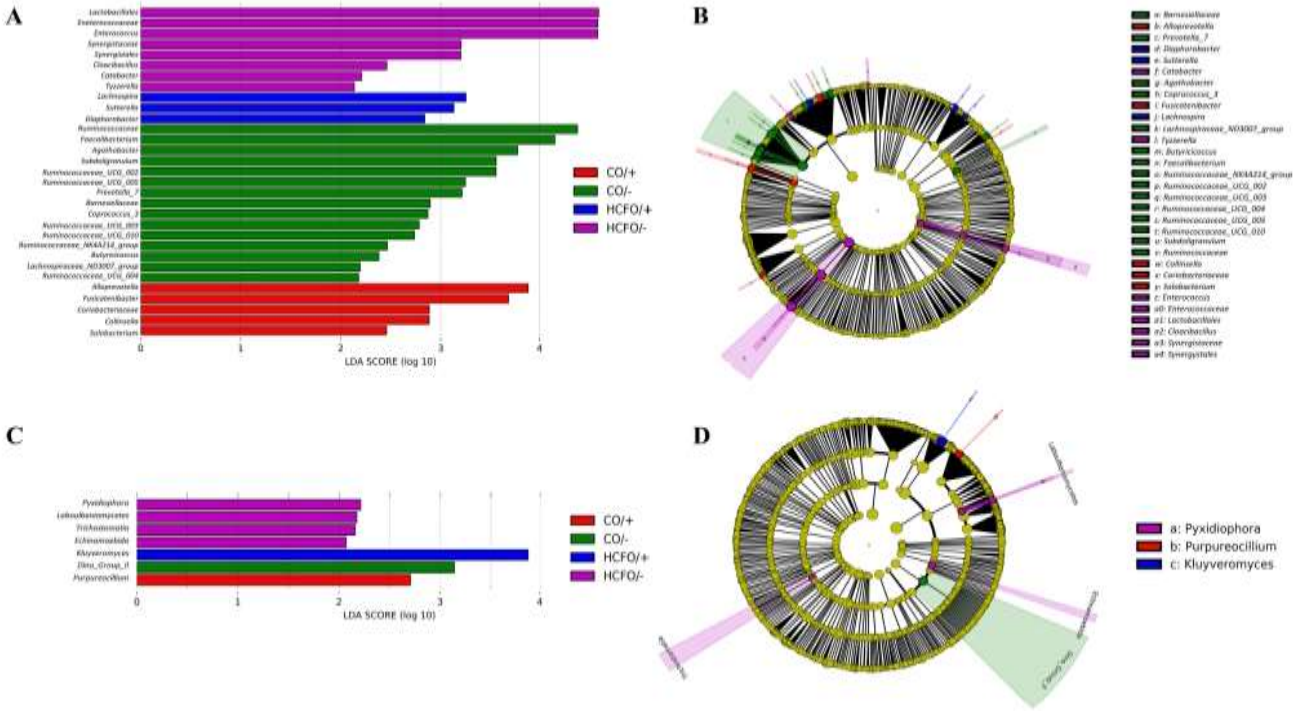

Figure 4. Effect of size measurements (LEfSe) at phylum and genus levels. Linear discriminant analysis (LDA) combined with LEfSe showing a list of possible biomarkers that enable discrimination between groups. LDA ≥ 2.0 and $p < 0.05$ were considered significant in Kruskal–Wallis and pairwise Wilcoxon tests. (**A**) Distinct bacterial phyla and genera biomarkers by group. (**B**) Cladogram reporting the bacterial taxa (highlighted by small circles and shading) showing different abundance values in the groups. Circles' diameters are proportional to the taxon's abundance, and the shadow size is proportional to the effect size. (**C**) Distinct eukaryotic phyla and genera biomarkers by group. (**D**) Cladogram reporting eukaryotic taxa showing different abundance values in the groups. Figure created on Galaxy platform[73,74].

In the HCFO/− group, the most abundant bacteria belonged to the order Lactobacillales, and the most important representative within this order was the genus *Enterococcus*, with an LDA score > 4 for both the order and genus. Finally, the HCFO/+ group was characterized by a greater abundance of *Lachnospira* bacteria; however, the lowest LDA scores and the least number of differentiating characteristic groups were found in this group (Fig. 4A,B). Finally, Archaea were not evaluated due to the low abundance.

In the case of eukaryotes, the number of biomarkers per group was considerably reduced. The CO/− group was characterized by an abundance of protists belonging to Dino Group II; while, for the CO/+ group, we evidenced an increase in fungi of the genus *Purpureocillium* with a moderate effect size. The HCFO/− group showed the smallest effect sizes in all its biomarkers, which were the most diverse, as they ranged from fungi such as *Pyxidiophora* to protists belonging to the Trichostomatia class. The HCFO/+ group was found to have the largest effect size with its unique biomarker, *Kluyveromyces* (Fig. 4C,D).

Discussion

In terms of the composition of the microbiota, an increase in the relative abundance of bacteria belonging to the phyla Bacteroidetes and Firmicutes was observed in CDI-positive patients with respect to CDI-negative patients (Fig. 1). This increase is contrary to what was previously discovered in various studies that found intestinal microbiota profiles were characterized by a decrease in these phyla[21–24]. This decrease is supported by the roles that these groups of microorganisms play in carbohydrate metabolism and the production of short-chain fatty acids (SCFAs), as well as their contribution to the regulation of the immune system, which hinders the development of vegetative forms of *C. difficile*. Therefore, a decrease in Bacteroidetes and Firmicutes facilitates the development of *C. difficile* infections[25,26]. This discrepancy in the results may be explained by the fact that a greater increase in the relative abundance of these phyla, especially Firmicutes, was seen in patients with community-acquired diarrhea (CO/+) (Fig. 1). This may account for the smaller impact on the intestinal microbiota of the circulating strains in this population, and the physical and nutritional conditions of patients with community-acquired diarrhea may provide a more stable intestinal microbiota composition and, therefore, less severe forms of CDI. However, since there are no clinical data associated with the patients, further research is needed to reveal the true impact of the source of the *C. difficile* infection and associated clinical factors.

Another important result in the composition of the microbiome was the increased abundance of Proteobacteria in patients with CDI compared with uninfected patients (Fig. 1). The increase these bacteria has been previously reported for CDI, suggesting that these microorganisms take advantage of the imbalance of the microbiota to proliferate and may even cause the exacerbation of symptoms[9,23,24]. In this sense, it is necessary to evaluate in

future studies the abundance of these phyla in a non-diarrheal control group, which was not the objective of our analysis. This is also corroborated with the diversity of both the bacterial and eukaryotic microbiota that was low in all groups studied (Figure S3). This can be related with diarrhea presentation.

In the case of eukaryotes, the predominance of fungi in the eukaryotic microbiome composition (Fig. 1) agrees with previous descriptions, as these organisms are the main component of the eukaryotic intestinal microbiota[27–29]. However, the absence of differences between the studied groups contrasts with previous reports in which an increased ratio between Basidiomycota/Ascomycota was associated with the pathogenesis of colorectal cancer and inflammatory bowel disease[30]. The absence of these changes could be due to the presence of diarrhea that may help to hide differences between the groups. This highlights the need to compare the findings with those from patients without diarrhea in future studies.

As previously discussed, the health status of patients who develop intrahospital diarrhea is usually marked by multiple treatments and interventions that can facilitate the emergence of various pathogens, such as *C. difficile*[9,23,31]. However, the absence of this pathogen in some individuals of the HCFO/– group suggests that alternative mechanisms may contribute to the homeostasis of the intestinal microbiota, which should be investigated in a future study. One of the proposed mechanisms is related to the marked increase in *Methanobrevibacter* in this group (Figure S2), involved in production of SCFAs from carbohydrates[32–34], some of which act as growth inhibitors of *C. difficile*[17]. This suggests that some archaea could contribute to supply the function of some bacterial families that produce these components and were found to be diminished in this group. Therefore, there may be a relationship between the increase in *Methanobrevibacter* (Figure S2) and protection against CDI, the mechanism of which should be addressed in future research. In addition, studies should consider other methanogenic Archaea, which contrastingly increased in the HCFO/+ group, denoting a possible genus-specific role not described so far.

Diarrhea, associated with an imbalance of microbiota, may contribute to the increase in certain opportunistic microorganisms. In this regard, *Candida* and *Malassezia* genera in the HCFO/+ and HCFO/– groups (Figures S2 and S4) could have taken advantage of the disruption of the microbiota to consolidate their populations, although these tend to be pathobionts and are innate components of the intestinal microbiota[30,35,36]. For example, more than 30 species of *Candida* were identified (Fig. 2 and Figure S2), prominently *C. albicans* and *C. glabrata*; the latter are recognized for their ability to alter the microbiota of immunocompromised individuals[37,38], and their growth is favored by the proliferation of aerobic bacteria, especially of the Enterobacteriaceae family[39], which were increased in the HCFO groups (Fig. 2). In a similar way, the *Malassezia* species, among which *M. restricta* was prominent (Figure S2), are known for their ability to exacerbate the severity of colitis in Crohn's disease without causing alterations to the microbiota[40]. This indicates a complex relationship between kingdoms in which the microbiota is altered by some bacteria and eukaryote groups to facilitate their proliferation while utilizing the energy sources available as a result of the decrease in other beneficial groups. Additionally, the modulation of immunity at the intestinal level by some bacteria could be used by opportunistic pathogens to proliferate, leading to a state of intestinal ecosystem imbalance, as occurs in other inflammatory diseases[38,41–43]. Similar phenomena can occur as a result of eukaryotes that facilitate infection by pathogenic bacteria[44].

Contrary to the findings described above, the presence of some eukaryotes, such as *Blastocystis*, has been associated with an increase in the diversity of the bacterial microbiota and of beneficial bacteria groups such as *Faecalibacterium* and *Roseburia*[45–47]. This positive modulation of the intestinal microbiota is related to the development of an anaerobic environment, which is necessary for the growth and development of this protozoan and is generated by beneficial bacteria through the production of SCFAs such as butyrate. The SCFAs are consumed by the colonocytes, increasing oxidative phosphorylation and, thus, decreasing the amount of oxygen available in the intestinal lumen[48]. This was exemplified by the CO/+ group, in which there was an increase in the relative abundance of *Blastocystis* (Figure S4) accompanied by an increase in beneficial bacteria such as *Faecalibacterium*, *Lachnospira*, *Prevotella*, and *Roseburia* (Fig. 2). The above suggests that the co-occurrence of *Blastocystis* and *C. difficile* attenuates the negative impact of CDI on the intestinal microbiota. The effects of this attenuation on clinical manifestations should be studied in depth in future research.

The co-relationships between bacteria and eukaryotes are marked by multiple interactions, both synergistic and antagonistic, and competition for energy sources[30,36,38]. The correlograms in this study represent the complex relationships between the various bacterial and eukaryotic genera (Fig. 3). These relationships in the CDI-negative groups may denote closer interactions, as occurs in ulcerative colitis[38,42]. Furthermore, the inverse correlations observed in the CDI-positive groups reinforce the previous hypothesis that some microorganisms proliferate by exploiting the energy sources available from the depletion of other microorganisms and suggest a greater antagonism between the different kingdoms, as occurs in Crohn's disease[49]. Imbalances in the microbiota similar to those shown here, which are usually associated with the deterioration of the immune system and metabolic homeostasis in critically ill patients at risk of developing sepsis[50], are generated mainly by the presence of patients at ICUs, which as mentioned above, can profoundly alter the delicate balance of the intestinal ecosystem. Moreover, polymicrobial interactions that occur within biofilms have been associated with the progression of some diseases, such as colorectal cancer, prostatitis, and cystic fibrosis[51–53], highlighting once again the importance of further studying their potential roles.

To differentiate between the studied groups based on the abundance profiles of gut microbiota members, prokaryote and eukaryote biomarkers were established (Fig. 4). As has been shown throughout the study, each group showed characteristic profiles that could account for the degree of homeostasis of the intestinal microbiota. An example of this occurred in the HCFO/– group, in which the increase in bacteria belonging to the Lactobacilalles, Enterococcaceae, and Enterococcus groups showed disruption of the microbiota characteristic of diarrhea associated with irritable bowel syndrome[22]. Whereas the increase in bacteria belonging to the Ruminococcaceae family, proposed as biomarkers for the CO/– group, suggest a greater degree of balance within the intestinal microbiota driven by the production of SCFAs, such as butyrate[10]. A similar profile occurred in the

other CDI-negative group, in which the presence of potentially beneficial bacteria was associated with the presentation of less severe symptoms and better resolution of the disease[54,55]. Finally, the increase in *Alloprevotella* noted in the HCFO/+ group agrees with findings from previous studies on other inflammatory bowel diseases, in which an increase in Prevotellaceae family members was associated with the development of inflammation and colitis, and *Alloprevotella* was suggested as a biomarker for the identification of these pathologies.

We found that most of the eukaryotic biomarkers corresponded to fungi not usually found at the intestinal level[56], which suggests that the number and diversity of eukaryotic sequences available in the databases should be increased to allow for more accurate taxonomic assignment based on the 18S-rRNA marker and thus achieve greater precision in the description of this community[55]. Despite the above, the suitability of *Kluyveromyces* as a biomarker of HCFO/+ group suggests immunosuppression is a key factor in the development of this type of opportunistic infection[57,58], as eukaryotic infections are associated with both the multiple clinical and therapeutic interventions to which patients may be exposed at the hospital, generating a general alteration of their health status.

The present investigation aimed to evaluate the roles of other microorganisms, such as eukaryotes, with unclear effects in CDI based on patients with diarrhea. Despite the absence of clinical and sociodemographic data, there were some characteristic differential profiles among the four evaluated groups that deserve to be studied in greater depth to reveal the roles of other microorganisms, such as viruses, and identify potentially relevant virulence and resistance markers. In the future, it is necessary to conduct additional analyses including a cohort of control patients (without diarrhea) to have a most complete view about the changes in gut microbiota composition under different scenarios. Additionally, the use of techniques such as metagenomic sequencing and interactome analysis will be required to complement the data presented here and to provide a more holistic understanding of CDI and the role of the intestinal microbiome in its establishment, development, and recovery.

Methods

Ethical considerations. The current project was conducted with the approval of the Research Ethics Committee of the Universidad del Rosario (Approval Act No. 339). This study was considered low risk according to Resolution 8430 of 1993 of the Ministry of Health of Colombia. The samples were coded to protect the identity of the patients in accordance with national ethical guidelines and the Declaration of Helsinki. The duration of diarrheal symptoms was the only data obtained from the clinical history of the patients and was directly associated with the coding of the sample. Informed consent was obtained for the use of the sample in research in accordance with what was authorized by the ethics committee. Data concerning age and sex were collected only from the HCFO groups.

Study population. A total of 98 DNA samples were selected from the biobank of the Microbiological Research Group—UR (GIMUR). These stool samples were obtained in the framework of the "*Clostridioides difficile* characterization in Colombia" project from patients with diarrhea, the main symptom of *C. difficile* infection (CDI). Each sample was assigned to a group according with the location of diarrhea acquisition, CO- or HCFO-acquired, according to the protocols of the Society for Healthcare Epidemiology of America and the Infectious Diseases Society of America[31].

Sample collection and transportation procedure is detailed at supplementary information section. DNA extraction protocol and CDI status identification were obtained from Muñoz et al.[59]. Briefly, DNA extraction was performed using the Stool DNA Isolation Kit (Norgen, Biotek Corporation, Thorold, Canada) following the manufacturer's instructions. Conventional PCR was performed for the detection of CDI using the markers 16S-RNA and glutamate dehydrogenase (GDH), as reported elsewhere[59]. The results were visualized by 2% agarose gel electrophoresis. Based on these results, the following groups were stablished:

Group 1: CDI-positive samples from HCFO (n: 30).
Group 2: CDI-positive samples from CO (n: 30).
Group 3: CDI-negative samples from HCFO (n: 18).
Group 4: CDI-negative samples from CO (n: 20).

DNA quality control and sequencing process. The extracted DNA was subjected to quality control by 2% agarose gel electrophoresis to verify the integrity of the DNA. Additionally, the concentration was evaluated using a NanoDrop/2000/2000c spectrophotometer (Thermo Fisher Scientific, Massachusetts, USA). A 260/280 relationship between 1.8 and 2.0 and a minimum concentration of 20 ng/µL was verified.

Paired-end sequencing was performed on the Illumina HiSeq platform (PE 250 Platform) with a depth of 100.000X at the facilities of Novogene Corporation Inc. (Shanghai, China) using primers targeting the hypervariable V4 region of the 16S-rRNA marker specific for bacteria and Archaea 515-F (5′-GTGCCAGCMGCCGCG GTAA-3′) and 806-R (5′-GGACTACHVGGGTWTCTAAT-3′)[60]. For the description of eukaryotic communities, we used primers targeting the hypervariable region of 18S-rRNA 528F (5′-GCGGTAATTCCAGCTCCAA-3′) and 706R (5′-AATCCRAGAATTTCACCTCT-3′)[61].

Taxonomic assignment. Initially, a quality control step was performed to determine the quality of the reads from the sequencing process. The average number of reads per sample, phred score, frequency of unassigned bases, and content of adapters in the samples were analyzed. Subsequently, the barcodes and primers were removed using the QIIME2 tool[62,63] before proceeding with taxonomic assignment using the DADA2 tool in R studio[64], following the default pipeline[65]. The assignment was performed by comparing the sequences obtained

for the 16S-rRNA marker against the 16S-rRNA SILVA version 132 database[66], and the sequences obtained for the 18S-rRNA marker against the Protist Ribosomal Reference database (PR2)[67]. Finally, rarefaction curves were performed to determine the sufficiency of the sequencing depth to ascertain the microbial diversity of the samples using the ranacapa package of the R Studio program[68].

Diversity analysis. Based on the ASVs resulting from the taxonomic assignment, we created relative abundance graphs (bar plots) of the different phyla and genera in both the CDI-positive and -negative patients. Subsequently, alpha (Shannon and Simpson) and beta (NMDS from Bray–Curtis similarity index matrices) diversity analyzes were performed using R studio's phyloseq package and were later graphed using ggplot2[69] and reshape2[70].

Heatmaps, correlations, and biomarker search. A heatmap was generated with the most relevant bacterial and eukaryotic genera at the intestinal level[19,20] to determine the relative abundances in each group. From the findings from the heatmaps, boxplots were created to determine the differences between the studied groups with respect to the genera investigated. The differences between the groups in terms of abundance of archaea and some genera of eukaryotes was determined by creating chord diagrams using the circlize package[71] in R studio. Statistically significant differences between the studied groups were evaluated using the Kruskal–Wallis test with respective post-hoc analyses with the Dunn test using Benjamini–Hochberg correction with a confidence level of 95%. Likewise, correlogram graphs were made between ASVs corresponding to the most abundant phyla (Bacteroidetes, Firmicutes, Proteobacteria, Ascomycota and Basidiomycota). Based on these, a filter of sub-represented data was carried out, eliminating all those ASVs corresponding to genera whose sum of reads was less than 1,000, as well as those ASVs corresponding to genera whose reads were not present in at least 25% of the samples. These filters were carried out to reduce potential technical bias and to ensuring that comparisons were made between ASVs that were present in the groups and not in single samples. The correlation matrix was constructed using the psych package on R software[72] applying the spearman method with Benjamini–Hochberg correction. We considered only strong correlation values greater than 0.7 and less that −0.7 (Spearman Rho strong correlation) and select statistically significant ($p < 0.05$) at the moment of establishing a correlation between the ASVs evaluated. Finally, we performed multiple comparisons among the different taxa of the groups to identify potential biomarkers using an LDA of effect of size (LEfSe), which was performed on the Galaxy platform[73,74], following the indications of the framework.

Data availability

The 16S-rRNA and 18S-rRNA gene sequencing data used in this study are available through the National Center for Biotechnology Information (NCBI) Sequence Read Archive: https://ncbi.nlm.nih.gov/sra under accession number PRJNA679727.

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Author contributions

G.H., J.D.R. and M.M. designed the study and drafted the manuscript. G.H. and M.M. carried out the processing of the samples. G.H., L.V. and M.M. performed the bioinformatics analyses. M.A.P., J.D.R. and M.M. substantively revised the manuscript. All authors read and approved the final version of the manuscript.

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The authors declare no competing interests.

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REVIEW

Updating changes in human gut microbial communities associated with *Clostridioides difficile* infection

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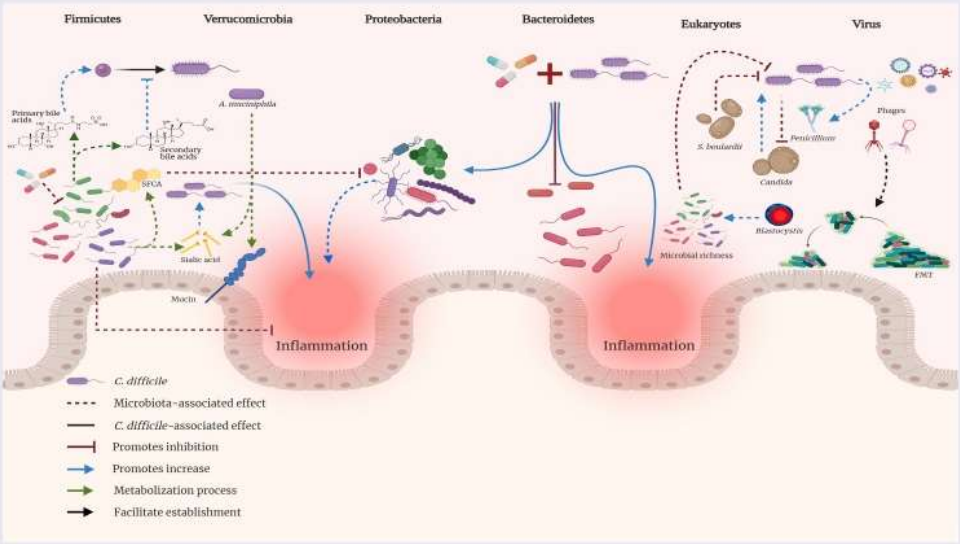
ABSTRACT

Clostridioides difficile is the causative agent of antibiotic-associated diarrhea, a worldwide public health problem. Different factors can promote the progression of *C. difficile* infection (CDI), mainly altered intestinal microbiota composition. Microbial species belonging to different domains (i.e., bacteria, archaea, eukaryotes, and even viruses) are synergistically and antagonistically associated with CDI. This review was aimed at updating changes regarding CDI-related human microbiota composition using recent data and an integral approach that included the different microorganism domains. The three domains of life contribute to intestinal microbiota homeostasis at different levels in which relationships among microorganisms could explain the wide range of clinical manifestations. A holistic understanding of intestinal ecosystem functioning will facilitate identifying new predictive factors for infection and developing better treatment and new diagnostic tools, thereby reducing this disease's morbidity and mortality.



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Introduction

Clostridioides difficile (CD) infection (CDI) is a healthcare-associated infection, which has a substantial global impact, including antibiotic-associated diarrhea.[1,2] This microorganism has a broad clinical spectrum, ranging from asymptomatic infections to complicated digestive tract illness that can lead to death.[1,2] CDI represents a serious public health problem in developed countries; morbidity and mortality rates have increased during recent years,

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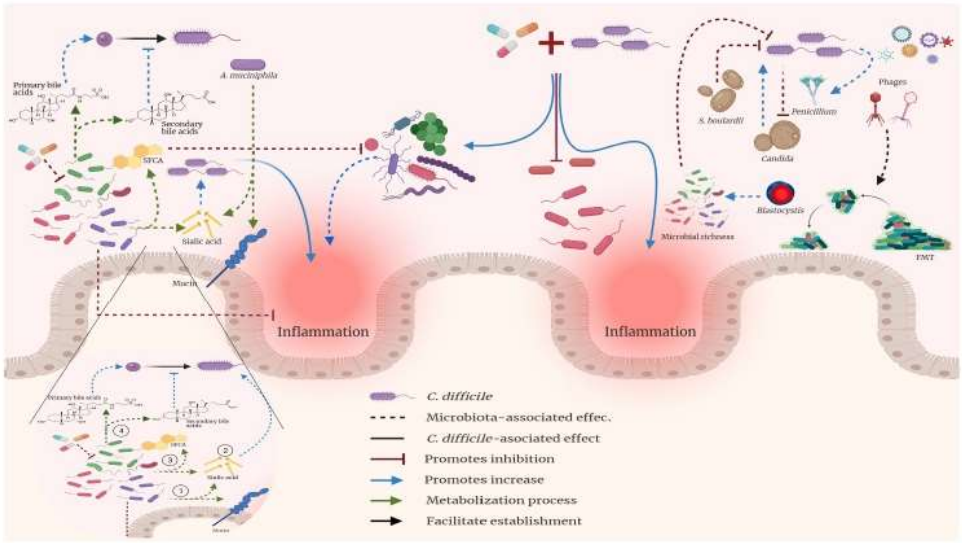

Figure 1. Interaction between different bacterial phyla during *Clostridioides difficile* (CD) infection (CDI). Firmicutes. This phylum plays a primary role in defense against CDI and inhibiting intestinal inflammation as its members are mostly responsible for sialic acid metabolism to short-chain fatty acids (SCFAs) which prevent CD spore germination. SCFAs inhibit the growth of some pathogenic members from the Proteobacteria phylum. Verrucomicrobia. *Akkermansia muciniphila*'s role in this phylum is striking, given its involvement in colonocyte mucin degradation which increases sialic acid production, provides nutrient availability for the CD vegetative form and results in increased inflammation. Proteobacteria. CD decreases bacterial group abundance, thereby promoting the pathogenic bacteria growth and exacerbating CDI's inflammatory symptoms. Bacteroidetes. Antibiotic use inhibits the growth of various members from this phylum, resulting in no increase in inflammation and the abundance of pathogenic bacterial phyla, such as Proteobacteria. Zoom panel: *Establishment of Clostridioides] difficile infection*. Under homeostasis, 1) intestinal bacteria metabolize carbohydrates from colonocyte glycoprotein membrane. 2) This results in sialic acid release which 3) is then degraded by commensal bacteria, generating SCFAs including butyrate (one of the main energy sources for colonocytes). 4) Primary bile acid conversion to secondary bile acid creates products preventing *C. difficile* spore passage to their vegetative form. However, these conditions are disturbed by antibiotic use – affecting commensal bacteria populations (in turn, protecting against intestinal inflammation), avoiding primary fatty acid conversion to secondary fatty acids and facilitating sialic acid availability which promotes *C. difficile* bacilli formation in any environment having little competition for energy sources.

resulting in multimillion-dollar costs for health systems.[3,4] Nevertheless, its impact on most developing countries remains unknown.

Patients undergoing antibiotic therapy in hospitals often suffer alterations in their intestinal microbiota as a result of treatment, thereby reducing the populations of beneficial microorganisms that compete for energy sources and metabolize primary bile acids into secondary bile acids, producing metabolites (i.e., taurocholic acid and glycolic acid) that inhibit CD growth (Figure 1).[3,5] Antibiotic therapy is also known to alter populations of Archaea, such as *Methanobrevibacter* involved in bile acid metabolism.[6]

Other factors such as age and the use of some drugs can have an impact on microbiota, thereby facilitating CDI development (mainly concerning recurrent disease).[7] However, intestinal homeostasis alterations are considered both a cause and of CD may promote indole production by pathogens, such as *Escherichia coli*, a bioactive molecule that inhibits protective gut microbiota growth and reconstitution during infection.[4]

The forgoing highlights the microbiota's preponderant role regarding protection against CD colonization and the development of CDI itself; this has prompted various studies focused on describing the intestinal bacterial communities of individuals suffering CDI.[8,9] Although studies have already highlighted intestinal microbiota components as effective tools for treating various diseases (i.e., CDI), extensive efforts are needed to understand its members' functions regarding the intestinal ecosystem.[10] An example would be the effectiveness of fecal microbiota transplantation (FMT) for restoring intestinal microbiota in patients suffering recurrent CDI, thereby leading to healing and a decrease in events associated with the disease,

schemes.[11,12] However, limitations regarding the role of other microbiome constituents have not led to fully elucidating the key factors involved in this type of intervention's success. The purpose of this review was thus to gather and discuss the main findings for a wide range of human gastrointestinal microbiota components and their relationship with CDI.

Intestinal microbiome

The human gastrointestinal microbiome is a complex system of multiple microorganisms, their gene products and corresponding physiological functions.[13] The microbiome includes bacteria, Archaea, viruses, and eukaryotic organisms constantly interacting with each other and with the target host.[14] Microbiota composition is highly dynamic and depends on a host's physical state, genotype, immune phenotype, and environmental factors, such as diet, antibiotic use, and lifestyle. Such environmental factors can adversely affect the intestinal ecosystem, their effects frequently being associated with increased susceptibility to infection and non-communicable diseases, such as obesity, metabolic syndromes (e.g., diabetes and cardiovascular disease), allergy, and other inflammatory diseases.[15] Emerging evidence from recent studies has also established a two-way communication pathway linking the gastrointestinal tract and microbiota with the brain, suggesting that such microorganisms may play a role in neurological disorders as well as host perception, behavior, and emotional responses.[16,17]

Antibiotic use is associated with microbiota variations causing decreased diversity, an abundance of microbial communities, affecting the recovery of identical microbiota to that prior to long-term treatment;[18] a differential effect has been observed regarding acute and recurrent CDI. It has been proposed that antibiotic treatment (or some other disturbance) significantly alters the composition of gut microbial communities,[19] thereby having an impact on the balance of primary and secondary bile acids promoting CD colonization.[3] Increased carbohydrate concentration (such as sialic acid) in the intestinal mucosa is a secondary effect of antibiotic therapy due to the disruption of carbohydrate-fermenting microbiota, which is exploited by CD during its proliferation.[20,21]

This has led to an increase in studies seeking to elucidate the relationship between the intestinal microbiota and CDI; most such studies have focused exclusively on bacterial populations.[3,4] Although these populations are the primary components of microbial communities, other members play a determining role in maintaining intestinal homeostasis (i.e., Archaea, eukaryotes, and even viruses) and thus identifying other members represents a challenge for a complete description of gastrointestinal microbiome composition. *Tritrichomonas musculis* would be an example of the impact of protozoan species on this ecosystem; it has recently been revealed to be related to an increased intestinal immune response. Such immune responses (despite conferring resistance against colonization by certain bacterial species) have promoted inflammatory disease and tumor development in a murine model.[22] *Blastocystis* subtype 7 has been seen to affect the microbiota by reducing the populations of beneficial bacteria, which could lead to an imbalance of the entire intestinal ecosystem.[23]

Advances in microbiota research

Studying the intestinal microbiome has undergone numerous changes concerning data collection techniques and the tools for its analysis.[24,25] Traditional and novel *in vitro* culture and techniques aimed at deciphering all microbiota components have recently led to the characterization of many bacteria in the gut.[26] However, the challenges of culturing some fastidious microorganisms and difficulties regarding the recovery of other members of the intestinal ecosystem (such as viruses and eukaryotes) highlight the need for alternative molecular techniques for characterizing them.[27] Next-generation sequencing and the advent of omics has led to the amount of microbiome studies increasing exponentially, thereby producing increasingly complex data regarding the members of this ecosystem and its homeostasis.

Table 1. Main findings from the study of *Clostridioides difficile*-associated bacterial microbiota.

Year	Main findings	Study description	Reference
1982	Six genera inhibited *C. difficile* multiplication, *Lactobacillus* and group D Enterococci being the most antagonistic	*In vitro* study: 23 genera of fecal bacteria vs toxigenic *C. difficile* strains	45
1982	*Streptococcus* inhibited *C. difficile* growth	*In vitro* study of 7 *Streptococcus* strains vs 34 *C. difficile* strains	46
1988	Competition between unknown microorganisms and *C. difficile* by SFCA metabolism	*In vitro* continuous-flow culture model	47
1994	*C. difficile* failed to establish itself in the intestines of mice colonized with human fecal microorganisms; neither toxin A nor B were detected in these animals' fecal pellets	Germ-free mice	48
1994	The combination of standard antibiotics and *S. boulardii* was shown to be an effective and safe therapy for patients suffering recurrent CDD; no *S. boulardii*-related benefit was demonstrated for those suffering an initial episode of CDD	A randomized placebo-controlled trial. 64 patients were enrolled having had an initial episode of CDD, and another 60 who had a history of at least one prior CDAD episode	49
2002	Altered composition of gut microbiota at species level in CDAD patients, characterized by greater diversity of facultative species, lactobacilli, and clostridia, but greatly reduced numbers of *Bacteroides, Prevotella and Bifidobacteria*	Identifying bacterial species isolated from healthy young adults and elderly subjects' fecal samples and elderly patients suffering CDAD	50
2008	Recurrent CDAD patients had a highly variable bacterial community composition and decreased diversity	Stool samples from 10 individuals (7 CDAD and 3 controls)	36
2010	An increase in Firmicutes and Proteobacteria and a decrease of Bacteroidetes were observed	Nested case-control. 25 CDAD and 50 controls. 16S rRNA microarray	32
2012	Mice precolonized with a murine Lachnospiraceae isolate had significantly decreased *C. difficile* colonization, but not those colonized with *E. coli* while mice colonized with both *C. difficile* and *E. coli* died after 48 h (80% mortality reduction after 2 days in mice precolonized with *Lachnospiraceae* isolate)	Germ-free mice. Murine *Lachnospiraceae* and *E. coli* isolates were cultured from wild-type mice	44
2013	Decreased microbial diversity and species richness driven primarily by a paucity of phylotypes within the Firmicutes phylum. Normally abundant gut commensal organisms, including the Ruminococcaceae and Lachnospiraceae families and butyrate producing C2 to C4 anaerobic fermenters, were significantly depleted in CDI and CDN groups	Culture-independent high-density Roche 454 pyrosequencing was used to survey the distal gut microbiota for 39 individuals having CDI, 36 subjects suffering (CDN), and 40 healthy control subjects	51
2015	CDI patients and asymptomatic carriers had microbial richness and diversity compared to healthy subjects, accompanied by a paucity of phylum Bacteroidetes and Firmicutes and overabundance of Proteobacteria. Some normally commensal bacteria, especially butyrate producers, were significantly depleted in CDI patients and asymptomatic carriers	25 participants (8 CDI patients, (asymptomatic *C. difficile* carriers) and 9 healthy individuals)	35
2016	Increased butyrogenic bacteria in both CDI and non-CDI patients. Increased *Akkermansia* and *Penicillium* in CDI patients. Decreased *Bacteroides* population density	24 inpatients with diarrhea (12 CDI vs 12 controls)	43
2016	Lower microbial diversity in CDI patients. CDI was associated with a significant under-representation of gut commensals having putative protective functionalities, including *Bacteroides, Alistipes, Lachnospira* and *Barnesiella*, and over-representation of opportunistic pathogens	Three groups of hospitalized elderly patients (age ≥ 65) following standard diet including 25 CDI-positive (CDI group), 29 CDI-negative exposed to antibiotic treatment (AB+ group) and 30 CDI-negative subjects not on antibiotic treatment (AB− group)	37
2016	A review highlighting risk factors for developing CDI. CDI patients had increased Proteobacteria and decreased commensal bacteria: *Ruminococcaceae, Lachnospiraceae* or *Bifidobacterium longum*	Review	7
2016	Metabolomics profiling (highly responsive to changes in physiological conditions) has shown promise in differentiating subtle disease phenotypes having a nearly identical microbiome community structure, suggesting metabolite-based biomarkers may be an ideal diagnostic tool for identifying CDI patients	Review	42
2017	The authors identified *C. difficile* in 131 of 156 CDI index cases (1.78% average abundance) and 18 out of 211 healthy controls (0.008% average abundance). Consistent negative association with *C. scindens* and multiple *Blautia* species	High-resolution method for 16S rRNA sequence assignment to previously published gut microbiome studies of CDI and other patient populations	52
2018	Microbiota-dependent alteration in innate immune response early on during infection may explain poor outcome in aged hosts suffering CDI	*in vivo* mouse model	53
2018	Compared to IBD patients without CDI, IBD patients with CDI had more pronounced dysbiosis with higher levels of *Ruminococcus gnavus* and *Enterococcus* OTUs and lower levels of *Blautia* and *Dorea* OTUs	56 IBD patients, including 8 having flares with concomitant CDI, 24 flares without CDI, and 24 in remission; 24 healthy subjects	54
2018	Supplementing with anti-inflammatory butyrate-supporting commensal bacteria and prebiotics may support innate immune responses and minimize bacterial burden and negative effects during antibiotic treatment and exposure to CD	*in vivo* mouse model	41
2019	A reduced abundance of *Bacteroides* was associated with a poor CDI prognosis, severe diarrhea, and high recurrence incidence	57 patients suffering diarrhea from nosocomial and community-acquired CDI	9

(*Continued*)

Table 1. (Continued).

Year	Main findings	Study description	Reference
2019	Several genera, such as *Phascolarctobacterium, Lachnospira, Butyricimonas, Catenibacterium, Paraprevotella, Odoribacter*, and *Anaerostipes*, were not detected in most CDI cases	79 tcdB positive patients and 20 controls	38
2020	Depletion of *Alistipes* and *Ruminococcus* species and reduced methionine biosynthesis were noted in *C. difficile* patients having undergone surgery	A prospective single-center study of 70 CD patients	40
2020	66 species inhibited *C. difficile*; species composition and blend size were important re inhibition	*C. difficile* coculture with 1,590 isolates from gut microbiota. 256 combinatorial community assemblies	55
2021	There was a significant association between *Blastocystis* and CDI	220 patients suffering diarrhea	56

SCFA: Short-chain fatty acids; CDD: *Clostridioides difficile*-associated disease; CDAD: *Clostridioides difficile*-associated diarrhea; CD: *Clostridioides difficile*; CDI: *Clostridioides difficile* infection; OUT: operational taxonomic units

Single-marker amplicon-based sequencing is one of the most widely used methods for identifying microbiome components.[28] This technique's speed, ease, and reproducibility have made it a fundamental tool and an almost necessary first step when studying microbial ecosystems from intestinal and other sources; this has led to the discovery of multiple microbial communities inhabiting environments regarding which there was no prior evidence regarding their presence.[29] This method is based on amplifying and sequencing marker genes' (i.e., 16S-rRNA, 18S-rRNA and/or ITS) highly conserved regions among all groups. The presence of polymorphisms enables the identification and differentiation of the members belonging to a microbial community.[29]

Despite the multiple benefits of single-marker amplicon-based sequencing, it has been shown that this technique has some shortcomings hampering full understanding of all gut microbiota elements. The metagenomic approach has emerged as an alternative; it consists of the random amplification and sequencing of all genetic content in a sample.[27,29] Metagenomics provides better taxonomic resolution and genomic information compared to single-marker techniques; it also facilitates the functional analysis and prediction of circulating genes.[30,31] This technique's cost can be prohibitive regarding the mass analysis of study populations and thus most studies involving this methodology have only used it on small population subgroups initially studied using just amplicon-based sequencing.[24,32]

CDI and its impact on gastrointestinal microbiota

CD induces alterations in microbiota balance, ranging from asymptomatic infections to intestinal homeostasis imbalances, which can lead to serious symptoms and even death.[1,2,33] Reduced diversity (i.e., different species in a sample) is one of the main alterations regarding intestinal microbiota; it is mainly caused by the decreased abundance of some groups of beneficial microorganisms and an increased abundance of pathogenic bacteria (Table 1).[34–36]

The phylum Firmicutes is one of the groups having decreased abundance following CDI; bacterial families such as Ruminococcaceae and Lachnospiraceae belong to it (Table 1).[8,37,38] Such bacteria are known for their role in butyrate production which is the preferred metabolic substrate for colonocytes; butyrate metabolism contributes to maintaining low oxygen levels, thereby suppressing pathogenic aerobic and facultative bacteria populations (Figure 1).[39] Reduced *Faecalibacterium* and *Bifidobacterium* counts are apparent in these groups and such changes have been associated with intestinal anti-inflammatory effects, which would explain their depletion in CDI.[40–42] Most individuals included in studies providing evidence of such alterations had been treated with antibiotics, probably leading to a reduction of these populations.[37,43] However, Reeves *et al.*,[44] have advanced an argument favoring these microorganisms' role through experiments using germ-free mice colonized with a Lachnospiraceae murine isolate in the absence of *E. coli*; they observed partial restoration of resistance to CDI (Table 1). Such findings have highlighted these microorganisms' preponderant role in CDI prevention.

Regarding the description of specific microorganism genera and their relationship with CDI, a finding in mice has indicated that CDI in a murine model was related to a decreased abundance of specific groups of microorganisms; this included *Clostridium scindens* (Table 1) a bile acid dehydroxylator acting as protector via the production of primary bile acid-derived metabolites, thereby making it a probiotic candidate for CDI treatment.[57] Another study reported increased *Akkermansia muciniphila* abundance in CDI patients.[43] This microorganism has frequently been associated with healthy intestinal microbiota, mainly in obesity studies.[58] This finding has been associated with this microorganism's ability to degrade mucin in the intestine's mucous layer; its metabolites are used by CD as an energy source, thereby enabling it to survive in the environment (Figure 1).[8,43]

There is usually a decreased abundance of some butyrate-producing bacterial genera in CDI patients, including *Dorea* and *Blautia* spp. (Table 1); their reestablishment in intestinal microbiota can thus prevent CD spore germination through primary bile salt metabolism.[34,52,54,59] These findings stress the fact that intestinal microbiome composition and its members' functions must be evaluated according to the global scenario being studied. The influence of inter-individual variations regarding these microorganisms' role and on the metabolic pathways in which their participation has been suggested must also be deciphered.

A similar situation has been observed in the phylum Bacteroidetes[32] within which such genera as *Alistipes, Prevotella*, and *Bacteroides* are usually associated with intestinal mucosa inflammation (Table 1).[37,40,50,53] Such reduction is usually accompanied by an increased abundance of members from the phylum Proteobacteria (Table 1) which are known for their role in disrupting intestinal homeostasis and mucosal inflammation. This results in exacerbating intestinal symptoms and leads to clinical complications that could eventually result in patient death (Figure 1).[35,51,60]

The aforementioned relationships between microbiota members denotes complex crosstalk systems within a competitive ecosystem (that could work in a bidirectional and dynamic manner) in which certain populations are constantly replaced by others. This would have an impact on these microbes' functions and consequences concerning tissues and affect the intestine's delicate balance. Intestinal regulation between inflammation and repair varies depending on the environment, nutrient availability, and their components.[61] This is reflected in the effects that small changes in certain groups of microorganisms have on such balance; maintaining intestinal homeostasis thus represents a challenge for modern science.

In search of the optimal microbial composition or restoration of intestinal homeostasis regarding CDI

CDI-related gastrointestinal microbiota studies have shed light on the affected components and their impact on microbiota balance; research has thus been focused on restoring gastrointestinal microbiota through FMT. The first studies were aimed at determining the microorganisms directly involved in protection against CDI to produce an adequate cocktail of microorganisms for restoring the balance of intestinal homeostasis. Some research has demonstrated that certain bacteria from the genera *Streptococcus, Lactobacillus*, and *Bacteroides* have inhibited CD growth, possibly through competitive effects on the monosaccharides released from mucin (Table 1).[45–47,62] Evidence has emerged regarding the role of intestinal microbiota as a barrier against CDI and the impact that antibiotics and other medications have on its balance to promote the microorganism's spore germination.[48,63]

Later, studies have focused on FMT from a healthy individual to one having CDI and its impact on microbiota restoration. One such study reported a dramatic change in the recipient's microbiota composition 14 days after transplantation; similarity with donor microbiota was achieved, leading to symptom resolution.[64] However, such interventions were unsuccessful in some patients; this led to further research regarding specific components and appropriate administration routes for this type of treatment. Different phyla and bacterial families were identified as potential CD biomarkers and antagonists.[6,43,55]

Lactobacillus and *Saccharomyces boulardii* administration has proved effective in CDI patients' treatment and recovery in some studies.[49,65,66]

However, subsequent studies did not corroborate such findings and even suggested that *Lactobacillus* administration as a probiotic is contraindicated for critically ill patients because of the risk of fungemia.[56] This finding led to the development of therapy guidelines indicating the types of patient for whom FMT can be suggested.[67–69]

The US Food and Drug Administration and the international consensus conference on stool banking for FMT in clinical practice determined the criteria that donor patients must meet, that is, the absence of sexually-transmitted infection, intestinal disorders, and other risk factors along with disorders and drug use that could alter intestinal microbiota. The absence of microorganisms, such as CD, common enteric pathogens, such as *E. coli*, *Salmonella*, *Shigella*, and *Vibrio*, and antibiotic-resistant bacteria, such as vancomycin-resistant *Enterococcus* and methicillin-resistant *Staphylococcus aureus* must be guaranteed. There had to be lack of some viruses (i.e., norovirus and rotavirus), helminths and protozoa (i.e., *Blastocystis, Dientamoeba, and Giardia*) and microsporidia.[68,69]

Despite such advances, identifying the right combination for restoring intestinal microbiota that can be administered to patients without causing side effects remains a challenge for the scientific community. Although several studies have suggested different FMT combinations, a recent meta-analysis has shown that a decisive aspect concerning this treatment's effectiveness was related to its administration route and the amount used.[70] The aforementioned findings suggest that other intestinal ecosystem components could be involved in FMT success or failure, preparing the way for new studies assessing previously unexplored intestinal microbiota components, such as viruses and eukaryotes.

Archaea: small populations having a significant impact

Archaea are a large and diverse group of abundantly-distributed, single-celled, intestinal microorganisms; their variations in terms of abundance are related to geographical and ethnic factors.[71] This domain's role was poorly studied for some time because of its low abundance and such microbes were considered to have a low impact on microbiota homeostasis.[71] The main members of this group account for less than 2% of intestinal microbiota methanogenic microorganisms, microbes, such as *Methanobrevibacter*, and halophilic ones including *Haloferax* and *Halococcus*.[72]

Heterogeneous roles such as gut microbiota have been associated with Archaea; their role as probiotics has been mentioned because they can metabolize intestinal products that can be harmful to health; the term 'archaebiotics' is consequently the subject of ongoing research.[72,73] Hydrogen consumption is another role associated with this

Table 2. Microbiota changes caused by human protozoa.

Protozoa	Bacterial group altered	Effect	Study type	Ref.
Giardia lamblia	Beneficial bacterial groups	Induces alterations aggravating *Giardia*-associated symptoms	*In vitro* cell culture and germ-free murine infection model	82
Giardia lamblia	*Clostridium, Lactobacillus* and *Bacteroides* in canines *Prevotella* and *Gammaproteobacteria*	Increased bacterial diversity and beneficial groups. Decreased potential pathogenic bacteria	Cross-sectional and data mining	83
Entamoeba histolytica	*Prevotella copri*	Increased bacterial group induced colitis	*In vivo*	84,85
Entamoeba coli	*Akkermansia*	Increased beneficial bacteria could have led to establishing healthy microbiota	Cross-sectional	86
Blastocystis	*Enterobacteriaceae*	Increased microbial diversity and lower abundance of potential pathogenic bacterial group	Cross-sectional	87
Blastocystis	-	No differences between *Blastocystis*-infected and control groups	Cross-sectional metataxonomic	88
Blastocystis	*Bacteroides* and *Faecalibacterium*	No statistically significant differences in microbiota composition	Cross-sectional	89
Blastocystis	*Bacteroides, Prevotella* and *Ruminococcus*	*Bacteroides*-driven enterotype could protect against *Blastocystis* infection	Metagenomic	90
Blastocystis	*Bifidobacterium* and *Lactobacillus*	*Blastocystis* subtype 7 could induce alterations in beneficial bacterial groups	*In vitro* and *in vivo*	23
Blastocystis	*C. difficile*	Co-infection with both microorganisms suggested alternative mechanisms for *Blastocystis* adaptation	Cross-sectional	56

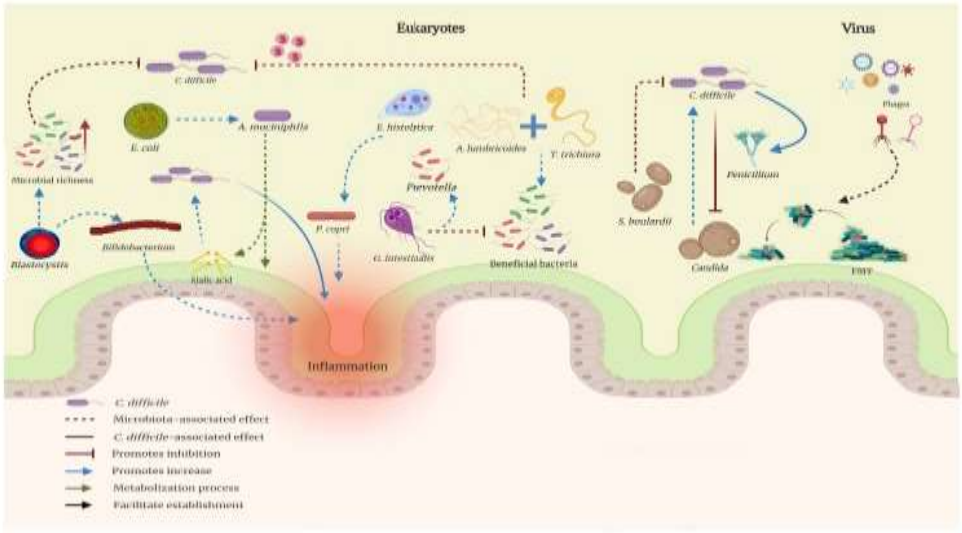

Figure 2. Interaction between different members of the eukaryotic and viral microbiota during *Clostridioides difficile* (CD) infection. *Blastocystis* has been associated with increased microbial richness, resulting in a state of protection against various intestinal diseases. It has also been associated with increased abundance of *Bifidobacterium*, a genus capable of triggering an increase in intestinal inflammatory activity. *Entamoeba coli* has been associated with an increased abundance of *Akkermansia muciniphila* and intestinal microbial structure comparable to that of healthy subjects. *E. histolytica* is associated with increased prevalence of *Prevotella copri* which is sometimes used as a biomarker for diarrheal disease in cases of amoebiasis and inflammatory bowel disease. *Giardia*. This protozoan has been associated with increased *Prevotella* prevalence and decreases in beneficial bacteria populations. Nematodes. Co-infection by *Ascaris lumbricoides* and *Trichuris trichiura* leads to increased abundance of beneficial bacteria; such change could lead to asymptomatic infection by other pathogens. Fungi. *Saccharomyces boulardii* has been associated with *in vivo* and *in vitro* CD growth inhibition. The opposite occurs with *Candida* (a genus that can enhance bacillus growth); however, it has also been shown that the bacillus inhibits the growth of different species of the fungus. *Penicillium* has been associated with increased CD, taking advantage of imbalance in microbiota caused by the bacillus. Virus. Although different viruses have been described as forming part of the intestinal microbiota (rotavirus, astrovirus, calicivirus, norovirus, hepatitis E virus, coronavirus, torovirus, and adenovirus predominating), their roles or intestinal interactions are unclear. Phages are associated with microbiota establishment after fecal microbiota transplantation.

domain's members; it could increase ATP availability generated by anaerobic bacteria, thereby creating an ideal setting for the growth of opportunistic populations and causing symptoms such as constipation.[72] It is worth highlighting *M. smithii*'s protective role regarding inflammatory bowel disease (IBD) due to its ability to produce short-chain fatty acids (SCFAs) which could act as CDI-related protectors.[6] An opposite effect has been observed for *Methanosphaera stadtmanae*, which is frequently associated with IBD.[61,74]

Despite this domain's low abundance, it has a great impact on gut microbiome as shown by studies regarding obesity, muscle abscesses, pneumonia, and urinary tract infection.[71] Research has shown that members such as *M. smithii* can play a protective role against CDI due to SCFA-associated mechanisms; the reduction in this Archaea's relative abundance has been related to diarrheal symptomatology and CD proliferation.[75,76] Inflammatory diseases associated with these populations' imbalance suggests so-far-unknown mechanisms, so future in-depth studies must ascertain microbiota components in CDI and other diseases.

Protists, helminths, and fungi: poorly explored territory?

Eukaryotic microorganisms residing in many vertebrate species' gastrointestinal tracts also affect host health/disease events; however, characterizing such microbiome components has lagged behind that for bacteria.[77] Some multicellular (i.e., helminths) and unicellular organisms (amoebae, some fungi, and certain protozoa) have been identified as members of the gastrointestinal microbiome. Many of these taxa have been investigated from a parasitological viewpoint for decades now, using microscopy and directed molecular approaches. It is thus considered that eukaryotic

microbiota diversity in the human intestine has not yet been systematically investigated from a community perspective.[78]

The relationship between eukaryotic microorganisms and intestinal diseases in humans has been established; however, recent advances have led to understanding that not all eukaryotes in the intestinal tract should be considered parasites as many of them increase bacterial diversity and interact with the immune system to prevent pathogens' intestinal colonization.[79] Although, the pathogenic roles of eukaryotic species have been documented (i.e., *Ascaris lumbricoides, Entamoeba histolytica, Cryptosporidium* spp. and *Strongyloides stercoralis*), recent evidence has suggested that other eukaryotic microorganisms commonly inhabiting the gastrointestinal tract may play significant ecological roles in intestinal homeostasis (i.e., *Blastocystis* and *Dientamoeba*).[78–81]

Blastocystis represents a special case deserving more detailed study because of contradictory research results (Table 2). Evidence of its pathogenic role regarding human health is extensive, as is the effect that it can have on intestinal microbiota. Colonization by this protozoan has been associated with healthy microbiota because increased intestinal microbiota diversity has been observed, together with less abundance of Enterobacteriaceae in *Blastocystis*-positive patients.[87] However, another study of irritable bowel syndrome (IBS) patients did not reveal differences regarding microbiota composition and diversity compared to that of healthy controls.[88] Recent research involving school-aged children in Colombia indicated that *Blastocystis* was accompanied by decreased abundance of *Bacteroides* and increased relative abundance of *Faecalibacterium*, although change in intestinal microbiota composition was not statistically significant.[89]

Metagenomic studies have attempted to resolve such contradictory findings. Andersen *et al.*, observed that people having *Bacteroides*-dominated microbiota were less prone to *Blastocystis* colonization than those whose microbiota was dominated by *Prevotella* and *Ruminococcus*.[90] These findings did not delve into the *Blastocystis* subtype involved in such infection. Some subtypes have been associated with intestinal manifestations, such as subtype 7; Yason *et al.*, described its impact on intestinal microbiota using *in vivo* and *in vitro* techniques. They observed negative effects on beneficial bacteria (i.e., *Bifidobacterium* and *Lactobacillus*) which could have led to microbiota dysbiosis, thereby facilitating the appearance of intestinal pathologies.[23]

Vega *et al.*, recently described *Blastocystis* in CDI patients, highlighting the adaptive mechanisms enabling this protozoan to survive in CD-related imbalance (Figure 2).[91] Vega *et al.*, (in other work) suggested that *Blastocystis* and CD co-occurrence could positively modulate intestinal microbiota, permitting increased beneficial bacteria abundance compared to patients without *Blastocystis*.[56] There were no differences between groups regarding eukaryotic microbiota abundance. This highlights the need to explore this important microbiota component because its impact remains unknown.

The helminths (generally considered pathogenic) have contributed to increased intestinal microbiota diversity which tends to disappear after therapy aimed at their removal;[92] as with protozoa, contradictory results have been found. A study of children in Ecuador co-infected with *Trichuris trichiura* and Ascaris *lumbricoides* observed decreases in Firmicutes abundance and reduced bacterial diversity, which did not occur in children only infected by *T. trichiura*,[93] denoting *A. lumbricoides* influence on intestinal microbiota modulation. Another study in Malaysia recorded increased bacterial diversity in samples from helminth-infected children and increased abundance of bacterial species belonging to the Paraprevotellaceae family in *T. trichiura*-infected individuals.[94] A study of celiac disease patients assessed the impact of *Necator americanus* infection; increased bacterial richness was observed.[95]

Such relationships suggest that hosts and parasites do not exist in an isolated manner but that they interact via co-evolution, enabling the balanced, co-existence of countless microorganisms in a niche benefiting all members, including the host[79] (i.e., a two-way relationship in which such interactions have a positive or negative impact on other members of the microbiota).[92] Such relationships could consequently explain CDI patients' clinical manifestations, since some eukaryotes' positive modulation of the microbiota could protect against inflammation and diarrhea, creating

a delicate balance resembling a healthy patient's microbiota.[35] It has been suggested recently that helminth infection could be a protective factor for CDI due to the type-2 immune response promoted in a host during such infection and eosinophil proliferation, which can reduce CD populations by still-unknown mechanisms.[96] Host immune response represents an interesting field of study having profound pathophysiological implications and even new therapeutic options for CDI as occurs with other inflammatory diseases, such as Crohn's disease and IBD where *Trichuris suis* use has been suggested as possible treatment.[97]

Intestinal mycobiome composition (microbiota fungal components) has been less extensively studied. The main mycobiome components have been identified as *Saccharomyces, Malassezia*, and *Candida* in a study involving a cohort of healthy patients; the role of *Candida* in CDI establishment and development, however, is not entirely clear because of conflicting results (Figure 2).[98–101] Some studies have found a correlation between great *C. albicans* abundance and reduced FMT efficacy,[102] while other research has recorded low *C. albicans* frequency in CD-colonized patients and described a probable protective role for *Candida* species overgrowth regarding CDI and its lethal effects.[103,104]

No evidence has been presented concerning the relationship of *Malassezia* with CDI; however, recent studies have suggested that intestinal conditions may promote its growth and colonization, which could lead to exacerbation of IBS symptoms.[105] The protective effect of *Saccharomyces boulardii* against colitis caused by CD has been demonstrated (i.e., one of the most common probiotics isolated from fruits); this is mediated by immunoglobulin A production (Figure 2).[106,107] Increased *Penicillium* abundance has been observed in CDI patients suggesting that intestinal fungal microbiota imbalance may contribute to CD (Figure 2).[55] However, no recent evidence has supported the role of this or any other fungi regarding CDI development.

The unexplored virome in CDI

The gut virome (defined as all viruses inhabiting the intestinal tract) consists of bacteriophages (phages) that infect bacteria, viruses that infect other cellular microorganisms (such as Archaea), eukaryotes (i.e., protozoa or human cells) and free viral particles as transients in food.[108] Such viruses (including DNA and RNA viruses) have become increasingly important regarding the gastrointestinal tract because of their contribution to microbial ecology, meaning that their diversity, abundance, and function in intestinal microbiota must be compared.[109]

A limitation of virome sequencing concerns suitable methods for purifying and enriching all ranges of virus-like particles from stool samples. There are also genome-related limiting factors (size and composition, especially regarding RNA viruses); the small percentage within microbial communities could result in underestimating their participation in such ecosystems.[110] These factors have led to standardizing sample processing methods, ascertaining evaluation of such particles' true representativeness and their characterization.[111]

Despite limitations, some studies have led to improving the intestinal virome's description; some representative groups have been identified, including double-stranded DNA and RNA viruses,[112–114] that could have an impact on microbial communities' modulation and consequent effects on host health. It is worth describing these particles' dynamics.[115]

Few CDI studies have focused on clarifying the relationship between viral microbiota and CD. CDI patients' intestinal virome was first characterized in 2018 in Asia (Table 1); a dysbiotic enteric virome was demonstrated in this study, mainly characterized by a decreased abundance of Microviridae family viruses.[116] Further studies were aimed at determining modifications and impact on the virome after FMT was used for CDI treatment. Several studies have shown that an intestinal viral core is specific for each donor's conditions despite modifications to the microbiota following FMT; this is characterized by a decrease in Caudovirales, though retaining a phage profile similar to that of unaltered individuals. This has suggested that this component contributes to the long-term establishment of donor microbiota (Table 1, Figure 2).[116–118]

The aforementioned research strikingly indicated that although phages are markers of inflammation, their abundance does not vary between donors and recipients. Future research must thus delve into the role played by these microbiota components. Phages' beneficial role has also been

observed regarding metabolism, motility regulation, and maintenance of the intestinal barrier against pathogens;[119–121] regardless of such evidence, this topic has not been extensively studied. Some viruses' ability to infect *Entamoeba* and *Giardia* (highly prevalent parasites worldwide) have been demonstrated recently;[122] however, such findings' impact must be clarified. This data supports the need to assess other intestinal ecosystem components' roles and the resulting interactions between members of the different kingdoms and their health–disease-related implications.

Interdomain complexities: a holistic view of intestinal ecosystem CDI

The intestinal ecosystem should not be viewed or analyzed as a sum of isolated components; rather, it should be understood as a complex network of interactions among its different elements. Established and speculated relationships between gastrointestinal microbiota members highlight the microbiome's complexity (Figure 2). Concerning CDI, evidence has been presented regarding interactions among the domains inhabiting the intestinal ecosystem, including interdomain communication pathways mediated by signaling molecules, such as indoles;[123] metabolites produced by some members of the microbiota promoting other members' survival has been highlighted. SCFAs represent one relevant example based on evidence of their use by both bacteria and eukaryotes using them as energy supplies.[124]

This complex interaction fulfills energy needs/functions; intestinal microbiota members' ability to modulate host immune response has also been shown, suggesting asymptomatic infections (as observed for some protozoa) or symptom exacerbation (as observed in IBS).[84,85,125] The above is especially important as intestinal microbiota can maintain a delicate balance with the mucosa's immune system by regulating antigen presentation, thereby enabling/ensuring the survival of many of this ecosystem's inhabitants.[126,127] This balance can be affected by many factors, such as parasites able to modulate the immune response thereby activating mechanisms (such as the inflammasome) ultimately exacerbating intestinal inflammatory symptoms due to commensals being recognized as foreign agents.[22] This also occurs for microorganisms directly affecting the intestinal mucosa, resulting in the release of immune system cells and pro-inflammatory molecules interacting with usual microbiota members. They consequently become targets for an aberrant immune response.[128,129]

The gastrointestinal ecosystem's complex relationships must be comprehensively explored for a better understanding of the findings. Antibiotic-associated diarrhea is a clear example of this as the fragmented study of a complex network of relationships and interactions does not provide a complete picture of the disease's pathophysiology. This constitutes a challenge for future research aimed at covering as many components of the intestinal ecosystem as possible (microbiome, metabolome, and interactome) and replicate its conditions in the most reliable manner possible to ensure obtaining accurate results to improve the health of millions of people worldwide. This challenge implies understanding the imbalance in microbiota that occurs during CDI from many perspectives, including biotic components and immunological and molecular factors that may be involved in the disease. Future studies focusing on these factors should lead to complete understanding of the phenomenon.[82,83,86]

Conclusions

Intestinal microbiota members' effects on homeostasis and diseases are highly variable and even contradictory. Many difficulties related to studying microbiota in relation to CDI arise from the impossibility of controlling the confounding factors, along with the approach used for conducting these studies. Such approaches usually examine small groups of microbiota members of a complex and constantly changing ecosystem. The microbiota is increasingly presented as a complex ecological niche of constantly evolving interactions, which must be reconsidered regarding its study and analysis. New perspectives must enable a vision encompassing most, if not all, of the parts encompassing the intestinal microbiota.

Most microbiota–CDI research has been limited to examining the role of bacteria in relation to CDI establishment and development. Although much of the knowledge regarding the disease's pathophysiology is derived from such studies, large gaps remain regarding a complete and multifactorial

understanding of intestinal imbalance because of the role played in intestinal diseases by other ecosystem elements. Although their role was assumed to be practically nil, current evidence has indicated that they could be main actors and even protagonists as noted in other intestinal diseases. Further studies are thus required to examine the roles of the different elements involved to enable a better approach to CDI.

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CAPÍTULO 2. "Identificación de otros componentes del microbioma intestinal (virus de ADN y conjunto de genes circulantes) mediante metagenómica"

Una vez obtenida la descripción de las comunidades microbianas del ecosistema intestinal mediante secuenciación profunda de marcador único, se procedió a determinar los demás componentes del microbioma intestinal, como son los virus de ADN, los factores de virulencia y los marcadores de resistencia, profundizando en los análisis funcionales. Para esto, se seleccionaron 48 de las 98 muestras previamente analizadas y fueron sometidas a secuenciación metagenómico (Illumina pair end, 4 Gb/muestra, 150 pb). Los datos producidos fueron analizados tanto para la composición taxonómica como para las características funcionales mediante un esquema de análisis bioinformático previamente establecido. Finalmente, se realizaron ensamblajes de genomas a partir de los datos metagenómicos, los cuales fueron analizados en función de la presencia/ausencia de los genes relacionados con el metabolismo de los ácidos grasos de cadena corta. Se observaron alteraciones taxonómicas principalmente en los grupos asociados con diarrea adquirida a nivel intrahospitalario, caracterizadas por disminución de bacterias benéficas, principalmente productoras de butirato. Se identificó un set de 51 especies diferencialmente abundantes, las cuales permitieron la agrupación espacial de acuerdo con los grupos de estudio. Adicionalmente se identificaron diferencias en la abundancia relativa de genomas ensamblados a partir de metagenómica (MAGs) con potencial para el metabolismo del acetato.

Como productos de este capítulo se generaron 2 artículos que se listan a continuación:

Artículo 5. Herrera, G., Arboleda, J. C., Pérez-Jaramillo, J. E., Patarroyo, M. A., Ramírez, J. D., Muñoz M. (2022). Microbial interdomain interactions delineate the disruptive intestinal homeostasis in *Clostridioides difficile* infection. Microbiol Spectr. 10(5), e00502-22

Artículo 6. Herrera, G., Castañeda, S., Arboleda, J. C., Pérez-Jaramillo, J. E., Patarroyo, M. A., Ramírez, J. D., Muñoz M. Metagenome-assembled genomes (MAGs) suggest an Acetate-driven protective role in gut microbiota disrupted by *Clostridioides difficile.* (Sometido)

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RESEARCH ARTICLE

Microbial Interdomain Interactions Delineate the Disruptive Intestinal Homeostasis in *Clostridioides difficile* Infection

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ABSTRACT *Clostridioides difficile* infection (CDI) creates an imbalance in the intestinal microbiota due to the interaction of the components making up this ecosystem, but little is known about the impact of this disease on other microbial members. This work has thus been aimed at evaluating the taxonomic composition, potential gene-associated functions, virulence factors, and antimicrobial resistance profiles of gut microbiomes. A total of 48 DNA samples obtained from patients with health care facility-acquired (HCFO) and community-onset (CO) diarrhea were distributed in the following four groups according to CDI status: HCFO/+ (n = 13), HCFO/– (n = 8), CO/+ (n = 13), and CO/– (n = 14). These samples were subjected to shotgun metagenomics sequencing. Although the CDI groups' microbiota had microbiome alterations, the greatest imbalance was observed in the in the HCFO+/– groups, with an increase in common pathogens and phage populations, as well as a decrease in beneficial microorganisms that leads to a negative impact on some intestinal homeostasis-related metabolic processes. A reduction in the relative abundance of butyrate metabolism-associated genes was also detected in the HCFO groups ($P < 0.01$), with an increase in some virulence factors and antibiotic-resistance markers. A set of 51 differentially abundant species in the groups with potential association to CDI enabled its characterization, leading to their spatial separation by onset. Strong correlations between phages and some archaeal and bacterial phyla were identified. This highlighted the need to study the microbiota's various components since their imbalance is multifactorial, with some pathogens contributing to a greater or lesser extent because of their interaction with the ecosystem they inhabit.

IMPORTANCE *Clostridioides difficile* infection represents a serious public health problem in different countries due to its high morbi-mortality and the high costs it represents for health care systems. Studies have shown the impact of this infection on intestinal microbiome homeostasis, mainly on bacterial populations. Our research provides evidence of the impact of CDI at both the compositional (bacteria, archaea, and viruses), and functional levels, allowing us to understand that the alterations of the microbiota occur systemically and are caused by multiple perturbations generated by different members of the microbiota as well as by some pathogens that take advantage of the imbalance to proliferate. Likewise, the 51 differentially abundant species in the study groups with potential association to CDI found in this study could help us envisage future treatments against this and other inflammatory diseases, improving future therapeutic options for patients.

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Clostridioides difficile (a Gram-positive bacillus) is considered the main pathogen causing health care-associated infections in countries worldwide; 15% to 45% infection frequency has been described regarding community-acquired/onset and hospitalized patients, leading to more than 25,000 deaths annually and multimillion-dollar costs for health systems (1–5). *Clostridioides difficile* infection (CDI) can produce multiple alterations in the intestinal microbiota of patients suffering from it; patient state is aggravated by many factors, such as age, antibiotic use, and other comorbidities (6–11). Such alterations occur more frequently in an intrahospital setting where patients are exposed to many therapies associated with their delicate state of health, leading to an adverse effect on intestinal ecosystem equilibrium and thereby facilitating some pathogens' growth and proliferation (11–13).

Recent studies have shown that *Faecalibacterium*, *Dorea*, and *Lachnospira* bacterial genera become reduced during CDI, as well as some prokaryotic archaea associated with protection against the disease (9). This has been accompanied by an increase in pathogens from the phylum *Pseudomonadota* (9–11, 13–15) and an increase in *Candida*, *Malassezia*, and *Blastocystis* (16–19). Such increase in pathogen populations creates suitable conditions for CDI maintenance and recurrence (7, 9, 13); this creates an ideal ecosystem for *C. difficile* development and proliferation due to a lack of commensal *Pseudomonas* able to produce short-chain fatty acids (SCFA) and secondary bile acids, leading to the exacerbation of symptoms and even death (13, 20).

Shotgun metagenomics sequencing, combined with other tools such metabolomics and metatranscriptomics, has enabled the detailed characterization of changes and relationships in the intestines of patients suffering inflammatory bowel diseases (IBD), such as Crohn's disease (CD), ulcerative colitis (UC), irritable bowel syndrome (IBD), and colorectal cancer (CRC). Such an approach highlighting taxonomic, functional, and biochemical alterations has enabled the identification of biomarkers for such diseases' diagnosis and treatment (21, 22). Most CDI studies have focused on delving into the taxonomic differences produced by *C. difficile*; this has led to some microorganisms being selected which have potential therapeutic use due to their protective role against CDI, as well as to exploring differences regarding fungal taxa abundance (10, 11, 23, 24).

However, studies concerning CDI-related intestinal microbiota disruption do not account for relationships among all the domains represented by a host's wild intestinal ecosystem. This results in a lack of understanding about the complex processes associated with such disruption, highlighting the need for focusing on the study of microbiomes and considering a broader range of elements making up such ecosystems for improving our understanding of what happens regarding CDI.

This study used shotgun metagenomics for determining the composition of microbial communities (archaea, bacteria, and viruses), their functional profiles, and the relationships between the members of the microbiota and intestinal virulence- and antibiotic-resistance-associated molecular markers in patients suffering community-onset (CO) and HCFO CDI-associated diarrhea, compared to CDI-free diarrheal patients. Taxonomic composition profiles were found which agreed with those described in the pertinent literature, along with sets of characteristic differentially abundant species in the groups with potential association to CDI. Some metabolic processes' functional profiling highlighted certain *Archaea* and *Faecalibacterium* species' potential role in butyrate metabolism and oxidoreduction. Each group's virulence and resistance profiles were determined; this led to increasing knowledge about the changes in microbial ecology potentially associated with CDI and improving a therapeutic approach to CDI patients.

RESULTS

The study groups presented differentially abundant bacterial and archaeal species. Samples were grouped according to previously defined groups for highlighting differences in terms of taxonomic composition; an average of 16.4 million reads were obtained

TABLE 1 General statistics of taxonomical assignment of shotgun metagenomic reads

			Data for bacteria			Data for viruses			Data for archaea		
Group	Total reads	No hits (%)	%	Mean	SD	%	Mean	SD	%	Mean	SD
CO/–	197,797,349	51	48	6,792,810	3,571,867	0.07	10,232	12,420	0.04	5,463	3,813
CO/+	213,065,122	53	47	7,640,430	2,404,422	0.20	39,495	109,192	0.05	7,414	12,654
HCFO/–	139,362,722	22	76	13,183,284	3,249,136	2.00	291,200	756,346	0.02	3,209	5,041
HCFO/+	214,001,657	26	71	11,732,144	5,394,188	0.40	66,681	149,711	0.02	2,531	3,504

per sample (>33 Phred score). After eliminating host sequences, 15.9 million reads per sample were obtained, with the *Bacteria* domain being the most abundant (47% to 76%) (Table 1). The large number of unidentified sequences (no hits) in all groups (22% to 53%) was striking; there were more in the CO groups (Table 1). The similarities between HCFO groups are worth noting, as they had lower percentages of unidentified sequences and similar relative frequency for each taxonomic group found, characterized by a high percentage of bacteria.

The bacterial population composition in each group described by 16S-rRNA marker reads had different profiles for each group. *Bacteroides*, *Lachnospira*, and *Oscillospira* dominated in the CO/– and CO/+ groups (Fig. 1A), while *Enterobacteriaceae* and *Pseudomonas* increased in the HCFO/ and HCFO/+ groups (Fig. 1A).

The relative abundance of differentially abundant species identified by metagenomic sequencing had characteristic patterns (Fig. 2). For instance, we highlight a marked increase in common pathogens such as *Klebsiella pneumoniae*, *Enterobacter cloacae*, and *Klebsiella variicola*, along with an overall increase of *Pseudomonadota* phylum-related reads in the CO/+ group (Fig. 1B, green box). Few beneficial species accompanied by *Eggerthella lenta* were found in the HCFO/– group (Fig. 1B, blue boxes). The CO/– and CO/+ groups were characterized by greater diversity of members which have been associated with a beneficial profile; species from the *Bacteroidota* and *Bacillota* phyla were found, such as *Odoribacter splanchnicus*, *Bacteroides uniformis*, *Roseburia faecis*, and *Roseburia inulinivorans* (Fig. 1B). On the other hand, *Akkermansia muciniphila* was a species with a high relative abundance in all evaluated groups.

The analysis of differentially abundant species revealed 51 bacterial and archaeal species in the groups and the absence of up to 80% of the microorganisms described in 5/8 HCFO/– group samples (Fig. 2A, blue box). Differentially abundant species-based principal-coordinate analysis (PCoA) showed that both the HCFO (R^2 = 0.12289, P = 0.001) and CO groups (R^2 = 0.07584, P = 0.001) tended to cluster separately (Fig. 2B and C).

External validation of differentially abundant species in the studied groups with potential association with CDI. Analysis of compositions of microbiomes with bias correction (ANCOM-BC) allowed us to deepen into the species with a relative differential abundance in the CDI+ groups (HCFO/+ and CO/+) to determine which microorganisms had a potential association with the presence of *C. difficile*. The ANCOM-BC was performed on the study samples and displayed 48 species with a differential abundance. Some of these species had been previously described in the MetaPhlAn analysis (see Fig. S1 in the supplemental material). We carried out a validation of these differentially abundant species with a potential association with CDI by employing 27 publicly available samples belonging to two different studies, which we analyzed separately. Initially, the five samples belonging to the study of Milani et al. (25) reported less than 1 million reads per sample, whereas the 22 samples belonging to the study of Verma et al. (26) ranged from 23 to 34 million reads per sample. The ANCOM-BC performed on the data set of Milani et al. along with the negative samples of the present study indicated a total of 13 differentially abundant species. In contrast, the same analysis carried out on the data from Verma et al. along with the negative samples of our study yielded 52 differentially abundant species (Fig. S1). *Clostridium clostridioforme* was identified as the common differentially abundant species for the three data sets (Herrera [this study], Milani et al. [25], and Verma et al. [26]) in the

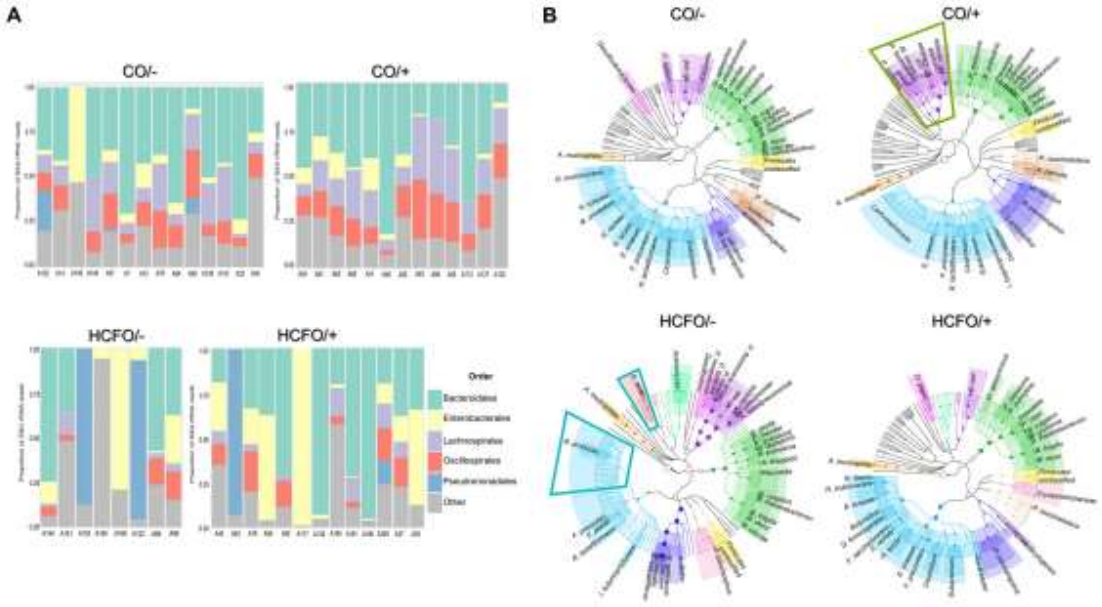

FIG 1 Taxonomic composition of HCFO and CO patients' gut microbiome. (A) Reconstruction of taxonomic composition of bacterial orders following extraction of 16S gene reads using shotgun metagenomics data. (B) Dendrograms created using metagenomic phylogenetic analysis (MetaPhlAn) identifying differentially abundant bacterial species and showing characteristic species distribution in each group studied here. Colors were used for facilitating comparison of members from the same bacterial family in the study groups.

samples positive for CDI. We found four different bacterial and archaeal species in Milani et al. and the other studies (two Milani and Herrera, two Milani and Verma), whereas we found six differentially abundant species herein and in the data set of Verma et al. (Fig. S1).

Viral populations did not display differences between groups. Viral communities accounted for 0.07% to 2% of all reads from the different groups, with the HCFO/– group having the highest percentage of these microorganisms (Table 1) (no statistically significant differences). Characteristic viral community profiles were observed in each group; IAS virus and *Faecalibacterium* phages predominated in the CO/– group, representing a third (33%) of this group's viral populations, while the CO/+ group composition was characterized mainly by members of the Siphoviridae family (80% of the reads identified as virus), and *Bacteroides* phages were the most abundant (Fig. S2). There was an increase in Siphoviridae and Autographiviridae family members in the HCFO/– group (62% of viral sequences), accompanied by a relatively high abundance of *Klebsiella* phages, coinciding with the previously described differentially abundant species composition. *Enterobacter* phages such as those for *Escherichia* and *Enterococcus* dominated in the HCFO/+ group. However, such differences between viral families and species when comparing groups, onset, and CDI state were not statistically significant.

Bacterial and viral populations depicted a strong correlation. Cooccurrence networks between viral families and archaeal and bacterial phyla revealed differences between groups (Fig. 3). Interestingly, the CO/– and HCFO/+ groups had fewer correlations, all being inverse ($\rho < -0.75$) in the group associated with intrahospital onset. For this type of onset, we observed an inverse proportional relationship between the abundances of some phages of enterobacteria, as well as other viruses with various bacterial families. The CO/+ group had numerous correlations, mainly between the Siphoviridae family and different bacterial and archaeal families, indicating the importance of this phage family and the wide range of hosts it can infect. Complex negative correlations were found in the HCFO/– group between viral families such as Picornaviridae and Microviridae with the same bacterial phylum such as Fusobacteria. A direct correlation between Siphoviridae

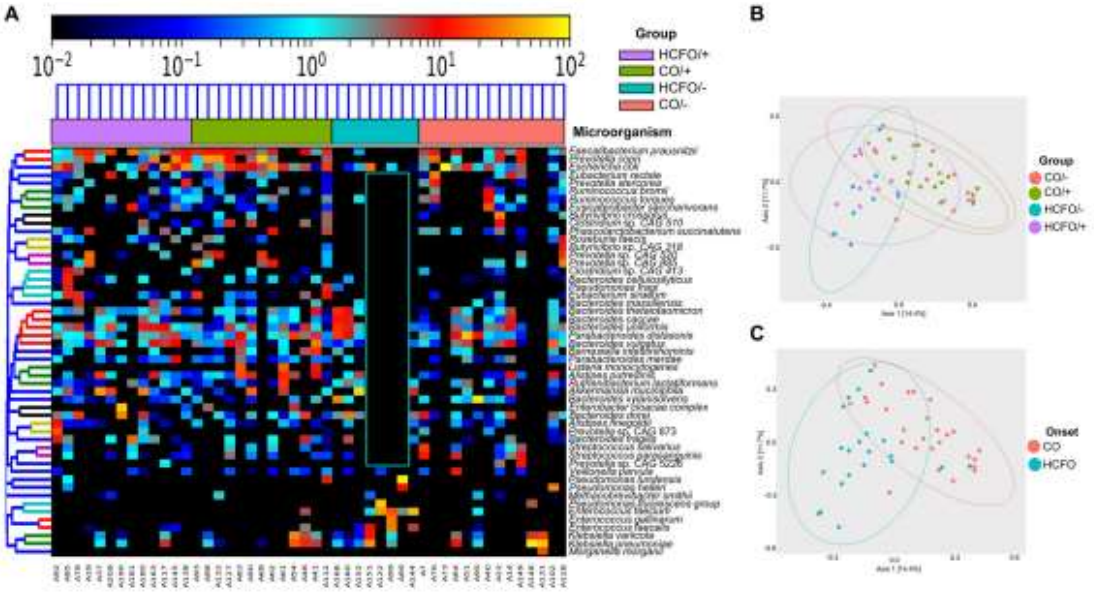

FIG 2 CDI and non-CDI diarrheic patients' differentially abundant bacterial and archaeal species contribute to special differentiation. (A) Heatmap of 51 differentially abundant archaeal and bacterial species found in the study groups. (B) Principal-coordinate analysis (PCoA) based on the 51 differentially abundant species found in the study, showing the samples' spatial separation for each group. (C) Principal-coordinate analysis (PCoA) showing sample separation according to onset.

and Adenoviridae with many archaeal and bacterial phyla was observed. Likewise, the complex correlations between the different families of *Archaea*, *Bacteria*, and viruses suggest an interaction between the domains, which may play a relevant role in the development of various diseases (Fig. 3).

Metabolic pathways exhibited no differences between groups. Multivariate analysis of the samples' functional profiles revealed differences between the groups regarding the genes associated with 17 pathways; 5 were related to biological processes, and the other 12 were related to metabolic functions (Table 2). There were marked differences between the CDI-positive and -negative community groups compared to the HCFO/– group, as there were statistically significant differences concerning all the genes (Table 2). Analysis of butyrate metabolism proved interesting due to its potential impact on CDI's natural history; there was an increase in bacteria contributing to such metabolic processes, mainly in the CO groups, accompanied by a reduction of all microorganisms potentially associated with butyrate metabolism in the HCFO/– group (Figure 4a; *P* values reported in Table 2).

Statistically significant differences were observed when comparing relative abundance between groups regarding genes and onset (Fig. 4B, Table 2). There were statistically significant differences regarding the microorganisms involved in such metabolic processes between groups and onset, i.e., *Coprococcus comes* (Kruskal-Wallis chi-squared value = 15.477, $P = 0.001451$; W = 422, $P = 0.0004825$), *Flavobacteria bacterium* (Kruskal-Wallis chi-squared value = 7.8338, $P = 0.04957$; W = 366.5, $P = 0.02415$), and *Gemmiger formicilis* (Kruskal-Wallis chi-squared value = 17.658, $P = 0.0005173$; W = 460.5, $P = 0.00008817$) (Fig. 4B and C).

Differences were found regarding the metabolic process associated with oxidoreductase activity concerning the abundance of genes associated with such processes between groups (Kruskal-Wallis chi-squared value = 12.542, $P = 0.005739$) and onset (W = 446.5, $P = 0.0007032$) (Fig. S3A). Statistically significant differences were found regarding the contribution of *Faecalibacterium prausnitzii* to this process in the HCFO groups (Kruskal-Wallis chi-squared value = 14.22, $P = 0.00262$) (Fig. S3B).

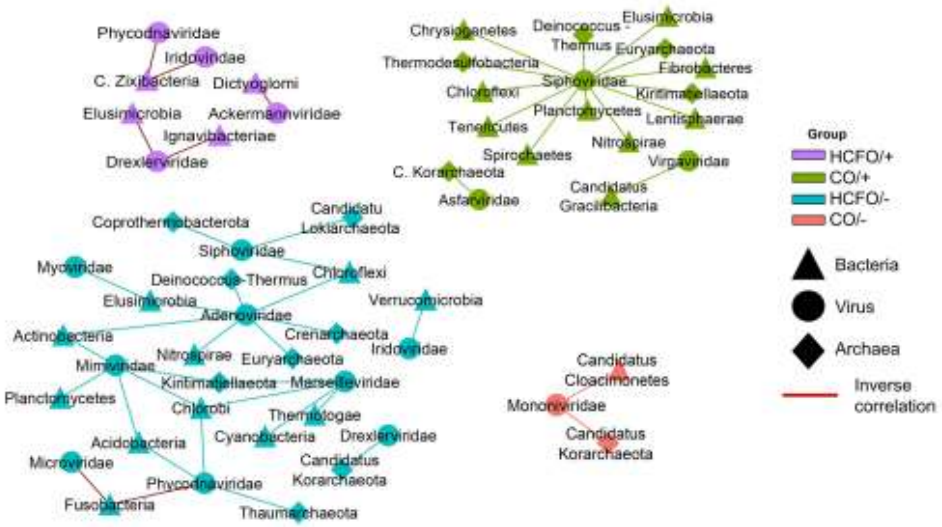

FIG 3 Positive and negative correlations between intestinal microbiota members from the patients being evaluated. Cooccurrence networks are shown for each study group with correlations higher than 0.75 and lower than −0.75.

***C. difficile* infection-positive groups had increased virulence factors.** Analysis of virulence factors (placing special emphasis on toxins) revealed an increase in toxin-related genes in CDI-positive groups, especially intrahospital-related ones (Fig. 5A), with the *Escherichia coli* toxins (*astA*) heat-stable enterotoxin 1 ($W = 405$, $P = 0.003821$) and (*rtxB*) RTX toxin transporter, and ATPase protein ($W = 378.5$, $P = 0.03206$) being different. *C. difficile*-encoding virulence factors, which were only found in the HCFO/+ and CO/+ groups, were analyzed (Fig. 5B). It was noted that the HCFO/+ group had the largest number of these virulence factors, including toxin A- and B-related genes which were not found in the CO/+ group.

The HCFO groups presented multiple antibiotic resistance genes compared to the other groups. Genomic and plasmid analysis of antibiotic-resistant genes revealed that the HCFO groups had more antibiotic resistance marker (ARM) reads, especially in the HCFO/+ group (not statistically significant) (Fig. 6). Analyzing ARM genomic composition (Fig. 6A) revealed that most markers were fluoroquinolone-resistant ARMs; however, there were no statistically significant differences regarding any of the ARMs analyzed, while statistically significant differences were observed when grouping samples according to CDI state between the percentages of ARM-encoding genes associated with aminoglycoside resistance ($P = 0.0096$), with CDI-positive groups having the largest number of these markers.

Plasmid ARMs were mostly cephalosporin- and aminoglycoside-resistant markers (Fig. 6D). An increase in the relative abundance of cephalosporin-specific ARMs was observed in the CDI-positive patient group ($P = 0.0224$). Differences were consistent with genomic findings, as no statistically significant differences were observed regarding the other ARMs (Fig. 6E and F).

DISCUSSION

The study groups' bacterial taxonomic composition (Fig. 1A) agreed with the information obtained by previous deep sequencing of a single 16S rRNA gene marker in a set of samples which included those analyzed in this study (9). Analysis of some 16S-rRNA hypervariable regions enabled precise characterization of the bacterial populations and accounted for *C. difficile*'s negative impact on beneficial bacterial populations (Fig. 1). This impact has been observed in many studies (10, 14, 15, 25, 27); it has been

TABLE 2 *P* values for multiple comparisons of potential gene-associated functions

Feature	Process	CO/− vs CO/+	CO/− vs HCFO/−	CO/+ vs HCFO/−	CO/− vs HCFO/+	CO/+ vs HCFO/+	HCFO/− vs HCFO/+
Butyrate metabolic process	Biological process	0.468	*0.002*	*0.000*	*0.040*	*0.008*	0.186
Cell wall assembly	Biological process	1.000	*0.001*	*0.000*	0.207	0.174	*0.022*
D-ribose catabolic process	Biological process	0.916	*0.002*	*0.002*	*0.007*	*0.008*	0.394
Regulation of apoptotic process	Biological process	0.425	*0.006*	*0.031*	0.120	0.346	0.152
Response to oxidative stress	Biological process	0.801	*0.001*	*0.001*	*0.013*	*0.010*	0.242
Acetone carboxylase activity	Metabolic function	0.452	*0.005*	*0.001*	0.476	0.174	*0.035*
Aryl-alcohol dehydrogenase (NAD+) activity	Metabolic function	0.956	*0.003*	*0.002*	*0.006*	*0.007*	0.417
Glucose-6-phosphate dehydrogenase activity	Metabolic function	0.600	*0.002*	*0.001*	0.215	0.100	*0.046*
Glutamate synthase (ferredoxin) activity	Metabolic function	0.682	*0.004*	*0.002*	*0.012*	*0.005*	0.437
Glycerophosphoinositol glycerophosphodiesterase activity	Metabolic function	0.997	*0.017*	*0.010*	0.062	0.051	0.331
Nonmembrane spanning protein tyrosine phosphatase activity	Metabolic function	0.952	*0.005*	*0.010*	0.189	0.222	0.122
Oligosaccharide reducing-end xylanase activity	Metabolic function	0.534	*0.006*	*0.002*	*0.022*	*0.005*	0.408
Oxidoreductase activity, acting on iron-sulfur proteins as donors	Metabolic function	0.989	*0.026*	*0.014*	0.068	0.054	0.384
Peptide-methionine (S)-S-oxide reductase activity	Metabolic function	0.501	*0.002*	*0.001*	0.482	0.206	*0.019*
Phosphatidylinositol-4-phosphate binding	Metabolic function	0.705	*0.002*	*0.004*	0.498	0.674	*0.013*
Tyrosine decarboxylase activity	Metabolic function	0.945	*0.045*	*0.030*	0.296	0.212	0.243
Uridylyltransferase activity	Metabolic function	0.484	*0.008*	*0.002*	*0.043*	*0.009*	0.336

described as altered microbiota regarding ecosystem balance, having reduced *Bacteroidota* and *Bacillota* phyla (11, 13), probably due to the administration of antibiotics, thereby producing an increase in inflammatory processes facilitating the proliferation of pathogenic bacteria from the *Pseudomonadota* phylum. This would sustain patients' adverse conditions, leading to a recurrence of CDI and making them susceptible to other infections (11).

The changes between taxonomic groups in this study could show that the CDI-related microbiota imbalance arises from the relationships between intestinal ecosystem elements, i.e., not being modulated by an isolated member. Evidence of this is the impact (even at the metabolic level) of relationships established between different beneficial markers, such as *Odoribacter*, *Faecalibacterium*, and *Roseburia* in community-associated groups. (Fig. 1B and Fig. S3); this is related to a positive influence on intestinal ecosystem homeostasis, bearing in mind the ability to produce SCFA (i.e., butyrate), which has been associated with triggering inhibitory signals concerning the expression of proinflammatory cytokine transcription factors creating an environment with low inflammation levels (28–33).

A reduction in the amount of these beneficial microorganisms has an impact on intestinal microbiota; this is taken advantage of by common pathogens such as *Pseudomonas*, *Morganella*, *Klebsiella*, and *Enterococcus* (as observed in the intrahospital groups: Fig. 1B), which have been associated with inflammatory states and the worsening of patients' states in other studies, thereby hampering their clinical and therapeutic management (34–37). The presence of other microorganisms such as *Eggerthella lenta* in the HCFO/− group (Fig. 1B) suggested a negative effect on intestinal microbiota, which has been associated with inflammatory diseases such as colitis and other complications such as bacteremia, even though its mechanisms of pathogenicity are poorly understood (38–41).

The observed profiles of the differentially abundant species with potential association with CDI between the data sets may be due to clinical and sociodemographic factors. Due to the absence of clinical data from our samples, we could not establish comparisons at this level with the other data sets. Thus, it is relevant to highlight that the absence of the factors here and the technical differences (sequence length and amount) represent the principal limitations of this investigation. Therefore, there is a need to deepen both the results obtained here and future comparisons based on clinical and population data. Consequently, this information will allow a more in-depth evaluation of the potential associations between CDI and microorganisms that contribute to the imbalance of the intestinal ecosystem, which occurs in CDI and other inflammatory pathologies. Moreover, the impact of individual and temporal variations on the intestinal microbiota composition (42) hinders the extrapolation of the

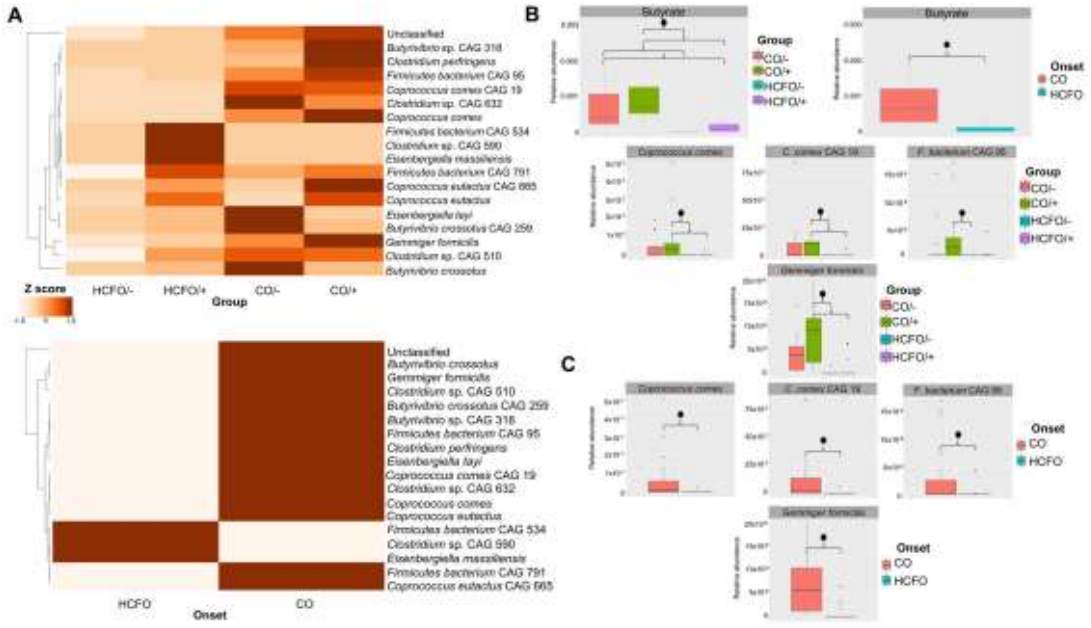

FIG 4 Loss of diversity regarding butyrate metabolism-associated bacteria in HCFO patients. (A) The contribution of each microorganism species in the samples by group and onset. (B) Comparison of the bacterial species contributing to butyrate metabolism in the study groups. (C) Box plots showing statistically significant differences between some species involved in butyrate metabolism by onset.

results to studies carried out in diverse populations such as European and American ones. For this reason, it is fundamental to increase the data at a regional scale to obtain more accurate comparisons that lead to promising results in the management and treatment of CDI.

Despite the lack of differences between the groups' viral communities' taxonomic composition, the cooccurrence networks indicated direct correlations in most groups (Fig. 3); this could have been related to the viral lysogenic cycle, suggesting provirus-related phage populations and that their increase resulted from an increase in *Bacteria* and *Archaea* populations which they infected. Recent reports suggest that this could have arisen from a reduction in available nutrients due to phage ability to obtain information from inside a host cell regarding the metabolic activity of the bacterial populations they infect, i.e., for determining whether such conditions might promote phage proliferation (43, 44).

Similarly, a model of interaction between phage P22 and *Salmonella enterica* serovar Typhimurium led to identifying subpopulations which were provisionally resistant to phage infection, enabling phage production without leading to a reduction of host populations (45). However, further studies are required for demonstrating the impact of such relationships, since little is known about the switch between lytic and lysogenic cycles in the intestinal microbiota.

The reduction of butyrate metabolism-associated genes found in this study, mainly in intrahospital groups (Fig. 4), was an extremely relevant finding, as this metabolite contributes to intestinal homeostasis regarding immune and inflammatory response modulation, intestinal barrier formation, and maintenance of colonocyte energy metabolism (28, 46). Such a reduction might be related to a deterioration in HCFO patients' condition compared to that of CO patients; this highlights the importance of controlling the intestinal microbiota balance for patients' gradual improvement.

The broad variety of microorganisms associated with butyrate metabolism found in all groups studied (Fig. 4) could have resulted from a broad group of commensal and

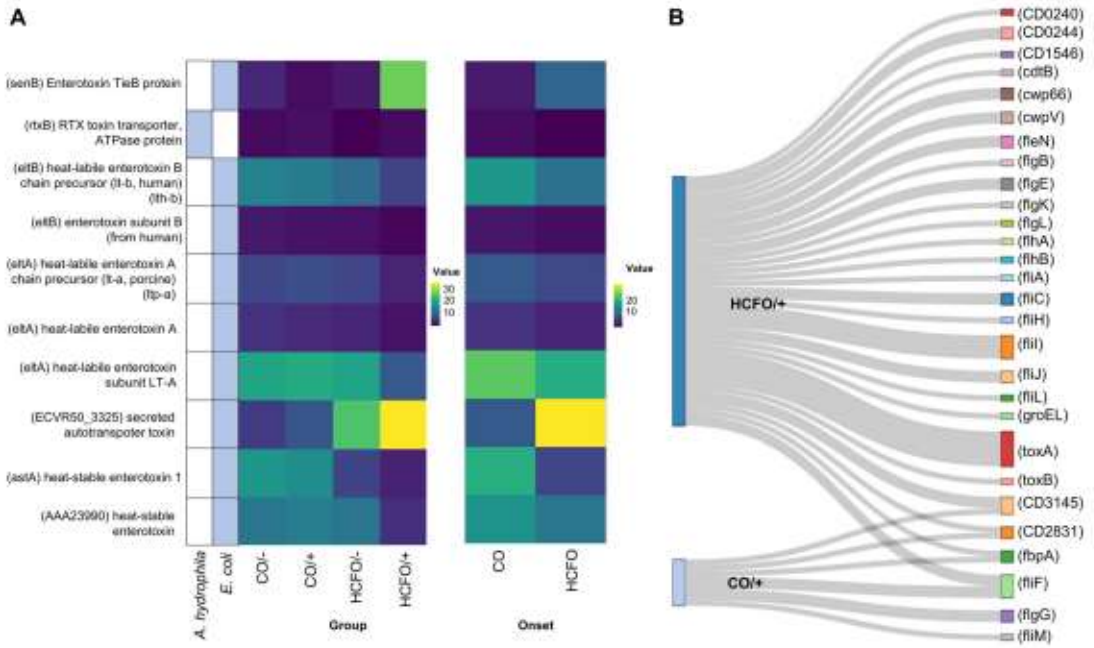

FIG 5 Toxins and virulence factors are more related to HCFO groups. (A) Heatmap of the toxins and associated microorganisms found in each group. (B) Distribution of *C. difficile*-specific virulence factors found in the groups positive for such microorganisms.

pathogenic bacteria's ability to produce this metabolite from different substrates (47). The forgoing is very important due to butyrate's many benefits regarding intestinal homeostasis and lipid and carbohydrate metabolism (46, 48–50), meaning that it must be maintained within the intestinal ecosystem for promoting microbiota equilibrium.

Virulence factor analysis revealed an increase in the genes encoding *Escherichia coli*-associated toxins in the HCFO/– group, mainly the secreted autotransporter toxin (Sat) (Fig. 5A) inducing cell damage during enteroaggregative infection by this microorganism (51), which could trigger complications for patients in this group. It is worth stressing the increase in sequences identified as *C. difficile* virulence factors in the HCFO/+ group compared to the CO/+ group (Fig. 5B). The HCFO/+ group had a higher degree of microbiota imbalance, which would have provided suitable environmental conditions for pathogenic microorganism proliferation and the transfer of genes playing an important role regarding health (52). This would support the hypothesis that the presence of *C. difficile* along with the imbalance caused in the microbiota produced by an increase in virulence factors leads to a worsening of patients' health-related conditions.

It is also worth noting that antibiotic administration could contribute to eliminating bacterial populations; this would create disturbances in their equilibrium due to an impact on many members' diversity and abundance, in turn contributing to the development of resistance to antibiotics among members of the microbiota by acquiring genes from the environment and other bacteria (53), representing a threat to public health. Factors which could be related to determined ARMs must thus be identified, as in this study the ARMs where identified in HCFO group (Fig. 6); however, future studies are needed to identify the factors that could be related to its presence in this population in developing countries as Colombia. The available works that have analyzed antimicrobial resistance in HCFO have provided an association between the environment of the patients and the multiple treatments to which they are subjected due to the diseases they suffer from (54, 55). Chromosome and plasmid resistance markers' differential patterns (Fig. 6) reveal the

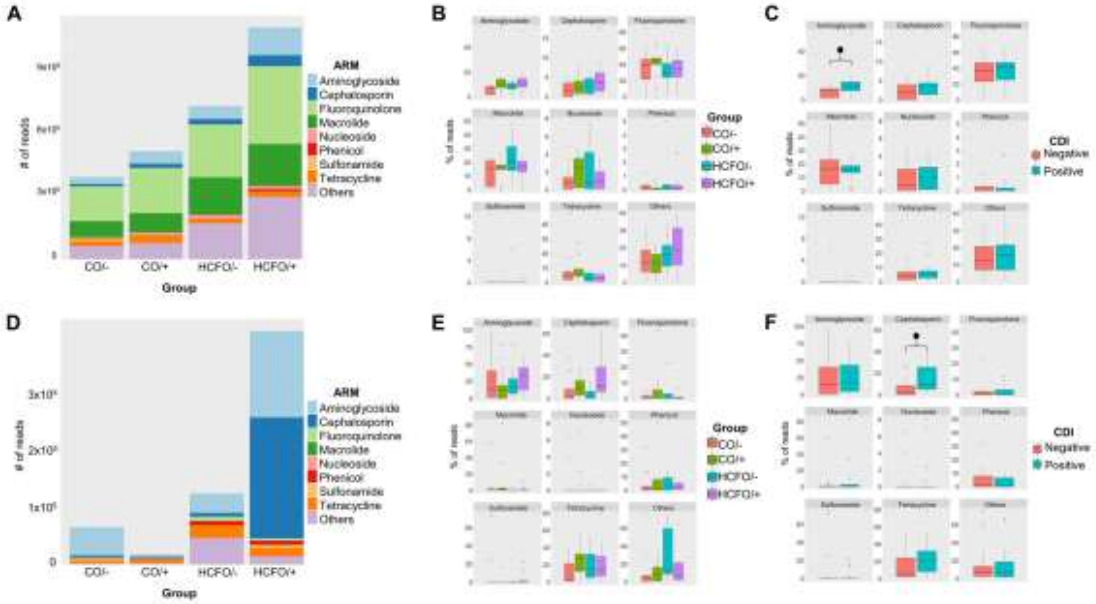

FIG 6 Increased genomic and plasmid ARMs in HCFO groups. (A) Distribution of genomic ARMs regarding antibiotic class per study group. (B and C) Boxplots of each antibiotic involved in the ARMs found in groups and by onset. (D) Distribution of plasmid ARM reads regarding antibiotic class by groups. (E and F) Boxplots of each antibiotic involved in plasmid ARMs found by group and onset.

imbalance in these patients' intestinal microbiota generated by many factors, such as the presence of *C. difficile*, which could contribute to the transfer of resistance genes among microorganisms, thereby worsening patients' clinical condition, limiting their treatment, and even placing their lives at risk.

This has thus been the first metagenomics study regarding the setting for *C. difficile*-associated diarrheic patients in Colombia. The results suggested that individual microbial members do not cause microbiota imbalance but, rather, that microbial ecology (the relationships established with other individuals and their environment [56]) plays an essential role, and thus any imbalance affects microbial communities' composition to different extents, including a possible metabolic impact and thus an impact on patients' health.

Further studies are required for determining the impact on the expression of the genes found here. Pharmacological surveillance of antibiotic treatment in the general population must be strengthened, as this could be triggering an increase in different microorganisms' resistance. These results should contribute to identifying pathogenic microorganism's characteristic of the imbalance produced by CDI and potentially beneficial ones that could counteract the infection's impact and which, therefore, might be candidates for probiotics; however, future research must be aimed at verifying differentially abundant species' roles regarding health and establishing these microorganisms' intestinal ecosystem homeostasis.

MATERIALS AND METHODS

DNA selection and shotgun metagenomic sequencing. A total of 48 DNA samples stored in the Universidad de Rosario's Centro de Investigaciones en Microbiología and Biotecnología (CIMBIUR) cryobank from a 2019 study by Muñoz et al., (57) were selected for this work. The samples had been classified into four groups according to the state of CDI and where the infection had been acquired, following Society for Healthcare Epidemiology of America and Infectious Diseases Society of America guidelines (58) as described in Muñoz et al., (59): community onset positive for CDI (CO/+, $n = 13$), community onset negative for CDI (CO/−, $n = 14$), health care facility-acquired positive for CDI (HCFO/+, $n = 13$), and health care facility-acquired negative for CDI (HCFO/−, $n = 8$). The samples forming the groups were randomly selected in line with the following technical requirements: amount of DNA, purity, and available

volume. Metagenomics sequencing was used for the selected samples (Illumina platform, Paired end (PE)150 Q30, >80%; 4G raw data/sample) at Novogene (Sacramento, CA, USA).

Evaluating data quality and filtering. FastQC (60) and MultiQC searches (61) were made of the data for ascertaining read quality; the Trimmomatic read-trimming tool for Illumina next-generation sequencing (NGS) data (62) was used for trimming low-quality sequences (Q score, <20) and those with less than 150-bp size. Bowtie 2 (63) was used for the decontamination step when aligning reads from human host sequences with the human genome reported in NCBI (Genome Reference Consortium Human Build 38 [GRCh38], accession number PRJNA31257).

Taxonomic binning and profiling and identification of differentially abundant species by group. Two approaches were used for describing the composition of the communities in the samples. The phyloFlash pipeline (64) was used for specifically describing the samples' bacterial and archaeal communities; this involved extracting 16S rRNA gene sequences. The Kraken (65) tool for assigning taxonomic labels to short DNA sequences was used for the samples' taxonomic binning. The gplots (66) programming tool was used for producing heatmaps; differences were evaluated by Kruskal-Wallis test and *post hoc* analysis using Dunn's test with Benjamini-Hochberg stepwise correction (67), with the 0.05 significance level in Rstudio software (68).

MetaPhlAn 3.0 software (69) was used for profiling the composition of microbial communities; GraPhlAn (70) was used for creating the graphics. The Kruskal-Wallis test and *post hoc* analysis were used for evaluating differences regarding differentially abundant species—abundance between groups. The phyloseq package (71) was used for importing, storing, analyzing, and graphically displaying already clustered phylogenetic sequencing data, along with beta diversity using the differentially abundant species found by a principal-coordinate graph based on Bray-Curtis dissimilarity. Permutational multivariate analysis of variance (PERMANOVA) was used for evaluating centroid differences, i.e., adonis (analysis and partitioning sums of squares using dissimilarities) and vegan functions (descriptive community ecology-related statistics package) (72).

External validation of CDI-associated species. We performed an analysis of compositions of microbiomes with bias correction (ANCOM-BC) to validate the bacterial and archaeal species that were differentially abundant in the CDI-positive groups. The ANCOM-BC is a robust analysis that controls the false-discovery rate (FDR) and presents a statistical approach that allows evaluating the reproducibility and reducing the bias associated with differences in sampling (73). For this analysis, we simultaneously compared the groups: CO/− and HCFO/− versus CO/+ and HCFO/+, considering a significance level of 5%.

The differentially abundant species associated with the CDI-positive groups observed here were compared to previously published data of Milani et al. ($n = 5$) (25) and Verma et al. ($n = 22$) (26). These two studies used shotgun metagenomics on their samples, where CDI-positive patients presented diarrheal symptoms similar to those of the present study. CDI-negative samples of these two studies were not considered, as they were patients without diarrhea symptoms; thus, they did not meet the inclusion criteria of our research.

For the validation, we retrieved the raw data belonging to CDI-positive samples from the Sequence Read Archive (SRA) and submitted it individually to the previously described preprocessing (quality control, filter and trimming, and decontamination). Subsequently, a taxonomic assignation was performed using MetaPhlAn, as described previously. Finally, each data set was compared to the CDI-negative samples of our study to determine the differentially abundant species present in the CDI-positive samples of each study. For this, we applied the ANCOM-BC with a significance level of 5%.

Bacteria and virus cooccurrence network. Spearman's nonparametric rank-order correlation with Benjamini-Hochberg correction was used for corelating viral families and archaeal and bacterial phyla, taking $P < 0.05$ values as being significant and strong correlations ($\rho < -0.75$ and $\rho > 0.75$) (R package psych). Correlations were then graphed in the Cytoscape 3.9.0 network visualization tool, data integration, and analysis software (R packages igraph, ggraph, and Rcy3).

Functional profiling. Humman3 was used for metabolic pathway functional profiling and reconstruction (69), using MaAsLin 2.0 (74) (Rstudio) for evaluating differences between groups by multivariate analysis; $P < 0.05$ values were taken as being significant. A Kruskal-Wallis test was used when association was identified, along with a Dunn test with Benjamini-Hochberg correction for *post hoc* analysis using multiple comparisons ($P < 0.05$ for significant associations).

Identifying virulence factors. The Basic Local Alignment Search Tool (BLAST) (75) was used for identifying virulence factors by aligning the decontaminated reads obtained with Bowtie 2 against reads from the virulence factor database (VFDB) (http://www.mgc.ac.cn/VFs/) (released 17 June 2021) (76). Results were given in terms of the highest percentage of identity, using a 95% cutoff point.

Identifying antibiotic resistance markers. The Comprehensive Antibiotic Resistance Database (CARD, version 3.1.3, released 5 July 2021) (77) Resistance Gene Identifier (RGI) tool was used for evaluating and predicting antibiotic-resistance markers and analyzing metagenomic reads. The Kruskal-Wallis test was used for evaluating the differences between marker reads by type of antibiotic used in the groups, along with *post hoc* analysis by Dunn test with Benjamini-Hochberg adjustment for multiple comparisons ($P < 0.05$ significance).

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

Data availability. The data are publicly available at the European Nucleotide Archive (ENA) repository under accession number PRJEB50313.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.5 MB.

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We declare that we have no competing interests.

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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 diarrhoea 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 signalling remained constant in microbiota members; characteristic patterns were observed in the MAGs along with genes involved in SCFA butyrate and acetate metabolic pathways in each study group. A decrease in genera and species, and relative MAG abundance with the presence of the acetate metabolism-related gene was evident in the HCFO/- group. An increase in antibiotic resistance markers (ARM) was observed in MAGs along with the genes involved in acetate metabolism. The results highlight the need for exploring the role of acetate in greater depth as a potential protector of the imbalances produced by CDI, as occurs in other inflammatory intestinal diseases.
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Metagenome-assembled genomes (MAGs) suggest an acetate-driven protective role in gut microbiota disrupted by *Clostridioides difficile*

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Summary

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 diarrhoea 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 signalling remained constant in microbiota members; characteristic patterns were observed in the MAGs along with genes involved in SCFA butyrate and acetate metabolic pathways in each study group. A decrease in genera and species, and relative MAG abundance with the presence of the acetate metabolism-related gene was evident in the HCFO/- group. An increase in antibiotic resistance markers (ARM) was observed in MAGs along with the genes involved in acetate metabolism. The results highlight the need for exploring the role of acetate in greater depth as a potential protector of the imbalances produced by CDI, as occurs in other inflammatory intestinal diseases.

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 [1–3]. CDI is caused by a sporulated anaerobic bacillus which is resistant to multiple antibiotic regimens, resulting in recurrent infections leading to pseudomembranous colitis, sepsis and even death [4,5]. *C.*

difficile can proliferate due to the action of antibiotics and other drugs; multiple interactions among intestinal microbiota members become altered, resulting in the exacerbation of symptoms and even in the proliferation of other opportunistic pathogens as a consequence of the imbalance caused by the decrease in commensal bacteria [6–8]. This highlights this intestinal microorganism ecosystem's importance regarding disease development and recurrence and the elements that could contribute to protection against it [4,6].

Shotgun metagenomics unveiling the gut ecosystem has led to reconstructing the taxonomic composition of the environments so studied, exploring functional diversity and comparing metagenomic-assembled genomes (MAGs) [9]. This has facilitated the description of new microorganism species [10], epidemiological surveillance of antibiotic resistance markers (e.g. in wastewater) [11,12] and the in-depth description of various species' microbiome composition and potential functioning [13–15].

Few CDI studies have shown commensal carbohydrate-degrading clostridial interaction with *C. difficile*; this inhibits bacillus growth, leading to a decrease in associated symptoms [16]. Other studies have proposed some short-chain fatty acids (SCFA) role, 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) [17].

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 [18–21].

It has been shown recently that acetate could be actively involved in intestinal processes, especially in a neutrophil-mediated response against *C. difficile* [17], highlighting the

72 importance of deepening understanding regarding this SCFA's role regarding inflammatory
73 diseases, especially CDI.
74 Few studies have used metagenomics tools for investigating CDI in Latin America; our
75 team's study has highlighted taxonomic alterations and differentially abundant species in
76 each study group, along with a decrease in the genes involved in butyrate metabolism, mainly
77 in the groups suffering hospital-acquired diarrhoea [22]. However, intestinal microbiota's
78 metabolic and functional profiles in Latin American CDI patients remain unknown.
79 The study's second stage was thus aimed at evaluating SCFAs' taxonomic and functional
80 profile, emphasising MAGs recovered from samples taken from patients with and without
81 CDI in two hospitals in Bogota, Colombia. An increase in MAGs' relative abundance with
82 the presence of genes involved in acetate metabolism in the groups having less microbiota
83 imbalance suggested an important role for this SCFA in intestinal homeostasis that should be
84 addressed in future research.
85
86 **Results**
87 *MAG quality*
88 The workflow regarding bin assembly and dereplication gave 437 drafts of high-quality
89 MAGs to be assembled from the samples; 130 (29.7%) were recovered from CO/- group
90 samples, 144 (33.0%) from the CO/+ group, 60 (13.7%) from the HCFO/- group and 103
91 (23.6%) from the HCFO/+ group. Table 1 summarises the quality of the recovered MAG
92 statistics. MAG sizes ranged from 1.4 to 7.0 megabases, 97 kb N50 average (3.7 kb to 1.5
93 Mb). The % GC ranged from 24.9 to 65%; 6 of the 437 MAGs had 100% completeness and
94 0% contamination (Figure S1A).
95 *MAG taxonomic assignation and functional annotation*

96 Taxonomically, high-quality draft MAGs belonged to 11 phyla, Bacillota (n=222),
97 Bacteroidota (n=109) and Pseudomonadota (n=61) being the most abundant (Figure 1A); the
98 Clostridia class was the most abundant (n=181). *Prevotella* (23), *Alistipes* (20) and
99 *Escherichia* (19) genera were most abundant. A slight increase in MAGs belonging to the
100 Verrucomicrobiota and Actinomycetota phyla was observed in the HCFO/- group (Figure
101 1B), although no statistically significant differences were observed. It was found that most
102 genes were involved in metabolism, followed by information processing and storage, when
103 analysing the MAGs according to COGs categories, having no differences between groups
104 (Figure 1C).
105 Enzymes buk and ackA were found in 229 MAGs (90 for both enzymes, 4 only for *buk* and
106 135 only for *ackA*) when ascertaining MAGs in SCFA metabolism-encoding genes; genes
107 encoding both enzymes were found in 5 phyla, Bacteroidota and Bacillota being the most
108 abundant (Figure S1B).
109 *Differential profile of acetate producers amongst groups*
110 MAG analysis of *ackA* and *buk* enzyme presence gave a differential microorganism profile
111 capable of metabolising SCFA for each study group (Figure 2A). The CO/- group had 25
112 species capable of metabolising acetate and 10 for butyrate, which were not found in other
113 groups (differentially present species); a greater percentage of MAGs identified as
114 *Butyrivibrio crossotus* and *Alistipes finegoldi* was observed.
115 The CO/+ group was characterised by 21 unique species having *ackA* and 11 with *buk*, the
116 genus *Prevotella* predominating. The intrahospital-associated groups had profiles, where
117 from the samples of the HCFO/- group 16 differentially present species metabolizing acetate
118 and 5 metabolizing butyrate were recovered, while the HCFO/- group had 25 differentially
119 present species with enzymes for acetate metabolism and 7 for butyrate, mainly characterised

120 by the presence of the genera *Bacteroides, Enterococcus* and *Alistipes* (Figures 2A and 2B).
121 Statistically significant differences were found when analysing the number of genera having
122 enzymes for metabolising SCFA in each group, specifically for genera having *ackA* in the
123 CO/- vs HCFO/- (p =0.0046), CO/+ vs HCFO/- (p =0.028) and HCFO/- vs HCFO/+ (p
124 =0.034) groups (Figure 2C). No differences were observed between groups regarding
125 butyrate.
126 *MAG relative abundance, resistance, and virulence profile*
127 MAGs' relative abundance varied markedly, the CO/+ and CO/- groups having the highest
128 relative abundance and the HCFO/- group low relative abundance re most MAGs (Figure
129 3A). *Faecalibacterium prausnitzii* (CO/+ vs HCFO/- p =0.036; HCFO/- vs HCFO/+ p
130 =0.045) and *Pseudomonas helleri* (CO/- vs HCFO/-, p =0.031; CO/+ vs HCFO/+, p =0.033)
131 had statistically significant differences. There were no statistically significant differences re
132 ARMs and virulence factors (Figure S1C).
133 However, when analysing ARMs re the class of antibiotics against which they conferred
134 resistance, differences were found concerning aminocoumarin (acetate vs both, p =0.022;
135 acetate vs none, p =0.032), aminoglycoside (acetate vs both, p =0.027; acetate vs none p
136 =0.035), phosphomycin (acetate vs none, p =0.018) and peptide (acetate vs none, p =0.015)
137 (Figure 3B). The same analysis discriminating by study group revealed differences re the
138 abundance of ARMs related to acridine dye (CO/- vs HCFO/+, p =0.022; CO/+ vs HCFO/+,
139 p =0.030; HCFO/- vs HCFO/+, p =0.02), diaminopyrimidine (CO/- vs HCFO/+, p =0.007;
140 CO/+ vs HCFO/+, p =0.010; HCFO/- vs HCFO/+, p =0.011) and lincosamide (CO/- vs
141 HCFO/+, p =0.003; CO/+ vs HCFO/+, p =0.010; HCFO/- vs HCFO/+, p =0.012) (Figure
142 3B).
143

Discussion

Shotgun metagenomics enabled exploring differences between taxonomic groups and functional changes which might have been occurring in the microbiota of the individuals from which the samples were collected. Despite MAGs taxonomic composition differences (Figure 1A and 1B), COGs analysis did not reveal marked variations regarding macro processes amongst groups related to genes encoding proteins involved in the most abundant metabolic processes (Figure 1C).

Such results agreed with previous studies which reported that most predicted genes were related to carbohydrate and amino acid metabolism in CDI positive and negative samples, i.e. such processes being of great importance for intestinal homeostasis maintenance [16,23]. This suggested that microorganisms inhabiting altered microbiota must maintain metabolism, transport and signalling, despite taxonomic changes in the microbiota; this is especially true for amino acids, as these are fundamental for intestinal ecosystem functionality and thus for their survival [24–26]. However, further studies are required for acquiring data regarding in-depth functional impacts of the changes observed here.

The patterns observed concerning relative MAG abundance and genes involved in acetate and butyrate metabolism (Figure 2A) suggested that differentially abundant microorganisms capable of SCFA metabolism could contribute to intestinal homeostasis maintenance in each group studied. Some microorganisms involved in SCFA metabolism could be considered pathogenic, such as *Enterococcus faecalis* in the HCFO/- group (Figure 2A). This was consistent with previous reports that enterococci group members (commensal and pathogenic lactic acid bacteria) can exchange genes by horizontal transfer and may act as an intestinal probiotic, despite being recognised as pathogenic [27,28].

167 This highlights the importance of microbiota resilience (understood as the capability to
168 maintain and recover after perturbations) promoted by multiple interactions and
169 interdependencies occurring amongst members [29]; pathogenic microorganisms could even be
170 involved in the metabolism and production of the elements necessary for their equilibrium.
171 However, this study's limitations hindered determining whether predicting MAG-related
172 gene functions may affect compounds' synthesis and metabolism (future research being
173 required to determine this).
174 The reduction in MAGs genera and species, along with genes involved in SCFA metabolism
175 in the HCFO/- group observed in this study (Figure 2B and 2C) contrasted with that reported
176 by Antharam *et al*., who found a high percentage of sequencing reads for acetogenic bacterial
177 genera, mainly in patients negative for CDI having nosocomial diarrhoea [18]. Such
178 discrepancies may have been due to clinical, sociodemographic and dietary factors directly
179 affecting SCFA production [30–32]. However, the lack of clinical data for patients in the present
180 study prevented establishing the cause of such differences.
181 Previous studies regarding these samples have shown taxonomic alterations in the HCFO/-
182 group characterised by a reduction in beneficial bacteria's relative abundance [8,22]; this could
183 have decreased SCFA metabolism, in turn negatively affecting immunoglobulin A (IgA)
184 production which would have facilitated pathogenic bacteria proliferation and decreased
185 beneficial bacteria maintenance capability [30,33–35]. However, since SCFA levels could not be
186 quantified in this study's samples, the impact of MAG reduction on the enzymes involved in
187 their metabolism could not be determined (requiring further research adopting a multi-omics
188 approach).
189 The large amount of genera and species, along with high relative MAG abundance with ackA
190 and bukA observed in the CDI groups (CO/+ and HCFO/+) (Figures 2 and 3) contrasted with

what was expected, since this pathogen is associated with altered microbiota characterised by decreased SCFA metabolism-associated beneficial bacteria [4,16,18,36]. However, despite the varied toxigenic profiles in *C. difficile* isolates recovered from samples, [37,38] our research revealed slightly altered microbiota in the target patients, characterised by beneficial bacteria's strong diversity and relative abundance [8,22]. 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 [30].

SCFA ability to inhibit *C. difficile* growth has been demonstrated; it has also been shown that SCFA can inhibit this potentially pathogenic bacteria's growth [6,39,40] and some studies have shown that these metabolites may exacerbate its toxin production [41,42] which, in turn, could be related to the varied toxigenic profiles mentioned above. However, further studies are required to establish the relationship between these elements.

C. difficile's survival mechanisms should be determined in microbiota having potential for SCFA metabolism, especially acetate as this fatty acid is closely related to a neutrophil-mediated response against the bacillus [17]. 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. Multicentre studies are required for largescale evaluation of 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 [24–26].

This has been the first study reporting MAG assembly from CDI samples in Colombia. A reduction in bacteria able to metabolise SCFA appears to affect intestinal microbiota's taxonomic composition and diversity, mainly in patients having diarrhoea associated with an in-hospital setting. The study was limited as clinical data could not be obtained regarding the

patients from whom the samples had been taken; this could help explain the results. Likewise, there was no control group without diarrhoea, which would have enabled more robust comparisons to be made.

This also emphasised that the presence of genes alone does not demonstrate microorganism ability to perform a given function. It should be noted that this first approach should be complemented by measuring serum and stool SCFA levels to improve understanding these metabolites in CDI and other inflammatory bowel diseases. Likewise, research involving the use of multiple omics is required for holistic understanding of the process occurring during CDI.

Limitations of the study

The clinical data could not be obtained regarding the patients from whom the samples had been taken. Likewise, there was no control group without diarrhoea.

Methods

DNA selection and shotgun metagenomic sequencing

Forty-eight DNA samples resulting from a study by Muñoz *et al*., [37], stored in the Universidad del Rosario's Microbiology and Biotechnology Research Centre's cryobank, were selected for the present research. Samples were randomly selected in compliance with technical requirements (DNA concentration, purity and volume) and classified according to the Society for Healthcare Epidemiology of America and Infectious Diseases Society of America guidelines [43], as described in Muñoz *et al*., [38]. Four populations were established: 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). Novogene (USA, Sacramento, CA) used the Illumina

sequencing platform for metagenomic sequencing of the samples (PE150, Q30>80% with 4 Gb of raw data per sample).

Data quality and filtering

FastQC [44] and MultiQC [45] were used for assessing sequence quality; the Trimmomatic tool [46] was used for filtering and cutting sequences (Q score <20, length <150bp). The Bowtie2 tool [47] was used for deleting human host reads using the reference genome reported by the National Center for Biotechnology Information (NCBI) (GRCh38, accession number PRJNA31257).

Metagenomic assembled genomes (MAGs) recovery and taxonomical assignation

The SPAdes assembler with the meta parameter (35) toolkit/pipeline for was used for filtering reads from the metagenomic data set, using 1500 nt as contig size. Bowtie2 and SAMtools sequencing alignment tools were used for mapping the decontaminated reads, using the MAGs as reference [48]. Maxbin [49], Metabat [50] and Concoct [51] were used for binning the assemblies. The CheckM tool [52] was used for checking bin quality and the DAS Tool [53] for refining the bins. Only high-quality drafts were selected for downstream analysis, defined by Bowers *et al.*'s parameters [54] (completion >90%, contamination <5%) (Figure S1). The Genome Taxonomy Database Toolkit (GTDB-Tk was used for taxonomic classification [55]; the PhyloPhLan integrated pipeline [56] 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 iTol v6 [57].

Functional annotation and MAG analysis

The eggNOG-mapper functional annotation tool [58] was used with high-quality MAG drafts for identifying clusters of orthologous groups (COGs). The COGs were grouped according to their functional category and plotted for each previously-defined working group. Prokka

263 [59] was then used for functionally annotating the MAGs for extracting the encoding genes for
264 the enzymes involved in SCFA metabolism, i.e. acetate kinase (ackA), butyrate kinase (buk),
265 butyryl-CoA:acetate CoA-transferase(but), methyl malonyl-CoA decarboxylase (mmdA),
266 lactoyl-CoA dehydratase (lcdA) and CoA-dependent propionaldehyde dehydrogenase
267 (pduP).
268 Each MAG's relative abundance was determined by calculating the total number of reads
269 mapped to MAG concerning the total number of reads in each sample (Hua *et al.,*)[60]. Abricate
270 [61] was used for searching for antibiotic resistance markers (ARM) and virulence factors by
271 comparison with the Comprehensive Antibiotic Resistance Database (CARD) (updated in
272 July 2022) [62] and the virulence factor database (updated in July 2022) [63].
273 *Statistical analysis*
274 A bubble plot demonstrated MAGs quality dispersion, using the aforementioned statistical
275 software. Mann-Whitney U and Kruskal-Wallis tests with Bonferroni correction were used
276 for multiple comparisons and Dunn's test in R studio software [64] for post hoc analysis, taking
277 < 0.05 *p*-values as being statistically significant.
278 *Data availability*
279 The raw data are publicly available at the European Nucleotide Archive (ENA) repository
280 under accession number PRJEB50313. The fasta files of MAGs are publicly available at
281 Github repository https://github.com/gio9024/Cdiff_mags.git
282
283 **Declaration of interests**
284 The authors declare no competing interests.
285
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Author contributions

MM, JDR and GH: study conception and design.

GH, SC, JCA and JEPJ: bioinformatics analysis.

GH and MM: interpretation of data.

GH, MM and JDR: drafted the work.

MM, JDR and MAP: substantial revision of manuscript.

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Figure legends

Figure 1. MAG taxonomic and functional profiles. A) Phylogenetic tree of the MAGs assembled from the samples. Branch colours denote the Phylum to which they belong, while outer ring colours 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.

Figure 2. Differences in the profiles of bacteria able to metabolise 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) o *buk* (butyrate) encoding genes. C) Boxplot with the amount of genera having *ackA* and *buk* enzymes for each group.

Figure 3. MAG relative abundance, ARM and virulence factor profiles. A) MAG relative abundance with only *ackA* calculated by mapping reads onto assembled MAGs. B) Boxplot with the amount of MRAs and virulence factors found in the MAGs discriminated by the presence of *ackA* and *buk* genes, as well as by study group.

Figure S1. A) Bubble plot showing assembled MAG quality based on their completeness and contamination. B) MAGs taxonomic composition with the simultaneous presence of *ackA* and *buk*. C) Boxplot showing the amount of MAG virulence factors and MRA according to *ackA* and *buk* enzymes.

CAPÍTULO 3. "Identificación de microbiota asociadas a garrapatas duras que se alimentan de humanos en España"

Uno de los productos generados durante el desarrollo de la presente tesis fue un esquema de análisis para datos de secuenciación profunda de marcador único, que permiten obtener información de manera rápida y sencilla sobre la composición taxonómica y la diversidad del microbioma. Este esquema ya se ha empleado para análisis de microbioma de bovinos en el contexto de la infección por *Fasciola hepatica,* mariposas del género *Heliconius* y su asociación con variables geográficas, así como en diferentes especies de triatominos. Teniendo en cuenta lo anterior y como complemento de la doble titulación en la Universidad de Salamanca, se dispuso la evaluación de la utilidad del esquema de análisis para determinar la composición y diversidad de la microbiota de garrapatas recolectadas en diferentes zonas de España. Se tomaron los ADN de 29 individuos de 5 especies de garrapatas (*Ixodes ricinus, Hyalomma marginatum, Dermacentor marginatus, Rhipicepalus bursa* y *Rhipicephalus sanguineus*) colectadas en la provincia de Castilla y León y fueron sometidos a secuenciación de la región V4 del ARNr-16S. Si bien no se encontraron diferencias taxonómicas a nivel de phylum, ni en cuanto a la alfa diversidad, las variaciones interindividuales permitieron la agrupación espacial de acuerdo con las especies, así como se evidenciaron complejas interacciones entre diferentes géneros de patógenos y endosimbiontes con bacterias de la microbiota, lo cual permitió realizar una primera descripción de la microbiota de estos géneros de garrapatas en el noroccidente de España.

A continuación, se describen los objetivos de este apartado:

Objetivo General

- Describir la composición de la microbiota de 5 especies de garrapatas duras (Acari:Ixodidae) colectadas en Castilla y León con evidencia previa de alimentación a partir de humanos

Objetivos específicos:

- Determinar la composición taxonómica de la microbiota 5 especies de garrapatas colectadas en Castilla y León con evidencia previa de alimentación a partir de humanos
- Establecer la diversidad de la microbiota de 5 especies de garrapatas colectadas en Castilla y León con evidencia previa de alimentación a partir de humanos
- Describir las correlaciones entre las diferentes bacterias de la microbiota de las 5 especies de garrapatas colectadas

Como producto de este capítulo se generó 1 artículo que actualmente se encuentra sometido y se lista a continuación:

Artículo 7. Herrera, Giovanny, Vieira, María Carmen, Páez-Triana, Luisa, Muro, Antonio, López-Abán, Julio, Muñoz, Marina, Ramírez, Juan David. Interrogating the gut microbiota from several human-biting tick species in Northwestern Spain. Journal of Medical Entomology (sometido)

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Please choose a section from the list:	Vector/Pathogen/Host Interaction, Transmission
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Interrogating the gut microbiota from several human-biting tick species in Northwestern Spain

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Abstract

Tick-borne diseases have increased significantly in Europe and Spain in recent years. One strategy explored for tick surveillance and control is the study of the microbiota. The focus is on understanding the relationships between pathogens and endosymbionts within the microbiota and how these relationships can alter these arthropods' vectorial capacity. Thus, it is pivotal to depict the bacterial communities composing the microbiota of ticks present in specific territories. This work aimed to describe the microbiota present in 29 individuals of 5 tick species collected from 4 provinces of Castilla y Leon in northwestern Spain from 2015 to 2022. DNA extraction and sequencing of the V4 hypervariable region of 16S-rRNA was performed, with subsequent analysis of diversity, taxonomic composition, and correlations between genera. There were no differences in the alpha diversity by species, nor were compositional changes evident at the phylum level. However, inter-individual differences at the genus level allowed spatial differentiation of the five species included in the study. Correlation analyses showed complex interactions between different genera of pathogens and endosymbionts with other microbiota members. These findings provide an initial insight into

the composition of the gut microbiota of various tick species in northwestern Spain, which can contribute to establishing surveillance and control measures to reduce diseases such as rickettsiosis, Lyme disease, and Crimean-Congo hemorrhagic fever.

Keywords: Tick microbiota, Tick-borne diseases, Ixodidae, Spain, emerging diseases.

Introduction

Ticks are hematophagous ectoparasitic arthropods able to transmit several pathogens, such as viruses, bacteria, and protozoa, to humans and animals, generating health risks and becoming a worldwide concern (Estrada-Peña et al., 2008; Ghosh et al., 2007; Parola & Raoult, 2001). Among the main microorganisms transmitted by ticks are *Rickettsia* spp., *Anaplasma* spp., *Borrelia* spp., *Ehrlichia* spp., *Babesia* spp., and the Crimean-Congo virus (Dantas-Torres et al., 2012; Rochlin & Toledo, 2020; Wu-Chuang et al., 2021), having significant economic effects due to morbidity and mortality rates, as well as severe implications for healthcare systems (Ghosh et al., 2007; Heyman et al., 2010).

More than 890 ticks species have been described worldwide, where the Ixodidae family has most of the vectors found in Europe, reaching 70 different species throughout the continent and five main genera: *Ixodes*, *Dermacentor*, *Haemaphysalis*, *Rhipicephalus* and *Hyalomma* (Estrada-Peña et al., 2018a; Gilbert, 2010). In the Iberian Peninsula, these five genera are potential vectors of the *Anaplasma, Rickettsia*, and Crimean-Congo virus (Fernández Soto, 2003). Likewise, a recent study showed fluctuating distribution patterns of ticks with their potential to transmit several pathogens throughout northwestern Spain. These findings demonstrate the importance of monitoring the dispersion of these arthropods and the transmitting pathogens (Vieira Lista et al., 2022). In this regard, it is worth noting that tick-borne diseases have increased in southern Europe, especially in Spain, where rickettsiosis, Lyme disease, and tick-borne encephalitis are considered emerging health problems with incidences of up to 0.36 cases/100,000 individuals (Dantas-Torres et al., 2012; Portillo et al., 2015) and Crimean-Congo hemorrhagic sporadic cases (Lorenzo Juanes et al., 2023)

Ticks are hosts to a broad range of commensal and symbiont microorganisms in their microbiota, involved in various physiological processes such as nutrition, reproduction, development, vectorial ability, and immunity (Bonnet et al., 2017; Dantas-Torres et al., 2011;

Pollet et al., 2020). The tick's microbiota exhibits substantial variation in its composition and diversity according to different factors such as tick species, sex, life stage, and environmental factors (Aivelo et al., 2019; Ponnusamy et al., 2014; Sperling et al., 2017; Van Treuren et al., 2015). Despite these variations, the ability of some tick-borne pathogens to modulate their microbiota stand out (Abraham et al., 2017; Adegoke et al., 2020; Narasimhan et al., 2017). Interactions with specific key taxa play a crucial role in modifying the microbiota, potentially leading to significant alterations in its structure and further affecting the ticks' vectorial ability (Maitre et al., 2022). Therefore, studying tick microbiota is considered fundamental for preventing and managing tick-borne diseases due to the impact that changes in the microbiota could produce on the transmission of pathogens or commensals/symbionts.

Despite the importance of this in both epidemiological surveillance and vaccine development, limited investigations have been conducted in the Iberian Peninsula to explore variations in the microbiota among different species of ticks. Thus, this study aimed to describe the composition and diversity of the microbiota of 5 ticks found in the Castilla y León between 2015 and 2022. This is the first study that reports the composition and diversity of the microbiota of ticks from northwestern Spain. The role of endosymbionts in the structure and development of interactions with other pathogenic microorganisms present in the microbiota.

Materials and methods

Tick Collection, Identification, and DNA extraction

A total of 29 adult ticks were included in the present study, stored at the University of Salamanca, Spain. The samples were collected and preserved using the protocol described by Vieira et al. (Vieira Lista et al., 2022). Briefly, the ticks were gathered between 2015 and 2022 from individuals who attended primary healthcare centers and hospital services for tick removal. After removal, the ticks were transported to the laboratory for coding and morphological identification using taxonomic keys (Apanaskevich et al., 2008; Estrada-Peña et al., 2004, 2018b; Gil-Collado et al., 1979). Once the ticks were identified, DNA extraction was performed using the NucleoSpin Tissue kit (Macherey-Nagel, Dueren, Germany) following the manufacturer's instructions.

DNA quality control and sequencing process

The extracted DNA was subjected to quality control by 2% agarose gel electrophoresis to verify the integrity of the DNA. Additionally, the concentration was evaluated using a NanoDrop/2000/2000c spectrophotometer (Thermo Fisher Scientific, Massachusetts, USA). A 260/280 relationship between 1.8 and 2.0 and a minimum concentration of 20 ng/μL was verified. Paired-end sequencing was performed on the Illumina NovaSeq platform (PE 250 Platform) with 0.1 million reads per sample at the facilities of Novogene Corporation Inc. (Shanghai, China) using primers targeting the hypervariable V4 region of the 16S-rRNA marker specific for bacteria and Archaea 515-F (5′-GTGCCAGCMGCCGCGGTAA-3′) and 806-R (5′-GGACTACHVGGGTWTCTAAT-3′) (Caporaso et al., 2011).

Taxonomic assignment

Initially, a quality control step was performed to assess the quality of the sequencing reads, including the average number of reads per sample, Phred score, frequency of unassigned bases, and presence of adapters in the samples. Subsequently, the barcodes and primers were removed using the QIIME2 tool (Bolyen et al., 2019, p. 2) before proceeding with taxonomic assignment using the DADA2 tool in R studio (R Core Team, 2013), following the default pipeline (Callahan et al., 2016, p. 2). The sequences were assigned, comparing the sequences obtained for the 16S-rRNA marker versus the 16S-rRNA using SILVA database version 138 (Quast et al., 2013) until obtaining the Amplicon Sequence Variants (ASVs). The ASVs are defined as each sequence obtained in the sequencing process, which may vary by a single nucleotide.

Taxonomical composition, diversity analysis, and correlation plot

The phyloseq package (McMurdie & Holmes, 2013) was used for importing, storing, analyzing, and graphically displaying already clustered phylogenetic sequencing data. With the resulting ASVs, the relative abundance of the different phyla was plotted for each tick genus. Subsequently, alpha (Shannon and Simpson) and beta diversities were analyzed using the ASVs by tick genera using a main coordinate plot (PCoA) based on Bray-Curtis dissimilarity. Permutational multivariate analysis of variance (PERMANOVA), i.e., adonis (analysis and partitioning of sums of squares using dissimilarities), and vegan functions

(descriptive statistical package related to community ecology) (Oksanen, 2011) were used to assess differences between centroids. Finally, the pathogens transmissible to humans and tick symbionts found in the samples were plotted for each tick genus using a chord diagram using the cyclize package (Gu et al., 2014).

Statistical analyzes

Statistically significant differences between the studied groups were evaluated using the Kruskal–Wallis test with respective *post hoc* analyses with the Dunn test using Benjamini–Hochberg correction with a confidence level of 95%. Likewise, correlogram graphs were obtained between ASVs corresponding to genera of the most abundant phyla (*Actinobacteriota*, *Bacillota*, *Bacteroidota*, and *Pseudomonadota*). Based on these, a filter of sub-represented data was carried out, eliminating all those ASVs corresponding to genera whose sum of reads was less than 1,000 and those ASVs corresponding to genera whose reads were not present in at least 25% of the samples. These filters were carried out to reduce potential technical bias and ensure that comparisons were conducted between ASVs in the groups and not in single samples. The correlation matrix was constructed using the psych package (Revelle, 2020) on R software, applying the spearman method with Benjamini–Hochberg correction. We considered only strong correlation values greater than 0.7 and less than − 0.7 (Spearman Rho strong correlation) and selected statistically significant ($p < 0.05$) during the process of establishing a correlation between the ASVs evaluated.

Results

Geographical distribution of collected ticks.

Of the 29 ticks studied, 28% were *H. marginatum* (6 male, two female), 24% were *D. marginatus* (5 female, two male), 17% to *I. ricinus* (5 female), 17% were *R. bursa* (4 female, one male), and 14% were *R. sanguineus sensu lato* (4 female). Most of the ticks were gathered in the provinces of Ávila (n=17) and Salamanca (n=5), most of them from the southern and western of Castilla y León (Figure 1).

Taxonomical composition and alpha and beta diversity

The alpha diversity analyses showed no statistically significant differences between tick species, with low average values of Shannon and Simpson's indices (Figure 2A). The opposite occurred when analyzing PCoA since spatial differences between tick species were evident (p-value = 0.017) (Figure 2B). In terms of the microbiota composition, there were no differences between groups, with the phylum *Pseudomonadota* as the most abundant, followed by the phyla *Bacillota* and *Bacteroidota* (Figure 2C). The analysis of tick symbionts and pathogens transmissible to humans showed an increase in the genus *Francisella* (K-W test=11.598, p-value=0.00087) in ticks corresponding to the genus *Hyalomma* versus individuals of the genera *Dermacentor* (p-value= 0.0086) and *Rhipicephalus* (p-value= 0.0275) (Figure 2D). Likewise, the years of the collection did not impact the results of beta diversity or the taxonomic composition (Figure S1). Also, it is noteworthy that the taxonomic assignment of microbiota members was limited to the genus level.

Complex correlation between bacterial genera in ticks

Finally, correlation analyses showed distinctive patterns for each tick genus (Figure 3). The genera *Hyalomma* (Figure 3B) and *Ixodes* (Figure 3C) showed the minor complex interactions between the different bacterial genera found, with a predominance of positive correlations, while the genera *Dermacentor* (Figure 3A) and *Rhipicephalus* (Figure 3D) displayed the most complex interactions with the presence of many negative correlations. The interactions between the genera of tick endosymbionts, pathogens transmissible to humans, and the other bacteria present in the microbiota are striking and characterized by positive correlations, with *Rickettsia, Coxiella,* and *Stenotrophomonas* as the genera with the highest number of interactions.

Discussion

The tick microbiota analyzed presented diverse profiles. The absence of differences in alpha diversity between the different tick genera contrasting with that reported by Portillo et al. (Portillo et al., 2019), who observed diverse alpha diversity between *I. ricinus* and *D. marginatus* collected in northern Spain. These discrepancies could be due to the presence of various pathogens in the samples, food preferences, and even the sex of the tick, which can

affect the diversity of the microbiota of these arthropods (Adegoke et al., 2020; Narasimhan et al., 2021; Swei & Kwan, 2017). Despite statistically significant differences in centroids, the spatial groupings of the different tick genera showed some overlap between *Rhipicephalus, Dermacentor,* and *Ixodes* (Figure 2B). Such spatial overlap was reported previously, suggesting that the composition of the microbiota of these genera is usually quite similar and is altered by sex and geographic distribution (Portillo et al., 2019).

Considering that the ticks herein included presented somewhat restrictive geographical distribution patterns (Figure 1), it is likely that this factor is a determinant of both the environmental conditions and the hosts on which they feed (Adegoke et al., 2020; Narasimhan et al., 2021). Although the study did not have samples of each species for each year, the temporal analysis showed no spatial clustering patterns (Figure S1), suggesting that annual seasonality does not generate significant differences beyond the inter-individual differences in tick microbiota. This contrasts with that reported by Lejal and collaborators (Lejal et al., 2021), who described recurrent differences in the microbiota of *I. ricinus* promoted by temporal dynamics. Such discrepancies may be caused by the difference in the seasonality of each of the individuals included in the different studies (monthly vs. annual and intermittent), the tick species (*Ixodes ricinus vs*. five different species), as well as the stage (nymphs *vs*. adults). This is supported by Pollet and collaborators, who argue that variability in the microbiota is associated with short periods (Pollet et al., 2020). Although both studies point to the strong influence of environmental factors in these differences, further research on the impact of temporal variations in the tick microbiota is required. In addition, we must highlight that the number of samples was low to support this statement.

The predominance of *Pseudomonadota* microorganisms in the taxonomic composition of the ticks evaluated in the present study agrees with previous studies carried out in Spain and Tunisia (Benyedem et al., 2022; Portillo et al., 2019). This microbiota composition is probably related to environmental factors, considering that most of the endosymbiont genera were identified, including *Coxiella* and *Francisella*, which are of great importance for the development of the ticks (Ben-Yosef et al., 2020; Gerhart et al., 2016), as well as some tick-borne pathogens, belonging to this phylum (Figure 2D). In this sense, the geographic distribution of ticks becomes essential, as it could influence both the composition and

diversity of the microbiota since many of the microorganisms found in these ticks are usually associated with the soils and water of the ecosystems where these arthropods develop (Narasimhan et al., 2021; Portillo et al., 2019). Likewise, it is worth noting the marked increase of the genus *Francisella* in ticks of the genus *Hyalomma*. A finding of great importance considering the role of this bacterium in the metabolism of vitamin B, folic acid, biotin, and riboflavin, which play a fundamental role in the tick's survival (Gerhart et al., 2016; Sjödin et al., 2012). This finding could denote the effect of competition among *Coxiella*, *Francisella*, and *Rickettsia* endosymbionts within the microbiota in northwestern Spain and their limited diet. However, further research with larger samples and temporal scales is required for a comprehensive understanding of the impact of these changes at the community level and to explore the potential causes.

The complex interactions observed within the microbiota of all the ticks analyzed suggest the development of close relationships between the different members of the microbiota, especially between endosymbionts and some pathogens. Our findings contrast with previous reports that have suggested the ability of some tick-borne pathogens to modify the microbiota structure (Adegoke et al., 2020; Narasimhan et al., 2021). Despite the above, these interactions could account for endosymbionts' fundamental role in the tick's vectorial capacity by regulating pathogen populations and generating suitable environments for their development. Understanding these processes is of great importance, considering that searching for crucial taxa is one of the strategies currently used to develop vaccines against tick-borne diseases (Mateos-Hernández et al., 2020).

Likewise, it is worth highlighting the large number of negative correlations observed in *Dermacentor*, *Hyalomma*, and *Rhipicephalus*, especially between some endosymbionts/pathogens transmissible to humans and other components of the microbiota, suggesting an increase in competition among the members of this ecosystem, in agreement with Lejal et al. (Lejal et al., 2021). Also, the few correlations found within the genus *Ixodes* are noteworthy, considering that *I. ricinus* is a notable species in Europe, known to transmit a wide range of pathogens, such as *Borrelia burgdorferi* (Aivelo et al., 2019). This low number of interactions between members of the microbiota could be due to the low number of individuals included in the present study and the low spatiotemporal variability in their

collection. It is worth noting that the present research, due to its exploratory nature, has some limitations, such as the low sample size and the absence of some species per year, the limited geographical area of collection, and the resolution of the taxonomic assignment.

Our results are of great relevance, considering that the region of Castilla y León has increased the number of reports of tick bites in humans in recent years (Vieira Lista et al., 2022). This region has geographical and climatological characteristics that favor the proliferation of multiple tick species and also have been reported the presence of *Francisella tularensis, Rickettsia* spp., *Borrelia* spp., and Crimean-Congo hemorrhagic fever virus (Mínguez-González et al., 2021; Monsalve Arteaga et al., 2020; Portillo et al., 2018). Considering both the taxonomic composition and the complex relationships established among the members of the tick microbiota exposed in the present study, the development of biological control strategies, as well as active epidemiological surveillance, could significantly impact the number of cases of tick-borne diseases, as well as could contribute to the immediate and adequate care of patients, considering the spatial distribution of pathogens in the territory. The results suggest that compositional differences allow spatial clustering of tick species, despite the absence of differences in diversity. Likewise, complex interaction networks between microbiota members with endosymbionts and tick-borne pathogens were observed.

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Conflict of interest

Authors declare no conflict of interest.

Data Availability

The data are publicly available at the European Nucleotide Archive (ENA) repository under accession number PRJEB59487.

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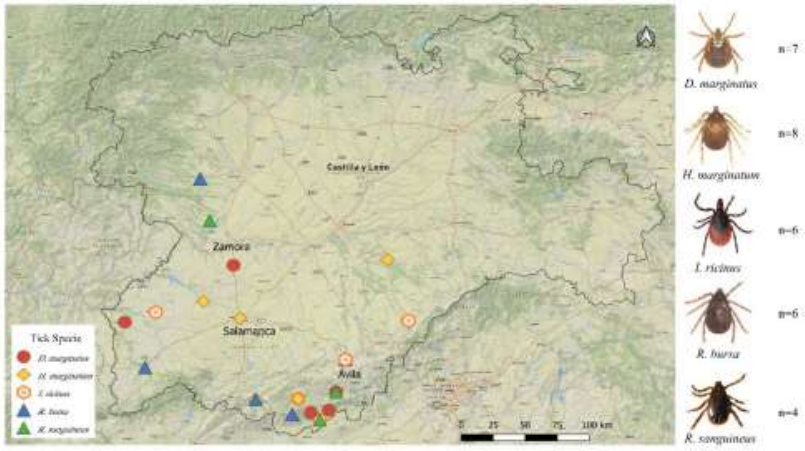
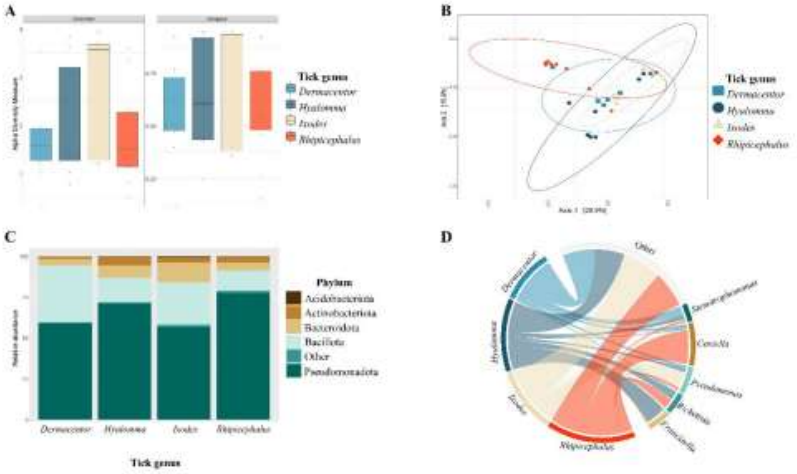


Figure 1. Geographical distribution of collected ticks. The map shows the different provinces where the samples were collected, and the number of each species included in the study



436

437 **Figure 2.** Diversity and taxonomical composition of ticks' microbiota. A. Shannon and
438 Simpson indices by tick species. B. Principal Coordinate Analyses by tick species. C.
439 Taxonomical composition of ticks' microbiota by phyla. D. Chord diagram of endosymbionts
440 and tick-borne pathogens by tick genera.

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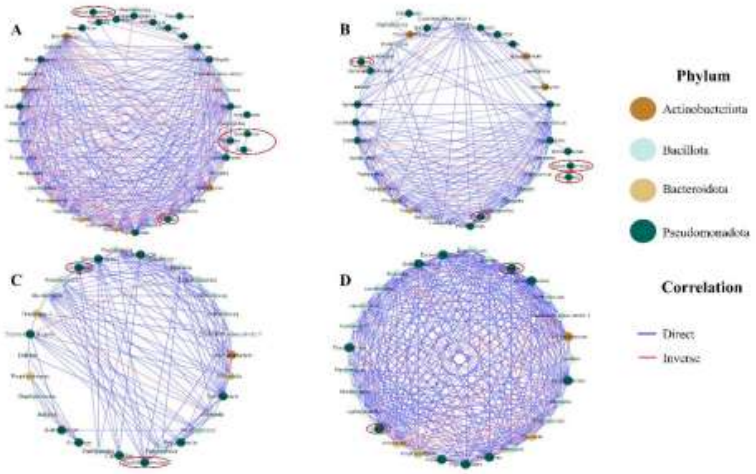

442

443 **Figure 3.** Complex correlations between different bacterial genera in ticks' microbiota. A.
444 Correlation plot of *Dermacentor*. B Correlation plot of *Hyalomma*. C. Correlation plot of
445 *Ixodes*. D. Correlation plot of *Rhipicephalus*.

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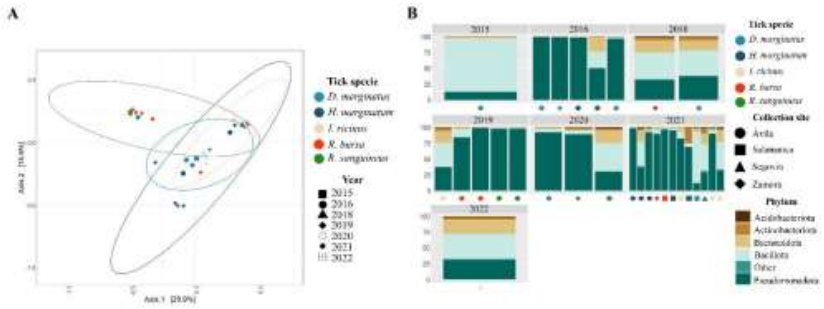

447

448 **Figure S1.** Influence of spatial-temporal patterns and tick sex over diversity and taxonomical
449 composition. A) Principal Coordinate Analyses by tick species including year of collection.
450 B) Principal Coordinate Analyses by tick species including sex C)Taxonomical composition
451 of ticks' microbiota by phyla sorted by year and place of collection.

Figure 1

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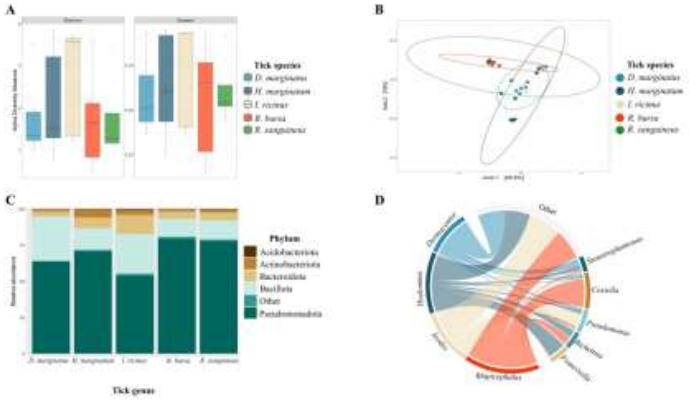


Figure 2

338x190mm (300 x 300 DPI)

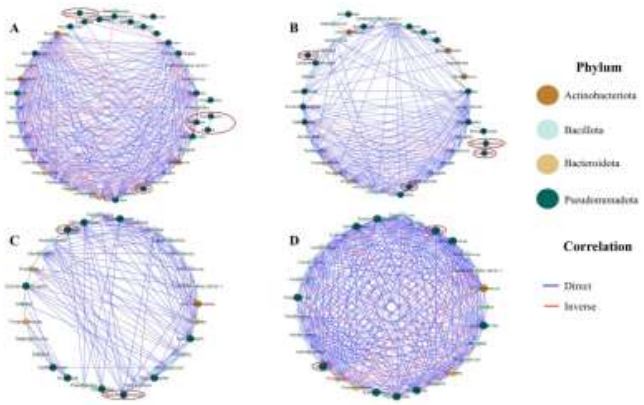
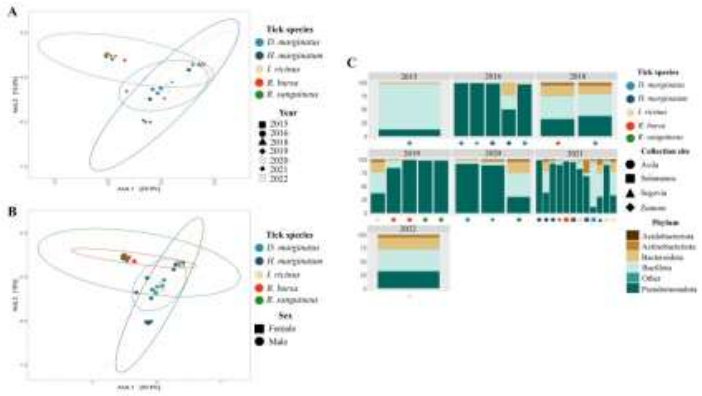


Figure 3

338x190mm (300 x 300 DPI)



338x190mm (300 x 300 DPI)

5. CONCLUSIONES

Capítulo 1.

- ✓ El lugar de adquisición de la diarrea juega un papel fundamental en la modulación del microbioma intestinal, siendo los individuos con diarrea adquirida a nivel intrahospitalario los que evidencian un mayor desequilibrio
- ✓ El desequilibrio de la microbiota observado en la ICD está más asociado con la ecología microbiana y las interacciones inter-dominios que con la acción individual de *C. difficile*
- ✓ La desregulación de la homeostasis intestinal afecta a la composición de las comunidades microbianas de diferentes maneras, incluyendo un posible impacto a nivel metabólico y funcional

Capítulo 2.

- ✓ Al igual que en el primer capítulo se evidenció que la composición de la microbiota intestinal se ve seriamente afectada por el lugar de adquisición de la diarrea
- ✓ Las interacciones entre los diferentes dominios revelan complejas relaciones dentro del ecosistema intestinal que podrían impactar la homeóstasis de este ecosistema
- ✓ El ambiente intrahospitalario favorece la circulación de marcadores de resistencia y virulencia en los miembros de la microbiota intestinal
- ✓ El desbalance de la microbiota intestinal no es causado únicamente por la presencia de individuos aislados, sino que es fruto de las complejas interacciones entre los diferentes miembros de la microbiota
- ✓ Las alteraciones en la composición de la microbiota podrían estar asociadas con desregulaciones funcionales de la misma
- ✓ El acetato podría desempeñar un rol de gran importancia sobre el mantenimiento del equilibrio de la microbiota intestinal

Capítulo 3.

- ✓ La composición de la microbiota de las 5 especies de garrapatas varió de forma significativa permitiendo su agrupación espacial.
- ✓ El estudio de la microbiota en garrapatas es una herramienta de gran potencial para la vigilancia y control de enfermedades transmitidas por garrapatas en España

6. PROSPECTIVA

- Evaluar el impacto de factores clínicos y sociodemográficos sobre las alteraciones en la microbiota intestinal

- Comparar los datos obtenidos contra pacientes sin diarrea con el fin de establecer una línea de base de personas sanas

- Evaluar la utilidad de las 51 especies diferencialmente abundantes encontradas en el presente estudio para el diagnóstico y seguimiento de personas con ICD

- Realizar estudios *in vitro* e *in vivo* sobre el impacto en la homeostasis de la microbiota intestinal del acetato de forma individual y en conjunto con el butirato

- Establecer biomarcadores para la ICD mediante la realización de estudios metagenómicos multicéntricos

- Determinar el impacto de bacterias benéficas descritas en el presente estudio sobre el equilibrio de la microbiota intestinal mediante estudios *in vivo*

- Realizar estudios con mayores marcos muestrales sobre las especies de garrapatas evaluadas

- Profundizar en las investigaciones de la microbiota de garrapatas mediante secuenciación de shotgun metagenomics

7. ANEXOS

7.1. *Productos de la tesis*

A continuación, se listan los productos generados durante el desarrollo de la presente tesis

7.1.1. *Lista de publicaciones*

Artículo 1. Vega, L., Herrera, G., Muñoz, M., Patarroyo, M. A., & Ramírez, J. D. (2020). Occurrence of *Blastocystis* in patients with *Clostridioides difficile* infection. *Pathogens*, *9*(4), 283.

Artículo 2. Vega L., Herrera G., Muñoz M., Patarroyo M. A., Maloney J. G., et al. (2021) Gut microbiota profiles in diarrheic patients with co-occurrence of *Clostridioides difficile* and *Blastocystis*. PLOS ONE 16(3): e0248185. https://doi.org/10.1371/journal.pone.0248185

Artículo 3. Herrera, G., Vega, L., Patarroyo, M.A. et al. Gut microbiota composition in health-care facility-and community-onset diarrheic patients with *Clostridioides difficile* infection. Sci Rep 11, 10849 (2021). https://doi.org/10.1038/s41598-021-90380-7

Artículo 4. Herrera, G., Paredes-Sabja, D., Patarroyo, M. A., Ramírez, J. D., & Muñoz, M. (2021). Updating changes in human gut microbial communities associated with *Clostridioides difficile* infection. *Gut Microbes*, *13*(1), 1966277.

Artículo 5. Herrera, G., Arboleda, J. C., Pérez-Jaramillo, J. E., Patarroyo, M. A., Ramírez, J. D., Muñoz M. (2022). Microbial interdomain interactions delineate the disruptive intestinal homeostasis in *Clostridioides difficile* infection. Microbiol Spectr. 10(5), e00502-22

Artículo 6. Herrera, Giovanny, Vieira, María Carmen, Páez-Triana, Luisa, Muro, Antonio, López-Abán, Julio, Muñoz, Marina, Ramírez, Juan David. Interrogating the gut microbiota from several human-biting tick species in Northwestern Spain. Journal of Medical Entomology (sometido)

Artículo 7. Herrera, G., Castañeda, S., Arboleda, J. C., Pérez-Jaramillo, J. E., Patarroyo, M. A., Ramírez, J. D., Muñoz M. Metagenome-assembled genomes (MAGs) suggest an Acetate-driven protective role in gut microbiota disrupted by *Clostridioides difficile.* (Sometido)

7.1.2. Índices de calidad de las publicaciones aportadas

<table>
<tr><td colspan="5">Publicado en Pathogens</td></tr>
<tr><td>Título</td><td colspan="4">Occurrence of Blastocystis in patients with Clostridioides difficile infection</td></tr>
<tr><td>Autores</td><td colspan="4">Vega, L., Herrera, G., Muñoz, M., Patarroyo, M. A., & Ramírez, J. D.</td></tr>
<tr><td>Volumen</td><td colspan="4">9</td></tr>
<tr><td>Número</td><td colspan="4">4</td></tr>
<tr><td>Páginas</td><td colspan="4">283</td></tr>
<tr><td>DOI</td><td colspan="4">10.3390/pathogens9040283</td></tr>
<tr><td rowspan="6">Indicadores de calidad de la revista</td><td colspan="4">WoS: Journal Citation Reports (JCR–SCIE edition)</td></tr>
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Microbiology – SCIE</td><td colspan="2">Ranking - Cuartil - Percentil:
58/137 Q2 58.03</td></tr>
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Infectious disease</td><td colspan="2">Ranking - Cuartil - Percentil:
151/295 Q2 48</td></tr>
</table>

<table>
<tr><td colspan="5">Publicado en PLoS One</td></tr>
<tr><td>Título</td><td colspan="4">Gut microbiota profiles in diarrheic patients with co-occurrence of Clostridioides difficile and Blastocystis.</td></tr>
<tr><td>Autores</td><td colspan="4">Vega L., Herrera G., Muñoz M., Patarroyo M. A., Maloney J. G., Santín, M., Ramírez, J. D.</td></tr>
<tr><td>Volumen</td><td colspan="4">16</td></tr>
<tr><td>Número</td><td colspan="4">3</td></tr>
<tr><td>Páginas</td><td colspan="4">e0248185</td></tr>
<tr><td>DOI</td><td colspan="4">10.1371/journal.pone.0248185</td></tr>
<tr><td rowspan="6">Indicadores de calidad de la revista</td><td colspan="4">WoS: Journal Citation Reports (JCR–SCIE edition)</td></tr>
<tr><td colspan="4">JIF 2021: 3.752</td></tr>
<tr><td colspan="2">Categoría:
Multidiscuplinary sciences</td><td colspan="2">Ranking - Cuartil - Percentil:
29/73 Q2 60.96</td></tr>
<tr><td colspan="4">SCOPUS y SCImago Journal Rank (SJR)</td></tr>
<tr><td>CiteScore: 5.6</td><td>SJR 2021: 0.852</td><td colspan="2">SNIP 2021: 1.368</td></tr>
<tr><td colspan="2">Categoría:
Multidisciplinary</td><td colspan="2">Ranking - Cuartil - Percentil:
15/120 Q1 87</td></tr>
</table>

Publicado en *Scientific Reports*			
Título	Gut microbiota composition in health-care facility-and community-onset diarrheic patients with *Clostridioides difficile* infection.		
Autores	Herrera, G., Vega, L., Patarroyo, M. A., Ramírez, J. D., Muñoz, M.		
Volumen	11		
Número	10849		
Páginas	NA		
DOI	10.1038/s41598-021-90380-7		
Indicadores de calidad de la revista	**WoS: Journal Citation Reports (JCR–SCIE edition)**		
	JIF 2021: 4.996		
	Categoría: Multidiscuplinary sciences	Ranking - Cuartil - Percentil: 19/73 Q2 74.66	
	SCOPUS y SCImago Journal Rank (SJR)		
	CiteScore: 6.9	SJR 2021:1.005	SNIP 2021: 1.389
	Categoría: Multidisciplinary	Ranking - Cuartil - Percentil: 11/120 Q1 91st	

Publicado en *Gut Pathogens*		
Título	Updating changes in human gut microbial communities associated with Clostridioides difficile infection	
Autores	Herrera, G., Paredes-Sabja, D., Patarroyo, M. A., Ramírez, J. D., Muñoz, M.	
Volumen	13	
Número	1	
Páginas	1966277	
DOI	10.1080/19490976.2021.1966277	
Indicadores de calidad de la revista	**WoS: Journal Citation Reports (JCR–SCIE edition)**	
	JIF 2021: 5.324	
	Categoría: Microbiology - SCIE	Ranking - Cuartil - Percentil: 44/137 Q1 68.25
	SCOPUS y SCImago Journal Rank (SJR)	

	CiteScore: 6.5	SJR 2021: 1.037	SNIP 2021: 1.296
	Categoría: Infectious diseases	Ranking - Cuartil - Percentil: 73/295 Q1 82	

Publicado en *Microbiology Spectrum*			
Título	Microbial interdomain interactions delineate the disruptive intestinal homeostasis in *Clostridioides difficile* infection.		
Autores	Herrera, G., Arboleda, J. C., Pérez-Jaramillo, J. E., Patarroyo, M. A., Ramírez, J. D., Muñoz M.		
Volumen	10		
Número	5		
Páginas	502 – 522		
DOI	https://doi.org/10.1128/spectrum.00502-22		
Indicadores de calidad de la revista	**WoS: Journal Citation Reports (JCR–SCIE edition)**		
	JIF 2021: 9.043		
	Categoría: Microbiology	Ranking - Cuartil - Percentil: 20/136 Q1 85.66	
	SCOPUS y SCImago Journal Rank (SJR)		
	CiteScore: 12.3	SJR 2021: 2.148	SNIP 2021: 2.22
	Categoría: Microbiology	Ranking - Cuartil - Percentil: 8/116 Q1 93	

7.1.3. Presentaciones en eventos

Nombre evento: Annual conference online 2021 Anaerobe 2021: the microbiota and beyond
Tipo de evento: Congreso
Ámbito: Internacional
Nombre ponencia: Gut microbiota composition in health-care facility-and community-onset diarrheic patients under *Clostridioides difficile* infection
Tipo de producto: Póster
Fecha: 15 – 16 de julio de 2021

Nombre evento: Jornadas científicas "Latinbiota 2021"
Tipo de evento: Conferencia
Ámbito: Internacional
Nombre ponencia: Gut microbiota composition in health-care facility-and community-onset diarrheic patients with Clostridioides difficile infection
Tipo de producto: Presentación oral
Fecha: noviembre 2021

Nombre evento: Seminarios "CIETUS"
Tipo de evento: Conferencia
Ámbito: Internacional
Nombre ponencia: Cambios en la microbiota intestinal asociados a la infección por Clostridioides difficile: Una aproximación metagenómica
Tipo de producto: Presentación oral
Fecha: 15 de diciembre de 2021

Nombre evento: XVIII Congreso Colombiano de Parasitología y Medicina Tropical
Tipo de evento: Congreso
Ámbito: Internacional
Nombre ponencia: Gut microbiota composition in health-care facility-and community-onset diarrheic patients with Clostridioides difficile infection
Tipo de producto: Presentación oral
Fecha: noviembre 2021

7.1.4. Pasantías

Lugar: Centro de Investigación de Enfermedades Tropicales de la Universidad de Salamanca (CIETUS)
Duración: 6 meses

7.2.*Becas y reconocimientos*

- Beca comité doctoral, Universidad del Rosario. 1er puesto entre aspirantes al doctorado
- Beca pasantía estudios doctorales, Universidad del Rosario. Asistente graduado

7.3.Publicaciones en índice de impacto sobre microbiomas

Ramírez, A. L., Herrera, G., Muñoz, M., Vega, L., Cruz-Saavedra, L., García-Corredor, D., ... & Ramírez, J. D. (2021). Describing the intestinal microbiota of Holstein Fasciola-positive and-negative cattle from a hyperendemic area of fascioliasis in central Colombia. *PLoS Neglected Tropical Diseases*, *15*(8), e0009658.

7.4.Publicaciones en índice de impacto sobre garrapatas

Páez-Triana, L., Muñoz, M., Herrera, G., Moreno-Pérez, D. A., Tafur-Gómez, G. A., Montenegro, D., ... & Ramírez, J. D. (2021). Genetic diversity and population structure of Rhipicephalus sanguineus sensu lato across different regions of Colombia. *Parasites & Vectors*, *14*, 1-11.

7.5.Otras publicaciones en índice de impacto

Velásquez-Ortiz, N., Hernández, C., Cantillo-Barraza, O., Ballesteros, N., Cruz-Saavedra, L., Herrera, G., ... & Ramírez, J. D. (2022). Trypanosoma cruzi Parasite Burdens of Several Triatomine Species in Colombia. *Tropical Medicine and Infectious Disease*, *7*(12), 445.

Velásquez-Ortiz, N., Herrera, G., Hernández, C., Muñoz, M., & Ramírez, J. D. (2022). Discrete typing units of Trypanosoma cruzi: Geographical and biological distribution in the Americas. *Scientific Data*, *9*(1), 360.

Villanueva-Saz, S., Martínez, M., Ramirez, J. D., Herrera, G., Marteles, D., Servián, M., ... & Fernández, A. (2022). Evaluation of five different rapid immunochromatographic tests for canine leishmaniosis in Spain. *Acta Tropica*, *229*, 106371.

Cantillo-Barraza, O., Torres, J., Hernández, C., Romero, Y., Zuluaga, S., Correa-Cárdenas, C. A., ... & Méndez, C. (2021). The potential risk of enzootic Trypanosoma cruzi transmission inside four training and re-training military battalions (BITER) in Colombia. *Parasites & Vectors*, *14*, 1-13.

Higuera, A., Herrera, G., Jimenez, P., García-Corredor, D., Pulido-Medellín, M., Bulla-Castañeda, D. M., ... & Ramírez, J. D. (2021). Identification of multiple Blastocystis subtypes in domestic animals from Colombia using amplicon-based next generation sequencing. *Frontiers in Veterinary Science*, *8*, 732129.

Castillo-Castañeda, A., Herrera, G., Ayala, M. S., Fuya, P., & Ramírez, J. D. (2021). Spatial and temporal variability of visceral leishmaniasis in Colombia, 2007 to 2018. *The American Journal of Tropical Medicine and Hygiene*, *105*(1), 144.

Ramírez, J. D., Florez, C., Muñoz, M., Hernández, C., Castillo, A., Gomez, S., ... & Paniz-Mondolfi, A. (2021). The arrival and spread of SARS-CoV-2 in Colombia. *Journal of Medical Virology*, *93*(2), 1158-1163.

Teherán, A. A., Ramos, G. C., De La Guardia, R. P., Hernández, C., Herrera, G., Pombo, L. M., ... & Ramírez, J. D. (2020). Epidemiological characterisation of asymptomatic carriers of COVID-19 in Colombia: a cross-sectional study. *BMJ open*, *10*(12), e042122.

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