Caracterización de *Clostridium perfringens* circulante en muestras fecales de humanos y animales en el altiplano Cundiboyacense Colombiano

Anny Jineth Camargo Mancipe

Documento de tesis presentado como requisito para optar al título de
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"Si he logrado ver más lejos,
ha sido porque he subido a hombros de gigantes".
Isaac Newton.

A mis maestros:
Mis padres y profesores

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1. LISTA DE PUBLICACIONES

Esta Tesis Doctoral corresponde a un compendio de artículos científicos publicados en revistas internacionales e indexadas en el Science Citation Reports. A continuación, se listan todos los artículos que fueron publicados, los cuales están adjuntos a este documento. Cualquier información suplementaria y/o tablas se incluirán en archivos comprimidos organizados según el número asignado a continuación:

Artículo 1: Camargo A, Páez-Triana L, Camargo D, García-Corredor D, Pulido-Medellín M, Camargo M, Ramírez J.D. and Muñoz M*. Carriage of *Clostridium perfringens* in Domestic and Farm Animals across the Central Highlands of Colombia: Implications for Gut Health and Zoonotic Transmission. Vet Res Commun. 2024.

Artículo 2: Camargo A., Bohórquez L., López D., Ferrebuz-Cardozo A., Castellanos-Rozo J., Díaz J., Rada M., Camargo M., Ramírez J. D. and Muñoz M. *Clostridium perfringens* in central Colombia: Frequency, Toxin Genes, and Risk Factors. (**SOMETIDO** en Gut Pathogens)

Artículo 3: Camargo A., Guerrero-Araya E, Castañeda S, Vega L, Cardenas-Alvarez MX, Rodríguez C, Paredes-Sabja D, Ramírez JD, Muñoz M. Intra-species diversity of *Clostridium perfringens*: A diverse genetic repertoire reveals its pathogenic potential. Front Microbiol. 2022 Jul 22; 13:952081. doi: 10.3389/fmicb.2022.952081. PMID: 35935202; PMCID: PMC9354469.

Artículo 4: Camargo A., Bohorquez L., Cáceres T., Ferrebuz-Cardozo A, Díaz J, Castellanos-Rozo J, Diaz J., Kiu R., Hall L. J., Rámirez J. D. and Muñoz M. Insights into *Clostridium perfringens* Dispersal Hotspots, Toxins, and Virulence Factors through Integrated Genomic and Phenotypic Profiling. (**EN CONSTRUCCIÓN**)

Artículo 5: Camargo A, Rámirez J. D., Kiu R., Hall L.J., Muñoz M. Unveiling the pathogenic mechanisms of *Clostridium perfringens* toxins and virulence factors. Emerg Microbes Infect. 2024 Apr 9:2341968. doi: 10.1080/22221751.2024.2341968. Epub ahead of print. PMID: 38590276.

Artículo 6: Herrera G, Vega L, Camargo A, Patarroyo MA, Ramírez JD, Muñoz M. Acquisition site-based remodelling of *Clostridium perfringens*- and Clostridioides difficile-related gut microbiota. Comp Immunol Microbiol Infect Dis. 2023 Nov; 102:102074. doi: 10.1016/j.cimid.2023.102074. Epub 2023 Oct 10. PMID: 37832162.

2. LISTA DE ANEXOS

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Artículo 6

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2.2.2 Presentación en eventos científicos
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3. LISTA DE FIGURAS

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Figura 2. Mecanismos de acción de las principales toxinas de *C. perfringens* utilizadas para la toxinotipificación.

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Figura 4. Enfermedades humanas causadas por *C. perfringens.*

Figura 5. Enfermedades animales causadas por *C. perfringens.*

4. LISTA DE ABREVIATURAS

ADN: Desoxirribonucleico
CDC: Center for Disease Control and Prevention
CPA: Alfa-toxina de *C. perfringens*
CPB: Beta-toxina de *C. perfringens*
CPE: Enterotoxina de *C. perfringens*
DAA: Diarrea asociada a antibióticos
ECN: Enterocolitis necrotizante
EN: Enteritis necrotizante
ETA: Enfermedades transmitidas por alimentos
ETX: Epsilon-toxina
GTP: Guanosín trifosfato
ITX: Iota-toxina
LSR: Receptor de lipoproteínas estimulado por lipólisis
MRA: Marcadores de resistencia a antibióticos
NETB: Necrotic enteritis B-like toxin
OMS: Organización Mundial de la Salud
PCR: Reacción en cadena de polimerasa
PFOA: Perfingolisina O
TSC: Triptosa Sulfito Cicloserina
UFC: Unidades Formadoras de Colonias

5. RESUMEN

Clostridium perfringens es una bacteria anaerobia Gram positiva, formadora de esporas y productora de toxinas, que puede encontrarse en el tracto intestinal humano y animal, así como en alimentos, suelo y agua en forma esporulada. Esta bacteria es reconocida como causante de brotes de intoxicación alimentaria, necrosis intestinal y mionecrosis grave en humanos y animales, ocupando el tercer lugar en incidencia de enfermedades transmitidas por alimentos (ETA) en los Estados Unidos.

Estudios recientes han revelado la presencia de aislamientos toxigénicos de *C. perfringens*, portadores de la toxina perfingolisina O (PFOA) en individuos sanos. Esta toxina se relacionó con toxicidad celular, hemólisis completa, respuestas proinflamatorias y una mayor capacidad de esporulación. La combinación de es estas características biológicas en aislamientos de *C. perfringens* se correlacionan con un avance desalentador de la patología intestinal, estando asociado con infección/enfermedad y una mayor propagación entre hospederos. Asimismo, se ha observado un aumento en los factores de virulencia y marcadores de resistencia a los antibióticos (MRA) que transporta esta especie bacteriana, lo que representa un desafío considerable tanto a nivel clínico como de salud pública a escala global.

A pesar de la relevancia para la salud pública, es evidente que en Colombia y en Suramérica existe un conocimiento limitado sobre esta bacteria. Por lo tanto, es crucial avanzar en la descripción de su arquitectura genómica, así como de sus factores de virulencia y resistencia antibiótica. Estos conocimientos son fundamentales para mejorar las estrategias de prevención y manejo de las enfermedades causadas por *C. perfringens*.

Por lo tanto, esta tesis doctoral tuvo como objetivo describir la frecuencia de detección, diversidad genética y principales factores de virulencia de *C. perfringens* en muestras fecales de humanos y animales en el altiplano Cundiboyacense Colombiano.

La tesis se dividió en tres capítulos:

El Capítulo I se enfocó en determinar la frecuencia de detección de *C. perfringens* en humanos y animales en el altiplano Cundiboyacense colombiano mediante pruebas moleculares. Las muestras fecales de humanos con y sin síntomas gastrointestinales, así como muestras fecales de diversas especies animales, fueron colectadas en los Departamentos de Cundinamarca y Boyacá. Se realizó la extracción de ADN y PCR dirigida al gen *16S-rRNA* y al gen de la toxina alfa (*cpa*) para la detección de *C. perfringens*.

Los resultados revelaron una alta frecuencia de detección de *C. perfringens* a nivel general en animales domésticos y cerdos. En humanos la toxina beta 2 de *C. perfringens* (CPB2) se asoció con la presencia de diabetes, lo que sugiere interacciones con el sistema inmunológico del huésped.

El Capítulo II tuvo como objetivo describir la estructura genética poblacional y los factores de virulencia de *C. perfringens* utilizando genomas públicos para evaluar la diversidad genética, linajes y toxinotipos circulantes en diferentes hospederos, así como los principales factores de virulencia a nivel global. Los análisis bioinformáticos revelaron que la mayoría de los genomas provienen de países desarrollados como EE. UU., Francia y China, cuyos aislamientos fueron establecidos principalmente de alimentos, aves y humanos. La clasificación filogenética mostró rutas de dispersión entre diferentes hospederos, además se encontró una alta frecuencia de toxinas como toxina alfa (CPA) y enterotoxina (CPE), así como el incremento en la detección de MRA asociados a tetraciclinas y macrólidos. Por otra parte, se detectó una escasa representación de aislamientos en países en desarrollo, destacando la necesidad de investigaciones locales para comprender mejor la diversidad genética y los mecanismos de transmisión y virulencia de este patógeno.

El propósito del Capítulo III fue caracterizar la arquitectura genómica, factores de virulencia y MRA de aislamientos colombianos de *C. perfringens*, obtenidos de muestras positivas de humanos y animales recolectadas en el Capítulo I. Además, se llevaron a cabo pruebas fenotípicas de hemólisis, inhibición del crecimiento celular, esporulación y susceptibilidad a antibióticos en un grupo representativo de aislamientos. El criterio de selección de los aislamientos se basó en su capacidad para producir la toxina PFOA, una toxina formadora de poros que juega un papel crucial en la patogenia intestinal, con el objetivo de evaluar su impacto biológico. El análisis microgeográfico de aislamientos obtenidos de diversas fuentes en una región central de Colombia, reveló una amplia diversidad genética y posibles eventos de dispersión entre humanos, y animales domésticos como perros y gatos. La presencia de toxinas como PFOA en aislamientos de individuos asintomáticos plantea un potencial riesgo de infección en dicha población, debido a su asociación con hemolisis, inhibición del crecimiento celular y mayor capacidad de esporulación. Además, se observó una reducción de la susceptibilidad a varios antibióticos, incluyendo gentamicina, eritromicina, metronidazol y tetraciclina.

En resumen, este estudio ha enriquecido el entendimiento de *C. perfringens* en Colombia al proporcionar datos de epidemiología molecular y genómica de *C. perfringens* circulante en la región de análisis. Además, ofreció el primer reporte de genomas obtenidos de gatos a nivel mundial e incluyó el mayor número de genomas registrados en América Latina hasta la fecha. Nuestros hallazgos permitieron fortalecer las acciones de promoción de la salud y prevención de la transmisión de enfermedades infecciosas a través de campañas educativas con la comunidad y las entidades de salud, con implicaciones significativas para mejorar la calidad de vida de poblaciones vulnerables.

6. MARCO TEÓRICO

6.1 Generalidades sobre *Clostridium perfringens*

C. perfringens (anteriormente conocido como *Bacillus aerogenes capsulatus*, *Bacillus perfringens*, *Bacillus welchii* o *Clostridium welchii*) es un bacilo Gram positivo, anaerobio que carece de flagelos pero exhibe motilidad de deslizamiento mediada por pili tipo IV [1], productor de toxinas y formador de esporas, lo que le permite sobrevivir en condiciones extremas o con pocos nutrientes, una característica que favorece su rápida dispersión [2].

Esta bacteria fue identificada y aislada por primera vez por William H. Welch en 1891 a partir de la autopsia de un hombre donde se observaron burbujas de gas dentro de sus vasos sanguíneos, signos posteriormente asociados con gangrena gaseosa presentada por soldados británicos durante la Primera Guerra Mundial [3].

C. perfringens es un enteropatógeno oportunista capaz de colonizar el intestino de humanos y animales sin causar síntomas (colonización). Sin embargo, cuando la presencia de esta bacteria se combina con otros factores de riesgo como enfermedades inflamatorias intestinales, cambios dietéticos a dietas ricas en proteínas, o la coexistencia de otros patógenos, puede desencadenar infecciones con síntomas como diarrea, dolor abdominal, vómitos y en los casos graves, necrosis intestinal y afectación sistémica [4, 5].

La producción de toxinas juega un papel crucial en el efecto patógeno de esta bacteria, predisponiendo a infecciones graves. Dentro de los factores de riesgo clave asociados con la producción de toxinas se destacan el uso de antiácidos y la edad avanzada pueden aumentar la susceptibilidad a estas infecciones, no obstante, los mecanismos exactos que influyen en el riesgo individual aún no están completamente claros [5, 6].

6.2 Epidemiología

Según el Centro de Control y Prevención de Enfermedades de los EE. UU., durante el periodo de 2009 a 2015 se presentaron más de 800 brotes de ETA que generaron 800 hospitalizaciones y más de 20 muertes [7]. Dentro de los organismos comúnmente asociados a ETA *C. perfringens* hace parte de las cinco causas principales junto a norovirus, *Salmonella*, *Campylobacter* y *Staphylococcus aureus* [8, 9].

En Inglaterra, se estima que entre el 8 y el 13% de los brotes gastrointestinales de origen alimentario están asociados a esta bacteria (90.000 casos de *C. perfringens* al año) [10]. Según el informe de la Autoridad Europea de Seguridad Alimentaria y el Centro Europeo para la Prevención y el Control de Enfermedades, donde se presentan los resultados de las actividades de seguimiento de las zoonosis llevadas a cabo en 2017 en 37 países europeos, se informaron 818 (16,1%) brotes de origen alimentario, asociados con toxinas bacterianas especialmente de *C. perfringens*, *Staphylococcus* y *Bacillus cereus* [11].

6.3 Características del genoma de *C. perfringens*

El tamaño del genoma de *C. perfringens* varía de 3.0 a 4.1 Mb, con un contenido de G-C relativamente bajo entre 27 y 28% y un potencial codificante de 2.500 a 3.600 genes aproximadamente [12]. Además, presenta un pangenoma compuesto por 11.667 genes (12,6% de genes centrales y 87,4% de genes accesorios), estas características representan un indicador de la alta plasticidad genómica de *C. perfringens* [13-15].

Los genomas de *C. perfringens* incluyen genes que codifican para homólogos del sistema feoAB de captación de hierro, un elemento esencial para la supervivencia bacteriana, así como genes asociados a actividades glucolíticas, lo que refleja su capacidad para adaptarse a diversas condiciones ambientales [16]. Además, los genomas de dichas poblaciones bacterianas contienen genes para factores putativos que facilitan la respuesta adaptativa al estrés, incluyendo la enzima superóxido dismutasa, reductasas de metionina sulfóxido y glutatión peroxidasas, contribuyendo a su resiliencia y supervivencia en ambientes hostiles [17].

6.4 Factores de virulencia de *C. perfringens*

6.4.1 Toxinas principales de *C. perfringens*

El impacto de las infecciones por *C. perfringens* se debe en parte al potencial de la bacteria para secretar múltiples toxinas extracelulares, incluyendo la alfa-toxina (CPA, gen *cpa/plc*), la beta-toxina (CPB, gen *cpb*), la epsilon-toxina (ETX, gen *etx*), la iota-toxina (ITX, genes binarios *iap* e *ibp*), la enterotoxina de *C. perfringens* (CPE, gen *cpe*) y Necrotic enteritis B-like toxin (*NetB*). La presencia diferencial de estas toxinas permite la clasificación en siete toxinotipos del A al G cada uno implicado con una enfermedad en un hospedero particular [18] (**Figura 1**).

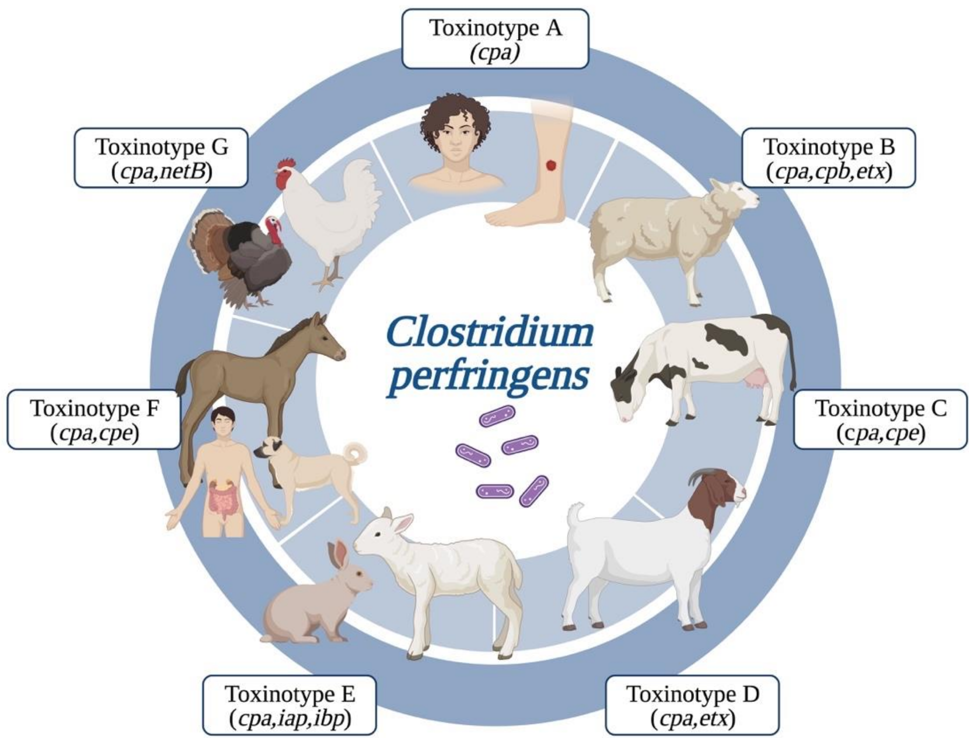

Figura 1. Representación esquemática de los principales hospedadores para cada toxinotipo de *C. perfringens*. Cada recuadro representa un toxinotipo diferente. Tomado de Camargo y colaboradores.

La toxina alfa (CPA), compuesta por 370 aminoácidos, es producida por todos los toxinotipos de *C. perfringens* y se asocia con mionecrosis clostridial en individuos inmunocomprometidos. Esta patología se caracteriza por edema subcutáneo, necrosis muscular, enfisema, shock y falla multiorgánica [19]. CPA actúa sobre la proteína de unión a Guanosín Trifosfato (GTP), desencadenando la apoptosis y necrosis celular [20]. La CPA también induce la activación de la vía del ácido araquidónico, generando prostaglandinas, tromboxanos y leucotrienos que contribuyen a la inflamación y a la disminución de la perfusión sanguínea [21, 22] (**Figura 2A**).

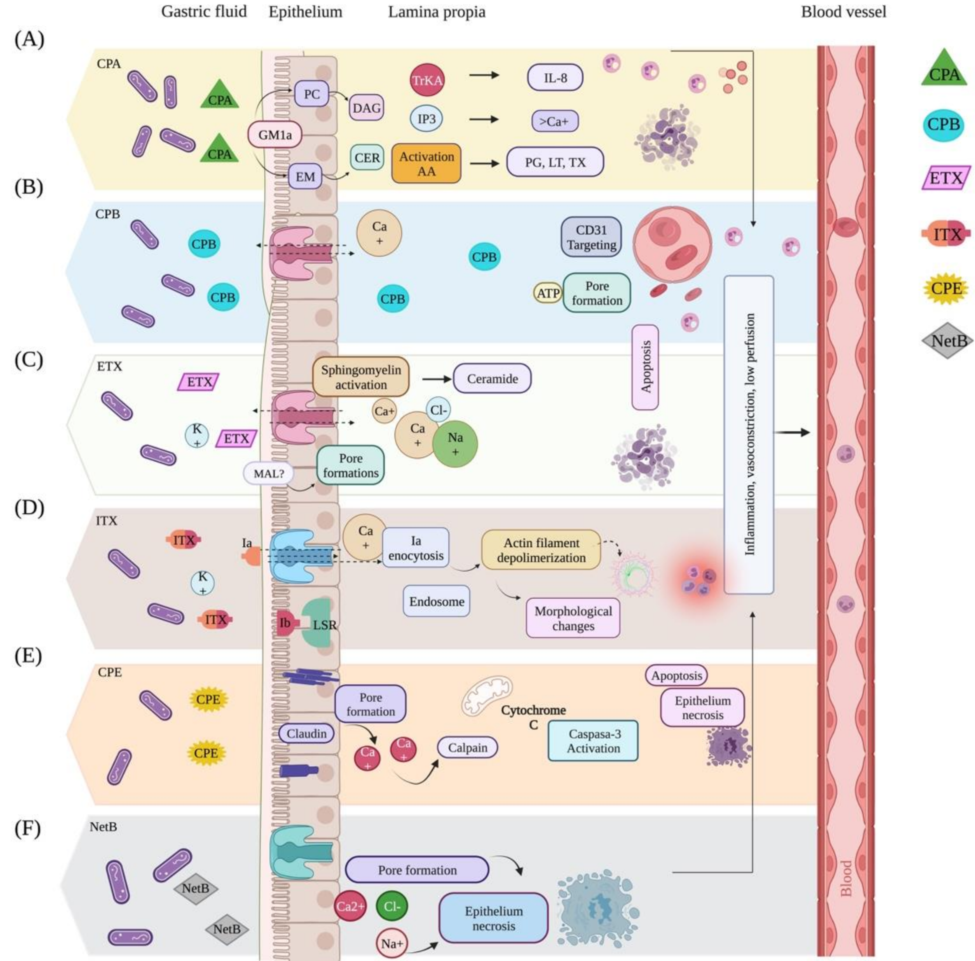

Figura 2. Mecanismos de acción de las principales toxinas de *C. perfringens* utilizadas para la toxinotipificación. Mecanismo de acción molecular de las principales toxinas de *C. perfringens*. **(A).** Toxina CPA: La toxina CPA interactúa con GM1a, hidrolizando la fosfatidilcolina (PC) y la esfingomielina (SM) dando lugar a la formación de diacilglicerol (DAG) y ceramida (CER) con la activación del receptor Tropomiosina quinasa A (TrKA) y desencadena la activación de una cascada de señalización intracelular con liberación de Interleucina - 8 (IL-8). Se produce la activación del fosfatidil inositol 3 (IP3) que promueve la entrada de calcio (Ca+) intracitoplasmático **(B).** Toxina CPB: La CPB se une a la molécula-1 de adhesión de las células endoteliales plaquetarias (PECAM-1) con la consiguiente liberación de trifosfato de adenosina (ATP) y formación de poros que permiten el intercambio de iones hacia y desde la célula **(C).** Toxina ETX: La toxina ETX interactúa con la proteína "mielina y linfocitos" (MAL) formando un poro activo que induce el transporte e intercambio de iones a través de la membrana celular **(D).** Toxina ITX: La unión de Ib al receptor de lipoproteínas estimulado por lipólisis (LSR) media su entrada en la célula huésped, promoviendo la formación de canales para la entrada de Ia por endocitosis con la consiguiente despolimerización de los filamentos de actina, generando cambios morfológicos y alteración de la permeabilidad celular **(E).** Toxina CPE: la toxina CPE se une a los receptores de claudina, contribuyendo a la formación de un poro en la

superficie celular con intercambio iónico y desequilibrio osmótico. **(F).** La toxina NetB reconoce regiones libres de colesterol en las membranas celulares formando poros hidrofílicos heptaméricos que permiten la entrada de iones como Na+, Cl- y Ca 2+. Tomado de Camargo y colaboradores.

La toxina beta (CPB), compuesta por 336 aminoácidos producida por *C. perfringens* tipo B y C, está vinculada con disentería hemorrágica en ovejas y enteritis necrótica en ganado y humanos [23, 24]. Su mecanismo de acción ocurre a nivel de la mucosa intestinal, generando poros en las membranas celulares y causando hemorragias y trombos fibrinosos en el intestino [31]. La patología de la enfermedad se caracteriza principalmente por hemorragias y trombos de fibrina que obstruyen la microvasculatura en la lámina propia del intestino, desencadenando necrosis del epitelio [25] (**Figura 2B**).

La toxina Épsilon (ETX), producida por *C. perfringens* tipo B y D, se asocia con enterotoxemias en rumiantes y puede provocar complicaciones cerebrales graves [25]. Su mecanismo de acción implica la formación de poros en la membrana plasmática, conduciendo a la muerte celular y a la alteración de la transmisión eléctrica en el cerebro [26]. Además, se ha demostrado que induce inflamación, necrosis, daño mitocondrial y alteraciones en la membrana celular [27, 28] (**Figura 2C**).

La Iota toxina (ITX), producida por el toxinotipo E, se vincula con enteritis hemorrágica. ITX es una toxina binaria implicada en daño al citoesqueleto celular y apoptosis [29, 30]. Su ingreso a las células se facilita a través del receptor de lipoproteínas estimulado por lipólisis (LSR), lo que conduce a la apoptosis celular y a un aumento de la permeabilidad de las monocapas de las células intestinales [29, 31, 32] (**Figura 2D**).

El gen *cpe*, que codifica la toxina CPE, se halla en el cromosoma de la mayoría de los aislamientos de *C. perfringens* asociadas a intoxicaciones alimentarias o en plásmidos conjugativos grandes, acompañado de secuencias de inserción que facilitan su movilización y diseminación [33]. La toxina CPE es producida durante la esporulación de *C. perfringens* tipo F, es crucial en intoxicaciones alimentarias y diarrea [34]. Actúa formando poros en las membranas celulares, desencadenando la muerte celular por necrosis del epitelio ileal y colónico humano a través de la liberación de citocromo C y activación de caspasa-3 [33, 35] **Figura 2E**.

Finalmente, la toxina de enteritis necrótica tipo B (NetB), es una toxina formadora de poros codificada por el gen *netB* ubicada en plásmidos conjugativos [36]. Es producida por *C. perfringens* tipo G [18]. Esta toxina está implicada en enteritis necrótica aviar, forma poros hidrófilos que causan lisis celular y destrucción de la lámina propia del intestino [37, 38] (**Figura 2F**).

6.4.2 Toxinas accesorias de *C. perfringens*

Perfingolisina O

La Perfingolisina O (PFOA), clásicamente conocida como toxina θ, es codificada por el gen *pfoA*, situado en el cromosoma de *C. perfringens*, y actúa como una toxina formadora de poros en las membranas que contienen colesterol. Aunque se ha sugerido que casi todos los aislamientos de *C.*

perfringens codifican el gen *pfoA*. Algunos estudios han revelado que la mayoría de los aislamientos productores de enterotoxinas asociadas a intoxicaciones alimentarias carecen de este gen [17, 39].

La toxina PFOA se asocia con citotoxicidad en células Caco-2 y hemólisis completa, lo que está vinculado con la patología de las células intestinales y posiblemente con los resultados de infección o enfermedad en neonatos con enterocolitis necrotizante. Por otro lado, los aislamientos de *C. perfringens* portadores de *pfoA* tanto en individuos sanos como en aquellos con enterocolitis necrotizante, muestran una mayor tolerancia al oxígeno y una capacidad de formación de esporas/germinación más pronunciada, lo que parece favorecer su propagación [40].

Además, la toxina PFOA se vincula con el desarrollo de la gangrena gaseosa en humanos, ya que parece actuar de forma sinérgica con la toxina CPA para afectar desde la leucostasis periférica hasta la mionecrosis y la coagulopatía intravascular [41, 42]. Asimismo, la PFOA puede aumentar la expresión de moléculas de adhesión, como CD11b / CD18 en leucocitos y la molécula de adhesión intracelular 1 (ICAM-1) y el factor activador de plaquetas en células endoteliales humanas, contribuyendo así a la trombosis y a la disminución del flujo sanguíneo en la gangrena gaseosa [43, 44].

Alveolisina

La alveolisina es una toxina poco estudiada que es dependiente de colesterol y guarda similitudes con la PFOA [45]. Se activa a través de compuestos reductores, específicamente mediante la activación de tiol, y su receptor principal es el colesterol. Esta toxina exhibe actividad lítica contra los eritrocitos de varias especies animales. Se ha descrito que esta toxina está implicada en la inducción de la expresión de IL8 en polimorfonucleares humanos y en poblaciones de células linfocitos-monocitos-basófilos [46].

A medida que se acumula más información sobre el papel de esta familia de toxinas como factores de virulencia importantes, se hace cada vez más evidente que sus efectos pueden ir más allá de la simple destrucción de eritrocitos mediante la formación de poros. Los efectos aparentes sobre la función de las células inmunitarias y la inducción de vías inflamatorias parecen ser su principal mecanismo de acción [46].

***Clostridium perfringens* beta 2**

Clostridium perfringens beta 2 (CPB2), es una toxina accesoria localizada en un plásmido que se ha asociado a enteritis porcina, equina y bovina [47].

La estructura y el receptor de CPB2 siguen sin estar claros. Sin embargo, se ha sugerido que sólo una fracción muy pequeña de segmentos de aminoácidos adopta una conformación α-hélice, lo que es insuficiente para atravesar una membrana. En contraste, se han identificado algunos segmentos de aminoácidos que podrían formar cadenas β transmembrana [48].

Esta toxina formadora de poros está implicada en la formación de canales selectivos de cationes de aproximadamente 1,4 nm de diámetro en bicapas lipídicas, lo que provoca una alteración del flujo de iones y un aumento de la permeabilidad intestinal que desencadena la sintomatología durante la enteritis [49].

Sialidasas

Las sialidasas, también conocidas como neuraminidasas y exosialidasas, son codificadas por los genes *nanH*, *nanI* y *nanJ*, y desempeñan un papel crucial en la patogénesis de bacterias patógenas. Estas enzimas promueven la colonización bacteriana, la adhesión, la internalización, la formación de biopelículas y la unión de toxinas a las células huésped al hidrolizar el enlace α-glucósido del ácido siálico terminal en glucoproteínas y glucolípidos [50, 51]

Los aislamientos de *C. perfringens*, producen diversas sialidasas como: NanH, NanI y NanJ, codificadas por genes ubicados en diferentes regiones del cromosoma [17, 52]. La producción de estas enzimas varía entre aislamientos, siendo NanH un posible factor de virulencia accesorio que aún continua en estudio, mientras que para NanI se ha demostrado que juega un papel crucial en la adherencia a células y tejidos del huésped, así como en la modulación de la actividad de toxinas como CPA, CPB, ETX y NetF. Estudios sugieren que estas enzimas pueden regular positivamente la producción de toxinas, mejorar su citotoxicidad, aumentar la adhesión bacteriana a las células huésped y proporcionar sustratos para el crecimiento bacteriano y el metabolismo [53-55].

6.4.3 Esporulación de *C. perfringens*

La capacidad de *C. perfringens* para formar esporas juega un papel clave durante la transmisión de esta bacteria, su papel en las enfermedades y su supervivencia en muchos nichos ambientales como el suelo, aguas residuales, heces y alimentos.

Las esporas de *C. perfringens* presentan una compleja estructura multicapa que desempeña distintas funciones esenciales para su supervivencia y reproducción. La capa más externa está formada por más de 50 proteínas específicas, las cuales ofrecen protección contra enzimas líticas [56] **Figura 3**.

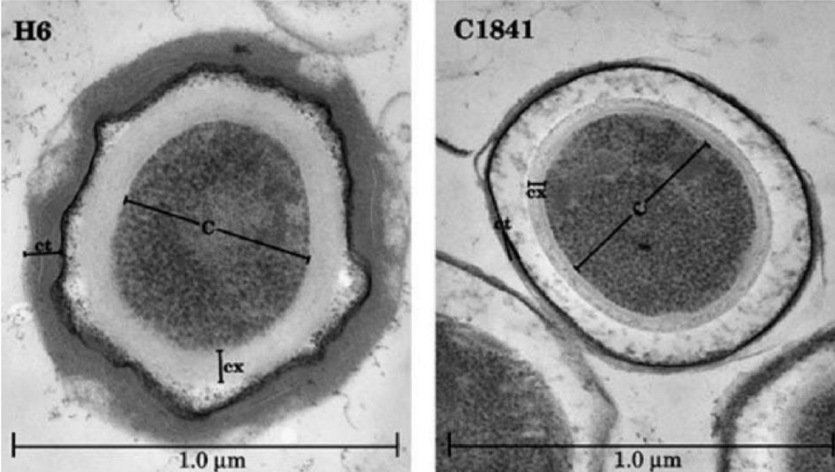

Figura 3. Microfotografías electrónicas de transmisión representativas de esporas de cepas de *C. perfringens* que contienen genes cromosómicos *cpe*. Cada microfotografía está etiquetada con la designación de la cepa respectiva. Los componentes estructurales medidos por tamaño están etiquetados: ct, capas de recubrimiento proteínico de la espora; cx, región de la corteza de la espora; y c, el núcleo de la espora con ribosomas que dan un aspecto granular. Las barras más bajas de cada micrografía representan 1,0 AM. Tomado de Novak y colaboradores [57].

Subsiguientemente, se encuentra el pelaje de la espora, seguido por la membrana externa. Aunque esta membrana no proporciona protección a las esporas inactivas, es fundamental para el proceso de formación de esporas. Bajo estas capas, se halla la corteza, compuesta principalmente de peptidoglicano, similar a la pared celular en crecimiento. Esta corteza es crucial para mantener la hidratación del núcleo de la espora, contribuyendo significativamente a la resistencia de las esporas frente al estrés ambiental y a agentes químicos [56]. Procediendo hacia el interior, se encuentra la pared de la célula germinal, la cual, a diferencia de las capas exteriores, no juega un rol en la resistencia de las esporas. Durante el proceso de germinación, esta pared se transforma en la pared celular activa.

La membrana interna, situada debajo de la pared de la célula germinal, actúa como una barrera protectora para el núcleo, que constituye la capa más interna de la espora. El núcleo alberga el ADN, el ARN y la mayoría de las enzimas críticas para las esporas, siendo esencial para su viabilidad y funcionamiento [58].

Las esporas mueren por daño a varios componentes diferentes, como el ADN, la membrana interna de la espora, las proteínas del núcleo de la espora, entre otros. Sin embargo, su resistencia está dada por el bajo contenido de agua del núcleo de la espora (20-50%), su capa protectora de peptidoglicano, sus altos niveles de ácido Ca-dipicolínico (Ca-DPA) (25%) y la saturación de ADN con pequeñas proteínas solubles en ácido (SASPs) [59, 60].

Durante la intoxicación alimentaria, causada por *C. perfringens,* las esporas germinan en alimentos con temperaturas de cocción muy bajas (<50 ºC) seguido de una rápida multiplicación de células vegetativas. Con un tiempo de duplicación muy corto de ~10 minutos, lo que le permite crecer

rápidamente en alimentos hasta alcanzar una carga bacteriana (>10^6 células vegetativas/gramo de alimento) necesaria para iniciar la enfermedad gastrointestinal [58].

Una vez ingeridos los alimentos contaminados, muchas de estas células mueren por exposición al pH ácido del estómago; sin embargo, algunas sobreviven y llegan al intestino delgado donde se inicia el proceso de esporulación en presencia de fosfato inorgánico en el medio ambiente. Durante la esporulación la toxina CPE involucrada en la patogenésis de la intoxicación alimentaria, se libera en la luz cuando la célula madre esporulante se lisa para liberar su espora madura [58, 61].

En los casos de mionecrosis, las células vegetativas o esporas de *C. perfringens* pueden ingresar al tejido muscular a través de una herida y germinar en condiciones de oxido – reducción (redox), las células vegetativas resultantes luego crecen rápidamente para reducir aún más las condiciones de Redox del tejido, promoviendo un crecimiento bacteriano adicional. Las células vegetativas producen CPA, CPE y PFOA que provocan necrosis local, daño del tejido, entre otros [62].

6.5 Impacto de *C. perfringens* en humanos

Se han descrito múltiples patologías humanas asociadas a *C. perfringens* (**Figura 4**).

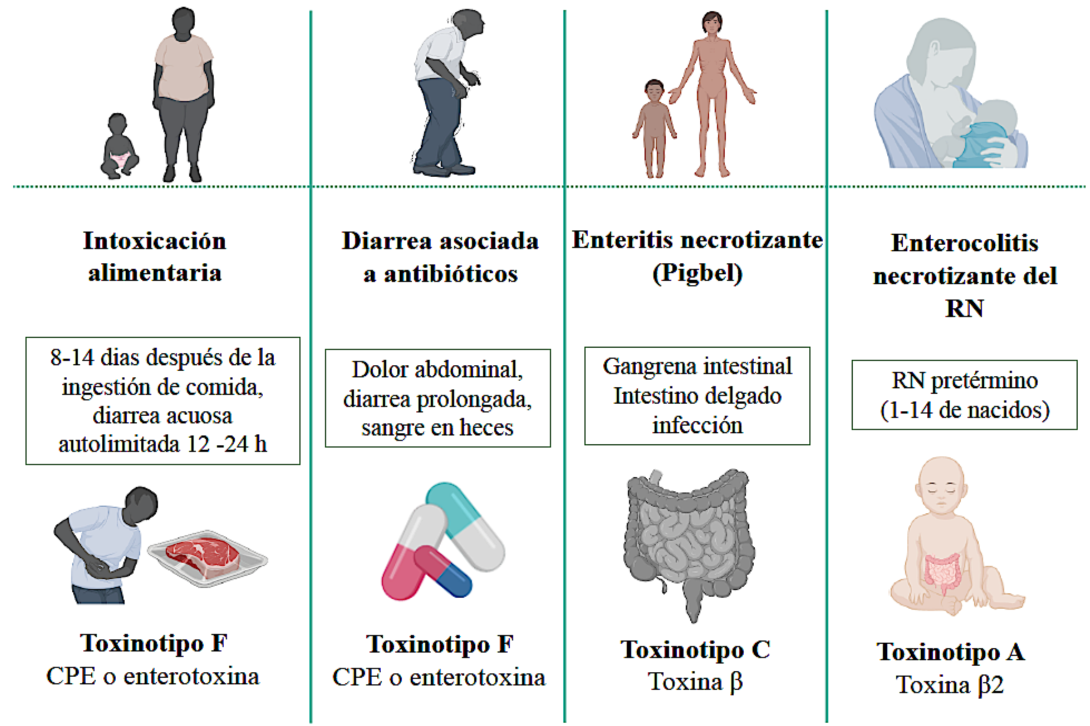

Figura 4. Enfermedades humanas causadas por *C. perfringens* [13, 23, 63]. **RN:** Recién nacido. **Fuente:** Autor

6.5.1 Intoxicación alimentaria

Cada año en todo el mundo se registran aproximadamente 600 millones de casos de enfermedades transmitidas por alimentos y 420.000 muertes. La intoxicación alimentaria por *C. perfringens* se encuentra entre las enfermedades gastrointestinales más prevalentes en países desarrollados [64].

La historia natural de la intoxicación alimentaria por *C. perfringens* ocurre cuando las esporas de las cepas tipo F que portan el gen *cpe* cromosómico sobreviven a la cocción inadecuada de alimentos (rango de temperatura de 10 a 50ºC), lo que permite la germinación de células vegetativas [65] [66]. Algunas células vegetativas ingeridas en alimentos contaminados sobreviven al pH ácido del estómago y pasan al intestino donde comienzan a esporular, la célula madre se lisa liberando la toxina CPE a la luz del intestino, donde actúa a través del mecanismo descrito anteriormente [33, 67-69].

Los síntomas clásicos de intoxicación alimentaria por *C. perfringens* ocurren dentro de las 8 a 14 h posteriores a la ingestión de alimentos contaminados con esporas y se caracterizan por la aparición de dolor abdominal, diarrea acuosa y deshidratación [13, 70]. El diagnóstico de intoxicación alimentaria por *C. perfringens* se basa en el aislamiento de colonias reductoras de sulfito que se vuelven negras en agar que contiene hierro y es suplementado con cicloserina; la presencia de *C. perfringens* es confirmada por microscopia [71].

6.5.2 Diarrea esporádica y diarrea asociada a antibióticos (DAA)

La DAA es una complicación grave que sigue siendo un problema sanitario importante, tanto en los pacientes hospitalizados como en la comunidad. Los aislamientos de *C. perfringens* tipo F que llevan el gen *cpe* en plásmidos de ~ 70-75 kb son asociadas con casos de enfermedad gastrointestinal no transmitida por alimentos como diarrea esporádica y DAA [34, 72, 73]. Se ha estimado que 5% de todos los pacientes con DAA están infectados por *C. perfringens* enterotoxigénica [74, 75].

La DAA se presenta como consecuencia del tratamiento antimicrobiano con penicilina, trimetroprim, cefalosporinas, o clotrimoxazol (Trimetroprim-sulfametoxazol), mientras que la diarrea esporádica se desarrolla independiente de cualquier tratamiento antibiótico. En contraste a las grandes cantidades de células bacterianas *cpe*$^{+}$ cromosómicas que deben ser ingeridas en alimentos contaminados para causar intoxicación alimentaria, la DAA y la diarrea esporádica son causadas por un pequeño inóculo de células positivas para *cpe* transmitidas por plásmido seguido de la transferencia conjugativa del plásmido *cpe*$^{+}$ a poblaciones intestinales de *C. perfringens cpe*$^{-}$ presentes en el entorno intestinal [70, 76, 77].

6.5.3 Gangrena gaseosa

Las infecciones de la piel y los tejidos blandos van desde leves, moderadas a graves con afectación sistémica, de progresión rápida, que requieren tratamiento médico de inmediato. Estas infecciones pueden ser mono o polimicrobianas causadas por microorganismos aeróbicos, anaeróbicos o una combinación de ambos, los cuales colonizan áreas expuestas de la piel debido principalmente a traumatismos, heridas penetrantes, intervenciones quirúrgicas o úlceras en pacientes diabéticos donde *C. perfringens* representa el 80-95% de las infecciones después de una lesión o cirugía [78, 79].

En los Estados Unidos, la incidencia de mionecrosis es de 1.000 casos por año, sin embargo, en los países menos desarrollados con menor acceso a la atención médica, tratamiento antibiótico y diagnóstico oportuno, la incidencia es probablemente mayor, pero se desconoce el número exacto [79].

La gangrena gaseosa causada por la invasión exitosa de heridas traumáticas por células vegetativas o esporas de cepas de *C. perfringens* productoras de CPA y PFOA, se caracteriza por mionecrosis grave (necrosis muscular), acumulación de leucocitos intravascular, trombosis significativa y hemólisis [13, 80]. En los casos más severos, la infección puede llevar a sepsis grave, lo que incluye choque séptico, síndrome de dificultad respiratoria del adulto, coagulación intravascular diseminada y anemia hemolítica [79, 81].

6.5.4 Enteritis necrotizante (EN)

La EN causada por *C. perfringens* tipo C productor de la toxina beta, es una enfermedad infecciosa potencialmente mortal que se caracteriza por necrosis segmentaria del yeyuno proximal [13, 82-85]. Los factores de riesgo son diabetes mellitus, desnutrición, ingesta repentina de comidas proteicas, ingestión de carne de cerdo contaminada con *C. perfringens* tipo C y altas cantidades de inhibidores de tripsina en la nutrición. La patogenia de la EN tipo C se atribuye principalmente a CPB, una exotoxina, que causa daño celular a través de la formación de poros multiméricos en las membranas plasmáticas de las células sensibles [86-88]. Los síntomas de la enfermedad son el dolor abdominal severo principalmente en el abdomen superior, vómito, fiebre, distensión abdominal, diarrea sanguinolenta y en los casos más severos puede progresar a sepsis, shock y muerte [89, 90].

6.5.5 Enterocolitis necrotizante del recién nacido

La enterocolitis necrotizante (ECN) es una de las principales causas de mortalidad en los recién nacidos prematuros (mortalidad de hasta un 35% en lactantes de peso extremadamente bajo al nacer <1000 g) [91], caracterizada por inflamación intestinal aguda con o sin perforación intestinal [92].

Se cree que la ECN es una enfermedad multifactorial que requiere un intestino inmaduro, alimentación enteral y colonización bacteriana para su desarrollo, además las preocupaciones sobre la infección intrauterina oculta que precipita el parto prematuro, la rotura prematura de membranas y la corioamnionitis a menudo provocan el inicio de un tratamiento antibiótico empírico en la primera semana de vida del lactante, con posible disrupción de la microbiota intestinal, lo que se asocia con un mayor riesgo de ECN [93-95].

El aislamiento de *C. perfringens* tipo A y C en lactantes con ECN sugiere su implicación en la fisiopatología de la enfermedad [96], donde la isquemia del intestino dada por suministro vascular reducido con baja tensión de oxígeno, podrían favorecer la conversión de esporas clostridiales en bacilos invasores productores de toxinas [97].

La presentación clínica de ECN puede variar entre los recién nacidos. Los signos clínicos característicos son distensión abdominal, llanto frecuente, heces con sangre, vómitos, ruidos

intestinales ausentes, en los casos más severos se observa celulitis en la pared abdominal, peritonitis, letargo, shock y la muerte [98-100].

6.6 Impacto de *C. perfringens* en animales

C. perfringens no sólo afecta humanos, sino que también, causa diversas enfermedades en hospedadores animales (**Figura 5**).

Hospedero	Enfermedad	Grupos afectados	Clínica	Toxinas asociadas
	Enteritis necrótica bovina	Terneros neonatales	Distensión de colon y necrosis intestinal	**Toxinotipo A** Toxina β2 Perfingolisina O
	Enteritis necrótica de aves de corral	Pollos neonatales 2-3 semanas	Lesiones gaseosas, necrosis intestinal, distensión de asas	**Toxinotipo A C y G** Toxina β NetB, TpeL
	Enterocolitis en cerdos	Lechones recién nacidos	Diarrea severa, necrosis intestinal, atrofia vellosidades	**Toxinotipo A/C** Toxina β2
	Gastroenteritis canina	n/a	Necrosis intestinal hemorrágica	**Toxinotipo A/F** CPE, Toxina β2
	Enterocolitis necrotizante equina	Potros neonatales	Diarrea hemorrágica, necrosis intestinal	**Toxinotipo A y F** Toxina CPE, β2 NetB, NetF, NetG

Figura 5. Enfermedades animales causadas por *C. perfringens* [3, 52, 109]. **Fuente:** Autor

6.6.1 Enteritis necrótica en cerdos

La EN grave y letal en lechones recién nacidos es causada principalmente por *C. perfringens* tipo C que se define por llevar los 2 genes de toxina de tipificación *cpa*$^{+}$ y *cpb*$^{+}$, además algunas cepas pueden portar el gen de la toxina CPE [18]. Las cepas de tipo C pueden producir otras toxinas, que, no se utilizan para la tipificación, como CPB2, PFOA y la toxina clostridial Tpel [18, 101].

C. perfringens tipo C causa enteritis con mayor frecuencia en animales recién nacidos como terneros, ovejas, cabras y, en particular, cerdos [102]. Esta enfermedad en cerdos puede propagarse rápidamente, los lechones con un curso clínico de subagudo a crónico más prolongado tienen diarrea no hemorrágica y crecimiento reducido [103-105].

6.6.2 Abomastitis y enteritis en ruminates

Diversas enfermedades como enterocolitis hemorrágica, enterotoxemia, enfermedad del riñón pulposo, abomastitis, entre otras son causadas por deferentes tipos de *C. perfringens.* El tipo A es un aislamiento cada vez más común asociado con abomasitis por clostridios en rumiantes, el tipo E también se ha aislado en casos de abomasitis, pero parece ser raro [106-108].

La tasa de letalidad de la abomasitis por clostridios parece ser alta cuando no se instituye un tratamiento rápido (75% -100%) [109]. Los signos clínicos incluyen distensión abdominal, letargo, distensión líquida del abomaso (último compartimento del complejo aparato estomacal de los rumiantes), timpanismo abdominal, cólicos y bruxismo (movimiento de diente contra diente), en los casos graves los animales pueden presentar fiebre, sepsis sistémica o peritonitis [106, 110].

C. perfringens tipo A también puede causar enterotoxemia en ovejas (enfermedad del cordero amarillo), un trastorno aparentemente raro pero muy fatal que se manifiesta como una enfermedad hemolítica aguda [111, 112]. El tipo C se asocia con enterocolitis hemorrágica en terneros y corderos y se sospecha que ocurre en raras ocasiones en cabras [106].

C perfringens tipo D es responsable de enterotoxemia en pequeños rumiantes de todas las edades, en ovejas es típicamente una enfermedad hiperaguda, y muchos casos simplemente se encuentran muertos, mientras que en terneros es muy rara [106]. Aunque el tipo E es supuestamente raro y aparece como una causa poco común de enterotoxemia en corderos, terneros y conejos, algunos estudios sugieren que el tipo E puede desempeñar un papel importante en la enteritis de terneros recién nacidos que pueden cursar con diarrea y muerte súbita, abomasitis y enteritis hemorrágica [113].

6.6.3 Síndrome de diarrea hemorrágica aguda canina

Se ha descrito que *C. perfringens* toxinotipo F productor de CPE asociado con la liberación de toxinas NetF, posiblemente junto con otras toxinas como NetE y NetG, es el responsable de la enterocolitis necrótica en perros, aunque se necesita más trabajo para comprender su epidemiología y los factores que predisponen a los perros a la infección [114] .

La enteritis necrótica canina se caracteriza por la aparición diarrea sanguinolenta severa, vómitos, y pérdida significativa de líquidos a través del tracto gastrointestinal [115, 116].

6.6.4 Enteritis necrótica en aves de corral

La enteritis necrótica en pollos es una enfermedad bacteriana común en la industria avícola, que impone una carga económica significativa debido a la reducción de la eficiencia de producción y el costo de las medidas de control. Es una enfermedad principalmente de pollos de engorde, pero también puede afectar a gallinas ponedoras y pavos [117].

En los estudios de lesiones de aves enfermas generalmente se han encontrado aislamientos de *C. perfringens* que portan el gen *netB*, mientras que el transporte del gen es menos frecuente en los

aislamientos de aves sanas. Las toxinas netB y TpeL se han asociado con la EN clínica en aves de corral [118, 119]. La enteritis necrótica clínica se caracteriza por un aumento de mortalidad repentino con tasas de letalidad que pueden llegar al 50%, los signos clínicos incluyen pérdida de peso, diarrea, intestino delgado distendido y daño o necrosis de la mucosa intestinal, lo que genera un aumento repentino de la mortalidad [117, 120, 121].

6.7 Detección y aislamiento de *C. perfringens*

Los métodos estándar para identificar *C. perfringens* tradicionalmente se basan en medios selectivos para el aislamiento (p. Ej., Triptosa-sulfito-cicloserina con agar yema de huevo [TSC-EYA][122], agar sangre con polimixina B [123] o placas de agar *C. perfringens* de membrana [mCP; Thermo Fisher Scientific, Reinach, Suiza])[124].

Los aislamientos de *C. perfringens* se caracterizan por colonias reductoras de sulfito, que aparecen negras en agar que contiene hierro complementado con cicloserina. En agar sangre, *C. perfringens* normalmente forma una zona doble característica de hemólisis. La zona interna de hemólisis completa es causada por la toxina PFOA; la zona exterior de hemólisis incompleta es causada por CPA [125].

Como alternativa práctica, rápida y sensible para la identificación de *C. perfringens,* se viene utilizando la detección molecular mediante la técnica de Reacción en Cadena de la Polimerasa (PCR). La detección molecular de *C. perfringens* dirigida a genes constitutivos de la especie resulta efectiva, debido a la elevada especificidad y sensibilidad analítica. Dentro de los genes más utilizados en este campo se incluyen el gen codificante para la toxina CPA (*cpa*), ubicado en una región estable del cromosoma y codificado universalmente por todos los aislamientos de *C. perfringens* [126], así como el gen de la subunidad ribosomal 16S (*ARNr 16S*). Este último es un gen conservado a nivel de especie, con múltiples copias en una misma célula, lo cual aumenta la sensibilidad analítica de la prueba [127]. Utilizar más de un marcador molecular para la detección de patógenos reduce sesgos asociados con la eficiencia de amplificación, uniones inespecíficas de los cebadores e incluso perdida de anillamiento por la alta plasticidad genética del agente.

Estudios realizados en países europeos y asiáticos han empleado cultivo in vitro y/o pruebas moleculares para detectar *C. perfringens,* revelando frecuencias de detección que oscilan alrededor del 38,4% en China [128], 25,0% en Reino Unido [40], 42,0% en Canadá [129] y 49,0% en Egipto [130]. Sin embargo, en países en vías de desarrollo, especialmente en América Latina, la investigación sobre la frecuencia de detección de *C. perfringens* es limitada, un estudio pionero en Colombia reportó frecuencias de infección del 32,7% en individuos de la comunidad [131].

6.8 Tratamiento de la infección por *C. perfringens*

El tratamiento de la intoxicación alimentaria por *C. perfringens* toxinotipo F es sintomático, principalmente se basa en el manejo del dolor abdominal e hidratación. El mejor enfoque para prevenir este tipo de enfermedades es cocinar bien los alimentos y mantenerlos a temperaturas adecuadas (<4ºC - >65ºC). En el caso de la EN, el único tratamiento es una cirugía intestinal rápida, para resecar la parte del intestino afectado [132].

El tratamiento de las enfermedades causadas por *C. perfringens* dependen del cuadro clínico que se presente. Sin embargo, algunas medidas son el manejo de la distensión abdominal, particularmente si la respiración está comprometida debido a la presión abdominal sobre el diafragma (en los casos de patología gastrointestinal), soporte sistémico y nutricional con líquidos intravenosos si está indicado, prevención de la proliferación bacteriana en curso mediante el uso de antibióticos como penicilina G, Clindamicina o Metronidazol, restaurar la microbiota intestinal normal y el manejo del dolor según sea necesario [106].

6.9 Resistencia a antibióticos de *C. perfringens*

Como patógeno emergente humano y animal que puede causar diversas enfermedades, *C. perfringens* ha ganado una atención creciente a nivel mundial en los últimos años debido especialmente a la gran cantidad de factores de virulencia y el aumento de resistencia a los antibióticos, lo que podría deberse a las presiones selectivas causadas por el uso inadecuado de antibióticos, tanto en animales como en humanos, especialmente durante los últimos 30 años [133].

La preocupación por la resistencia a los antibióticos en *C. perfringens*, se fundamenta en: i) el aumento de la resistencia a los antibióticos más comúnmente utilizados en el ámbito clínico para tratar infecciones en humanos, como la tetraciclina, clindamicina, gentamicina y eritromicina, que favorece su capacidad de proliferación y daño, siendo una de las características de este patógeno oportunista, y ii) el incremento cada vez mayor de la resistencia a antibióticos específicamente empleados en el tratamiento de infecciones por clostridiales, como la penicilina y el metronidazol. Estos fenómenos se atribuyen principalmente a la combinación del uso indiscriminado de antibióticos junto a la capacidad intrínseca de las bacterias para desarrollar mutaciones espontáneas en sus genomas, así como para llevar a cabo la transferencia horizontal de genes de resistencia a antibióticos [134].

Muchos MRA pueden moverse entre el cromosoma bacteriano y los plásmidos, dentro de la misma especie o entre diferentes especies o incluso entre géneros, a través de diferentes mecanismos de movilización como conjugación, transducción y transformación [135]. Aunque los mecanismos de transferencia horizontal de genes a menudo se consideran los principales mediadores de la resistencia a los antibióticos, la emergencia y fijación de mutaciones representa un fenómeno complejo, que puede afectar la unión del antibiótico al receptor, evitar el acceso del antibiótico a la célula diana o la proteger los blancos de los fármacos [136, 137], lo cual induce mecanismos de resistencia a antibióticos convirtiéndose en una amenaza continua a los tratamientos eficaces.

La integración de datos genómicos que reflejen la emergencia y propagación de mutaciones/genes asociados a resistencia antibióticos, con procesos de caracterización fenotípica podrían aportar información clave para fortalecer los regímenes de tratamiento, la vigilancia y el diseño de fármacos para ayudar a combatir la diseminación de la resistencia a los antibióticos [138].

Los plásmidos conjugativos son moléculas autorreplicativas que se transfieren entre aislamientos, a través del sistema de secreción tipo IV. El plásmido de resistencia a la tetracicilina 47 kb pCW3 de *C. perfringens* es un plásmido que comprende 47.263 pb, con un contenido de G + C del 27,6%,

similar al del cromosoma de *C. perfringens* [139], que porta dos genes de resistencia a la tetraciclina: proteína de resistencia al flujo de tetraciclina (*tetA (P)*) y proteína de resistencia a la tetraciclina (*tetB (P)*) [140, 141]. *tetA(P)* codifica una supuesta proteína transmembrana de 46 kDa que media la salida activa de tetraciclina de la célula, mientras que *tetB(P)* codifica una supuesta proteína de 72,6 kDa que tiene una similitud significativa con las proteínas de resistencia a tetraciclina similares a TetM [142].

Diversos estudios han encontrado en aislamientos de *C. perfringens* marcadores de resistencia a cloranfenicol [*cat(P), cat(Q)*], macrólido-lincosamida-estreptogramina B (MLS_B) [*erm(B), erm(Q)*], tetraciclina [*tetA(P), tetB(P), tet(M)* and *tet(Q)*] y un marcador recientemente descubierto de resistencia a los macrólidos [*mef (A)*], por lo tanto, el tratamiento con estos antibióticos se considera ineficaz [143-146].

6.10 Estrategias de prevención de la transmisión de *C. perfringens*

Según el Center for Disease Control and Prevention (CDC), *C. perfringens* puede encontrarse en el medio ambiente y en el tracto intestinal de animales y humanos, y participa frecuentemente en el desarrollo de enteritis. Las heces de animales infectados y la carne contaminada son las fuentes más comunes de infección. Los alimentos mantenidos a temperaturas inseguras entre 4ºC y 60ºC facilitan la rápida multiplicación bacteriana y la germinación de esporas, convirtiéndose en bacterias activas que se propagan en los alimentos.

Los alimentos más comúnmente asociados con brotes de intoxicación alimentaria son el pavo, el pollo, la carne de res o cerdo, las salchichas y las salsas. Por lo tanto, es esencial que las personas cocinen estos alimentos a temperaturas superiores a 60ºC y los refrigeren a -5ºC dentro de las 2 horas posteriores a la cocción. Además, los alimentos refrigerados deben ser calentados nuevamente a temperaturas superiores a 60ºC antes de su consumo [147].

Así mismo, se recomienda mantener separadas las carnes de aves, pescados y mariscos crudos de otros alimentos en el refrigerador de casa. Colocar estos alimentos en recipientes sellados o bolsas de plástico en el estante inferior del refrigerador evita que los jugos crudos goteen sobre otros alimentos.

Los alimentos refrigerados pueden durar un máximo de 2-3 días, y en el congelador a -18ºC, un máximo de 8-12 meses. Además, otras medidas de higiene, como lavarse las manos correctamente, disponer adecuadamente de los desechos, limpiar las superficies con toallas de papel y el manejo adecuado de excretas, pueden prevenir el riesgo de contaminación cruzada y la propagación de bacterias. Implementar estas prácticas de prevención en la comunidad contribuirá significativamente a la seguridad alimentaria, y prevención de la transmisión de *C. perfringens* [147].

7. OBJETIVOS

OBJETIVO GENERAL:

Describir la frecuencia de detección, diversidad genética y principales factores de virulencia de *Clostridium perfringens* en muestras fecales de humanos y animales en el altiplano Cundiboyacense Colombiano.

OBJETIVOS ESPECÍFICOS:

1. Determinar la frecuencia de detección de *C. perfringens* en humanos y animales en el altiplano Cundiboyacense Colombiano mediante pruebas moleculares.

2. Describir la estructura genética y factores de virulencia de *C. perfringens* a partir de genomas públicos.

3. Caracterizar la arquitectura genómica, marcadores de resistencia y factores de virulencia de aislamientos de *C. perfringens* obtenidos de las poblaciones de estudio.

4. Describir los perfiles de hemolisis, citotoxicidad, esporulación y susceptibilidad a antibióticos de *C. perfringens* en las poblaciones de estudio.

8. INTRODUCCIÓN A LOS CAPÍTULOS

C. perfringens, un microorganismo que reside en los intestinos de humanos y animales tiene la notable capacidad de proliferar y liberar una variedad de toxinas, desempeñando un papel crucial en la aparición de diversas enfermedades de tejidos. Este patógeno se ha identificado como uno de los cinco principales causantes de ETA en países desarrollados [8, 9], representando hasta 90.000 casos anuales de brotes gastrointestinales en Inglaterra [10] y 1000 casos por año de gangrena gaseosa solo en los EE.UU. [148]. Sin embargo, en países en vías de desarrollo, como Colombia, los estudios son limitados desconociéndose datos epidemiológicos y trabajos experimentales publicados que involucren a este patógeno.

En un estudio pionero reportado en Colombia se recolectaron un total de 220 muestras de heces de pacientes con diarrea, durante el período de septiembre de 2015 a abril de 2017, en dos centros de atención de salud ubicados en la ciudad de Bogotá, Colombia. Este estudio encontró que 32.7% de los participantes estaba infectado con *C. perfringens*. Es de particular interés que la frecuencia de infección fue considerablemente más alta en pacientes de la comunidad que a nivel intrahospitalario, lo que sugiere una prevalencia significativa de este patógeno en el entorno comunitario [131].

Este hallazgo subrayó la necesidad de investigar la frecuencia de detección de *C. perfringens* en humanos y animales a nivel microgeográfico e identificar factores de riesgo para la colonización o desarrollo de infección y características genómicas mediante estudios de secuenciación de genoma completo. Los estudios de genómica en bacterias permiten una caracterización detallada de los patógenos, revelando aspectos críticos como las relaciones filogenéticas, los factores de virulencia y marcadores de resistencia a antibióticos.

El análisis de 206 genomas públicos de *C. perfringens* ha arrojado luz sobre la complejidad genética de este microorganismo, revelando la existencia de cinco filogrupos distintos. Este descubrimiento subraya la notable diversidad genómica y la capacidad de adaptación de *C. perfringens*, evidenciando su alta plasticidad genética. Estos hallazgos son cruciales para comprender mejor la dinámica de este patógeno y su relación con diversas enfermedades [149].

A pesar de estos avances, existe una marcada escasez de investigaciones a nivel microgeográfico en países en desarrollo, lo que pone de manifiesto la urgente necesidad de estudios focalizados en la diversidad genética de *C. perfringens*, que aporten en la descripción de una diversidad potencialmente desconocida de este patógeno. Especialmente, es de interés enfocarse en la investigación de factores de virulencia y los MRA circulantes en diferentes contextos poblacionales, lo que podría aportar conocimiento sobre las variaciones en la patogenicidad y la resistencia a tratamientos.

Adicionalmente, la reciente detección, de aislamientos toxigénicos de *C. perfringens* que producen PFOA en bebés sanos y en neonatos con ECN en Inglaterra, y su correlación directa con toxicidad celular, hemólisis completa, respuestas proinflamatorias y mayor tolerancia al oxígeno, destacan la importancia de PFOA en la patología intestinal, en el establecimiento de la infección y en el desarrollo de enfermedad [40]. Estos rasgos plantean un desafío importante para las medidas de control de

infecciones y subrayan la necesidad de un examen detallado de *C. perfringens* en poblaciones aparentemente sanas que habitan en comunidad, para monitorear y prevenir la transmisión de aislamientos virulentos.

Esto incluiría además de estudios genómicos, evaluaciones fenotípicas de la hemólisis, capacidad de esporulación y viabilidad de las esporas, así como pruebas de susceptibilidad a los antibióticos. Dicha investigación no solo ampliaría nuestra comprensión de *C. perfringens*, sino que también contribuye significativamente al desarrollo de medidas de prevención y manejo más efectivas.

Este enfoque integral hacia la investigación de *C. perfringens* es esencial para enriquecer nuestro conocimiento sobre este patógeno, lo que, a su vez, fortalecerá las estrategias de salud pública dirigidas a mitigar las infecciones gastrointestinales asociadas a este microorganismo, tanto en Colombia como en otros países.

Por estas razones, se identificó la necesidad de describir la frecuencia de detección, diversidad genética, principales factores de virulencia, MRA y características fenotípicas de aislamientos de *C. perfringens* obtenidos a partir de muestras fecales de humanos y animales en el altiplano Cundiboyacense Colombiano. Para alcanzar los objetivos planteados, esta tesis fue dividida en tres capítulos. A continuación, se describen los capítulos y los objetivos a los que impacta cada uno de ellos:

CAPÍTULO I: Determinar la frecuencia de detección de *C. perfringens* en humanos y animales en el altiplano Cundiboyacense Colombiano mediante pruebas moleculares **(Objetivo específico 1).**

CAPÍTULO II: Describir la estructura genética y factores de virulencia de *C. perfringens* a partir de genomas públicos (**Objetivo específico 2**).

CAPÍTULO III: Caracterizar la arquitectura genómica y perfiles fenotípicos de hemolisis, citotoxicidad, esporulación y susceptibilidad a antibióticos de aislamientos colombianos de *C. perfringens* (**Objetivos específicos 3 y 4**).

CAPITULO I: Determinar la frecuencia de detección de *C. perfringens* en humanos y animales en el altiplano Cundiboyacense Colombiano mediante pruebas moleculares.

El Capítulo I se centra en la determinación de la frecuencia de detección de *C. perfringens* en humanos y animales en el altiplano Cundiboyacense colombiano usando pruebas moleculares. Se recolectaron muestras fecales de 114 humanos, tanto con síntomas gastrointestinales como sin ellos, así como 347 muestras fecales de diversas especies animales sin síntomas, que incluyeron perros, cerdos, gatos, bovinos, cabras y ovejas en los Departamentos de Cundinamarca y Boyacá. Todas las muestras se transportaron inmediatamente en recipientes estériles al laboratorio de procesamiento, tras la disrupción mecánica, se procedió a la extracción de ADN y a la PCR dirigida al gen *16S-rRNA* y al gen de la toxina alfa (*cpa*) para la detección de *C. perfringens.*

Los resultados revelaron una frecuencia de detección de *C. perfringens* en todos los animales del 22,1% (n=77/347), siendo más frecuente en gatos (34,1%), perros (30,0%) y cerdos (22,0%). Por otro lado, los rumiantes presentaron una frecuencia de detección menor (<11,1%). La mayor frecuencia de detección en animales domésticos podría estar relacionada con factores predisponentes, como la alteración de la microbiota intestinal debido a dietas ricas en proteínas y carbohidratos, o la posible infección entérica por otros patógenos.

En cuanto a los humanos, la frecuencia general fue del 19,3% (n=22/114) con un 21,2% en individuos asintomáticos y un 16,6% en sintomáticos. Se observó una asociación entre la presencia de la toxina CPB2 y la diabetes, lo que sugiere interacciones con el sistema inmunológico del huésped. Este estudio resalta una frecuencia de detección elevada de *C. perfringens* en comparación con otros países, además de una asociación entre la diabetes o enfermedad autoinmune y el transporte de la *C. perfringens* toxigénico.

Estos resultados fueron consolidados en dos artículos científicos derivados de este capítulo.

Artículo 1: Camargo A, Páez-Triana L, Camargo D, García-Corredor D, Pulido-Medellín M, Camargo M, Ramírez J.D. and Muñoz M*. Carriage of *Clostridium perfringens* in Domestic and Farm Animals across the Central Highlands of Colombia: Implications for Gut Health and Zoonotic Transmission. Vet Res Commun. 2024.

Artículo 2: Camargo A., Bohórquez L., López D., Ferrebuz-Cardozo A., Castellanos-Rozo J., Díaz J., Rada M., Camargo M., Ramírez J. D. and Muñoz M. *Clostridium perfringens* in central Colombia: Frequency, Toxin Genes, and Risk Factors. (**SOMETIDO** en Gut Pathogens)

CAPÍTULO II: Describir la estructura genética y factores de virulencia de *C. perfringens* a partir de genomas públicos.

El Capítulo II describe la estructura genética poblacional y los factores de virulencia de *C. perfringens* utilizando genomas públicos. Para esto, se descargaron 370 genomas de *C. perfringens* de bases de datos públicas, además se incluyeron 2 genomas provenientes de Chile secuenciados en este estudio. Mediante herramientas bioinformáticas, se realizó el ensamblaje de los datos y se llevó a cabo un análisis de MLST para tipificar los aislamientos, identificar complejos clonales e inferir posibles rutas de dispersión. Se elaboró un análisis de pangenoma y se construyó un árbol filogenético basado en el alineamiento completo del genoma central para definir grupos filogenéticos. Asimismo, se identificaron factores clave de virulencia, toxinotipos y MRA.

La mayoría de los genomas reportados tuvieron su origen geográfico en países desarrollados como EE. UU., Francia y China, y en gran parte correspondieron a fuentes de alimentos, aves y humanos. El 53% de los genomas se asignaron al toxinotipo A, seguido del F (32%) y G (7%). Se identificaron toxinotipos circulantes en humanos poco comunes, como los es el tipo G asociado con enfermedades en aves, junto con la presencia de genomas del toxinotipo D y E vinculados a enfermedades en rumiantes. Mediante el análisis de MLST y el alineamiento del genoma central, se identificaron 5 filogrupos. Se encontraron factores de virulencia como fosfolipasa C (*plc)*, sialidasa (*nanH*), alfa-clostripaína (*ccp*) y colagenasa (*colA*) implicados en el daño a la membrana celular endotelial, y un mayor número de MRA a tetraciclinas, macrólidos y aminoglucósidos.

El hallazgo en humanos de los toxinotipos D, E y G, los cuales generalmente se asocian con enfermedades en animales, junto con los resultados del análisis filogenético basado en MLST y en el alineamiento del genoma central que evidenció una estrecha relación entre aislamientos de diferentes hospederos, señala la adaptabilidad de *C. perfringens* a diferentes hospederos y resalta su potencial zoonótico. Además, el alto contenido de factores de virulencia y de MRA en los genomas de *C. perfringens* destacan su capacidad para adquirir y transmitir estos elementos, planteando preocupaciones sobre el tratamiento antibiótico eficaz. Por ende, se enfatiza la necesidad de una vigilancia continua para implementar medidas preventivas efectivas.

Los hallazgos de este capítulo resaltan además la importancia de fortalecer la vigilancia epidemiológica y mejorar la recopilación de datos genómicos en países en vías de desarrollo a través de un esfuerzo conjunto para ampliar la disponibilidad de información acerca de la arquitectura genómica de microorganismos de interés para la salud humana y animal.

Como producto de este capítulo se adjunta el siguiente artículo científico:

> **Artículo 3:** Camargo A., Guerrero-Araya E, Castañeda S, Vega L, Cardenas-Alvarez MX, Rodríguez C, Paredes-Sabja D, Ramírez JD, Muñoz M. Intra-species diversity of *Clostridium perfringens*: A diverse genetic repertoire reveals its pathogenic potential. Front Microbiol. 2022 Jul 22; 13:952081. doi: 10.3389/fmicb.2022.952081. PMID: 35935202; PMCID: PMC9354469

CAPÍTULO III: Caracterizar la arquitectura genómica y perfiles fenotípicos de hemolisis, citotoxicidad, esporulación y susceptibilidad a antibióticos de aislamientos colombianos de *C. perfringens.*

El objetivo del Capítulo III fue caracterizar la arquitectura genómica y los perfiles fenotípicos de aislamientos colombianos de *C. perfringens*, obtenidos a partir de muestras positivas de humanos y animales colectadas en el Capítulo I.

Se cultivaron un total de 55 muestras positivas para *C. perfringens* en agar selectivo Triptosa Sulfito Cicloserina (TSC), obteniendo entre dos y cinco colonias por muestra, para un total de 185 aislamientos. El ADN extraído fue secuenciado mediante la plataforma Illumina. A través de análisis bioinformáticos, se realizaron análisis genómicos microgeográficos utilizando los 185 aislamientos colombianos. Además, se realizó un análisis comparativo a nivel global que incluyó 617 genomas desreplicados, es decir, se incorporaron genomas representativos y de mayor calidad, de los cuales 71 fueron colombianos y 546 de acceso público.

Se identificaron rutas de dispersión, marcadores de virulencia y MRA a nivel genómico, y se llevaron a cabo pruebas fenotípicas de hemólisis, citotoxicidad, esporulación y susceptibilidad a antibióticos en un grupo representativo de aislamientos. El criterio de selección de los aislamientos se basó en su capacidad para producir la toxina PFOA, una toxina formadora de poros que juega un papel crucial en la patogenia intestinal, con el objetivo de evaluar su impacto biológico.

El análisis de la estructura poblacional de *C. perfringens* a nivel microgeográfico reveló una alta diversidad genética y sugirió eventos de dispersión zoonótica entre caninos, felinos y humanos, destacando la necesidad de mejorar las prácticas de higiene efectivas para prevenir la transmisión comunitaria. Además, la comparación con genomas globales mostró una agrupación de aislamientos de fuentes humanas y alimenticias, resaltando el papel de *C. perfringens* en las ETA y la necesidad de garantizar una manipulación segura de alimentos para evitar la propagación de esta bacteria.

Asimismo, en individuos asintomáticos se detectaron aislamientos de *C. perfringens* portadores de *pfoA*, que a nivel fenotípico indujeron hemolisis completa, inhibición del crecimiento celular y mayor eficiencia de esporulación. Estos hallazgos subrayan la importancia de comprender mejor el impacto de las toxinas accesorias y el potencial riesgo de transmisión de aislamientos toxigénicos que puedan desencadenar la enfermedad a nivel comunitario.

Por último, la vigilancia genómica y las pruebas fenotípicas revelaron una alta prevalencia de MRA y una susceptibilidad reducida a gentamicina, eritromicina, metronidazol y tetraciclina. Estos resultados resaltan la importancia de ajustar las estrategias terapéuticas según las características locales y enfatizan la necesidad de realizar más estudios epidemiológicos para abordar y comprender mejor la resistencia a los antimicrobianos en *C. perfringens.*

Los resultados del análisis genómico y fenotípico de aislamientos colombianos de *C. perfringens* se encuentran depositados en el siguiente artículo:

Artículo 4: Camargo A., Bohorquez L., Cáceres T., Ferrebuz-Cardozo A, Díaz J, Castellanos-Rozo J, Diaz J., Kiu R., Hall L. J., Rámirez J. D. and Muñoz M. Insights into *Clostridium perfringens* Dispersal Hotspots, Toxins, and Virulence Factors through Integrated Genomic and Phenotypic Profiling (**EN CONSTRUCCIÓN**).

Carriage of *Clostridium perfringens* in Domestic and Farm Animals across the Central Highlands of Colombia: Implications for Gut Health and Zoonotic Transmission

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22 **Abstract**

23 *Clostridium perfringens* inhabits the guts of humans and animal species. *C. perfringens* can
24 proliferate and express an arsenal of toxins, promoting the development of multiple gut
25 illnesses. Healthy animals carrying *C. perfringens* represents a risk of transmission to other
26 animals or humans through close contact and an increased likelihood of acquisition of toxin
27 plasmids. The aim of this study was to evaluate the frequency of *C. perfringens* carriage in
28 domestic and farm animals in the central highlands of Colombia.

29 *C. perfringens* was detected in six animal species using PCR targeting alpha toxin (*cpa*) and
30 16S ribosomal RNA (*16S-rRNA*) genes from 347 fecal samples collected in two
31 Departments: 177 from farm animals of Boyacá and 170 from domestic animals of both
32 Cundinamarca and Boyacá.

33 The overall frequency of *C. perfringens* detection was 22.1% (n=77/347), with the highest
34 frequency observed in cats 34.2% (n=41/120), followed by dogs 30.0% (n=15/50). The
35 lowest frequency was detected in ruminants: goats 11.1% (n=3/27), sheep 8.0% (n=4/50) and
36 cattle 6.0% (n=6/50).

37 Domestic animals showed a higher frequency of *C. perfringens* carriage than farm animals.
38 This difference could be associated with dietary patterns, as domestic animals have diets rich
39 in proteins and carbohydrates, while ruminants have low-carbohydrate diets, resulting in high
40 production of endopeptidase-type enzymes and differences in pH due to the anatomy of
41 gastrointestinal tract, which can influence bacterial proliferation. These findings indicate a
42 potential risk of transmission of *C. perfringens* among animals and from animals to humans
43 through close contact.

44

45 **Keywords**

46 *Clostridium perfringens*; bacterial carriage; healthy animals; domestic animals; farm
47 animals.

48 **Introduction**

49 *Clostridium perfringens* is an anaerobic bacterium that can produce a diverse range of toxins
50 (Uzal et al., 2014). The presence of toxin-encoding genes is the basis of the intra-taxa
51 diversity classification scheme for this species, which is supported by seven toxinotypes (A-
52 G) of clinical, epidemiological, and diagnostic importance (Kiu & Hall, 2018).

53 In humans and animals, *C. perfringens* toxinotype A can be a commensal of the intestinal
54 microbiota. However, the loss of homeostasis due to inflammatory diseases, a sudden change
55 to a protein-rich diet, or even enteric infection by other pathogens or systemic diseases are
56 predisposing factors for developing *C. perfringens* enterotoxemia (Silva & Lobato, 2015;
57 Turk et al., 1992).

58 *C. perfringens* is an important opportunistic pathogen in domestic and farm animals, with
59 prevalence rates in healthy animals of 76.0% (44/58) in dogs (Marks, Kather, Kass, & Melli,
60 2002), 63.0% in cats (34/54) (Queen, Marks, & Farver, 2012), 47.1% (33/70) in cattle, 58.0%
61 (29/50) in goats, and 65.4% (36/55) in sheep (Hamza, Dorgham, Elhariri, Elhelw, & Ismael,
62 2018) reported in the United States, India, and the United Kingdom. However, there is limited
63 information available on the frequency of carriage of this bacterial pathogen in developing
64 countries such as Colombia. This knowledge gap limits the design of control measures to
65 control the spread of infection among different hosts, representing a potential risk of
66 implications for human and animal health.

67 Therefore, this study aims to evaluate the frequency of *C. perfringens* in apparently healthy
68 domestic and farm animals by molecular testing. This work provides an accurate description

69 of the epidemiological overview of this bacterium in six animal species (cattle, goats, sheep,
70 pigs, canines, and felines) sampled in the departments of Boyacá and Cundinamarca in the
71 central highlands of Colombia.

72 **Materials and methods**

73 **Study population**

74 For this purpose, a total of 347 stool samples were collected from apparently healthy
75 domestic and farm animals in two departments of Colombia: i) Boyacá department, located
76 in the central-eastern part of the country, which has a large rural area with farming
77 communities focused on livestock and food production, and ii) Cundinamarca department,
78 specifically the city of Bogotá, the capital of Colombia, characterized for being a large, highly
79 populated urban area (see **Supplementary fig. 1**). Fecal sampling was distributed as follows:
80 120 cats (60 from Boyacá and 60 from Cundinamarca), 50 dogs (25 from Boyacá and 25
81 from Cundinamarca), and all other animal species were sampled exclusively from Boyacá
82 department, as follows: 50 cows, 50 pigs, 50 sheep and 27 goats. All fecal samples were
83 collected in sterile recipients with airtight seal (to avoid direct exposure to oxygen) and
84 without transport medium for preservation. Samples were stored frozen -30°C until
85 processing. This study was approved by the Research Ethics Committee of Universidad del
86 Rosario (CEI-UR).

87 **DNA extraction from feces and molecular detection of *C. perfringens***

88 DNA extraction was conducted using the Stool DNA Isolation Kit (Norgen), following the
89 manufacturer's instructions. DNA concentration was measured using the
90 NanoDrop/2000/2000c spectrophotometer (Thermo Fisher Scientific, Massachusetts, USA).
91 Subsequently, *C. perfringens* carriage was detected through conventional PCR, utilizing two
92 primer sets targeted specific genes: i) *16S-rRNA* gene, and ii) *cpa* gene. Both PCR reactions

93 were carried out under conditions that have been reported previously (Roussan, Shaheen,
94 Totanji, Khawaldeh, & Al Rifai, 2012). The positive control used was the *C. perfringens*
95 ATCC® strain 13124, while primers targeting the V4 hypervariable region of the bacterial-
96 and Archaea-specific 16S-rRNA marker (Caporaso et al., 2011) were employed as an internal
97 amplification control to ensure that negative samples were not inhibited. Presence of an
98 amplified product for the two molecular markers, 481 base pairs (bp) for *16S-rRNA* and 400
99 bp for *cpa*, was considered a positive sample for *C. perfringens.*

100 **Statistical analysis**

101 Quantitative variables were described in terms of percentages and were analyzed using a Chi
102 square test or Fisher's exact test depending to the observed frequencies. Binomial logistic
103 regression models were carried out to evaluate a possible association between the collected
104 variables (origin, animal use, and animal species) and the identification of *C. perfringens*.
105 All analyses were two-tailed with a statistical significance value of <0.05, using the
106 STATA17® program.

107

108 **Results**

109 The results showed an overall frequency of *C. perfringens* in all animals of 22.1% (n=77).
110 The frequency for domestic animals was higher (32.9%; 25.9-40.5 95%CI) compared to farm
111 animals (11.6%; 7.4-17.5 95%CI), indicating a statistically significant difference (p= 0.001,
112 Chi2 tests).
113 At the species level, the frequency of *C. perfringens* was higher in cats (34.2%; 25.7-43.3
114 95%CI) and dogs (30.0%; 17.8-44.6 95%CI), compared to cows, that presented the lowest
115 frequency of *C. perfringens* (6.0%; 1.2-16.5% 95%CI). This difference was statistically
116 significant (p=0.001, Fisher's exact test) (**Fig. 1A**).

117 Additionally, the frequency of *C. perfringens* for domestic animals according to the
118 geographical origin (Boyacá and Cundinamarca with 25 dogs and 60 cats for each
119 department), did not show significant differences for dogs (p= 0.75, Chi2 tests). However,
120 for cats, a significantly higher carriage was found in Cundinamarca (50.0%; 36.8-63.2
121 95%CI) compared to those from Boyacá (18.3%; 9.5-30.4 95%CI) (**Fig. 1B**)

122 A logistic regression analysis was carried out to establish the strength of association between
123 the characteristics of the population analyzed (origin and use of the animal) and the
124 identification of *C. perfringens* in the population. The results showed that domestic animals
125 had a positive association (aOR: 2.77; 1.36-5.61 95.0% CI) in *C. perfringens* identification
126 with respect to farm animals. In contrast, a lower probability of carriage was found in animals
127 from Boyacá (aOR: 0.38; 0.19-0.73 95.0% CI) (**Table 1**).
128 Finally, the strength of association between *C. perfringens* presence and animal species was
129 evaluated (**Table 1**). The results showed that pigs (aOR: 4.55; 1.18-17.50 95%CI), cats (aOR:
130 4.96; 1.37-17.87 95%CI), and dogs (aOR: 4.05; 1.02-16.01 95%CI) were the animal species
131 that showed a positive association with bacterial detection.
132 **Discussion**
133 These findings provide the first epidemiological overview of frequency of *C. perfringens*
134 carriage in several animal species in Colombia. The study detected a high frequency of this
135 pathogenic bacterium in domestic animals (**Fig. 1A**), consistent with research conducted by
136 other groups in Brazil where detection frequencies in animals without diarrhea of up to 55.0%
137 in dogs (22/40) (Silva et al., 2013) and 47.9% (112/234) in cats have been reported (Silva et
138 al., 2020). These findings may be related to predisposing factors such as altered intestinal
139 microbiota in these species due to their high protein and carbohydrate diets or potential

140 enteric infection by other pathogens (Sabshin et al., 2012; Silva & Lobato, 2015; Turk et al.,
141 1992).
142 The higher frequencies of *C. perfringens* in cats from Cundinamarca compared to those from
143 Boyacá (**Fig. 1B**) reported here may be due to sociodemographic differences in the collection
144 sites. Samples in Boyacá were collected mostly in rural areas where the animals are free-
145 living and acquire food through hunting and/or human food remains. In contrast, the vast
146 majority of samples in Cundinamarca were collected in urban areas where the animals
147 probably have access to processed food rich in carbohydrates and proteins that are broken
148 down by proteinases into peptides and amino acids before being absorbed by the intestinal
149 villi, which are important for bacterial growth (Bermingham, Maclean, Thomas, Cave, &
150 Young, 2017; Huang, Pan, Yang, Bi, & Xiong, 2020).
151 Regarding farm animals, pigs presented a higher carriage of *C. perfringens* than ruminants
152 (**Fig. 1A**). These results could be correlated with dietary factors in rural areas of the
153 Department of Boyacá. The vast majority of animals in these areas are fed with food remains
154 that could carry resistant spores from microbial species, fats, and high levels of crude protein.
155 These factors can affect the intestinal microbiota by increasing the availability of nitrogen as
156 well as amino acids, favoring the proliferation of pathogenic bacteria such as *C. perfringens*
157 (Duarte & Kim, 2022; Yu, Zhu, & Hang, 2019).
158 In contrast, ruminants (cows, sheep, and goats) that are polygastrophic animals showed low
159 *C. perfringens* carriage (**Fig. 1A**). The reduced circulation of *C. perfringens* in ruminants
160 could be due to factors such as diet based on a high content of fodder and pasture that induces
161 a higher transport of Gram-negative bacteria compared to Gram-positive bacteria in the
162 rumen (Plaizier et al., 2018), low consumption of carbohydrates and proteins that provide a
163 nutrient-poor environment for *C. perfringens* growth, and the consumption of poor quality

164 silage that generates excessive accumulation of lactic acid in the digestive system, causing
165 acidosis and alteration of the intestinal microbiota (Cholewińska, Górniak, & Wojnarowski,
166 2021). Additionally, mechanisms that facilitate nutrient digestion and absorption (microbial
167 fermentation versus enzymes in the mammalian intestinal tract), nutrient, as well as the
168 morphology and histology of the gastrointestinal tract may influence discrepancies in *C.*
169 *perfringens* carriage between domestic and farm animals such as ruminants
170 (Abdolmohammadi Khiav & Zahmatkesh, 2021; Constable, Hinchcliff, Done, & Grünberg,
171 2016; Nazki et al., 2017).

172 The results presented in this study provide an initial insight into the context of the frequency
173 of *C. perfringens* carriage in healthy farm and domestic animals in Colombia. The high
174 frequency of domestic animals and pigs carrying *C. perfringens*, as indicated in this study,
175 highlights the risk of transmission of this bacterium not only between animals, but also from
176 animals to humans through meat consumption or contact with feces of carrier animals that
177 share the same living environment as humans, as indicated above (Song et al., 2013).

178 According to the World Health Organization (WHO) (Narayan, Sinha, & Singh, 2023),
179 zoonoses represent a growing global threat as diseases naturally transmitted from animals to
180 humans. Approximately 60% of infectious agents are pathogenic to humans, 75% of
181 pathogens associated with emerging diseases are zoonotic, 25% of zoonoses originate in
182 domestic animals, and according to etiology bacteria account for the majority of zoonotic
183 diseases (Rahman et al., 2020).

184 This makes pathogen surveillance through molecular epidemiology, especially in domestic
185 animals such as dogs and cats, crucial because of their critical role in transmission and
186 increased interaction with humans (Meyer, Gastmeier, Kola, & Schwab, 2012; Tomori &
187 Oluwayelu, 2023).

188 Furthermore, it is relevant to note that low- and middle-income countries, particularly in rural
189 areas with closer contact with domestic and farm animals, face additional challenges. These
190 challenges include lack of access to safe drinking water, inadequate food refrigeration and
191 environmental conditions conducive to the transmission of bacteria such as *C. perfringens*.
192 These regions not only face higher zoonosis burdens, but also have limited capacity to
193 effectively address disease risk (Worsley-Tonks et al., 2022).
194 This, highlighting the urgent need for awareness and focus on One Health to protect both
195 animal and human health, including improved surveillance of potential pathogen transport in
196 animal hosts (Qiu et al., 2023; Worsley-Tonks et al., 2022).
197 Finally, it is worth noting that most studies on the biology and genomics of pathogens such
198 as *C. perfringens* focus on isolating strains from diseased individuals. However, studying the
199 evolutionary adaptation of strains reveals the role of *C. perfringens* in the healthy gut
200 microbiota, emphasizing the need to develop studies that investigate genome-scale metabolic
201 patterns and the differential presence of toxin-bearing strains and other virulence factors in
202 the gut of healthy animals.
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Author contributions

A.C., J.D.R. and M.M. designed the study and drafted the manuscript. A.C. and L.P. carried out the processing of the samples. A.C, M.C and M.M. performed the epidemiological analyses. A.C, D.C, L.P, D.G.C and M.P.M, helped in collection of samples. J.D.R. and M.M. revised the manuscript. All authors read and approved the final version of the manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

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325 pigs. *9*(4), 180. https://doi:10.3390/ani9040180.
326

327 **Figure legends**

328 **Figure 1. Carriage of *C. perfringens* in animal species from central Colombia**
329 **(Cundinamarca and Boyacá). A.** Overall carriage of *C. perfringens* in cats (*n=120*), dogs
330 (*n=50*), pigs (*n=50*), goats (*n=27*), sheep (*n=50*), cattle (*n=50*). **B.** Frequency of carriage in
331 domestic animals sampled in the departments of Boyacá (cats *n=25* and dogs *n=25*) and
332 Cundinamarca (cats *n=25* and dogs *n=25*).

333

334 **Tables**

335 **Table 1.** Logistic regression analysis of the association between the features of analyzed
336 population and *C. perfringens* carriage.

337

338 **Supplementary Material Legends**

339 **Supplementary Figure 1.** Schematic representation of the map of Colombia showing the
340 departments of Boyacá and Cundinamarca located in the center of the country, where
341 sampling was carried out.

1 **Micro-Geographical Insights into *Clostridium perfringens*: Frequency, Toxin-**

2 **Encoding Genes, and Risk Factors in a Colombian Population**

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19

20 **Abstract**

21 *Clostridium perfringens* is an opportunistic bacterium that causes intestinal diseases in both
22 humans and animals. This study aimed to assess the frequency of *C. perfringens* and the
23 presence of toxin-encoding genes in fecal samples from individuals with or without
24 gastrointestinal symptoms in the Department of Boyacá, Colombia. Additionally, risk factors
25 associated with colonization and disease development were analyzed.

26 A total of 114 stool samples were analyzed using a molecular test based on specific
27 polymerase chain reaction (PCR) targeting 16S-rRNA and alpha toxin (*cpa*) genes. Bacterial
28 isolates obtained from positive samples were screened for toxin-encoding genes related to
29 gastrointestinal diseases. In addition, sociodemographic, and clinical data from 77
30 individuals were also analyzed.

31 The overall frequency of *C. perfringens* was 19.3%, with an infection frequency of 16.6%
32 among symptomatic individuals and a colonization frequency of 21.2% among asymptomatic
33 individuals. All 56 isolates obtained carried the *cpa* gene, while *cpb2* was present in 10.7%
34 (n=6/56); *cpe* and *cpb* genes were not detected. Notably, diabetes/autoimmune disease
35 demonstrated a significant association with an increased risk of *C. perfringens* (adjusted OR
36 8.41: 95% CI 1.32-35.89). This study highlights an elevated frequency of *C. perfringens* and
37 the presence of *cpb2* gene in asymptomatic individuals compared with their symptomatic
38 counterparts.

39 These findings offer insights into the distribution and virulence factors of *C. perfringens* at a
40 micro-geographical level. This information supports the need for developing tailored
41 prevention strategies based on local characteristics to promote active surveillance programs
42 based on molecular epidemiology.

43 **Key words:** *Clostridium perfringens*, colonization, infection, symptomatic, asymptomatic,
44 toxins.

45 **Introduction**

46 *Clostridium perfringens*, an opportunistic enteropathogenic bacterium affecting both humans
47 and animals, has been linked to multiple intestinal and systemic diseases, including food
48 poisoning, antibiotic-associated diarrhea (AAD), and intestinal necrosis (1, 2). Colonization
49 by opportunistic bacterial pathogens, such as *C. perfringens*, capable of releasing toxins,
50 often precedes infections characterized by disease development (3). In many instances, the
51 factors influencing or mitigating this risk in individual patients remain unclear. Some studies
52 have indicated that colonization by *C. perfringens*, along host-associated risk factors such as
53 the use of antacids (1) and older age (>50 years) (4), may predispose individuals to infection
54 development.

55 Understanding predisposing host factors in specific contexts is essential for designing
56 tailored preventive and therapeutic strategies, thereby enhancing public health efforts to
57 address the incidence and impact of these diseases more effectively. Similarly, risk factors
58 associated with the microorganism, such as a rapid growth rate, production of high-
59 temperature resistant spores, the release of toxins, and antibiotic resistance mechanisms,
60 influence the development of severe infections that are difficult to control (5).

61 In this context, it is known that *C. perfringens* can codify a diverse array of toxins, including
62 pore-forming toxins such as perfringolysin O (*pfoA*), enterotoxin (*cpe*), and necrotic enteritis
63 B-like toxin (*netB*) (3). Based on toxin production, *C. perfringens* isolates are classified into
64 7 toxinotypes, labeled A to G, each associated with a specific disease in a particular host (5,
65 6). The CPA, CPE, CBPB, and CPB2 toxins are linked to gastrointestinal disease (6),
66 highlighting the importance of their routine surveillance and the implementation of improved

67 control measures for circulating strains carrying these toxins. Therefore, molecular
68 epidemiology studies could contribute to the understanding of the clinical,
69 sociodemographic, and biological factors that influence the development of diseases caused
70 by *C. perfringens* and other pathogens.
71 Although molecular epidemiology has explored the detection of *C. perfringens* in
72 symptomatic individuals in Europe, Asia, and North America, reporting infection frequencies
73 ranging from approximately 5 to 20% in cases of antibiotic-associated diarrhea (AAD) and
74 sporadic non-foodborne diarrhea (1, 7-11), comparative studies between the colonization
75 frequency in asymptomatic individuals and infection among symptomatic patients, as well
76 as information about predisposing factors, especially in developing countries, are limited.
77 In Colombia, a reported 32.7% infection rate was observed in patients with diarrhea, but this
78 data lacks detailed sociodemographic and clinical information, hindering the establishment
79 of significant clinical associations (12). The absence of relevant data poses challenges to
80 effectively contributing to the implementation of public health measures aimed at reducing
81 the disease burden. Despite the significance of *C. perfringens* in public health due to its
82 extensive toxigenic arsenal and involvement in various diseases, there is a clear lack of
83 epidemiological data on detection frequency, with few studies characterizing this bacterium
84 in asymptomatic patients (approximately 5%) (13, 14).
85 Therefore, we aimed to identify the presence of *C. perfringens* in fecal samples from
86 individuals with gastrointestinal symptoms and asymptomatic individuals in a central region
87 of Colombia. Additionally, we identified potential circulating toxins and collected relevant
88 sociodemographic and clinical data, aiming to understand the characteristics of the
89 population colonized by *C. perfringens*. By exploring these dimensions, our goal was to
90 contribute to the understanding of the relationship between the presence of *C. perfringens*,

91 toxin circulation, and population characteristics. The results of this study contribute to the
92 local understanding of the molecular epidemiology of *C. perfringens* in Colombia.

93 **Methods**

94

95 **Study Population**

96 Between May and September 2022, a total of 114 fecal samples were collected from adults
97 aged between 26 and 84 years. These samples were obtained from individuals seeking
98 healthcare services at three hospitals located in the Department of Boyacá, Colombia (**Figure**
99 **supplementary 1**). Participant selection included individuals with gastrointestinal symptoms
100 such as diarrhea, abdominal pain, fever, or vomiting, who were referred for stool sample
101 collection due to their symptoms. Asymptomatic participants were also included in the study,
102 selected as part of routine examinations, primarily screening tests (fecal occult blood test)
103 due to risk factors such as advanced age (>60 years), chronic diseases like diabetes,
104 hypertension, hypothyroidism, gastritis, or autoimmune diseases (**Supplementary table 1**)
105 Comprehensive data were collected from 77 out of the 114 participants, with a few choosing
106 not to disclose information due to privacy concerns or time constraints in responding to the
107 survey. This included sociodemographic details (gender, education level, access to clean
108 water, food refrigeration, presence of animals at home, and age), as well as clinical
109 information such as antibiotic consumption in the last 3 months and the presence of chronic
110 diseases.

111 **DNA extraction and molecular detection of *C. perfringens***

112 DNA extraction was performed from a 300 mg fecal sample aliquot using the Norgen fecal
113 DNA isolation kit following the manufacturer's instructions. DNA concentration was
114 measured with the NanoDrop/2000/2000c spectrophotometer (Thermo Fisher Scientific,
115 Massachusetts, USA). Subsequently, the presence of *C. perfringens* was detected through

116 conventional PCR using two primer sets targeting the genes: i) *16S rRNA* gene, and ii) *cpa*
117 gene. Both PCR reactions were carried out under conditions described in previous studies
118 (15). *C. perfringens* ATCC® strain 13124 was included as a positive control. The presence
119 of an amplified product for both genes, *16S rRNA* (product size 481 bp), and *cpa* (product
120 size 400 bp), was considered a positive result for *C. perfringens.*
121
122 **Bacterial culture and identification of potential toxins**
123 From samples that tested positive for *C. perfringens* during PCR, a second aliquot of the
124 sample was used for *in vitro* cultivation on Tryptose Sulfite Cycloserine agar (TSC),
125 incubated for 24 hours at 37°C under anaerobic conditions. Subsequently, 2-5 colonies per
126 sample were selected. Colonies were checked by Gram staining; the biomass was increased
127 on blood agar and bacterial genomic DNA was extracted using Promega's Wizard genomic
128 DNA purification kit. DNA concentration was measured with the NanoDrop/2000/2000c
129 spectrophotometer (Thermo Fisher Scientific, Massachusetts, USA).
130 The presence of genes encoding CPA, CPE, CPB, and CPB2 toxins on *C. perfringens* isolates
131 was evaluated by PCR using specific primer pairs detailed in **Table 1**. Amplification
132 reactions were carried out in a 12.5 µL volume, with a template DNA volume of 1.5 µL (100
133 ng/µL), following the previously described conditions (15, 16). PCR products were
134 visualized through electrophoresis on a 1.5% agarose gel with a 1000 bp molecular weight
135 marker.
136

Table 1. Primers used in the PCR for the detection of potential toxins from *C. perfringens.*

Toxin	Primer name	Sequence (5′–3′)	Gene	Product size (bp)	Ref

CPA	CPAF	TGCATGAGCTTCAATTAGGT	*Alpha-*	400	(15)
	CPAR	TTAGTTTTGCAACCTGCTGT	*toxin*		
CPE	CPEL	GGGGAACCCTCAGTAGTTTCA	*Entero-*	506	(16)
	CPER	ACCAGCTGGATTTGAGTTTAATG	*toxin*		
CPB	CPBL	TCCTTTCTTGAGGGAGGATAAA	*Beta-*	611	(16)
	CPBR	TGAACCTCCTATTTGTATCCCA	*toxin*		
CPB2	CPB2L	CAAGCAATTGGGGGAGTTTA	*Beta2 -*	200	(16)
	CPB2R	GCAGAATCAGGATTTTGACCA	*toxin*		

Statistical analysis

Given the distribution of the quantitative variable of age, we chose to use the median, and reported its measure of dispersion, the interquartile range (IQR). The qualitative variables (detailed in **Supplementary Table 1**.), such as sex, presence of animals in the home, access to drinking water, hypertension, gastritis, diabetes, and hypothyroidism, were presented in terms of frequencies and percentages.

To evaluate associations between *C. perfringens* detection, identified toxin, and sociodemographic and clinical variables, conditional logistic regression was employed. The model provided estimates of odds ratios (OR) in crude and adjusted form, accompanied by their 95% confidence intervals (CI). All two-tailed statistical tests were performed using the STATA 14 program, with p values < 0.05 being considered statistically significant.

Results

This study involved the collection and analysis of 114 stool samples from individuals seeking care at three hospital centers in the Department of Boyacá, Colombia. Sociodemographic and clinical data from 77 participants were obtained through a detailed survey (**see Supplementary Table 1**).

155 Globally, the detection frequency of *C. perfringens* was 19.3% (n=22/114). The metadata
156 for the 77 individuals revealed that 64.9% (n=50/77) were women, 20.7% (n=16/77) had
157 completed secondary education, and the average age was 57.2 years. The infection frequency
158 among symptomatic individuals was 16.6% (n=5/30), while the colonization detection
159 among asymptomatic individuals was 21.2% (n=10/47) (**Figure 1A)**.
160 Among the infected individuals, the average age was 56.6 years, 80.0% (n=4/5) were women,
161 and all were over 50 years old. Additionally, 40.0% (n=2/5) had consumed antibiotics in the
162 last 3 months, lived with animals, and had hypertension as the most common comorbidity.
163 Colonized individuals had an average age of 62.1 years, with 40% being women (n=4/10).
164 Additionally, 20% (n=2/10) had taken antibiotics in the last 3 months, and 40% (n=4/10)
165 lived with animals. Associated comorbidities included hypertension (50%, n=5/10), diabetes
166 (30%, n=3/10), hypercholesterolemia (20%, n=2/10), and gastritis or hypothyroidism (10%,
167 n=1/10).
168 The analysis of associated comorbidities in the population revealed an increased odds ratio
169 for Diabetes/Autoimmune Disease (adjusted OR 8.41: 95% CI 1.32-35.89) (**Figure 1B,**
170 **Supplementary Table 2**). No statistically significant associations were found between the
171 variables and the group of patients infected versus colonized.
172 A total of 56 isolates of *C. perfringens* were obtained from 15 stool samples. The *cpa* gene
173 was detected in all isolates, while *cpb2* was present in 10.7% (n=6/56); the *cpe* and *cpb* genes
174 were not detected. Individuals carrying the CPB2 toxin showed no gastrointestinal
175 symptoms. Importantly, a strong association was found between the detection of the CPB2
176 toxin and Diabetes/Autoimmune Disease (adjusted OR 27.52: 95% IC 1.68-50.67).
177 **Discussion**

178 The human intestine harbors a wide diversity of microorganisms and symbiotic bacteria that
179 play an essential role in promoting immune responses and preventing the colonization of
180 opportunistic pathogens (17). However, the alteration of the microbiota, caused by
181 environmental and genetic factors, creates a conducive environment for opportunistic
182 pathogens such as *C. perfringens* to develop survival strategies detrimental to the host's
183 health. This includes colonization by *C. perfringens* strains carrying cytotoxic toxins (18, 19)
184 acquired through zoonotic transmission, consumption of contaminated food, or person-to-
185 person transmission. Additionally, gut-resident toxinotype A strains of *C. perfringens* can
186 acquire other virulence factors through mobile genetic elements and release toxins. This
187 process triggers inflammatory intestinal and tissue diseases, underscoring the critical
188 importance of preventive strategies.
189 In this context, understanding the local epidemiology of these pathogens through micro-
190 geographical studies is crucial for anticipating and effectively addressing public health
191 threats. Therefore, comparing the detection frequencies of *C. perfringens* with studies
192 conducted in other countries highlights variability, emphasizing the importance of assessing
193 epidemiology locally.
194 While the infection frequencies reported in this study (16.6%) (**See Figure 1A**), are lower
195 than those found in patients with diarrhea in Bogotá, Colombia (32.7%) (12), they fall within
196 the range reported in other regions of the world. In Europe and Asia, the frequencies of *C.*
197 *perfringens* infection range from 5% to 20% in cases of antibiotic-associated diarrhea (AAD)
198 and sporadic non-foodborne diarrhea (1, 7, 8). In the United States, *C. perfringens* ranks as
199 the second leading cause of foodborne bacterial illnesses (11, 20).
200 Despite the infection percentage in Colombia being high compared to studies in countries
201 such as Germany (4.1%) (21) and India (8.6%) (22), it is similar to infection frequencies in

202 China (13.8%) (23) and Iran (22.4%) (8). These data significantly emphasize the relevant
203 presence of *C. perfringens* in the central region of Colombia, highlighting the pressing need
204 to consider the local context when interpreting detection frequencies in epidemiological
205 studies. The variability in these data among different regions could be attributed to factors
206 such as hygiene practices, environmental conditions, and demographic characteristics unique
207 to each region, emphasizing the complexity of the epidemiology of *C. perfringens*.
208 Regarding the frequency of colonization in asymptomatic individuals, our study revealed a
209 rate of 21.2% **(Figure 1A),** which is lower than that reported in healthy individuals in
210 Germany (40.0% - 20/50), North America (51.0% - 22/43) (24), and northern Mexico (63.0%
211 - 126/200) (25). The low colonization frequency reported here, compared to studies in other
212 countries, could be attributed to differences in innate and acquired immune responses and to
213 determinants of the host's genetic susceptibility to specific enteric infections (26).
214 The analysis of colonization frequency in asymptomatic individuals provides a unique
215 perspective that underscores the imminent risk this population faces when exposed to virulent
216 strains. Similarly, the higher frequency of colonization observed in this study compared to
217 infection emphasizes the significance of these findings. This is particularly crucial when
218 considering that colonized patients have a significantly greater propensity to experience
219 episodes of invasive infection compared to non-colonized individuals.
220 Furthermore, future research should take into account recent information from another study
221 suggesting that the presence of *C. perfringens* at the intestinal level in asymptomatic
222 individuals can trigger brain inflammation, oxidative stress, apoptosis, and cell damage (27).
223 This aspect becomes particularly relevant when exploring different age groups, especially in
224 the context of the presence of metabolic disorders.

225 The significant association between the detection of the CPB2 toxin of *C. perfringens* and
226 Diabetes/Autoimmune Disease (adjusted OR 27.52: 95% IC 1.68-50.67) reveals possible
227 pathways of interaction between the microorganism and the host's immune system. The
228 hypersensitivity of diabetic tissues to colonization factors due to low levels of UDP-glucose
229 (28) and the cytotoxic effects of the CPA and CPB2 toxins (29), raise questions about the
230 specific mechanisms involved, which should be studied.
231 Although we did not find other statistically significant associations, the most common
232 underlying comorbidities in patients where *C. perfringens* was detected were cardiovascular
233 diseases, diabetes, and/or hypercholesterolemia—findings similar to other studies (8, 23).
234 Probably, the dysfunction of the reticuloendothelial system in cardiovascular diseases and
235 high cholesterol levels, precursors to the production of bile acids important for spore
236 germination, may play a key role in colonization and infection by *C. perfringens* (30). These
237 data provide valuable information for guiding preventive measures in the colonized
238 population.
239 The identification of some limitations in our work, such as the sample size and specific
240 microgeographic representation, acknowledges the need for broader and more diversified
241 future investigations. Exploring additional factors that may predispose to symptomatic
242 disease and the genomic and phenotypic characterization of virulent strains carrying toxins
243 like pfoA and hypovirulent or "commensal-like" strains that significantly encoded fewer
244 virulence factors and plasmids, underscores research areas that can deepen our understanding
245 of microbial interactions and their clinical consequences.
246 In conclusion, this study explores the results within an international context, delving into
247 factors associated with patients experiencing intestinal symptoms. It underscores the
248 significance of colonization in individuals without intestinal symptoms. Moreover, it

249 indicates future directions, emphasizing the necessity to investigate complex microbial
250 interactions in the human intestine and to consider specific preventive measures for the at-
251 risk population.
252 The presented findings contribute to the local understanding of *C. perfringens* epidemiology
253 in Colombia, offering valuable insights for public health management and prevention
254 strategies in the central region of the country.
255
256 **Declarations**
257 **Ethical Considerations**
258 This study was conducted with the approval of the Research Ethics Committee of the
259 Universidad del Rosario (Approval Law No. 339). The study was deemed low risk according
260 to Resolution 8430 of 1993 from the Ministry of Health of Colombia. Samples were coded
261 to protect the identity of the participants following national ethical guidelines and the
262 Declaration of Helsinki. Informed consent was obtained for the use of samples in research,
263 in accordance with the committee's authorization. Data and information about individuals
264 were collected through a data collection instrument endorsed by the ethics committee.
265
266 **Conflict of interest**
267 The authors declare that they have no conflict of interest.
268
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277
278 **Authors' contributions**
279 A.C., J.D.R. and M.M. designed the study and drafted the manuscript. A.C., L.B., and J.D.
280 carried out the processing of the samples. M.C and M.R. performed the epidemiological
281 analyses. A.C, D.P.L, A.F and J.C, helped in collection of samples. J.D.R. and M.M. revised
282 the manuscript. All authors read and approved the final version of the manuscript.
283
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287
288 **Legends:**
289 **Figure 1. A)** Frequency of detection of *C. perfringens* in symptomatic (infected) vs
290 asymptomatic (colonized) individuals. **B.)** Schematic representation of the adjusted Odds
291 ratio (OR) together with its corresponding 95% confidence interval, which measures the
292 strength of association between the risk factors evaluated and the detection of *C. perfringens.*
293
294 **Figure Supplementary 1.** Sampling areas in the Department of Boyacá Colombia. The
295 hospitals included in the study are part of the municipalities highlighted in orange.
296

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Intra-species diversity of *Clostridium perfringens*: A diverse genetic repertoire reveals its pathogenic potential

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Clostridium perfringens is the causative agent of many enterotoxic diseases in humans and animals, and it is present in diverse environments (soil, food, sewage, and water). Multilocus Sequence Typing (MLST) and Whole Genome Sequencing (WGS) have provided a general approach about genetic diversity of *C. perfringens*; however, those studies are limited to specific locations and often include a reduced number of genomes. In this study, 372 *C. perfringens* genomes from multiple locations and sources were used to assess the genetic diversity and phylogenetic relatedness of this pathogen. *In silico* MLST was used for typing the isolates, and the resulting sequence types (ST) were assigned to clonal complexes (CC) based on allelic profiles that differ from its founder by up to double-locus variants. A pangenome analysis was conducted, and a core genome-based phylogenetic tree was created to define phylogenetic groups. Additionally, key virulence factors, toxinotypes, and antibiotic resistance genes were identified using ABRicate against Virulence Factor Database (VFDB), TOXiper, and Resfinder, respectively. The majority of the *C. perfringens* genomes found in publicly available databases were derived from food (n=85) and bird (n=85) isolates. A total of 195 STs, some of them shared between sources such as food and human, horses and dogs, and environment and birds, were grouped in 25 CC and distributed along five phylogenetic groups. Fifty-three percent of the genomes were allocated to toxinotype A, followed by F (32%) and G (7%). The most frequently found virulence factors based on >70% coverage and 99.95% identity were *plc* (100%), *nanH* (99%), *ccp* (99%), and *colA* (98%), which encode an alpha-toxin, a sialidase, an alpha-clostripain, and a collagenase, respectively, while *tetA* (39.5%) and *tetB* (36.2%), which mediate tetracycline resistance determinants, were the most common antibiotic

resistance genes detected. The analyses conducted here showed a better view of the presence of this pathogen across several host species. They also confirm that the genetic diversity of *C. perfringens* is based on a large number of virulence factors that vary among phylogroups, and antibiotic resistance markers, especially to tetracyclines, aminoglycosides, and macrolides. Those characteristics highlight the importance of *C. perfringens* as a one of the most common causes of foodborne illness.

KEYWORDS

***Clostridium perfringens*, intra-species diversity, multilocus sequence typing, genomic epidemiology, toxinotypes**

Introduction

Clostridium perfringens is an anaerobic, Gram-positive, spore-forming bacillus capable of surviving extreme conditions such as high temperature (>60°C) and low nutrient levels (Hassan et al., 2015). In 2002, the first *C. perfringens* genome was sequenced showing 2,660 protein-codifying regions, ten rRNA genes, and a low G+C content (28.6%; Shimizu et al., 2002). Since then, many studies have revealed the presence of genes that codify to multiple virulence factors involved in the pathogenicity of this species (Li and BA, 2014; Revitt-Mills et al., 2015; Kiu et al., 2017; Abdel-Glil et al., 2021), such as the alpha (CPA), beta (CPB), epsilon (ETX), iota (ITX), enterotoxin (CPE), and necrotic B-like (NetB) toxins (Awad et al., 1995; Sarker et al., 1999; Keyburn et al., 2008; Garcia et al., 2013; Rood et al., 2018), that contribute to neurologic, histotoxic, and intestinal infections in animals and humans and are used to classify strains in seven different toxin types (A to G; Kiu and Lindsay, 2018).

Despite the usefulness of toxin typing in epidemiology and diagnosis, Multilocus Sequence Typing (MLST) has been implemented as an alternative approach for *C. perfringens* typing (Chalmers et al., 2008; Deguchi et al., 2009; Verma et al., 2020). This method is based on the presence and combination of internal fragments of eight housekeeping genes, which in *C. perfringens* includes *colA*, *groEL*, *sodA*, *plc*, *gyrB*, *sigK*, *pgk*, and *nadA*. The sequences are assigned as distinct alleles creating a unique allelic profile or sequence type (ST). Using the goeBURST algorithm, the STs are classified into groups of genetically related organisms called clonal complexes (CC), which provide insights about evolution and diversification processes and allow intra-species analyses (Larsen et al., 2012; Uzal et al., 2014; Page et al., 2017) such as epidemiological and phylogenetical associations (Maiden, 2006). In addition, the identification of shared STs allows the assessment of possible transmission routes between hosts, a third shared source, and genetic stability in the lineage (Xiao et al., 2012).

Other methods, like Whole Genome Sequencing (WGS) have enabled a better understanding of bacterial pathogens through a high-resolution characterization of their genetic variation and evolution (Raskin et al., 2006). Recently, a genomic analysis allocated 206 publicly available *C. perfringens* genomes into five phylogroups (I–V) linked to different disease outcomes and hosts. Phylogroup I included human food poisoning strains and phylogroup II mostly grouped isolates from enteric lesions in horses and dogs. Phylogroup III was the most abundant and heterogeneous group, containing a variety of strains from different hosts causing multiple diseases, while phylogroups IV and V were less abundant (Abdel-Glil et al., 2021). Additionally, the use of WGS has allowed the identification of virulence factors such as sialidases and hyaluronidases along with other toxins associated with clinical outcomes (Kiu et al., 2017; Abdel-Glil et al., 2021). Furthermore, over the last few years molecular markers linked to antibiotic resistance to tetracycline, rifamycin, and aminoglycoside among others, have been recognized as a potential risk for treatment of the infections caused by *C. perfringens* (Kiu and Lindsay, 2018).

So far, *C. perfringens* genomic studies have been limited to a few geographic locations or to a small number of genomes. Hence, to better understand the global diversity of *C. perfringens*, we conducted a comparative genome analysis of 372 genomes from multiple locations and sources. Our goal was to determine the intra-species diversity and phylogenetic relationships of *C. perfringens*, as well as to identify and characterize key molecular markers associated with its pathogenicity, virulence, and antibiotic resistance from whole genome analyses.

Materials and methods

Strain and genome collection

A total of 372 *C. perfringens* genomes were included in our analysis. Two strains sequenced specifically for this study were recovered from human feces and water, in 2013 and 2019, respectively. Briefly, samples were collected in sterile screw cap containers to avoid direct oxygen exposure (Siah et al., 2014) and were grown on tryptose sulfite cycloserine (TSC) agar (Merck) under anaerobic conditions by using anaerobic jars with anaerobic atmosphere generation pouches (AnaeroGen, Thermo Scientific, Oxoid) at 37°C for 24h. Genomic DNA was extracted using the Wizard Genomic DNA Purification Kit (Promega). The quantity of the extracted DNA was assessed using a Qubit 2.0 Fluorometer.

DNA library preparation and paired-end whole genome sequencing (2 × 150 bp) were conducted on the Illumina NextSeq platform 2000 at the Microbial Genome Sequencing Center (MiGS). The assembled genomes were deposited at DDBJ/ENA/GenBank as part of a Bioproject under the accession number PRJNA836622.

Publicly available sequence data of 370 *C. perfringens* genomes was also included in our study for comparative analyses. These data comprised 197 genome assemblies from the Pathosystems Resource Integration Center (PATRIC; Wattam et al., 2014; accessed on May 20th, 2021), 40 assemblies from the Public databases for molecular typing and microbial genome diversity (PubMLST; Jolley et al., 2018; accessed on May 14th, 2021), and 133 reads that were retrieved from the European Nucleotide Archive (ENA) database[1] using the keywords "*C. perfringens*" (accessed on Jun 20th, 2021). Metadata and access numbers were verified to avoid duplicates (Supplementary Table S1).

Taxonomic classification, genome assembly and annotation

Quality stats of reads were collected using FastQC.[2] The 133 raw read pairs from ENA were processed with Trimmomatic v. 0.38 to remove low-quality bases below *Phred* 30 and adapter sequences (Bolger et al., 2014). *De novo* assembly was conducted using SPAdes v. 3.14.1(Bankevich et al., 2012) with the default settings. The quality of the resulting assemblies was assessed with Quast v5.0.2 (Gurevich et al., 2013).

The taxonomic classification of the assemblies was verified using Kraken v1.1.1 (Wood and Salzberg, 2014) with default parameters and using standard databases. Additionally, average nucleotide identity (ANI; Richter and Rossello-Mora, 2009) was calculated with pyANI.[3] An average identity percentage above 95% denotes strains that belong to the same species. *C. perfringens* ATCC 13124 was used as a reference strain for this analysis. The assemblies generated by SPAdes and the ones obtained from public databases were annotated with PROKKA v. 1.11 (Seemann, 2014) with the default parameters and using the UniprotKB (SwissProt) database (Research UCJNa, 2017), considering kingdom specific databases for Bacteria. Annotated assemblies were taken to calculate the pangenome using Roary (Page et al., 2015). Genes present in 95% of the genomes with at least 95% of identity were designated as core genes.

In silico MLST assignment

To assign isolates to sequence type (ST), *in silico* MLST was performed using FastMLST v0.0.14[4] (Guerrero-Araya et al., 2020) with the default parameters. Concatenated sequences of the eight housekeeping genes of the scheme were extracted (*colA*, *groEL*, *sodA*, *plc*, *gyrB*, *sigK*, *pgk*, and *nadA*) and novel STs were submitted to the PubMLST database for identification (accessed on May 14th, 2021). Global optimal eBURST (goeBURST)[5] was used to visualize CC, defined in this study as closely related STs that differ from a common founder at up to two of the eight loci used in the MLST scheme (DLV, double locus variant; Francisco et al., 2012).

Phylogenetic reconstruction based on MLST and core genome gene sequences

Concatenated sequences of the eight housekeeping genes belonging to the MLST scheme were used as input for a multiple alignment. Likewise, core genes were concatenated and aligned using MAFFT v7.407. The best-fit model for base substitution in IQ-TREE v2.0.3 was selected to infer phylogenetic relationships by maximum likelihood (ML) and ultrafast bootstrap (1,000 repetitions; Nguyen et al., 2015). A clade was considered well supported when the bootstrap was ≥ 80%, as previously indicated (Wróbel, 2008; Minh et al., 2013). The resulting trees were visualized and edited using iTOL v.5 (Letunic and Bork, 2021). To confirm the phylogenetic clades generated by IQ-TREE, a NeighbourNet network was reconstructed in SplitsTree4 software v4.17.0 (Huson and Bryant, 2006) from a core genome alignment and producing a splits graph representing sequence distances (Huson, 1998).

Reference genomes were included to classify isolates into phylogenetic groups. For Phylogroup I, C. perfringens Darmbrand NCTC8081 (ERR1656460) was selected, which is responsible for human enteritis Necroticans cases in Germany in the 1940s and is genetically related to strains carrying chromosomal cpe (Lindström et al., 2011; Ma et al., 2012). For Phylogroup II, strains involved in necrotizing enteritis in foals and hemorrhagic gastroenteritis in dogs, and denoted with the prefix JFP were chosen (GCA_001949795.1, GCA_001949805.1, GCA_001949775.1; Abdel-Glil et al., 2021). For Phylogroup III, *C. perfringens* ATCC3626 (GCA_000171155.1) and *C. perfringens* ATCC13124 (GCA_000013285.1) were used, and for Phylogroup IV, *C. perfringens* type D (GCA_006385425.1) isolated from a sheep. Phylogroup V was established based on the strain *C. perfringens* Tumat (GCA_003990265.1), Which was isolated from the mummified remains of an ancient puppy found in Siberian Permafrost (Abdel-Glil et al., 2021).

In silico identification of virulence and antimicrobial resistance genes

Toxin detection (CPA, CPB, ETX, IAP, CPE, and *netB*) and typing of the 372 isolates were conducted with ABRicate v. 0.5

1 https://www.ebi.ac.uk/ena/browser/home
2 http://www.bioinformatics.babraham.ac.uk/projects/fastqc/
3 https://github.com/widdowquinn/pyani
4 https://github.com/EnzoAndree/FastMLST
5 http://goeburst.phyloviz.net

using the TOXIper database[6] (available until October 20th, 2020). Additional virulence factors such as sialidases, collagenases, and secondary toxins like *pfo*, *iam*, and *cpb2* were screened using the Virulence Factor Database (VFDB; March 17th, 2017; Chen et al., 2016). The Resfinder database (available until July 8th, 2017; Zankari et al., 2012) was used for the Antimicrobial Resistance (AMR) evaluation since it was the most complete database available with 2,228 AMR sequences at the moment of the analysis.

Results

Long-range dispersion of *Clostridium perfringens*

A total of 372 genomes from different sources were collected for our study: two *de novo* sequenced genomes from Chile, 237 previously assembled sequences, and 133 raw reads (Supplementary Table S1). The number of contigs among the assemblies was variable, with an average of 82 contigs per genome. On overage, the genome size was 3.3 Mb, with a low GC content of around 28% (Supplementary Table S2). In addition, ANI values were invariably > 95% for the 372 genomes when compared to the reference strain *C. perfringens* ATCC 13124, confirming their classification as *C. perfringens* (Supplementary Figure S1). Most of the publicly available genomes were obtained from the US (123/372, 33.1%), France (48/372, 12.9%), and China (44/372, 11.8%; Figure 1A), and were recovered mainly from food (85/372, 22.8%), birds (85/372, 22.8%), and humans (74/372, 19.8%; Figure 1B).

Wide toxinotype diversity among multiple hosts

The seven different toxinotypes defined until now were seen among our 372 isolates. Toxinotype A was the most frequently found (198/372, 53%), followed by toxinotype F (120/372, 32%), and G (24/372, 7%; Figure 1C). Interestingly, toxinotype distribution varied among hosts. In this regard, 60% (45/74) and 32% (24/74) of the human isolates were classified as toxinotype A and F, respectively. Two human isolates (2.7%) belonged to toxinotype E, and toxinotype C, D, and G had one (1.3%) isolate each. Food isolates were also distributed in a similar way, as 32% (27/85) were toxinotype A, 67% (57/85) F, and 1% (1/85) G.

As for animals, 72% (61/85) of the bird isolates were classified as toxinotype A, 26% (22/85) as G, and 1% as C (1/85) and F (1/85). Fifty percent of the swine isolates (8/16) corresponded to toxinotype A, 43.7% (7/16) to C, and 6.3% (1/16) to F. Likewise, the ovine isolates were classified as toxinotype A (2/15, 14%), B (3/15, 20%), C (5/15, 33%), and D (5/15, 33%). Isolates from canines (16/22, 73%) and equines (15/16, 94%) belonged to toxinotype F and only a few to toxinotype A (6/22, 27% and 1/16, 6%, respectively). The high diversity of toxinotypes in the multiple evaluated hosts is an indicator of heterogeneous toxins that can support the differential pathogenic effect of *C. perfringens* populations circulating in each of these species.

Clostridium perfringens clones of heterogeneous origins

Multilocus sequence typing (MLST) was used to classify *C. perfringens* genomes. A total of 195 STs were identified among 322 genomes. One or more genes from the MLST scheme could not be recovered from 50 genomes, thus they were not assigned an ST. *cpa*, *colA*, and *nadA* were the genes with the most alleles (84, 84, and 73 respectively) compared to other MLST genes, which had less than 50 alleles (Supplementary Table S3).

MLST loci from the 322 genomes, were concatenated and aligned to generate a phylogenetic tree (Figure 2A), where five clusters were identified using the phylogenetic relationships and biological features as criteria, as reported before for *C. perfringens* (Abdel-Glil et al., 2021) Overall, Cluster III isolates were more frequent in our dataset (226/322, 70%). This cluster grouped isolates from diverse sources, including animals (120/226, 53%), humans (44/226, 20%), food (34/226, 15%), unknown sources (21/226, 9%), and environment (21/226, 3%). Cluster I (67/322, 20%) grouped mostly isolates from food (42/67, 63%) along with isolates from human (19/67, 28%), animal (3/67, 4.5%), and unknown sources (3/67, 4.5%). Interestingly, Cluster II (23/322, 7%) only grouped isolates from animals (19/23, 83%) and humans (4/23, 17%), whereas Cluster IV was formed by 3 isolates (3/322, 1%) from animal origin, and Cluster V by 3 isolates (3/322, 1%) from animal (2/3, 66%) and food (1/3, 34%) sources (Figure 2A).

Of the 195 STs detected, five were the most common: ST-147, -248, -80, -251, and ST-73 (Supplementary Figure S2). Isolates within these STs tended to have the same toxinotype, however, they were located across different clusters. For example, 14 toxinotype F isolates from food and human sources from the US and Italy isolated during 2017 and 2019 were classified as ST-147 and grouped in Cluster I. In the same way, 13 toxinotype F isolates from dogs and horses from Canada and the US, were classified as ST-80 located in Cluster II. Furthermore, ST-251 included 11 isolates from toxinotype F in cluster I recovered in the US. In contrast, ST-73 grouped 11 isolates from toxinotypes G and A from birds, all of them grouped in Cluster III.

The MLST-based minimum spanning tree built using the *goe*BURST algorithm led to evolutionary inferences. By identifying founder STs in the 25 CC and 95 singletons (Figure 2B; Supplementary Figure S3). Overall, CC1 was the most commonly represented CC. It was found within Cluster III, grouping 23 STs from 50 genomes mostly from birds (ST-73, -106, -118), human stool and blood samples (ST-5, -262 y ST-271), and food (ST-5, -195, -225, -299 y ST-302). The founder ST for this group was ST-225, which is associated with food.

6 https://github.com/raymondkiu/TOXIper

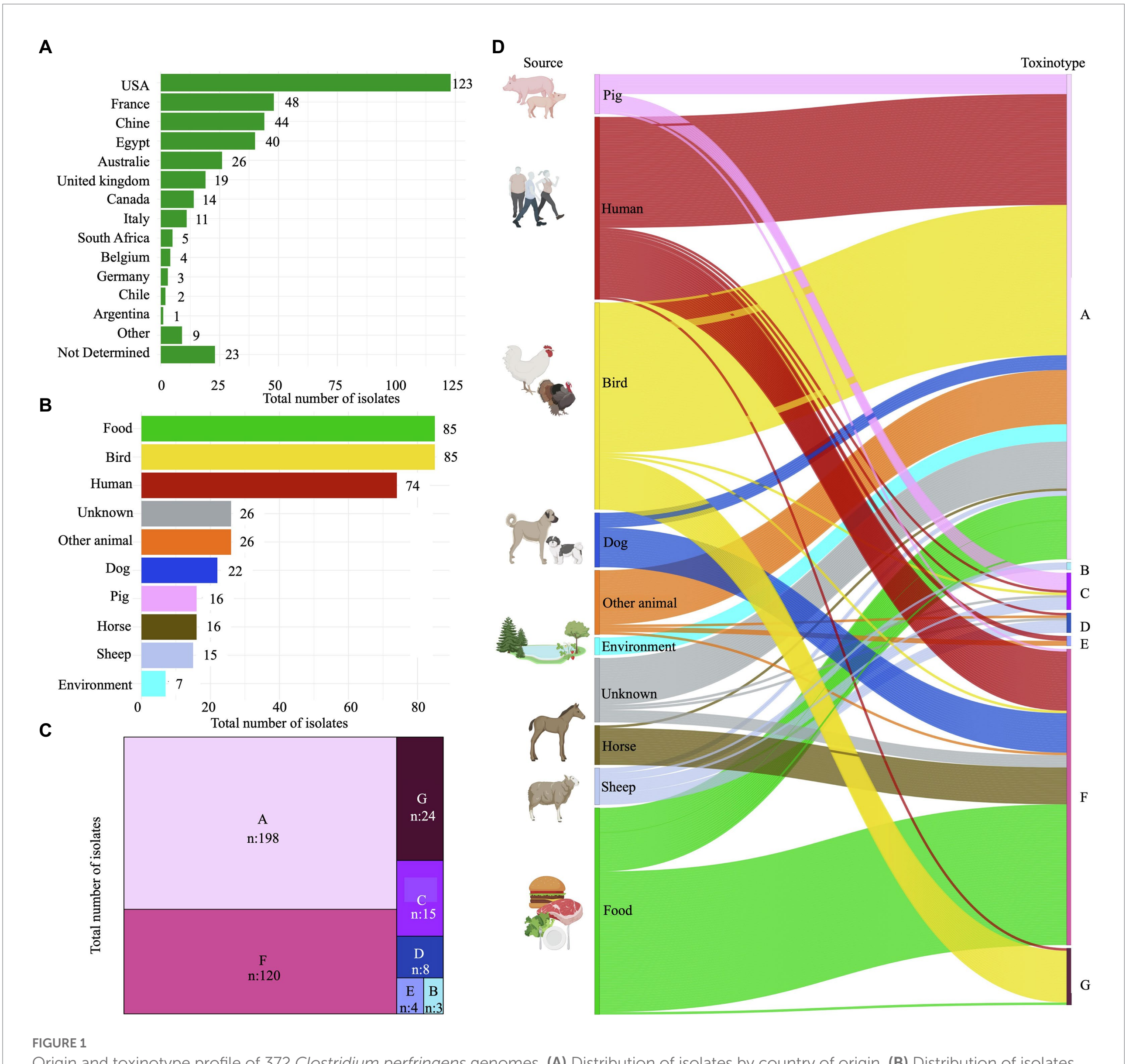

FIGURE 1
Origin and toxinotype profile of 372 *Clostridium perfringens* genomes. **(A)** Distribution of isolates by country of origin. **(B)** Distribution of isolates by source. **(C)** Distribution of isolates by toxin type. **(D)** Toxinotype diversity among hosts. All figures were created with R software.

Usually, the predicted founder corresponds to the most predominant ST in a CC (Feil et al., 2004), however this ST contained only one isolate whereas DLV descendant ST-73 contained 11 isolates (Supplementary Figure S3). This interesting finding might be caused by an origin bias due to either the relatively low number of samples used or by the natural selection pressure within the population leading to the emergence of strains with a strong adaptive advantage (Feil et al., 2004).

Most of the isolates from human stools and food were grouped in Cluster I as CC-2 along with ST-139 as founder ST. ST-39 and ST-253 from swine farms in China were classified as CC-18 in Cluster III, and founder ST-80, -93, and -79 from canine and equine isolates were in cluster II as CC-9. Some of the STs varied in terms of hosts, for instance ST-80 and ST-78 included isolates from dogs and horses, ST-5, -33, -132, -139, -147, -248, and -251 included human and food isolates, and ST-39 grouped isolates from swine and birds, while ST-143 grouped birds and environment, and ST-215 birds and food (Figure 2B).

Five *Clostridium perfringens* phylogroups identified by core genome-based phylogenetic analysis

A total of 35,876 genes are included in the pangenome of this dataset. Of them, 34,917 (97.3%) genes are accessory and only 959 (2.7%) are considered part of the core genome. In the resulting tree, five main branches were observed matching the

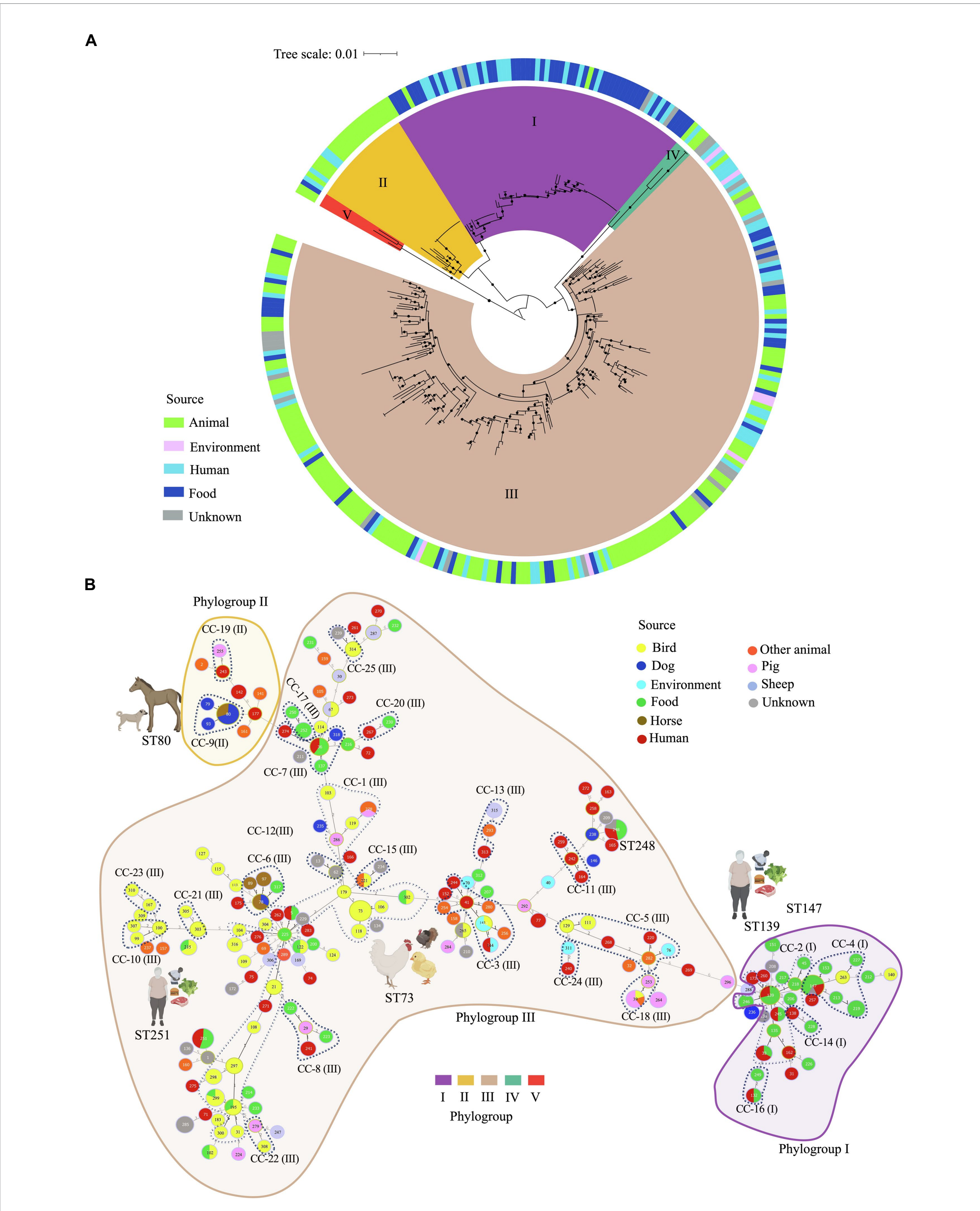

FIGURE 2
Multilocus sequence typing (MLST)-based phylogeny and goeBURST full minimum spanning tree of 195 *C. perfringens* MLST profiles among 322 genomes. **(A)** MLST-based phylogeny tree obtained with fastMLST. The outer ring shows the origin of the isolates as indicated in the legend. Clusters are represented by different colors on the inside of the ring. **(B)** MLST-based minimum spanning tree obtained with *goe*BURST. Sequence types (STs) are displayed as circles. Founder STs were defined as the STs with the greatest number of single-locus variants. Circle size indicates the number of isolates in every particular ST, with each color representing a different source type. Lines represent closely related isolates and line length illustrates STs that vary by one, two, or more alleles in their MLST profile. Clonal complexes (CC), defined as closely related STs that differ up to two of the eight loci used in the MLST scheme from a common founder (DLV, double locus variant), are designated by dashing lines.

clusters that were established by using the reference genomes and a bootstrap ≥80% (Figure 3A). The core genome-based phylogenetic tree matches with the MLST-based topology, as well as with the phylogenetic network generated using neighbor-joining, confirming the classification of *C. perfringens* population in five distinct clusters as reported previously (Figure 3B).

Phylogroup I (*n* = 69) clustered most of the isolates from humans and food from toxinotype F (*n* = 57) that were *cpa* and *cpe* positive, along with some isolates from toxinotype A (*n* = 7) from humans and animals. Phylogroup II (*n* = 36) grouped canine and equine isolates from toxinotype F (*n* = 26), as well as one cattle and two human isolates from toxinotype E. Phylogroup III (*n* = 257) was the most diverse group, where the novel isolates recovered from water and a human in Chile were located. In this phylogroup isolates from birds, food, environment, and humans classified as toxinotype A (*n* = 177), toxinotype F (*n* = 31), toxinotype G (*n* = 24), and in less proportion toxinotype C (*n* = 14), D (*n* = 7), and B (*n* = 3) were also found. Phylogroup IV (*n* = 3) and V (*n* = 7) included a majority of toxinotype A isolates (Figure 3A).

Clostridium perfringens is a highly versatile pathogen with a large number of virulence factors

A total of 372 WGS were analyzed to evaluate the distribution of virulence genes. The alpha-toxin gene *plc* associated with gangrene in humans and several animals, and possibly involved in enterotoxemia and gastrointestinal disease in ruminants, horses, and swine was present in most of the isolates, along with genes encoding extracellular enzymes such as alpha-clostripain (*ccp*), hyaluronidases (*nagH*, *nagI* and *nagJ*), and alpha-clostripain collagenase (*colA*), as expected (Canard et al., 1994; Sakurai et al., 2004; Goossens et al., 2017; Geier et al., 2021). Other genes present in the majority of the isolates were *pfoA* (perfringolysin O), *tpeL* (toxin perfringens large), and *cpb2* (beta2 toxin), which are protein coding genes involved in gastrointestinal outcomes and gangrene (Coursodon et al., 2012; Bueschel et al., 2013; Chen and McClane, 2015), and the sialidases *nanH*, *nanI* y *nanJ*, which play an important role in colonization and immunomodulation (Figure 4A).

The presence of virulence genes varied among the phylogroups. For example, all of the isolates in phylogroup I carried *nanH*, however *nanI, nanJ*, *nagH*, *pfoA,* and *cpb2* were absent in this group, in contrast to phylogroup II, where these genes were found in all of the isolates along with *nagI* and *nagK*. Additionally, 64% (23/36) of the isolates in this phylogroup also carried *netE* and 58% (21/36) *netF*. While the presence of these virulence genes in phylogroup III was variable, this was the only group where *TpeL*, a member of the large clostridial toxin (LCT) family involved in cell cytotoxicity was present, especially in isolates of toxinotype A, B, C, and G. However, this gene was not detected in type D or F isolates carrying the *cpe* and *itx* toxin genes. This difference in toxin profiles can be attributed to a potential incompatibility between plasmids carrying these genes (Chen and McClane, 2015).

Isolates in phylogroup IV carried the coding genes for the hyaluronidases *nagH*, *nagI,* and *nagJ*, as well as *nanI* and *pfoA*. Phylogroup V, which cluster toxinotype A isolates carried *nanI* and *pfoA*, unlike the toxinotype A isolates in phylogroup I (Figure 4A). These findings suggest that the differential presence of virulence factors in phylogroups may be due to selective advantage conferred by determinants in different niches (Sawires and Songer, 2006), routine treatment with clostridial toxoids especially in ruminants, or even environmental differences in the geographical regions (Simpson et al., 2018).

Prevalence of antibiotic resistance genes in *Clostridium perfringens*

AMR gene were found in 72.8% (271/372) of the genomes, with *tetA* (107/271, 39.5%) and *tetB* (98/271, 36.2%) involved in tetracycline resistance, being the most frequent and commonly found in phylogroup III isolates (Figure 4B). Interestingly, the water isolate from Chile harbors genes for tetracycline resistance (*tetA*), while the one from human stool possesses genes that encoded tetracycline (*tetA, tet44*) and aminoglycoside resistance (*ant(6)-Ib1*). Likewise, this approach identified genomes carrying *ermQ*, (28/372, 7.5%) an erythromycin resistance methylase gene mainly found in birds and environment isolates grouped in phylogroup III and classified as toxinotype A. *ant(6)-Ib1* genes encoding aminoglycoside resistance were found in some toxinotype A and C isolates from swine and birds from phylogroup III (Figure 4B).

Discussion

Clostridium perfringens is a clinically relevant pathogen due to its presence across several host species and its capacity to cause numerous medically important intestinal diseases in humans and animals (Mehdizadeh Gohari et al., 2021). To assess the genetic variation within the species, as well as to establish the phylogenetic relatedness, we collected the publicly available genomes of 370 isolates collected between 2010 and 2020 from different countries, with a majority of the isolates obtained in developed countries such as the US, France, and China (Figure 1A). There is a poor representation of isolates from developing countries in our dataset, possibly due to limited epidemiologic surveillance and genomic data collection, especially in South America, from where only one genome from Argentina was found and two more from Chile were added in this study.

The dataset also has a high percentage of strains isolated from stool samples of animals used for human consumption as well as isolates from food origin (Figures 1B, 2B), which along with the evidence of food products such as milk, meat, poultry, and pork

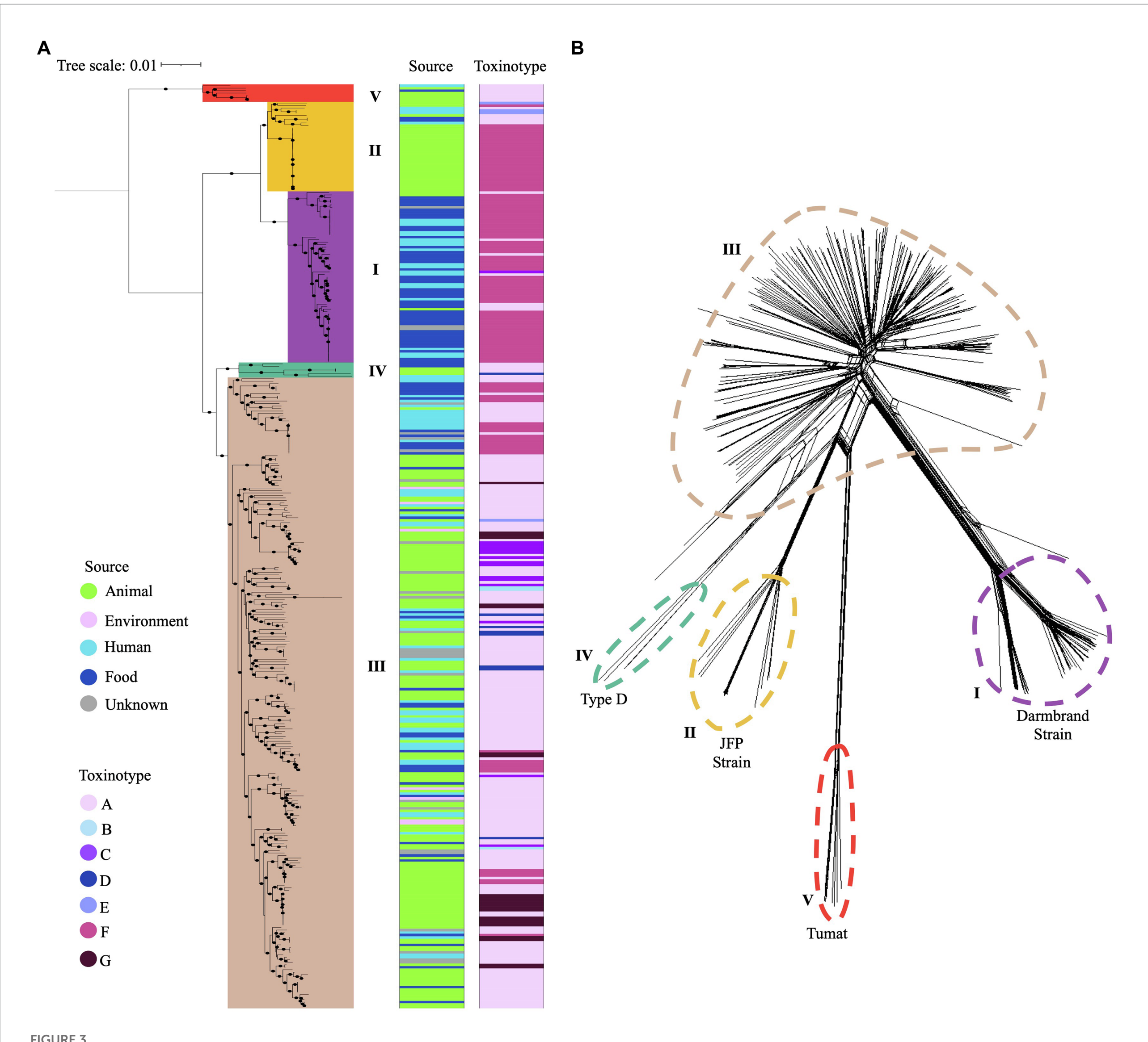

FIGURE 3
Phylogenetic grouping of *C. perfringens* using two different approaches. **(A)** Phylogenetic tree based on the core genome of 372 genomes. Five main phylogroups are highlighted in different colors. Source type and toxinotype are shown on the right panels. **(B)** Five phylogenetic networks based on the Neighbor-Joining (NJ) algorithm are shown with different colors.

among others as a source of infection, confirm the key role that *C. perfringens* plays as one of the most common causes of foodborne illness (Brynestad et al., 1997; Grass et al., 2013; Bintsis, 2017; Xu et al., 2021). However, despite the high percentage of isolates of human and food origin, it should be noted that environmental or commensal isolates are scarce (Figure 1B). In addition, some metadata variables are unknown, such as the possible association of genomes with outbreaks, which could underestimate the diversity of *C. perfringens* and affect the results of AMR prevalence, emphasizing the need for further studies with a greater number of genomes.

Clostridium perfringens toxinotypes are associated with heterogeneous diseases such as clostridial myonecrosis (gas gangrene) or gastrointestinal infections in humans and animals (Rood et al., 2018). Toxinotype G, associated with necrotic enteritis and gangrenous dermatitis in birds (Yang et al., 2019; Kiu et al., 2019a), and toxinotypes D and E that cause illness in sheep and cattle (Layana et al., 2006; Uzal and Songer, 2008; Nazki et al., 2017; Rood et al., 2018), have not been described in humans before, however, we identified one isolate from a raw chicken patty and another from human blood as toxinotype G (Figure 1D). Likewise, we found toxinotype D and E strains isolated from human stools. These findings support previous studies that have reported *C. perfringens* type D and E strains harboring *etx* and *iap* in humans (Al-Shukri et al., 2021), which can explain potential routes of transmission in subjects that have been in contact with infected animals or have consumed contaminated food

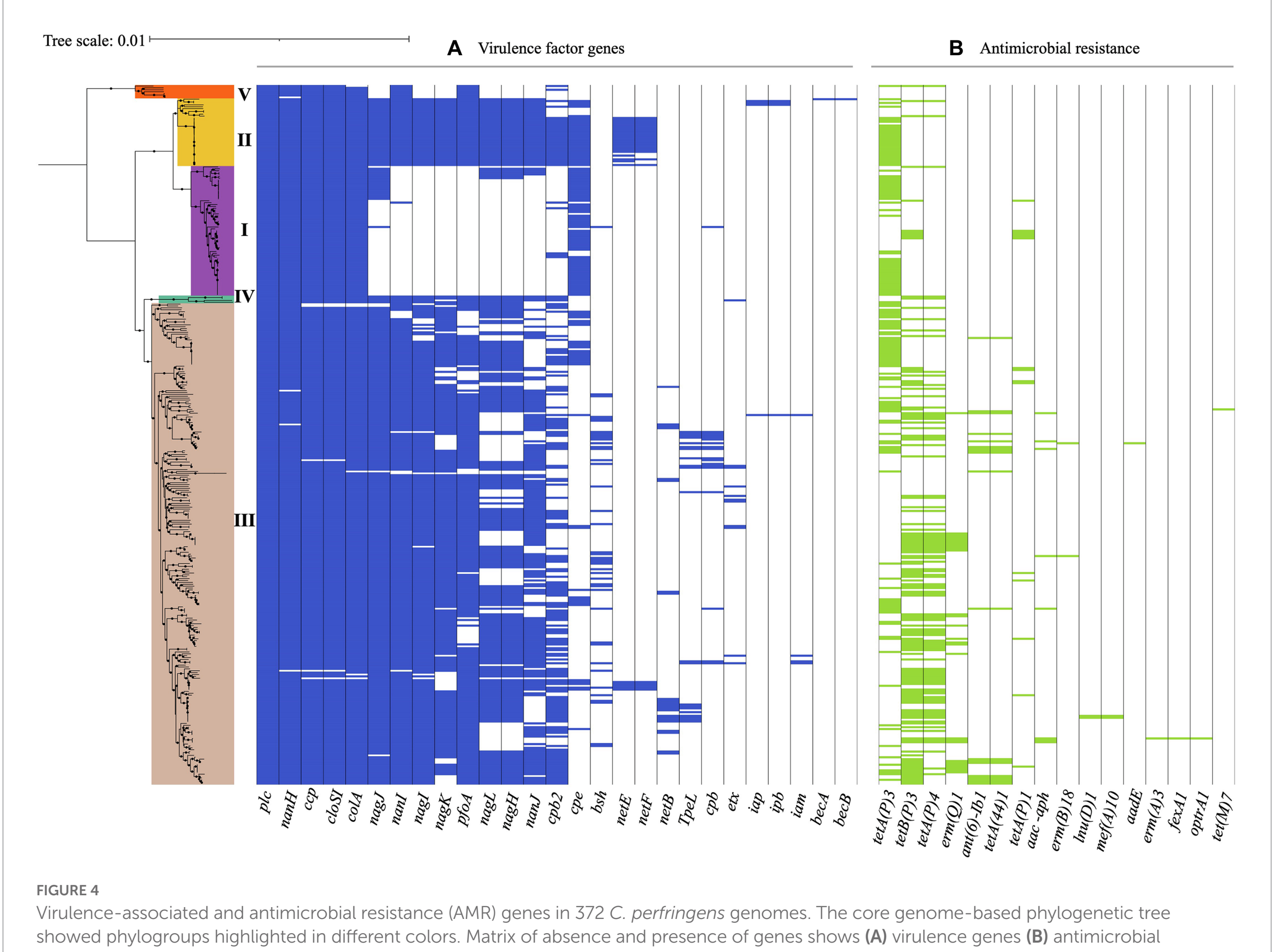

FIGURE 4
Virulence-associated and antimicrobial resistance (AMR) genes in 372 *C. perfringens* genomes. The core genome-based phylogenetic tree showed phylogroups highlighted in different colors. Matrix of absence and presence of genes shows **(A)** virulence genes **(B)** antimicrobial resistance (AMR) genes. The minimum coverage threshold needed for detection of these genes was 70% and the percentage of identity was 99.95%. The resulting trees were visualized and edited using iTOL v.5 (Letunic and Bork, 2021).

(Songer, 2010; Kiu et al., 2019b). Furthermore, these findings might be related to *C. perfringens* strains considered as normal intestinal microbiota that are in contact with acquired strains carrying conjugative plasmids that are often associated with insertion sequences that can mobilize toxin genes between different strains. This could lead to the conversion into virulent toxin-producing strains and the emergence of specific toxinotypes in new hosts (Freedman et al., 2015). Future studies should include genomes assembled with a standard pipeline that includes an approach to recover extrachromosomal elements in order to describe the plasmidome of *C. perfringens* and thus contribute to the biological knowledge of this species.

MLST and geoBURST analyses have not been widely used for *C. perfringens*: However, the identification of the founder STs, that differs from other STs at only one locus, provide a tool for epidemiological and evolutionary investigations of emergent pathogens (Feil and Enright, 2004). Furthermore, the identification of 25 CC in this work allowed us to compare the distribution of *C. perfringens* isolates from animals, humans, food and environment (Figure 2B; Supplementary Figure S2), evidencing a close relationship between isolates from different hosts. These findings support the strong association with foodborne diseases and suggesting their zoonotic potential and high diversification of this specie, as previously described (Verma et al., 2020; Hassani et al., 2022; Xiu et al., 2022).

The CC including toxinotype G isolates from human and birds, and the evidence of the distribution of human, food, and animal isolates in the same ST or CC matches the zoonotic potential of *C. perfringens* as demonstrated by other published studies (Immerseel et al., 2004; Mwangi et al., 2019; Verma et al., 2020). Population structure analyses based on MLST (Figure 2A) revealed five clusters in line with those generated here using the core genome (Figures 3A,B), and with results from previous studies (Abdel-Glil et al., 2021). Although MLST has a limited ability to establish phylogenetic relations since it only uses fragments from eight housekeeping genes, it is still a useful tool for interspecies *C. perfringens* typing due to its reproducibility, high discriminatory power, and easy accessibility (Pérez-Losada et al., 2013; Jolley et al., 2018; Guerrero-Araya et al., 2020). Despite these advantages, WGS has emerged as a more robust and complete tool contributing to the investigation of phylogenetic

relatedness among isolates and allowing a deeper understanding of transmission dynamics, emerging clones, key virulence loci, and the presence of AMR genes (Salipante et al., 2015; Quainoo et al., 2017; Pightling et al., 2018).

The pangenome analysis of *C. perfringens* conducted in this study showed an accessory genome of 97.32%, an extremely high percentage in comparison with other closely related bacteria such as *C. paraputrificum*, species with an accessory genome of 67%, *C. tertium* with 37.6% of accessory genes (Muñoz et al., 2019), or *C. baratii* with an accessory genome of 24.43% (Silva-Andrade et al., 2022). This high level of genome plasticity is similar to the one found in *Clostridioides difficile*, which has an accessory genome of 87.2% (Knight et al., 2021). *C. difficile* and *C. perfringens* are part of the normal intestinal flora but can become gastrointestinal pathogens, which can be explained by the ability to express different phenotypes in response to particular environmental conditions. The high genome plasticity of *C. perfringens* can give rise to the emergence of populations carrying new toxigenic profiles by acquired virulence factors due to horizontal gene transfer (HGT) leading to rapid genetic evolution. Thus, this genomic plasticity of *C. perfringens* is a determinant in the adaption to different hosts, as well as in the increase of its pathogenic potential and survival in different environments (Brüggemann, 2005).

The importance of the acquisition of different virulence factors in *C. perfringens* is given by their adaptation within different disease niches. When exploring the virulence factors present in the *C. perfringens* genomes, we found that *plc, colA,* and *nanH* are present in the majority of the genomes, hence they cannot be considered markers with high host discrimination capacity (Goossens et al., 2017; Mahamat Abdelrahim et al., 2019). The identification of isolates carrying *netE* and *netF* toxins in two animal species (dogs and foals) of phylogroup I, as well as previous reports of the prevalence of these two toxins in *C. perfringens* isolates from dogs with acute diarrhea hemorrhagic syndrome (Sindern et al., 2019), suggest their adaptability to these specific hosts. Likewise, the presence of three sialidases (*nanH*, *nanI*, and *nanJ*) in isolates from the same clinical outcomes highlights the role of these enzymes in increasing the adhesion of *C. perfringens* to host cells (Carman, 1997; Chiarezza et al., 2009; Li and McClane, 2021) and suggests an important role in the intestinal pathogenesis in these hosts, as was previously reported (Li et al., 2015; Li and McClane, 2021). However, a better understanding of the role and specificity of these molecules in canine acute hemorrhagic diarrhea and necrotizing enteritis of foals is required.

Another virulence-related gene, *pfoA*, was also found in most of the genomes included here (Figure 4A), however, it was absent in toxinotype F isolates clustered in the phylogroup I, which correlates with prior studies that have revealed that some strains that produce enterotoxins and therefor cause food poisoning lack *pfoA* (Myers et al., 2006; Deguchi et al., 2009). This suggests that this cytolytic toxin is not the main cause of most gastrointestinal outcomes in humans, however, it could be associated with the enteritis necro-hemorrhagic or bovine enterotoxemia, as recent studies have shown (Verherstraeten et al., 2013a,b). Thus, the high number of virulence-related genes in the 372 genomes analyzed, especially in the novel isolates from Chile included in our study (Figure 4A), reveals the need to continue with the epidemiological surveillance and the molecular study of virulence factors, mainly in unexplored countries, to provide more data for a deeper knowledge of the global diversity of *C. perfringens*.

Another group of molecular markers of importance in health is the one associated with antibiotic resistance. We found that a high percentage of the genomes (72.8%, 270/372) harbored some type of AMR gene (Figure 4B), where a large number of isolates carried genes putatively linked to resistance to tetracycline, macrolides, and aminoglycosides. The presence of a high number of AMR genes in isolates from pigs and birds could be a consequence of the use of antibiotics as growth promoters in these animals and may be related to the appearance of resistance in zoonotic pathogens (Osman and Elhariri, 2013). Although WHO has questioned the use of additives due to the risk of antibiotic residues in meat products for human consumption, several countries continue with this practice that can be related to the increase of antibiotic resistant strains (Salvarani et al., 2012). Many factors such as the production of high concentrations of antibiotics in the global industry (Nijsingh et al., 2019; Tell et al., 2019), the indiscriminate use of antibiotics in the community (Graham et al., 2019), the contamination of natural sources by hospital waste (Larsson and Flach, 2021), the impact of intrahospital infections caused by multidrug resistant strains (Avershina et al., 2021), the use of antibiotics for animal growth, the poor management of organic waste and the use of animal excrement in the agricultural sector (Tang et al., 2017), and the antimicrobial drugs overused during the first wave of COVID-19 (Manesh and Varghese, 2021), pose a high risk for possible pathways of antimicrobial resistance (Chokshi et al., 2019).

The analysis of 372 genomes conducted here, is the largest effort to snapshot the global genomic diversity of *C. perfringens* to date. The genomic plasticity of this microorganism due to its low GC content (~28%; Uzal et al., 2014), its short doubling time (~7 min; Maiden, 2006), and a high percentage of horizontally transferred toxin encoding genes (Xiao et al., 2012; Uzal et al., 2014) contributes to the spread of existing toxinotypes to new hosts, as well as to the increase of food poisoning outbreaks and the growth of AMR. The use of MLST and WGS in a "One Health" framework that connects the health of humans and animals in a shared environment represents an optimal approach to advance knowledge of the global genetic diversity of *C. perfringens*. Our findings emphasize the need for further studies using a larger number of isolates from different ecological niches to elucidate the genetic characteristics, diversity, and zoonotic potential of *C. perfringens* and to improve strategies to reduce the growing threat to public health by this pathogen.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary Material.

Author contributions

AC, EG-A, and MM designed the study and performed the data analyses. AC and MM performed methodology, formal analysis, data curation, and visualization and wrote the original draft preparation. JDR, DP-S, CR, SC, LV, and MC-A validated the results and revised and edited the manuscript. JDR and MM supervised the study and contributed to review and editing the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2022.952081/full#supplementary-material

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1 **Insights into *Clostridium perfringens* Dispersal Hotspots, Toxins, and Virulence Factors**
2 **through Integrated Genomic and Phenotypic Profiling**
3

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21 **Abstract**

22

23 *Clostridium perfringens*, an anaerobic bacterium known for causing intestinal and tissue diseases in
24 humans and animals, requires local insights into its virulence factors, antibiotic resistance markers
25 (ARM), and dispersal dynamics as a critical step to develop effective prevention and management
26 strategies. This study conducted whole genome sequencing (WGS) of *C. perfringens* isolates obtained
27 from symptomatic and asymptomatic individuals and animals in the Colombian Cundiboyacense
28 highlands in 2022. A comparative genomic analysis was carried out at the microgeographic level with
29 185 Colombian genomes and another at the global level with 617 representative genomes, of which
30 546 were publicly available and 71 were Colombian. The main objective was to evaluate dispersal
31 pathways, AMR and virulence factors. In addition, a phenotypic characterization of 30 representative
32 isolates from all phylogroups was performed, taking into account the presence of perfingolysin O
33 (*pfoA*) coding. These tests included the evaluation of hemolysis, cell growth inhibition, sporulation
34 capacity and antibiotic sensitivity.

35

36 Comparative genomic analyses at the microgeographic level identified eight phylogroups exhibiting
37 wide genetic diversity in the region. Phylogroup I comprised 55 genomes from humans, canines, and
38 felines, while phylogroups II, IV, and VI had lower representation, including isolates from humans
39 and multiple animal species. Phylogroup VII comprised genomes obtained from cats, which lacked
40 *pfoA* and a multi-peptide resistance factor (*mprF*). These deficiencies might correlate with heightened
41 susceptibility to aminoglycosides/defensins and reduced tolerance to acidic environments.
42 Additionally, *pfoA*, complete hemolysis, and increased cell growth inhibition rate were identified in
43 asymptomatic individuals, underscoring the potential risk of carrying toxigenic isolates in this
44 population. At the genomic level, we detected MRAs associated with aminoglycosides and
45 macrolides, which resulted in lower susceptibility to antibiotics such as gentamicin (87.0%),
46 erythromycin (20.0%) and metronidazole (17.0%). These results underscore the urgency of strict
47 regulation of antibiotic use in low- and middle-income countries to curb the proliferation of antibiotic
48 resistance. The integration of genomic, phenotypic, and epidemiological analyses allows us to better
49 understand the virulence factors and antibiotic resistance of locally circulating *C. perfringens* strains.
50 This knowledge is essential to improve public health interventions at the local level.

51

52 **Keywords**

53 *Clostridium perfringens*, comparative genomics, perfringolysin O, virulence factors, antibiotic
54 resistance markers, cytotoxicity, sporulation.

55 **INTRODUCTION**

56

57 *Clostridium perfringens*, an anaerobic and sporulating bacterium, has emerged as a global health
58 threat. It is recognized as one of the top five causes of foodborne illnesses (FBD) in the United States
59 (US) [1]. Despite extensive research on its impact in developed countries, the need for detailed
60 statistics in developing countries represents a crucial challenge.

61

62 *C. perfringens* is classified into seven toxinotypes (A-G) based on its ability to produce toxins,
63 including alpha (CPA), beta (CPB), enterotoxin (CPE), epsilon (Etx), iota (Itx), and necrotic enteritis
64 B-like (NetB) toxin, each with specific clinical implications. Toxinotype A, which carries the CPA
65 toxin, is ubiquitous and naturally inhabits the human and animal intestines. In vulnerable populations
66 with risk factors such as chronic diseases or immune deficiencies, this opportunistic pathogen can
67 quickly multiply, acquire, and release toxins capable of destroying epithelial tissue, thereby
68 contributing to the progression of intestinal and tissue diseases [2]. This toxinotype has been
69 implicated in pathologies such as clostridial myonecrosis [3, 4] and necrotizing enterocolitis in
70 premature infants [5, 6]. Toxinotype B isolates are associated with dysentery in sheep [7]. Toxinotype
71 C is associated with necrotizing enteritis and enterotoxemia in humans, sheep, foals, and piglets [8].
72 Toxinotype D is related to symptoms of enterotoxemia and enterocolitis in sheep, goats, and, rarely,
73 cattle [9]. Toxinotype E is associated with enterotoxemia in calves and lambs [10]. Toxinotype F,
74 carriers of CPE toxin, are mainly related to human food poisoning, non-foodborne diarrhea, and
75 antibiotic-associated diarrhea (AAD) [11, 12]. Finally, toxinotype G is associated with avian necrotic
76 enteritis [13].

77

78 While most studies have concentrated on detecting *C. perfringens* and characterizing major toxins
79 and virulence factors in symptomatic individuals, the specific role of this pathogen in asymptomatic
80 people, including the circulating accessory toxins and their contribution to disease development, still
81 needs to be completed. Some genotyping studies of *C. perfringens* carried out in healthy populations
82 have revealed the presence of toxigenic isolates in asymptomatic adults [14, 15] and premature
83 neonates [16], implying a potential risk as a reservoir of toxigenic isolates implicated in the
84 progression of natural history of diseases caused by this bacterium.

85

86 Approaches based on WGS enable the analysis of dissemination dynamics and the evaluation of
87 potential risks posed by circulating isolates to human and animal health [17]. Genomic studies of *C.*
88 *perfringens* have revealed the existence of five phylogroups, with clustering between isolates

89 involved in FBD from food and human sources and between isolates from intestinal lesions in foals
90 and canines, suggesting key relationships between host species [18]. In turn, the carriage of virulence
91 genes and ARM, as an evolutionary strategy to adapt to a specific habitat, may profoundly impact
92 both the phylogeny and the ability of this opportunistic pathogen to inhabit different ecological niches
93 [13].
94
95 Complementary to WGS-based approaches, biological characterization of pathogenic bacteria is
96 crucial in understanding circulating strains' ecological and physiological attributes [19]. Phenotypic
97 analysis of isolates from the pediatric population revealed the role of toxins such as PFOA in diseases
98 such as necrotizing enterocolitis, impacting cellular toxicity, hemolysis, and increased
99 proinflammatory responses [20, 21]. This shows the importance of phenotypic testing for
100 characterizing *C. perfringens* toxins.
101
102 The increasing spread of virulence factors, the increase of genomic AMR over time [22] , and the
103 rising emergence of phenotypic resistance to antibiotics such as aminoglycosides, macrolides, and
104 nitroimidazoles [23] complicate the implementation of effective treatments aimed at eradicating
105 infectious diseases. This complexity is compounded by the limited biological knowledge of *C.*
106 *perfringens*, particularly in developing countries, and the population's need for more information on
107 antibiotic resistance markers. These factors converge to create a significant barrier that impedes the
108 implementation of effective preventive measures and treatments.
109
110 Integrating genomic data with experimental validation through phenotypic testing deepens the
111 understanding of *C. perfringens* biology. This study aimed to characterize 185 *C. perfringens* isolates
112 collected from symptomatic humans and asymptomatic animals in a central region of Colombia at
113 the genomic level, addressing the need for more data in developing nations. These findings were
114 contextualized globally through comparative genomics analysis with reported data worldwide.
115 Additionally, phenotypic tests were conducted to assess distinctive biological traits such as hemolysis
116 capacity, cytotoxicity, sporulation, and antibiotic susceptibility across representative phylogroups of
117 *C. perfringens*. This research expands the understanding of this bacterium's genomic and phenotypic
118 features to a microgeographic model, shedding light on hotspots and highlighting its threat to human
119 and animal health in the Colombian region.
120

121 **MATERIALS AND METHODS**

122 **Sample Collection**

A total of 59 *C. perfringens*-positive fecal samples were analyzed, previously identified by a specific polymerase chain reaction (PCR) targeting the *16S-rRNA* and alpha toxin (*cpa*) genes [24]. These samples were collected from various sources in the Colombian Cundiboyacense highland region, encompassing 18 humans (14 asymptomatic and 4 with gastrointestinal symptoms) and 41 asymptomatic animals (29 cats, nine dogs, one goat, one sheep, and one pig) (**Supplementary Table 1**).

Ethics Approval and Consent to Participate

The current study received approval from the Research Ethics Committee of the Universidad del Rosario (CEI-UR approval number 449). It was classified as low risk by Resolution 8430/1993 of the Colombian Ministry of Health.

Isolate Establishment, Whole Genome Sequencing (WGS), and Assembly

C. perfringens isolates were established by directly culturing fecal samples on selective medium Tryptose Sulfite Cycloserine (TSC) agar through streaking technique [25], incubating for 24 hours at 37ºC under anaerobic conditions. Between 2 and 5 colony forming units (CFUs) per sample exhibiting black appearance were selected, resulting in 185 isolates. The biomass of each CFU was amplified on blood agar. Microscopic confirmation was achieved through Gram staining. Genomic DNA was extracted from pure colonies matching the macroscopic and microscopic characteristics using the Wizard Genomic DNA Purification Kit (Promega) following the manufacturer's instructions. Quantification of the extracted genomic DNA was performed using a Qubit 2.0 Fluorometer.

The whole-genome paired-end library (2x150 bp) was sequenced on Illumina HiSeq500. The quality of the reads (fastq files) and trimming was performed with Fastp v.0.20.0 [26]. De novo assembly was performed using Unicycler v 0.4.9b [27]. Contigs <500 bp in each assembly were filtered out before subsequent analyses, and the quality of the assemblies was assessed with sequence-stat v1.1 (https://github.com/raymondkiu/sequence-stats). The taxonomy of the assemblies was confirmed with GTDB-TK v.1.5.1 [28]. Subsequently, the assemblies that presented contamination of less than 10% and completeness greater than 80% using CheckM V.1.1.3 [29] were selected to be included in the subsequent analyses.

Public data set for comparative genomics analysis

156 The comparative genomic analyses included 1,083 public *C. perfringens* assemblies. These
157 assemblies, in fasta format, were compiled from the NCBI assembly database, accessible at
158 https://www.ncbi.nlm.nih.gov/assembly/?term=Clostridium+perfringens. Quality control was
159 implemented following the previously described methodology.
160 The complete dataset, comprising 1,268 genomes (1,083 public and 185 Colombian), underwent
161 analysis in dRep v.3.2.2 [30] to remove genomes with a 99.9% or higher similarity. This filtering
162 process yielded a final set of 617 genomes, consisting of 546 publicly accessible genomes and 71 of
163 Colombian origin.
164
165 **Phylogenomic analyses based on the core genome and whole genome SNPs**
166 The genomes were annotated with Prokka v.1.14.6 [31], and the resulting .gff files were employed as
167 input for pangenome analysis using Panaroo v.1.2.8. The core genome was identified with an identity
168 threshold of 98% and presence in at least 98% of the compared genomes [32]. Single Nucleotide
169 Polymorphisms (SNPs) were extracted from the core-genome alignment using snp-sites-2.3.3 [33].
170
171 Alignments were conducted for two datasets: one at a microgeographic level, comprising all
172 Colombian *C. perfringens* isolates (n=185) to analyze potential local transmission hotspots, and the
173 other at a global level, containing the public genomes along with dereplicated Colombian genomes
174 (n=617) to assess the phylogenetic relationships between the Colombian genomes and those reported
175 globally, we utilized data representative of the diversity. Maximum likelihood phylogenetic trees
176 were constructed using IQtree v.2.0.5 [34] for each dataset, employing the ultrafast bootstrap
177 (UFBoot) function (-bb option) and the SH-aLRT test (-alrt 1000) to define a reliable clade with SH-
178 aLRT >= 80% and UFboot >= 95%. The resulting phylogenetic trees were visualized using iTol [35],
179 and clusters were identified using an R package that implements the hierBAPS algorithm, a method
180 for hierarchically clustering DNA sequence alignments to reveal nested population structures [36].
181
182 **Antimicrobial resistance and in-silico virulence factors**
183 The presence of AMR-associated genes and virulence factors, specifically toxins, was determined
184 using Abricate v.1.0.1 (Seemann T, Abricate, GitHub https://github.com/tseemann/abricate) with the
185 CARD database [37], the VFDB database [38], and the Toxiper database (Kiu R, TOXIper: rapid
186 toxinotyping of *Clostridium perfringens* genomes, GitHub https://github.com/raymondkiu/
187 TOXIper), respectively. Sequence identification was conducted with a percentage of identity greater
188 than or equal to 90% and coverage exceeding 80% based on previous work [5].
189

190 ***In vitro* phenotypic tests**

191 Selection of isolates and hemolysis assay

192 Thirty representative isolates from the eight phylogroups were selected, ensuring a proportional
193 representation of each phylogroup in the total number identified. This approach aimed to adequately
194 capture the evaluated isolate set's genetic diversity. To analyze phenotypic traits related to the PFOA
195 toxin found in asymptomatic individuals, associated with necrotizing enterocolitis and linked to
196 increased cellular toxicity in intestinal cell lines as well as complete hemolysis [39], isolates were
197 classified according to the presence of the *pfoA* gene.
198 The isolates were cultured on blood agar plates for 24 hours at 37ºC under anaerobic conditions.
199 Complete hemolysis was assessed manually by observing the presence of transparent halos formed
200 inside and around the colonies.
201

202 Sulforhodamine B cytotoxicity assay

203 A cellular cytotoxicity assay using sulforhodamine B (SRB) was performed. This assay measures
204 cellular biomass by utilizing SRB's capability to bind to cellular proteins, forming complexes under
205 acidic conditions. Upon release in basic environments, dye release correlates with cell biomass, which
206 is quantified at 565 nm. This assay is independent of metabolic activity, thereby minimizing
207 interference [40].
208 The SRB assay was conducted following previously documented procedures [40]. Vero-CCL-81 cells
209 sourced from ATCC were cultured for 24 hours at 37°C and 5% CO2 in Roswell Park Memorial
210 Institute (RPMI) 1640 medium supplemented with 5% fetal bovine serum. After forming a confluent
211 monolayer within 24 hours, the cells were rinsed, and bacterial supernatants (diluted 1:5) that had
212 been filtered (0.22 μm filter) were added for 48 hours. Air-dried plates were then stained with 100 μl
213 of 0.04% SRB and incubated for 20 minutes at room temperature. Unbound dye was eliminated by
214 washing rapidly five times with 200 μl/well of 1% acetic acid. Subsequently, 100 μl of 10 mM Tris
215 base was added to the wells to solubilize the dye. Absorbance was measured at a wavelength of 525
216 nm using a SPECTROstar nano microplate reader from BMG Labtech [41].
217

218 Spore count and viability

219 Assays were conducted following previously outlined protocols [42-44]. In brief, 0.1 ml of the
220 bacterial culture was retrieved and inoculated into 10 ml of liquid thioglycollate (FTG) medium,
221 followed by incubation at 37°C until reaching an optical density at 620 nm (OD600) of 0.5 for all
222 inoculum to normalize bacterial biomass.
223

224 The spores were obtained by centrifuging at 16,000 × g at four °C for 20 minutes, followed by three
225 washes and resuspension in Phosphate Buffer Saline (PBS). Subsequently, the spores were stored at
226 -20°C for at least 48 hours to induce the lysis of vegetative cells. Density gradient separation was
227 then conducted using a 50% iso-osmotic Accudenz® solution [45-47]. Confirmation of spore
228 extraction and removal of vegetative cells was achieved through microscopic observation using the
229 Schaeffer-Fulton staining method [48]. Mechanical disaggregation of spores was performed with
230 Tween 80, and the spores were diluted 20 times in Guava® ViaCount™ reagent and quantified by
231 flow cytometry. Spores were identified as particles with a diameter of ≤1 µm and granularity of ≤ 3
232 µm (potentially aggregating up to three spores) [49]. To assess spore viability, 100 µL of spores were
233 plated on Brain Heart Infusion (BHI) agar plates supplemented with 0.1% taurocholate (a potent
234 germinant for spores). These agar plates were anaerobically incubated for 48 hours before counting
235 the CFU to determine viability.

236

237 Antibiotic susceptibility testing

238 The minimum inhibitory concentration (MIC) tests were conducted for a panel of nine antibiotics:
239 ceftriaxone, clindamycin, chloramphenicol, erythromycin, gentamicin, imipenem, metronidazole,
240 penicillin, and tetracycline. These antibiotics were selected based on their usage in humans and
241 animals and previous literature on antibiotic resistance in *C. perfringens* [50]. Upon reaching an
242 optical density at 620nm (OD600) of 0.5, bacterial inoculum was plated on Brucella agar
243 supplemented with hemin (5 µg/mL), vitamin K, and lamb blood. Commercial e-test strips were then
244 placed on the plates, which were incubated for 48 hours under anaerobic conditions. Resistance was
245 determined following the Clinical and Laboratory Standards Institute (CLSI) 2022 guidelines. For
246 antibiotics without established breakpoints for *C. perfringens*. *Staphylococcus aureus* was used as a
247 reference, consistent with previous reports [51].

248

249 **Results**

250

251 **Microgeographic analysis of *C. perfringens* reveals eight globally maintained phylogroups and**
252 **critical dispersal hotspots**

253

254 Genomic Analysis at a microgeographic level uncovers dispersal hotspots of 185 *C. perfringens*
255 isolates from humans and animals in the Colombian Cundiboyacense highlands, revealing an average
256 genome size of 3.4 mb, with 51.8 contigs and a GC Percentage of 27.9% (Supplementary Table 1).

257

SNP-based phylogenetic reconstruction reveals eight clusters corresponding to phylogroups, with a loss of clustering by host. It highlights critical relationships between human hosts and domestic animals at the local level. Phylogroup I was the most representative, including 55 isolates from humans, canines, and felines, while Phylogroup VI contained only two genomes, from a canine and a human. Phylogroup VII comprised 41 *C. perfringens* genomes isolated exclusively from cats (Figure 1).

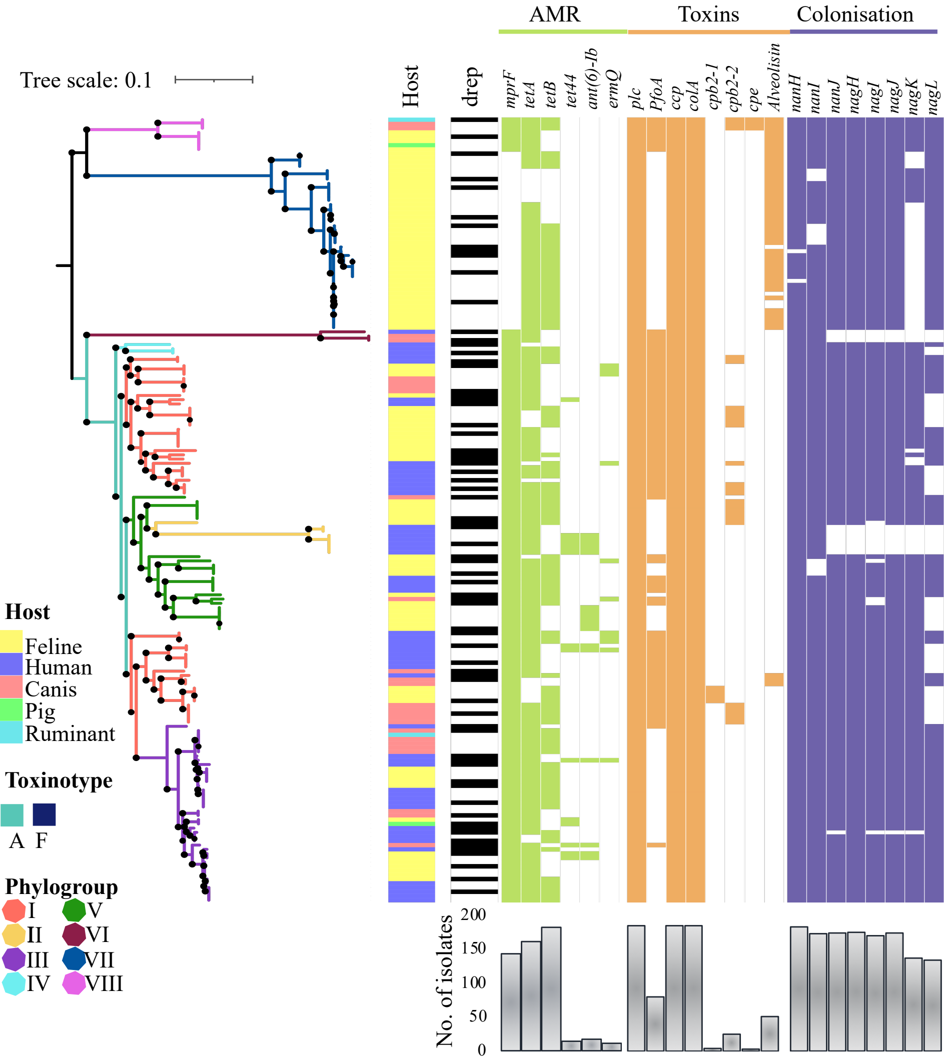
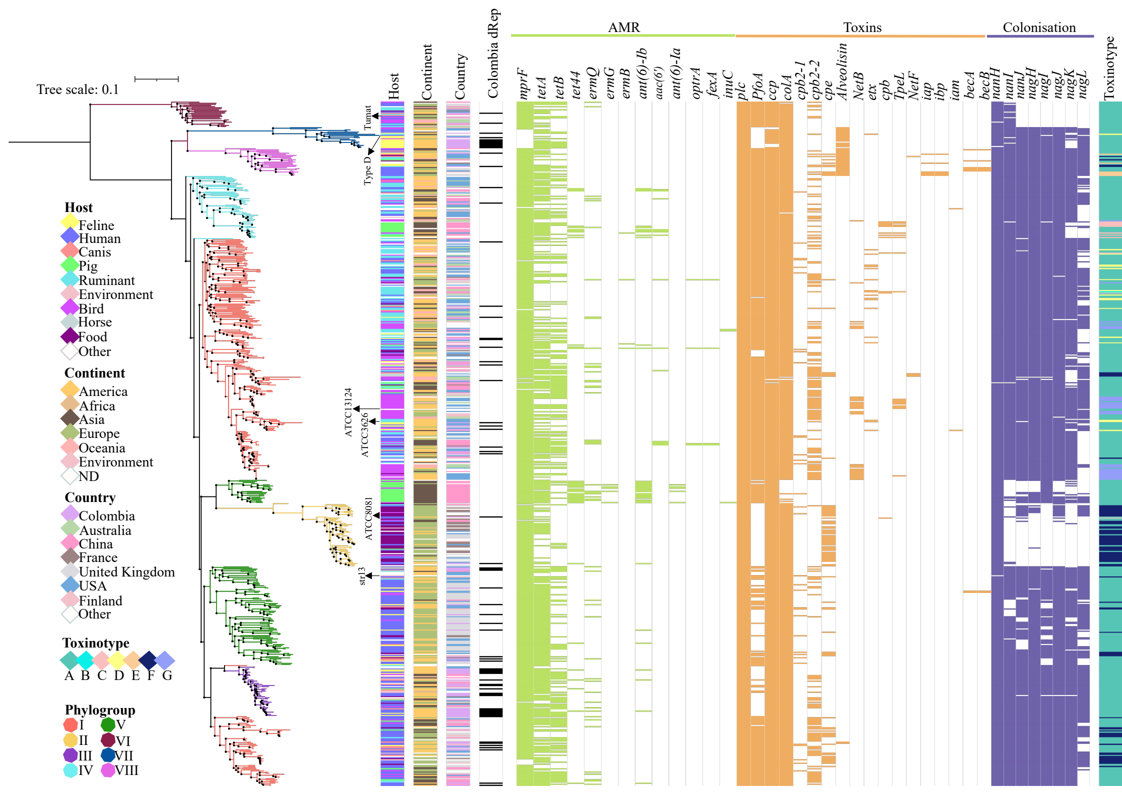


Figure 1. Maximum likelihood tree representing phylogenomics relationships of 185 *C. perfringens* genomes from humans and animals at a microgeographic level. Eight phylogroups were identified by hierBAPS. The first column indicates the host, with unique (dereplicated) genomes highlighted in black in the following column. A presence-absence matrix of antibiotic resistance markers (AMR), toxins,

269 virulence factors, and associated genes, as well as colonization, is presented in the final column. The
270 bottom of the figure shows the number of isolates carrying each inspected molecular marker.
271
272 A dereplicated dataset comprising 546 public genomes and 71 Colombian genomes as representative
273 of *C. perfringens* diversity was used to evaluate the phylogenomic relationships from Colombia
274 compared to Global Data. The pangenome of this global dataset (n=617) comprised 17,590 genes,
275 with 1,875 core genes (10.6%) shared by 99% of the genomes. The resulting phylogeny based on core
276 genome SNPs identified eight distinct phylogroups (Figure 2).
277
278 Insights into phylogroup distribution in *C. perfringens* were inferred from this phylogenomic
279 reconstruction. Phylogroup I was the most frequently detected, with 281 genomes primarily sourced
280 from humans, ruminants, birds, and some felines. Phylogroup II comprised genomes from humans
281 and food sources, whereas phylogroups III, IV, and VI included genomes isolated from multiple
282 species. Phylogroup V featured a significant presence of pig and human genomes, while a
283 predominant association among human, feline, and ruminant genomes characterized phylogroups VI,
284 VII, and VIII. The genomes recovered from Colombian samples were distributed across all
285 phylogroups, with a higher concentration of cat genomes in phylogroup VII. Conversely, genomes of
286 human origin remained dispersed alongside other animal sources in different phylogroups without
287 distinctive clustering by countries or continents. This loss of clustering by biological characteristics
288 supports the high frequency of circulation among hosts and the diversification of this species.
289



290

291 **Figure 2.** Maximum likelihood tree derived from SNP alignment of core genomes exploring
292 phylogenomic relationships of 617 dereplicated genomes. This comprehensive view of Colombian and
293 Global Representatives reveals eight phylogroups identified through hierBAPS. The tree includes a
294 column specifying the host, followed by the continent and country of isolation. The locations of the 71
295 dereplicated Colombian genomes are highlighted in black. Furthermore, the tree presents information on
296 antibiotic resistance markers (AMR), toxins, virulence factors, and genes associated with colonization and
297 toxinotype.

298

299 **Exploring the distribution of virulence factors and other distinctive characteristics across**
300 **phylogroups through phylogenomic reconstruction**

301

302 The impact of *C. perfringens* infections is attributed to its vast repertoire of virulence factors. These
303 include major toxins, which are crucial for classifying the bacteria into toxinotypes associated with
304 disease development, as well as accessory toxins, sialidases, and hyaluronidases, which facilitate
305 colonization and play an essential role in the pathogenesis of this bacterium.

306

307 The genomic identification of main toxin genes at a microgeographic level showed that the
308 phospholipase C (plc) toxin gene, associated with clostridial myonecrosis in immunocompromised

309 patients, was present in 100% (n=185) of the Colombian isolates. In contrast, the enterotoxin (*cpe*)
310 gene, linked to gastrointestinal disease in humans, was found in only 1.63% of the isolates and showed
311 no association with symptoms in carrier individuals. No other main toxins were identified, indicating
312 that 98.3% of the isolates were classified within toxinotype A, while the remaining 1.63%
313 corresponded to toxinotype F.
314
315 All analyzed isolates contained genes encoding for Alpha Clostripain (*ccp*) and Collagenase A
316 (ColA), two accessory toxins known for their role in tissue inflammation induction in clostridial
317 myonecrosis cases. Screening the distribution of other accessory toxins by each phylogroup revealed
318 distinctive patterns. In phylogroup I, the presence of the *pfoA* gene was identified, linked to the
319 pathogenesis of necrotizing enterocolitis and intravascular hemolysis in humans, along with the
320 presence of sialidases (*nanH*, *nanI*, *nanJ*) and hyaluronidases (*nagH*, *nagI*, *nagJ* and *nagK*) associated
321 with increased cytotoxicity and toxin spread, respectively. Furthermore, 29.0% (n=16/55) of the
322 isolates located in phylogroup I harbored the *C. perfringens* beta 2 gene (cpb2), a pore-forming toxin
323 implicated in the pathogenesis of necrotic enteritis. Phylogroup II was composed of hypovirulent
324 isolates lacking several toxins and virulence factors, only with the presence of the sialidase genes
325 *nanH* and *nanI* involved in bacterial colonization [52]. Phylogroups III and V were characterized by
326 carrying several sialidase and hyaluronidase genes. All genomes included in phylogroup VII obtained
327 exclusively from cats lacked *pfoA*, like phylogroups II and III, and showed alveolsin (alv), a
328 chromosomal toxin with hemolytic activity considered crucial in the damage of the eukaryotic cell
329 membrane [53, 54].
330 Phylogenomic analyses at a global scale revealed that Colombian isolates were distributed across all
331 phylogroups, exhibiting similar virulence characteristics to public genomes. For instance, in
332 Phylogroups I, IV, VI, VII, and VIII, the Colombian isolates were identified alongside others from
333 different countries carrying the *pfoA* gene. Conversely, Phylogroup VII contained isolates from
334 Colombian cats, humans, and other sources worldwide carrying *alv* but lacking *pfoA*. The single
335 isolate carrying *cpe* clustered with *C. perfringens* isolates from human and food sources.
336
337 ***C. perfringens* isolates carry AMR associated with tetracycline, macrolides, and**
338 **aminoglycosides**
339
340 Genomic surveillance of AMR from genomes obtained at a microgeographic level allowed the
341 identification that 93.5% of genomes (n=173/185) carry tetracycline resistance (*tet*) genes, being
342 distributed throughout all phylogroups. Genes that confer resistance to multiple peptides and human

defensins (*mprF*) were identified in 77.2% (n=143/185) of the genomes, except those from cats located in phylogroup VII. Furthermore, 9.1% of the isolates (n=17), located mostly in phylogroups II and V, exhibited the aminoglycoside O-nucleotidyltransferases gene (ant(6)-Ib), indicative of resistance to aminoglycosides. In a smaller proportion, 5.9% of the genomes (n=11) carried the erythromycin ribosome methylase Q (*ermQ*) gene, associated with resistance to macrolides, lincosamides, and streptogramins B, grouped mostly in phylogroup I (Figure 1).

Globally, the *mprF* gene was present in 92.0% of the genomes analyzed (n=586/617). The *tet44*, *tetA*, and *tetB* genes were found in 70.6% (n=436), followed by the *ermQ* gene, which was identified in 9.2% (n=57) of the genomes (Figure 2). Regarding geographical and species distribution, isolates from pigs in China, mainly located in phylogroup V, showed the highest amount of AMR, with between four and five AMR per genome. In contrast, most genomes from phylogroups I and II, which include isolates from birds, ruminants, humans, and foods, had a lower AMR load, with between one and two per genome.

PFOA toxin is associated with complete hemolysis and inhibition of cell growth

A recent pediatric study suggests that *C. perfringens* isolates producers of PFOA toxin may induce hemolysis and heightened cellular toxicity [21]. Considering this, we opted to assess the hemolytic capacity of blood agar and the impact of culture supernatants on Vero cells using isolates from asymptomatic individuals, including both carriers and non-carriers of PFOA.
The hemolysis profiles of the 30 representative isolates for the phylogroups identified in this study were examined, classifying them based on the presence or absence of the *pfoA* gene. These analyses revealed that all isolates carrying *pfoA+* demonstrated complete hemolysis on blood agar (n=16), while isolates lacking *pfoA-* exhibited partial hemolysis. When assessing the biological impact of toxin supernatants from each isolate (1:5 dilution) on Vero cells, diverse behaviors were observed among them. Those situated above the IC50 line showed a growth inhibitory effect at 48 hours (Figure 3A). Regarding the presence of the *pfoA* gene, a significant increase ($p<0.05$) in the percentage of growth inhibition was observed in isolates carrying *pfoA+* (Figure 3B).

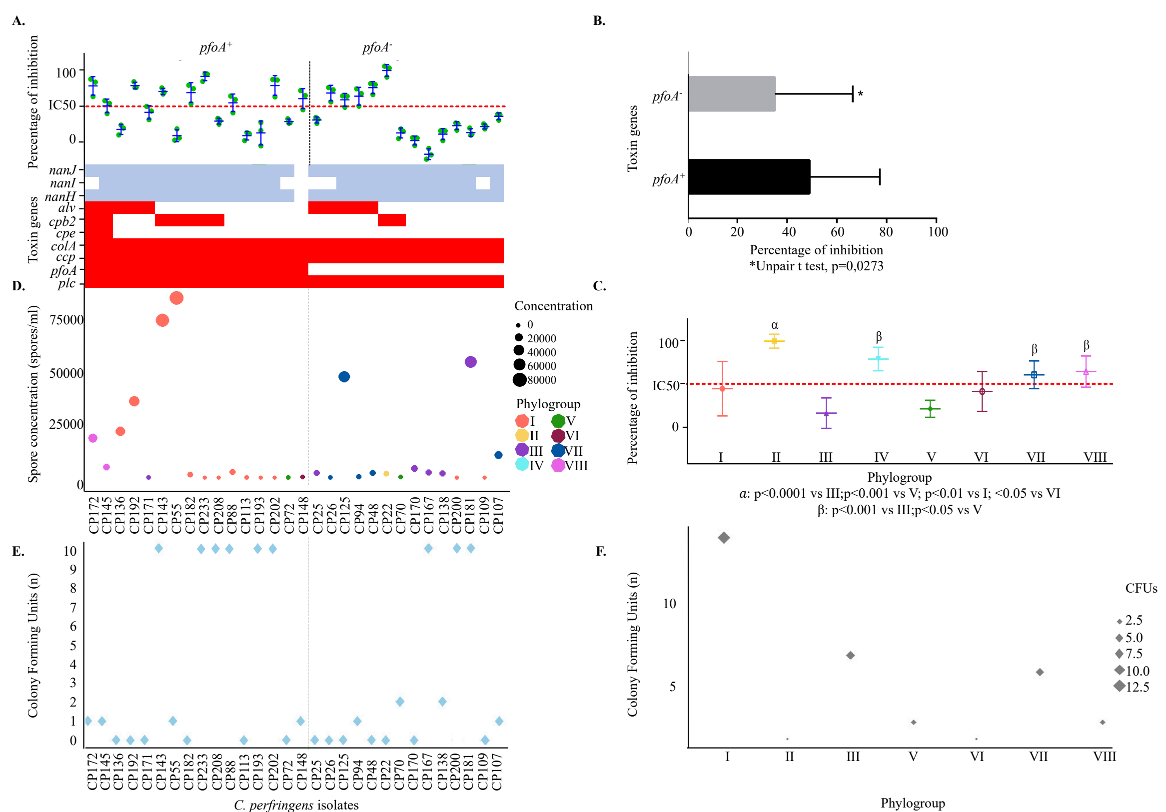
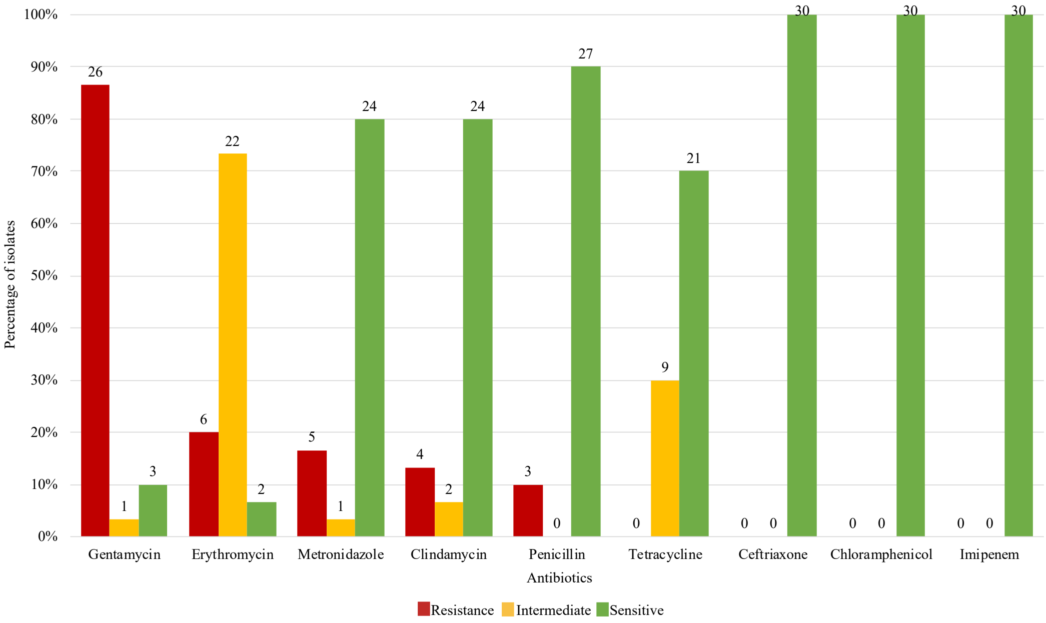


Figure 3. Phenotypic characteristics of cytotoxicity and sporulation were assessed in 30 *C. perfringens* isolates obtained from various sources in a central region of Colombia. (A) activity of culture supernatants (1:5 dilution) for 30 representative isolates of *C. perfringens* lineages to assess their potential toxigenic effect on Vero cells. The comparison was made between *pfoA*+ (n=16) and *pfoA*− (n=14) isolates. *pfoA*+ isolates encode the PFOA toxin, while *pfoA*− isolates do not. **(B)** Cellular toxicity of *C. perfringens* isolates was evaluated using the Sulphorodamine B assay, comparing between the *pfoA*+ and *pfoA*− groups. **(C)** Percentage of growth inhibition caused by toxin supernatants of isolates from different phylogroups on cell growth. **(D)** Sporulation efficiency of *C. perfringens* isolates. **(E)** The count of colony-forming units (CFU) was detected after spore purification and subsequent growth induction on a medium supplemented with taurocholate.

The behavior of isolates from different phylogroups on cell growth was analyzed through an ANOVA analysis with the Holm-Sidak multiple comparisons test. The isolates in phylogroups II, IV, VII, and VIII are responsible for the biological effect observed in the tests. It should be noted that phylogroups II and IV exhibit a more pronounced biological effect than the other phylogroups (Figure 3C).

The ability to sporulate and resist antibiotics confers propagation potential to *C. perfringens*

390 Phenotypic evaluation of sporulation efficiency revealed that isolates from phylogroups I, carrying
391 *pfoA*$^{+}$, showed higher levels of sporulation compared to other phylogroups (**Figure 3D**). Specifically,
392 isolates CP55 and CP143, obtained from a cat and a dog, respectively, that belong to phylogroup I
393 and carry both *pfoA*$^{+}$ and *cpb2*, demonstrated the highest sporulation efficiency.
394
395 Spore viability was measured as the isolates' ability to generate new CFU after purification and i*n*
396 *vitro* cultivation (Figure 3E). It was observed that 56.6% (n=17/30) of the isolates formed CFU after
397 48 hours of incubation. Isolates from phylogroup I, which exhibited a wide distribution among diverse
398 sources such as humans, dogs, and cats, showed higher spore viability (**Figure 3E**), a characteristic
399 that can contribute to their survival in many environmental niches facilitating the dispersal of *C.*
400 *perfringens* among different hosts.
401
402 Finally, the antibiotic susceptibility profiles of 30 *C. perfringens* isolates were evaluated against a
403 panel of nine antibiotics from multiple classes, including aminoglycosides, macrolides, lincosamides,
404 penicillins, third-generation cephalosporins, carbapenems, tetracyclines, amphenicols, and
405 nitroimidazoles (Figure 4). The detection of antibiotic susceptibility followed the criteria established
406 by CLSI in 2022 for *C. perfringens*.
407



408
409 **Figura 4.** Antibiotic susceptibility pattern of *C. perfringens* isolates. Percentage of antibiotic
410 susceptibility (resistant, intermediate or sensitive) of 30 isolates of *C. perfringens* collected from a
411 central region of Colombia.

412 All isolates demonstrated sensitivity to Imipenem, Chloramphenicol and Ceftriaxone, and 90.0% and
413 80.0% showed sensitivity to Penicillin and Clindamycin respectly.
414
415 On the other hand, 87.0% of the isolates resisted Gentamicin, while 20.0% resisted Erythromycin and
416 17.0% to Metronidazole. Additionally, 10.0% resisted Penicillin, as detailed in **Figure 4**. Although
417 70.0% of the isolates showed sensitivity to tetracycline, 30% showed intermediate susceptibility.
418
419 **Discussion**
420
421 Genomic studies of *C. perfringens* have primarily focused on isolates from limited geographical
422 regions in developed countries, creating an information gap in developing nations [55]. To address
423 this gap, our study obtained and characterized 185 genomes of *C. perfringens* from humans and
424 animals in the Altiplano Cundiboyacense, a central region of Colombia with a significant agricultural
425 impact.
426
427 Studying the population genetic structure of potentially pathogenic bacteria, coupled with
428 understanding their ecology, epidemiology, and geographical distribution, is a crucial tool for
429 unraveling their dispersal pathways. This study revealed a significant genetic diversity and two types
430 of profiles: i) groupings by animal species, such as in the case of phylogroup VII, and ii) signals of
431 potential zoonotic dispersal events between canines, felines, and humans, as evidenced in
432 phylogroups I and III (Figure 1). The dispersal of *C. perfringens* among animals or from animals to
433 humans can occur through three main pathways. Firstly, direct transmission, where most bacteria
434 multiply in the intestine and are excreted in feces, while spores present can contaminate surfaces in
435 contact with other individuals. Secondly, the presence of *C. perfringens* spores in tissues of
436 decomposing dead animals represents focal points of environmental contamination and increases
437 exposure for humans. Thirdly, transmission through animal meat products for human consumption
438 [56]. These findings highlight the evolutionary capacity that clostridial have developed to obtain
439 nutrients from multiple hosts, allowing them to adapt to a heterogeneity of niches. The high adaptive
440 capacity of *C. perfringens* is a concern, particularly in low- and middle-income developing countries
441 like Colombia, where factors such as increased exposure to domestic animals within the household,
442 lack of access to clean water and basic sanitation, as well as limited food refrigeration due to lack of
443 access to electricity, contribute to increased vulnerability to the spread of emerging high-impact
444 zoonoses.
445

446 In comparative bacterial genomics, analyzing organism spread globally stands out for identifying
447 potential health risks and establishing a baseline for evaluating intervention effectiveness [57].
448 Utilizing phylogenomic analysis at a microgeographic level allowed for identifying possible
449 dissemination routes between canines and felines, apparently asymptomatic domestic animals in a
450 region with significant agricultural activity, revealing the potential risk of transmission through direct
451 contact of humans with animals carrying *C. perfringens*, underscoring a possible threat to human
452 health. Comparing *C. perfringens* genomes studied locally with globally available information
453 revealed that phylogroup I encompassed the highest number of cosmopolitan isolates originating from
454 diverse sources. Meanwhile, phylogroup II featured isolates from human and food sources, and
455 phylogroups IV and V from pigs and humans (Figure 2), supporting the role of *C. perfringens* in
456 foodborne diseases (FBD) [13] and its spread between human and animal sources [12]. These findings
457 emphasize the importance of safe food handling practices and the need to promote hygiene measures
458 such as handwashing and surface disinfection to prevent transmission. Remarkable geographic
459 differences could be due to specific genomic traits, such as the clustering of pig isolates from Asia,
460 cats from South America, and food sources in Europe. However, limitations in sampling *C.*
461 *perfringens* isolates in other geographic regions restrict our global understanding. This highlights the
462 urgency of further sampling efforts to trace the origins and spread of infectious diseases, especially
463 in developing countries in South America, Oceania, and Africa.
464
465 *C. perfringens* can release toxins, such as CPA, CPE, CPB, ETX, ITX, and NETB, in the intestinal
466 tract of humans and animals. These main toxins classify *C. perfringens* into seven toxinotypes linked
467 to intestinal and tissue diseases, especially in symptomatic hosts [1, 10, 18]. Although the impact of
468 accessory toxins and toxigenic isolates in asymptomatic populations is not fully evidenced, recent
469 studies suggest that the PFOA toxin associated with gas gangrene and necrotizing enterocolitis in
470 newborns [5, 58, 59], this could increase the risk of intestinal damage in asymptomatic carriers [5].
471 This study revealed the presence of isolates carrying the *pfoA*+ gene in asymptomatic humans and
472 animals, which demonstrated complete hemolysis induction and exhibited a significant increase
473 ($p<0.05$) in the percentage of in-vitro cell growth inhibition compared to *pfoA*- isolates (ver Figura
474 3A y 3B). These findings are supported by previously reported results of toxigenic *C. perfringens*
475 isolates producing PFOA in healthy babies and neonates with NEC in England, which directly
476 correlated with cellular toxicity, complete hemolysis, proinflammatory responses, and increased
477 oxygen tolerance. This highlights the importance of PFOA in intestinal pathology, infection
478 establishment, and disease development [21]. On the other hand, alveolysin, a poorly studied
479 chromosomal toxin activated by thiol, which binds to cholesterol and shares a high identity (~86%

480 nucleotide identity) with PFOA, was predominantly present in isolates from Colombian cats located
481 in phylogroup VII (Figure 1). Its unique group specificity in asymptomatic domestic cats at a
482 microgeographic level, the high percentage of cell growth inhibition found in this study (Figure 3A),
483 and its presence in isolates from dogs with hemorrhagic diarrhea and foals with necrotizing enteritis
484 reported in North America and Switzerland [53, 60], highlight the importance of expanding
485 knowledge about its potential virulence in domestic animals.
486
487 The risk of *C. perfringens* transmission among individuals is due to its unique ability to survive in
488 adverse environments, forming spores resistant to high temperatures and hostile conditions, which
489 becomes a crucial factor during the dissemination of this bacterium [49, 61]. The increased
490 sporulation efficiency in *pfoA+* carrying isolates (Figure 3D) implies a higher risk of transmission of
491 toxigenic isolates capable of generating intestinal pathologies in previously healthy populations. On
492 the other hand, the differences in cellular compositions of the spores related to surface proteins,
493 mineralization, and central water, as well as nutritional requirements and the time required for
494 germination, may explain the variation in germination rates at 48 hours (Figure 3E and 3F) [62].
495
496 Phylogenomic studies are also crucial for monitoring AMR's mobilization, persistence, and
497 abundance in microbial populations [63]. Genomic studies in *C. perfringens* have revealed a
498 conserved presence of the gene encoding the multiple peptide resistance factor (MprF) [64, 65], which
499 confers resistance to antimicrobial peptides [66]. Despite being described as conserved, this study
500 identified a notable absence of MprF in isolates from cats in phylogroup VII (Figure 2). This absence
501 could be attributed to differences in selective pressures among hosts and may also indicate a
502 competitive disadvantage against other bacteria or the host's immune response in these isolates [67],
503 warranting further investigation.
504
505 Penicillin and clindamycin remain relevant for treatments of clostridial infections, with penicillin
506 currently being the drug of choice. Our study highlights that rates of resistance to penicillin and
507 clindamycin are notably low, similar to Hungary, Slovenia, and northern Taiwan [68]. However, the
508 presence of circulating isolates with reduced susceptibility to these antibiotics may lead to therapeutic
509 failures in treating serious pathologies caused by *C. perfringens*. Nevertheless, the reduction in
510 susceptibility to metronidazole in Colombia (17.0%) (Figure 4) contrasts markedly with other
511 countries such as Pakistan [69], Australia [50], and Canada [70], where susceptibility to
512 metronidazole is lower, raising questions about differences in prescription practices or specific local
513 factors such as diversity in the intestinal microbiota of hosts, genetic characteristics, and

514 sociodemographic factors. Since metronidazole is crucial for treating anaerobic and protozoal
515 infections, its resistance threatens the effectiveness of conventional treatment, underscoring the need
516 for more epidemiological studies to adapt therapeutic strategies to local realities [50, 71].
517
518 Our study has some limitations, including the loss of precise correlation between genotype and
519 phenotype for AMR, which may be due to variability in gene expression under culture conditions,
520 limitations of the databases used for identification, and even the existence of alternative mechanisms
521 of bacterial persistence [72, 73]. However, it is noteworthy that such studies transcend local
522 epidemiology, contributing to understanding the evolution and spread of *C. perfringens* and
523 identifying relevant genomic markers. Vero cells were used to evaluate the cytotoxic effect of *C.*
524 *perfringens*, which may not directly reflect the intestinal scenario. Hence, cells are proposed to model
525 the intestinal epithelial barrier or an enteroid model to compare our results [74]. Future research
526 should include a broader and more diverse variety of samples from different ecological niches, such
527 as humans, animals, soil, water, and food. These studies will improve the accuracy in detecting and
528 understanding this microorganism and discover more efficient and strategic methods to mitigate its
529 growing impact on health.
530
531 **Conclusions**
532 This study underscores the relevance of WGS for understanding the evolution and spread of *C.*
533 *perfringens*, identifying relevant genomic markers, and overcoming limitations in local
534 epidemiology. By integrating genomic and experimental analyses, results allow for more precise
535 implementation of treatments and prevention measures for infectious diseases. The findings reported
536 here indicate, for the first time, the genetic diversity and presence of virulence and antimicrobial
537 resistance factors in *C. perfringens* in Colombia, highlighting notable dissemination between humans
538 and domestic animals and differences in toxin distribution such as PFOA and alveolysin. The
539 correlation of phenotypic traits of the PFOA toxin and its presence in asymptomatic individuals
540 suggests a potential risk to intestinal health. The observation of reduced susceptibility to antibiotics
541 such as gentamicin, erythromycin, and metronidazole underscores the importance of genomic and
542 phenotypic analyses that allow tracking of clonal expansion and transmission of existing and
543 emerging pathogens, as well as AMR that capture and propagate antibiotic resistance. The
544 comprehensive approach of genomic and phenotypic data analysis within the One Health framework
545 is essential for understanding the ecology and epidemiology of pathogens of public health concern,
546 enabling the identification of markers associated with virulence and antimicrobial resistance and
547 contributing to understanding interactions with the environment and hosts. This multidisciplinary and

548 collaborative approach acknowledges the interconnectedness between human, animal, and
549 environmental health, providing valuable information to develop more precise and sustainable
550 strategies to mitigate these challenges at the community level.
551
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553 The authors declare that they have no potential conflicts of interest
554
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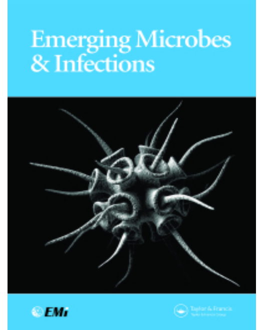

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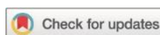

Unveiling the pathogenic mechanisms of *Clostridium perfringens* toxins and virulence factors

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Abstract

Clostridium perfringens causes multiple diseases in humans and animals. Its pathogenic effect is supported by a broad and heterogeneous arsenal of toxins and other virulence factors associated with a specific host tropism. Molecular approaches have indicated that most *C. perfringens* toxins produce membrane pores, leading to osmotic cell disruption and

apoptosis. However, identifying mechanisms involved in cell tropism and selective toxicity effects should be studied more. The differential presence and polymorphisms of toxin-encoding genes and genes encoding other virulence factors suggest that molecular mechanisms might exist associated with host preference, receptor binding, and impact on the host; however, this information has not been reviewed in detail. Therefore, this review aims to clarify the current state of knowledge on the structural features and mechanisms of action of the major toxins and virulence factors of *C. perfringens and* discuss the impact of genetic diversity of toxinotypes in tropism for several hosts.

Keywords: *Clostridium perfringens*, toxin type, genetic diversity, host, enteritis, toxinotypes.

Introduction

Clostridium perfringens is a Gram-positive anaerobic bacterium that can form spores that are crucial during transmission. *C. perfringens* spores are exceptionally resistant to stressful environments, such as high temperatures, the presence of oxygen, or low nutrient levels [1]. These features facilitate its survival in different environmental niches, including soil, faeces, sewage, food, and the intestinal tract of humans and animals [2]. This 'survivability' means that *C. perfringens* has been associated with many infections and diseases, including being the second leading cause of foodborne bacterial disease in the United States (causing one million illnesses yearly) and Europe's fourth leading cause [3, 4, 5]. In England, 8-13% of foodborne gastrointestinal outbreaks are estimated to be associated with this bacterium (90,000 cases of *C. perfringens* per year) [6].

The impact of *C. perfringens* infections is due in part to the bacterium's potential to secrete multiple extracellular toxins, including alpha-toxin (CPA, *cpa/plc* gene), beta-toxin (CPB, *cpb* gene), epsilon-toxin (ETX, *etx* gene), iota-toxin (ITX, *iap,* and *ibp* binary genes), *C. perfringens* enterotoxin (CPE, *cpe* gene) and Necrotic enteritis B-like toxin (*NetB*). These toxins correspond to essential toxin genes used in the current toxinotyping scheme (toxinotypes A-G) (**Figure supplementary 1**) [7].

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Each toxinotype is associated with specific diseases. For example, toxinotype A, characterised by the presence of the *cpa*$^{+}$ toxin genes, is linked to gas gangrene in humans and animals. Toxinotype B carries the *cpa*$^{+}$, *cpb*$^{+,}$ and *etx*$^{+}$ genes, and it is associated with haemorrhagic enteritis in calves, foals, and sheep, as well as dysentery in lambs. Toxinotype C, with the toxin genes, *cpa*$^{+}$, *cpb*$^{+}$, and *cpe*$^{+/-}$, is linked to enterotoxaemia in sheep and necrotising enteritis in humans (pigbel), pigs, calves, goats, and foals. Toxinotype D, featuring *cpa*$^{+}$, *etx*$^{+,}$ and *cpe*$^{+/-}$, is associated with enterotoxaemia in lambs (pulpy kidney disease), goats, and cattle. Toxinotype E, containing *cpa*$^{+}$, *itx*$^{+,}$ and *cpe*$^{+/-}$ genes, is linked to enterotoxaemia in calves and lambs. Toxinotype F, with *cpa*$^{+}$ and *cpe*$^{+}$, is associated with food poisoning and antibiotic-associated diarrhoea (AAD). Finally, toxinotype G, presenting *cpa*$^{+}$ and *netB*$^{+}$, is associated with avian necrotic enteritis [7].

Additionally, *C. perfringens* produces other clinically relevant accessory toxins, such as Perfringolysin O or theta toxin (PFO) and *C. perfringens* beta 2 toxin (CPB2). Although not used for toxinotyping, these toxins can act synergistically with extracellular toxins, impacting other toxins' expression, production levels, and virulence factors, thereby influencing overall disease progression [8].

The diversity of clostridial toxins and other virulence factors remains a critical study point. Therefore, in this review, we present a brief updated description of the structural and molecular characteristics, mechanisms of action, and genetic diversity of the main toxins and virulence factors associated with the tropism of *C. perfringens* toxinotypes across a diversity of hosts, including links to specific disease phenotypes.

C. perfringens toxins: Biological properties, function, host tropism and diversity

Localisation of toxins of *C. perfringens* and plasmids families

The virulence of *C. perfringens* largely depends on its ability to produce toxins, which can be encoded on plasmids, chromosomally, or both. So far, seven groups of plasmids have been described for *C. perfringens*: transfer of clostridial plasmids (Tcp), pCP13 *C. perfringens* (Pcp), pIP404, phage-like, small plasmids or an unclassified group [9], and

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recently the botulinum conjugation in *C. perfringens* (Bcp) group of plasmids encoding a new putative conjugation locus.

The first significant group of plasmids carries the Tcp conjugation locus and genes encoding clinically relevant toxins such as *cpb2* and *cpe*, iota binary toxin genes *iap* and *ibp*, as well as antibiotic resistance genes, including chloramphenicol, clindamycin, erythromycin, bacitracin, and lincomycin [10, 11]. Although a *C. perfringens* strain can carry up to five similar plasmids, it was recently demonstrated that the stability and inheritance of these plasmids are favoured by the ResP recombinase that catalyses the multimer resolution system and by differences in the type II partitioning systems (ParMRC) [13].

A second major group of *C. perfringens* conjugative plasmids is the pCP13-like plasmid family, which shares a highly conserved sequence at the Pcp locus [14, 15]. These plasmids carry a *cpb2* consensus variant associated with disease in horses and piglets [16]. They also have the novel C. perfringens binary enterotoxin (BEC/CPILE) [14], detected in the faeces of gastroenteritis patients, food [17], and healthy UK children [18].

The PiP404 plasmid family consists of small non-conjugative plasmids that encode a bacteriocin; however, they have not been identified to carry any toxin genes [19]. The newly identified Bcp plasmids contain a novel putative conjugation locus (Bcp) with a sequence like *Clostridium botulinum* plasmids and contigs that have the same plasmid mobilisation gene, mobC and the same zeta toxin-encoding gene as pJGS1984_5. Additionally, these plasmids encode VirB4 homologous proteins and VirD4-like conjugation proteins distinct from the variants encoded by the Tcp and Pcp plasmids [20].

The presence of toxin-carrying plasmids and antibiotic-resistance genes in *C. perfringens* has been associated with its survival ability in multiple environments. Moreover, the high rate of plasmid transfer in this bacterium (2.9×10^{-1} to 3.8×10^{-2}) also allows it to overcome fitness costs and segregation loss. This influences the transformation of toxinotypes in a given environment, especially within the gastrointestinal tract, implying impacts on disease outcomes [21].

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However, in some cases, the metabolic load and additional energy resources required by plasmid replication, disruption of essential host genes by plasmid gene integration, and plasmid-encoded molecules may negatively impact native host proteins, leading to decreased fitness in bacterial cells [21, 22].

In addition to plasmid-localized toxins, other toxins genes such as *cpa* (also *plc*) and *pfo* are located only on chromosomes [9]. Furthermore, *cpe* can be located on the chromosome, as shown in food poisoning isolates or on a large plasmid, as observed in non-foodborne gastrointestinal disease and veterinary isolates.

The location of toxin-associated genes is expected to influence disease outcomes, as plasmid-encoded toxins can be transferred by conjugation to strains of *C. perfringens* toxinotype A that reside as part of the 'normal' intestinal microbiota, giving rise to new strains with enhanced colonisation and virulence traits. Conversely, chromosomal variants may be lost over evolutionary time due to mutation or deletion events and may cause shorter disease durations due to low colonisation levels and spread [21].

Toxin structure

C. perfringens toxins are diverse and widely distributed. Structurally, clostridial toxins may have similar binding domains but different catalytic domains, suggesting possible recombination in toxin evolution. This recombination could occur through horizontal plasmid transfer or insertion-deletion processes, serving as adaptation, survival, and tropism mechanisms. This recombination could occur through horizontal plasmid transfer or insertion-deletion processes, serving as adaptation, survival, and tropism mechanisms [23]. As outlined below, some toxins are composed of different domains. In addition to allowing flexibility and dynamics in the toxin, these domains promote and facilitate the recognition of specific substrates in the target cell.

Major toxins of *C. perfringens*

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CPA is composed of 370 amino acids (aa) and is divided into two domains: the catalytic N-domain (CP1-249) and the membrane-binding C-terminal domain (CP247-370) [24] (**Figure 1a**). It involves cell membrane colonisation, haemolytic activity, and damaging action [24, 25, 26, 27].

CPB toxin, a 336 aa protein (**Figure 1b**) in the alpha-haemolysing family, is suggested to bind to its receptor or form the oligomer via its C-terminus (although the exact structure-function relationship is still under study) [28].

ETX toxin is composed of three domains: i) the amino-terminal domain, involved in the receptor binding process; ii) the central region domain, responsible for membrane insertion plus channel formation; and iii) the carboxy-terminal domain, involved in the activation of proteolysis [29] (**Figure 1c**).

ITX is a binary toxin composed of two components: an enzymatic Ia (454 amino acids) and a binding Ib (875 amino acids), separated by 243 non-coding nucleotides. Both components, Ia and Ib, exhibit cytotoxic properties [30] (**Figure 1d**).

CPE is a single-chain polypeptide consisting of 319 amino acids and three domains: I, II, and III. Domain I binds to the claudin-specific receptor, while domains II and III constitute the N-terminal region associated with pore-forming activity [31] (**Figure 1e**).

NetB, a putative toxin gene encoding a 323 aa protein (**Figure 1f**), including a 30 aa secretion signal sequence, which has similarity to CPB toxin (38% identity), leading to its naming as necrotic enteritis β-like toxin, NetB [32].

Another clinically relevant accessory toxins

PFO has 500 aa residues and a 27-residue signal peptide. It is composed of four domains, with domain four (D4) mediating the toxin's binding to the eukaryotic cell's plasma membrane [33].

The structure and receptor of CPB2 remain unclear. It is suggested that only a small fraction of amino acid segments adopt an α-helix conformation in both beta toxins (atypical and consensus), which is insufficient to cross a membrane. However, in the case of consensus

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CPB2, some amino acid segments have been identified that could form transmembrane β-chains [34].

Mechanisms of action of *C. perfringens* toxin

C. perfringens is a pathogen of significant clinical and veterinary importance, attributed to its capacity to induce disease in various hosts by producing different toxins (**Table 1. Section A, B**).

Sphingomyelin and phosphatidylcholine degradation

CPA, a toxin in all toxinotypes, is the primary determinant of virulence in clostridial myonecrosis caused by toxinotype A. The process is initiated by the toxin binding to the ganglioside receptor GM1a [35] (**Figure 2a**). This binding results in the degradation of sphingomyelin and phosphatidylcholine in the cell's plasma membrane, triggering proinflammatory responses during the early phases of gas gangrene [36]. This inflammatory process may advance to aggravated tissue infection, accompanied by secondary gas crepitation in the tissue and the development of necrotic in humans [37] (**Figure 3**).

Formation of pores

Pore-forming toxins (CPB, ETX, ITX, CPE, and NetB) allow Ca2+, Na+, Cl- entry and K+ loss with subsequent cellular electrochemical gradient depolarisation, ionic homeostasis alteration, and cell death [38].

CPB is the primary virulence determinant in necrotising human enteritis and enterotoxaemia caused by toxinotype C in sheep. It is also involved in haemorrhagic dysentery in sheep caused by toxinotype B (**Figure 3**). CPB binds with platelet endothelial cell adhesion molecule 1 (PECAM-1, also known as CD31) to trigger the formation of pores in the endothelial cell membrane. This process causes damage to the endothelial cells that make up the vasculature of the intestinal mucosa (**Figure 2b**), resulting in haemorrhagic and necrotising enteritis [39, 40].

The ETX toxin, found in toxinotypes B and D, is linked to enterotoxaemia in ruminants (**Figure 3**). It binds to receptors dependent on cholesterol and sphingomyelin present on the

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outer membranes of endothelial cells lining the intestine and vascular cells in organs such as the brain, kidneys, and liver in ruminants. While the exact identity of the receptor is unknown, it is confirmed that ETX forms active pores, causing direct damage to intestinal endothelial cells. This process increases vascular and intestinal wall permeability, leading to intestinal epithelial cell detachment and necrosis, triggering enterotoxaemia, especially in goats (**Figure 2c**) [41, 42].

For ITX, present only in toxinotype E and associated with haemorrhagic enteritis in cattle (**Figure 3**), it is described that the lipolysis-stimulated lipoprotein receptor (LSR) mediates the entry of toxin Ib into the host cell [43], allowing the formation of functional channels for ion movement and entry of Ia by endocytosis [44]. This interaction leads to degenerative changes in the mucosal epithelium of the small intestine, increased permeability of intestinal cell monolayers, and haemorrhagic lesions in the serosa and mucosa characteristic of enteritis **(Figure 2d)** [45].

The CPE toxin, found in toxinotype F, is recognised as the primary toxin causing food poisoning and gastrointestinal illnesses in humans, including AAD (**Figure 3**). Its mechanism of action involves binding to claudin receptors, crucial components of the tight junctions between epithelial or endothelial cells at the cell surface. This binding facilitates the formation of pores in the plasma membrane [46], removing claudins from the cell membrane and disrupting tight junctions between cells. Consequently, this disruption increases barrier permeability in the small intestine and colon, impairing absorption and diarrhoea **(Figure 2e)** [47, 48].

NetB, found in toxinotype G of *C. perfringens* and associated with avian necrotic enteritis (**Figure 3**), recognises cholesterol-free regions in the membrane of intestinal epithelial cells. It forms heptameric hydrophilic pores that allow the entry of ions, resulting in increased permeability. This process leads to focal, multifocal, or coalescent necrosis of enterocytes and, in severe cases, coagulative necrosis of the entire superficial mucosa separating the intestinal lamina of birds **(Figure 2f)** [49].

PFOA is encoded in most disease-associated *C. perfringens* strains, except for strains carrying *cpe* on the chromosome [50]. PFOA interacts with cell membrane cholesterol to

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form pores [33]. It acts synergistically with CPA toxin to affect leukostasis [51] and promotes the expression of adhesion molecules and platelet-activating factors. These toxin-associated features contribute to developing myonecrosis (gas gangrene), haemorrhagic enteritis in calves, and septicaemia (intravascular haemolysis) in humans, including neonates [52, 53, 54]. Most recently, it was described that *pfoA*+ strains were associated with preterm infants, including those with necrotising enterocolitis, and were shown *in vitro* to cause significantly more intestinal cell damage than *pfoA*- strains [11].

CPB2 is an accessory toxin produced by *C. perfrignens* associated with porcine, equine, and bovine enteritis. While the structure of CPB2 and the identification of residues responsible for its antigenicity and association with the membrane of intestinal epithelial cells are under study [55], CPB2 has been reported as a pore-forming toxin. Its action mechanism involves forming cation-selective channels approximately 1.4 nm in diameter in lipid bilayers, leading to altered ion flux and increased intestinal permeability [56].

Genetic diversity and host specificity of *C. perfringens* toxins

Understanding the molecular patterns influencing interspecies transmission and host adaptation (**Figure 3 and Table 1**) is crucial for disease prevention and control.

The amino acid sequence diversity of the CPA toxin from 15 strains of *C. perfringens* suggests variation within the signal sequences (six positions), the N-domain (10 positions), C-domain (three positions), and the N- to C-domain linker peptide (one position), with most of these changes conserved potentially impacting the toxin's resistance to degradation and biological activity [57]. The limited variability of the CPA toxin enhances its affinity for endothelial cells in the host, destroying the endothelial structure; this disruption in peripheral circulation results in myonecrosis.

The study of CPB diversity in isolates of toxinotypes B and C has identified that some isolates of type C have four conserved amino acid changes, resulting in two natural variants of the toxin. The sequence modifications in CPB found in toxinotype C isolates lead to increased toxin sensitivity to trypsin, a higher affinity phenotype for host endothelial cells, and increased cytotoxicity. As a result, the molecular diversity of CPB in toxinotype C may

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contribute to enterotoxaemia and necrotising enteritis in hosts deficient in trypsin, such as neonatal animals, individuals with diets rich in trypsin inhibitors, and those with pancreatic dysfunction [59].

The genetic diversity of the ETX toxin still needs to be fully elucidated. It has been described that the N-terminal amino acid sequence of ETX is identical in all strains examined to date [60]. Amino acid mutations within domain II of ETX affect pore characteristics and result in changes in cytotoxicity on renal distal tubule cells, leading to pulpy kidney disease in ruminants [61].

The diversity analysis of the ITX components, Ia and Ib, obtained from E-type strains from a calf and a human in France, reveals a very high identity, above 99%, at the amino acid level [62]. Although both toxins show conserved sequences, studies on iota toxin diversity are limited, and a better understanding of their contribution to enterotoxaemia in lambs, rabbits, and calves is required.

The genetic diversity analysis of the CPE toxin gene located on the chromosome, associated with food poisoning, and on plasmids linked to AAD reveals identical nucleotide sequences with similar cytotoxic activity, suggesting a physiological basis for the genotype-disease relationship [63]. Strains harbouring the CPE toxin on the chromosome exhibit superior heat resistance compared to non-food human gastrointestinal disease strains carrying the plasmid CPE gene. This resistance favours their survival in food and contributes to the development of food poisoning [64].

Analysis of NetB sequence diversity in different avian isolates reveals a high degree of conservation despite the presence of an amino acid variant, A168T, in some isolates. This variant does not significantly affect the physical properties of the encoded protein and does not differ in cytotoxicity. These genetic characteristics of the NetB toxin provide additional evidence that NetB constitutes an essential virulence factor in the pathogenesis of necrotic enteritis in birds [65].

Although the genetic diversity of the PFOA toxin and its influence on disease development is not well understood, the nucleotide sequence of the *pfoA* gene has been reported to exhibit

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approximately 86% identity with a membrane-damaging thiol-activated alveolysin detected in *C. perfringens* isolates IQ2 (type E) and IQ3 (BEC-positive) [18]. This toxin also shares a 60% identity with the streptolysin O gene and a 48% identity with the pneumolysin gene [66].

The expression of CPB2 toxin in *C. perfringens* strains varies according to host species, with two variants identified: consensus in porcine strains and atypical in non-porcine species. While the genotype-phenotype correlation is high in the consensus variant, the atypical variant shows only a 50% correlation. Furthermore, the atypical genes in types D and E are more similar to each other than isolates for non-porcine kinds A, B, and C, suggesting divergent evolution and a possible explanation for tropism by the host [67].

A detailed description of other accessory toxins is presented in **Table 1—section B.**

Conclusions

In recent years, efforts have been made to understand how chemical structures underpin the molecular and cellular mechanisms of action of bacterial toxins, including the regions involved in host cell recognition and the receptors that confer specificity to each toxin in different hosts, leading to the development of the pathological state. However, although *C. perfringens* is part of the 'resident' microbiota, studies need to probe the genetic diversity of each toxin in healthy animals and humans. This is crucial in understanding whether changes in the toxins render them less toxic or lead to their deactivation, ultimately contributing to a more 'commensal' lifestyle.

Future research could focus on additional sampling of isolates from healthy and diseased hosts and ecological niches to unravel broader evolutionary aspects, phylogenetic relationships, and genetic diversity of this bacterium, including a comprehensive examination of toxin-associated gene makeup. Further elucidation of the precise association between toxin-specific molecular and structural changes linked with host affinity will enhance knowledge about the diversity of this important pathogen.

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In addition to the above, gut microbiota studies are needed to understand how they may influence resistance to *C. perfringens* colonisation, competition for space and specific nutrients, or toxin function.

Understanding the interactions, genetic diversity, precise targets, and selective toxicity mechanism of *C. perfringens* toxins may open new avenues for preventative and therapy development. This could involve the rational design of potent pharmacological inhibitors/compounds and identifying new vaccine targets to counteract the effects of toxins, ultimately improving human and animal health.

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Conflict of interest

The authors declare that they have no conflict of interest.

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Table 1. Classification of toxins and other virulence factors of *C. perfringens*

Virulence factor or toxin	Gene	Biological Properties and Function	Associated disease	Reference
Section A. Toxins used for *C. perfringens* typing				
alpha-toxin	*plc, cpa*	- Modifies cell membranes by enzymatic activity. - Zinc-dependent phospholipase C which degrades phosphatidylcholine and sphingomyelin, in the eukaryotic cell membranes.	- Gas gangrene. - Necrotic enteritis in chickens. - Enteritis in calves and in piglets. - Sudden infant death syndrome (SIDS). - Inflammatory diseases.	[37]
Clostridium perfringens enterotoxin	*cpe*	- Intestinal damage, severe villus shortening, along with epithelial necrosis and desquamation.	- Human foodborne illness - Antibiotic-associated diarrhoea - Human enteritis necroticans	[68]

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			- Sporadic diarrhoea - Enteric diseases in swine, cattle, horses, sheep and goats and wild animals such as deer and bears.	
Alpha-clostripain	*ccp*	- Processing of secreted proteins - Potential to affect the levels of active extracellular toxins.	- Possible Clostridial myonecrosis -associated	[69]
Epsilon toxin	*etx*	- Aerolysin-like b-pore-forming toxin family. - Intestine: Epithelial cell detachment and cell necrosis, lamina propria haemorrhaging and polymorphonucleocyte infiltration. - Affect renal system, brain, cardiorespiratory system, and pleura. - Hemolysin.	- Enterotoxaemia in domestic ruminants (sheep and goats) - Illness in humans related to the epsilon toxin?	[42]

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		- Blood pressure elevation, increased contractility of smooth muscle, vascular permeability increase.		
Beta toxin	*cpb*	- Beta-barrel pore-forming toxin family and forms oligomeric pores in several susceptible immune cell lines. - Vascular necrosis and marked inflammatory reactions result from a direct interaction of CPB with vascular endothelial cells. - Destruction of jejunal and ileal villous tip epithelium.	- Necrotizing enteritis (NE) in pigs, sheep, goats, calves, and humans.	[70]
Iota toxin	*itx*	- Necrosis of the superficial epithelium with relative sparing of	- Lethal necrotizing - Enteritis and sudden death	[71]

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		the crypt epithelium, and submucosal haemorrhage and transmural haemorrhage.	in beef calves.	
Section B. Accessory toxins				
Clostridium Perfringens beta 2 toxin	*cpb2*	- Haemorrhage and necrosis of the small and large intestines. - Degenerated and necrotic desquamated epithelial cells, cell debris, inflammatory cells.	- Enteric diseases in swine, cattle, horses, sheep and goats and wild animals such as deer and bears.	[72]
Perfringolysin O	*pfoA*	- Tissue destruction and an anti-inflammatory response. - Vascular accumulation of leukocytes within blood vessels and the extracellular matrix of host tissues. - Disruption of endothelial, local	- Myonecrosis (gas gangrene), haemorrhagic enteritis in calves and, septicaemia (intravascular haemolysis) in humans. - Associated with	[11]

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		edema and systemic shock and multiorgan failure.	Necrotising Enterocolitis in human neonates.	
Necrotic enteritis E-like toxin	*netE*	- Pore-forming toxin within the Leukocidin/Hemolysin superfamily.		[73]
Necrotic enteritis F-like toxin	*netF*	- Putative beta-pore-forming toxin. - Pore-forming toxin within the Leukocidin/Hemolysin superfamily.	- Canine haemorrhagic gastroenteritis. - Foal necrotizing enteritis.	[38]
Necrotic enteritis G-like toxin	*netG*	- Putative beta-pore-forming toxin. - Cytotonic effects, such as proinflammatory effects.	- No confirmed association with disease.	[73]
Necrotic enteritis B-like toxin	*netB*	- Pore-forming toxin.	- Necrotic enteritis of chickens.	[49]
Toxin *C. perfringens* large cytotoxin	*TpeL*	- Ras-specific glucosyltransferase activity inactivating the Ras	- Associated with avian necrotic enteritis.	[74]

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		signalling pathway leading to apoptosis. - Cytotoxic effects (morphological changes such as enlargement and the rounding of Vero cells).		
Collagenase	*ColA*	- Degradation of the extracellular matrix due to their ability to digest native collagen.	- Associated with avian necrotic enteritis.	[75]
Binary enterotoxin of *C. perfringens*	*becA,becB*	- Enterotoxic activity. - Fluid accumulation in mice.	- Acute gastroenteritis in humans.	[17]
Neuramidases				
Secreted neuramidase	*nanJ*	- Increase CPE-induced cytotoxicity and CH-1 pore formation in Caco-2 cells.	- Contribute to food poisoning caused by F-type c-cpe strains carrying the *nanH* and *nanJ* genes.	[76]

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Secreted major neuramidase	*nanI*	- Synergy with CPA toxin, ETX toxin, CPB-toxin and CPE toxin - Sialidase activity. - Promotes the colonization of *C. perfringens* in the intestinal tract and enhances the cytotoxic activity.	- Gas gangrene?	[76]
Non-secreted neuramidase	*nanH*	- Enhance CPE cytotoxicity. - Reducing host cell surface charge repulsion during CPE binding or removing sialic acid residues that sterically interfere with CPE binding.	- Intestinal pathology.	[77]
Hyaluronidases				
Hyaluronidases	*nagI, nagJ, nagH, nagK nagL*	- Facilitates the spread of the major tissue-damaging α-toxin, thereby potentiating its cytolytic activity.	- Gas gangrene	[78, 79]

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		- Degrade hyaluronate cell surface coatings. - Act on connective tissue during gas gangrene. - Increased permeability of the connective tissues.

Figure legends

Figure 1. Schematic representation showing domain structure of *Clostridium pefringens* main toxins (A). CPA toxin **(B).** CPB toxin **(C).** ETX toxin **(D).** ITX toxin **(E).** CPE toxin and **(F).** NetB toxin are shown. Numbers indicate amino acids that mark domain boundaries.

Figure 2. Action mechanisms of the primary *C. perfringens* toxins used for toxin typing. Molecular mechanism of action of the major toxins of *C. perfringens.* **(A). CPA toxin:** CPA toxin interacts with GM1a, hydrolysing phosphatidylcholine (PC) and sphingomyelin (SM), resulting in the formation of diacylglycerol (DAG) and ceramide (CER) with Tropomyosin kinase A receptor (TrKA) activation and triggers the activation of an intracellular signalling

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cascade with Interleukin - 8 (IL-8) release. The activation of phosphatidyl inositol 3 (IP3) promotes intracytoplasmic calcium (Ca^{+}) entry **(B). CPB toxin:** CPB binds to platelet endothelial cell adhesion molecule-1 (PECAM-1) with subsequent release of adenosine triphosphate (ATP) and formation of pores that allow ion exchange to and from the cell **(C). ETX toxin:** ETX toxin interacts with protein "myelin and lymphocytes" (MAL), forming an active pore that induces ion transport and exchange across the cell membrane **(D). ITX toxin:** The binding of Ib to the lipolysis-stimulated lipoprotein receptor (LSR) receptor mediates its entry into the host cell, promoting the formation of channels for the entry of Ia by endocytosis with subsequent depolymerization of actin filaments, generating morphological changes and alteration of cell permeability **(E). CPE toxin:** the CPE toxin binds to claudin receptors, contributing to the formation of a pore on the cell surface with ion exchange and osmotic imbalance. **(F). NetB toxin** recognizes cholesterol-free regions in cell membranes by forming heptameric hydrophilic pores that allow the entry of ions such as Na^{+}, Cl^{-} y, and Ca 2^{+}.

Figure 3. Schematic representation of principal hosts for each *C. perfringens* toxinotypes—each box representing a different toxinotype.

Supplementary material

Figure supplementary 1. Toxinotyping scheme of *C. perfringens.* The main toxins are used as the primary typing scheme in seven toxin types (A-G).

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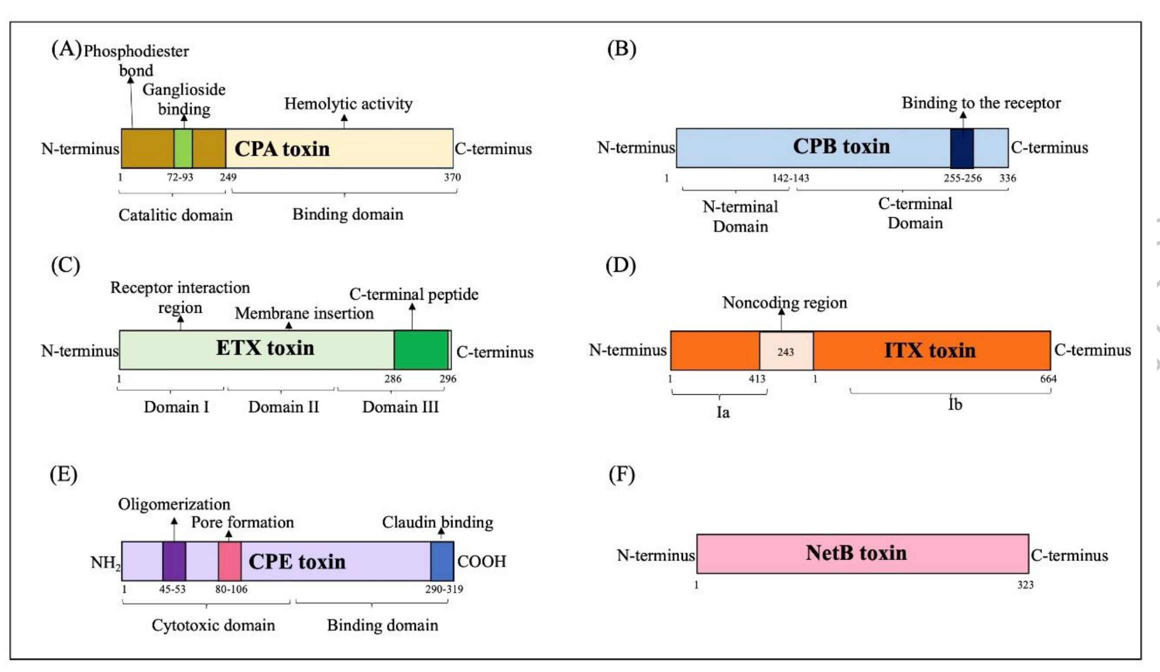

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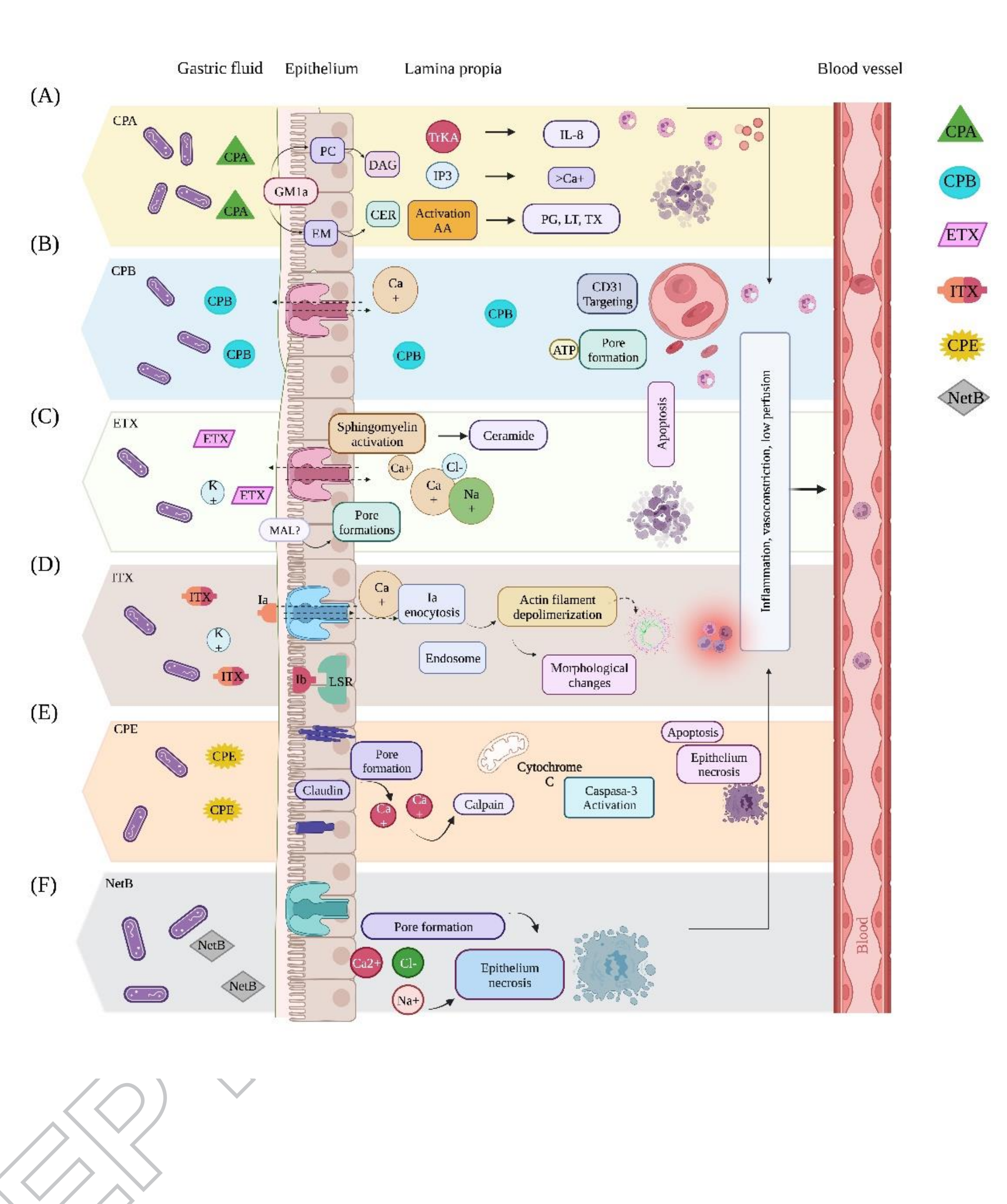

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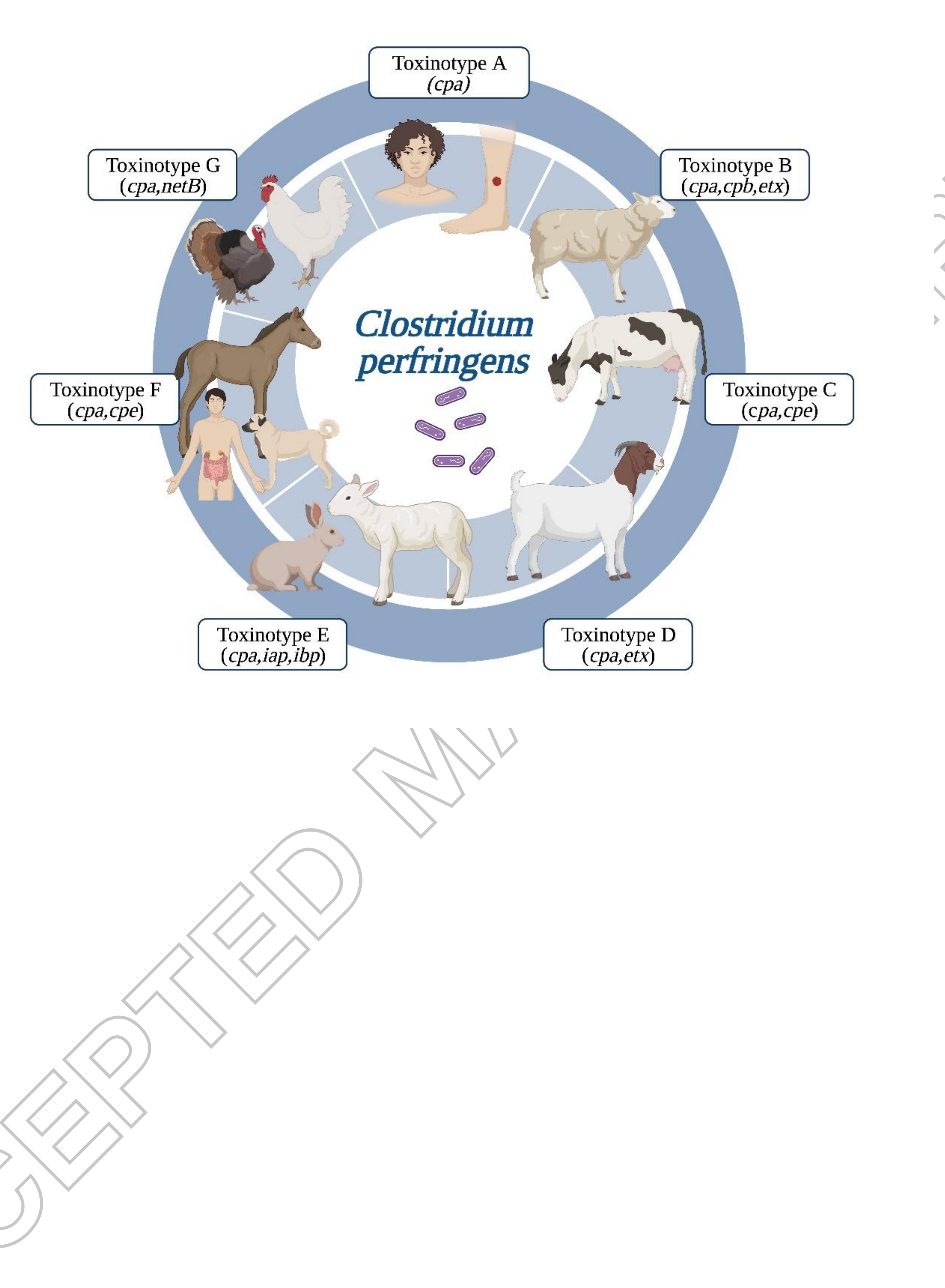

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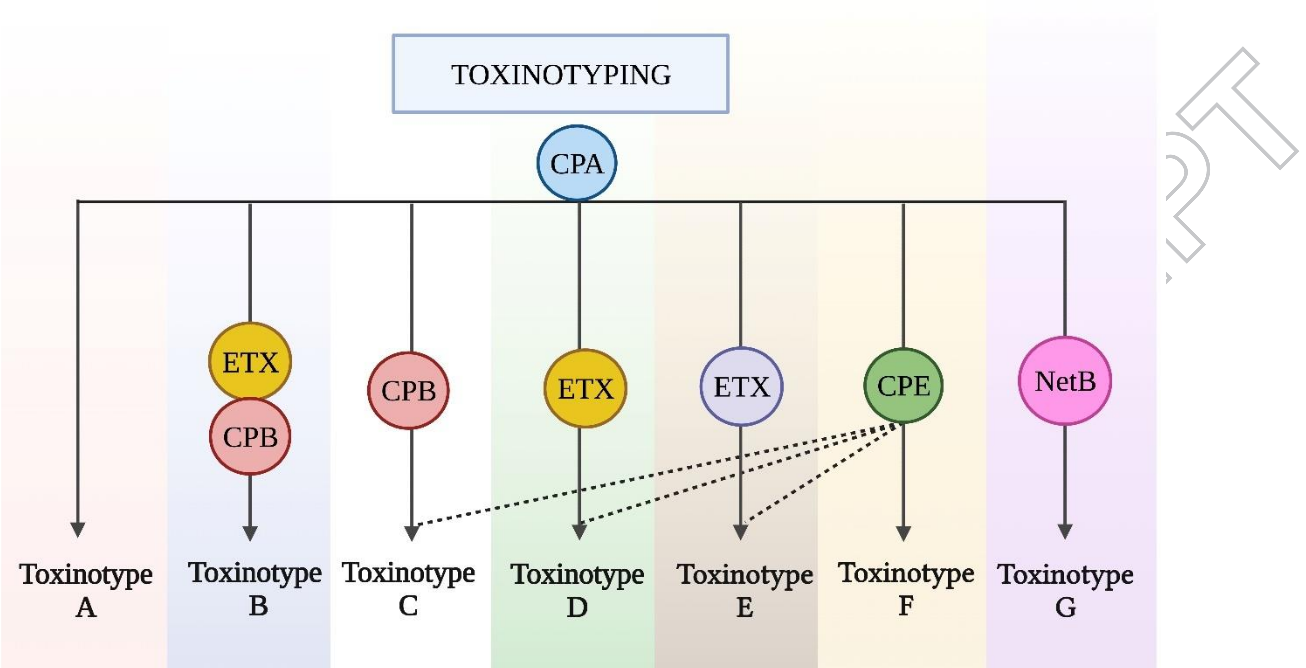

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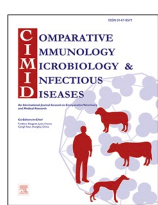

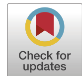

Acquisition site-based remodelling of *Clostridium perfringens*- and *Clostridioides difficile*-related gut microbiota

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ABSTRACT

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

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

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

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

1. Introduction

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

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

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

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

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

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

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

2. Materials and methods

2.1. Sample selection and group consolidation

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

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

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

2.2. Quality assessment and sequencing

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

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

2.3. Taxonomic assignment

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

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

2.4. Alpha and beta diversity analysis

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

2.5. Statistical analysis

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

3. Results

3.1. C. perfringens infection and microbiota composition

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

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

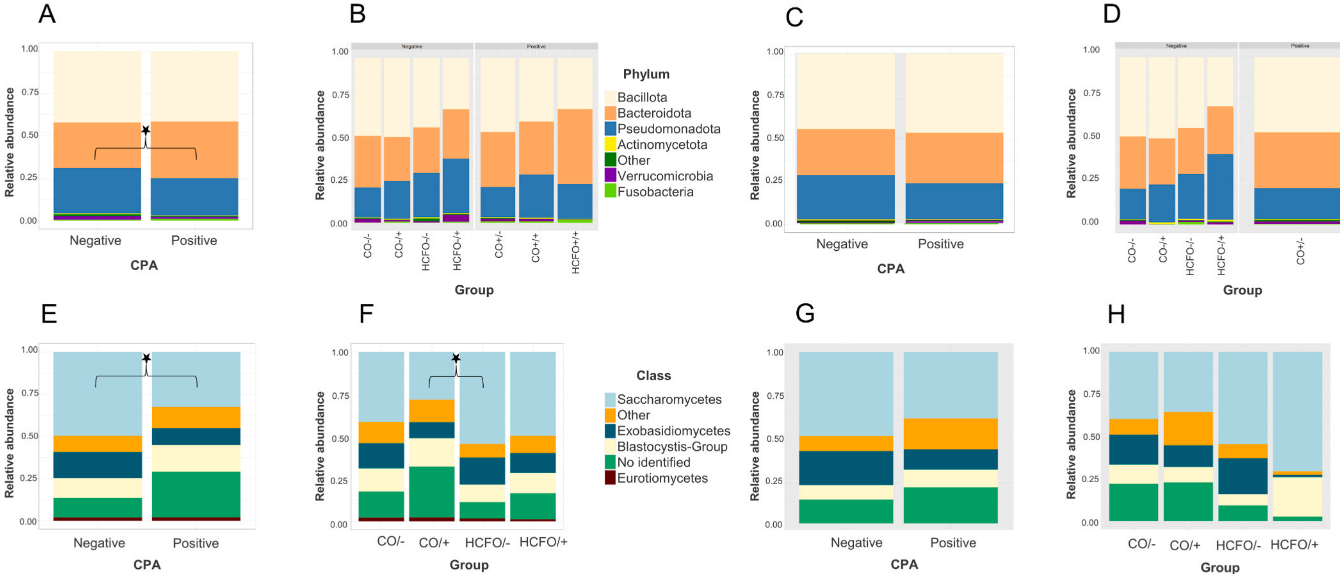

Fig. 1. Taxonomic composition of bacterial and eukaryotic microbiota by *C. perfringens/C. difficile* coexistence status and by group. A) Distribution of bacterial phyla involving *C. perfringens*; B) 16S-rRNA taxonomical composition by group (mixing diarrhoea acquisition site and *C. perfringens* and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

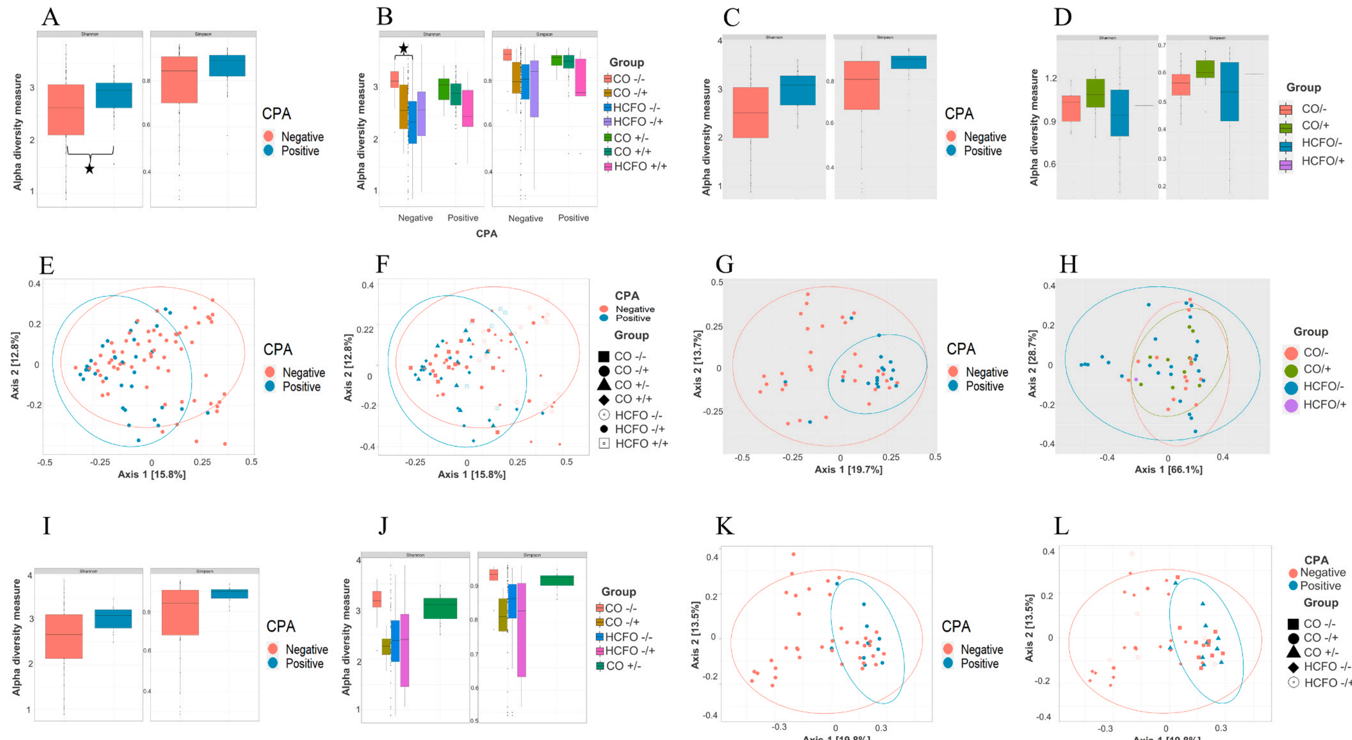

Fig. 2. Alpha and beta diversity measurement differences: statistically significant differences between groups are represented by a star; A) Alpha diversity indexes by bacterial sequence-based *C. perfringens* detection status; B) Alpha diversity indexes by bacterial sequence-based group (mixing diarrhoea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

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

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

3.2. Differences in alpha and beta diversity

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

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

4. Discussion

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

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

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

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

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

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

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

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

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

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

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

Funding source

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

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

to thank the Universidad del Rosario's Academic Affairs Office and Natural Sciences Faculty for granting Giovanny Herrera a graduate assistant scholarship.

Ethical approval statement

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

CRediT authorship contribution statement

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

Declaration of Competing Interest

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

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Appendix A. Supporting information

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

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9. CONCLUSIONES

CAPÍTULO I:

- El primer panorama epidemiológico sobre la frecuencia de detección de *C. perfringens* en varias especies animales en Colombia revela una elevada presencia de esta bacteria en animales domésticos y cerdos.
- La relación de la toxina CPB2 de *C. perfringens* con la diabetes, sugiere interacciones con el sistema inmunitario del huésped. Factores de riesgo como la inmunidad comprometida, la hiperglucemia, resaltan la importancia de comprender esta dinámica en pacientes diabéticos.
- Comprender la epidemiología local de *C. perfringens* mediante estudios microgeográficos es crucial para anticiparse a las amenazas para la salud pública y hacerles frente con eficacia.

CAPÍTULO II:

- La representación de genomas públicos de *C. perfringens* en países en desarrollo es escasa, probablemente debido a la falta de vigilancia epidemiológica y a la limitada recopilación de datos genómicos.
- Los análisis basados en MLST y el genoma central, utilizando genomas públicos de *C. perfringens*, revelan una estrecha relación entre aislamientos de diferentes hospederos. Estos hallazgos respaldan la marcada asociación de *C. perfringens* con las ETA y sugieren su potencial zoonótico.
- La identificación de toxinotipos de *C. perfringens* poco comunes en humanos, como el tipo G asociado con enfermedades en aves, junto con la presencia de aislamientos de tipo D y E vinculados con enfermedades en rumiantes, refleja la adaptabilidad de *C. perfringens* a diferentes hospedadores y resalta su papel zoonótico.
- Se requieren llevar a cabo estudios de caracterización genómica de microorganismos relevantes para la salud pública, particularmente en países en desarrollo, con el fin de comprender la diversidad genética, los patrones de dispersión y los marcadores de virulencia circulantes, que permitan instaurar medidas sanitarias y tratamientos adaptados a la realidad local.

CAPÍTULO III:

- El análisis microgeográfico de la estructura poblacional a partir de genomas colombianos de *C. perfringens*, reveló una amplia diversidad genética, sugiriendo posibles eventos de dispersión entre caninos, felinos y humanos. Estos hallazgos resaltan la necesidad de incrementar prácticas de higiene eficaces, especialmente en los cuidadores de animales domésticos con el objetivo de prevenir la transmisión a nivel comunitario.

- La presencia de alveolisina en aislamientos de *C. perfringens* de gatos asintomáticos en la región colombiana, junto con su alta capacidad de inhibición del crecimiento celular in vitro, sugiere un potencial significativo de virulencia en animales domésticos. Se requieren investigaciones adicionales para comprender mejor el papel de la alveolisina en la patogénesis de *C. perfringens*.

- Los aislamientos de *C. perfringens* portadores de la toxina PFOA, relacionada con la enterocolitis necrotizante, están presentes en individuos asintomáticos. Las pruebas fenotípicas, revelan su capacidad para generar hemólisis completa, inhibición del crecimiento celular *in vitro* y una mayor capacidad de esporulación. Esto sugiere un potencial riesgo de transmisión e infección a nivel comunitario y destaca la necesidad de comprender mejor el impacto de las toxinas accesorias a través de la caracterización fenotípica.

- Los hallazgos de MRA a aminoglucósidos, macrólidos y tetraciclinas a nivel genómico, junto con la reducida susceptibilidad fenotípica de aislamientos de *C. perfringens* a gentamicina, eritromicina, tetraciclina y metronidazol, representan una amenaza para el tratamiento adecuado de las infecciones causadas por esta bacteria. Esta capacidad para desarrollar un fenotipo resistente a múltiples fármacos de uso convencional supone un riesgo en el abordaje clínico y subraya la necesidad de realizar estudios microgeográficos para adaptar medidas terapéuticas eficientes a nivel local.

10. PERSPECTIVAS

- ✓ La mayor detección de *C. perfringens* en animales domésticos en comparación con los rumiantes resalta la importancia de factores como la dieta, la coinfección por otros patógenos y el papel de la microbiota intestinal. Por ende, los estudios futuros deberán incorporar datos acerca de hábitos alimenticios, factores demográficos y perfiles de dichas comundiades microbianas para profundizar en el entendimiento de estas complejas interacciones.

- ✓ La inclusión de un mayor número de participantes, tanto individuos de la comunidad asintomáticos como pacientes hospitalizados con síntomas gastrointestinales, de diferentes regiones de Colombia, es esencial para obtener una visión más completa y representativa de epidemiología de *C. perfringens*.

- ✓ La ampliación de investigaciones futuras incluyendo una variedad más amplia y diversa de muestras de diferentes nichos ecológicos como humanos, animales, muestras de suelo, agua y alimentos permitirá avanzar en la comprensión del potencial de transmisión de *C. perfringens.* Estos estudios, aportarán a mejorar la precisión en la detección y entendimiento de este microorganismo y descubrir métodos más eficientes y estratégicos para mitigar su creciente impacto en la salud.

- ✓ La asociación entre la presencia de aislamientos de *C. perfringens* portadores del gen de la toxina CPB2 y diabetes mellitus se convierte en un interesante campo de investigación. Profundizar en la frecuencia de detección de *C. perfringens* entre la población diabética y examinar muestras de tejido necrótico de úlceras diabéticas podría transformar el diagnóstico y tratamiento de estas afecciones, marcando un avance significativo en el manejo clínico de los pacientes afectados.

- ✓ El análisis de los datos de secuenciación de genomas completos de *C. perfringens* deberá incluir el análisis funcional de genes centrales y accesorios, para comprender mejor la plasticidad genómica de esta bacteria. Este proceso permitirá identificar genes relacionados con aspectos cruciales como el metabolismo, los mecanismos de defensa, la recombinación y los procesos de reparación, lo que a su vez enriquecerá el entendimiento sobre su elevada plasticidad genómica.

- ✓ Investigar el rol patológico de los factores de virulencia de *C. perfringens*, posiblemente a través de modelos murinos o modelos de colon humano, puede ofrecer información valiosa sobre cómo este patógeno afecta al organismo y cómo podría ser contrarrestado de manera más efectiva.

- ✓ Evaluar los niveles de expresión mediante ensayos de PCR cuantitativa en tiempo real de toxinas como CPA, CPE, CPB2 y PFOA y su asociación con el daño sobre células Caco2, podría aportar un mayor conocimiento acerca de la patogénesis de aislamientos toxigénicos de *C. perfringens*

- ✓ Las condiciones de esporulación afectan significativamente el crecimiento, la germinación y la resistencia de las esporas de *C. perfringens*. Por lo tanto, se deberá investigar los efectos de la temperatura, el pH y la actividad de desinfectantes sobre el crecimiento y la germinación de las esporas de *C. perfringens.*

- ✓ Es fundamental llevar a cabo investigaciones genómicas enfocadas en la población pediátrica, prestando especial atención a los recién nacidos prematuros con bajo peso al nacer que padecen de enterocolitis necrotizante por *C. perfringens* en las Unidades de Cuidados Intensivos. El objetivo principal podría estar enfocado a caracterizar los aislamientos microbianos implicados en la patogénesis de esta condición, con la intención de contribuir significativamente al desarrollo de tratamientos más efectivos.

11. PRODUCTOS DE LA TESIS

Los productos generados durante el desarrollo de la presente tesis doctoral se listan a continuación:

LISTA DE PUBLICACIONES:

1. **Artículo 1:** Camargo A, Páez-Triana L, Camargo D, García-Corredor D, Pulido-Medellín M, Camargo M, Ramírez J.D. and Muñoz M*. Carriage of *Clostridium perfringens* in Domestic and Farm Animals across the Central Highlands of Colombia: Implications for Gut Health and Zoonotic Transmission. Vet Res Commun. 2024.

2. **Artículo 2:** Camargo A., Bohórquez L., López D., Ferrebuz-Cardozo A., Castellanos-Rozo J., Díaz J., Rada M., Camargo M., Ramírez J. D. and Muñoz M. *Clostridium perfringens* in central Colombia: Frequency, Toxin Genes, and Risk Factors. (**SOMETIDO** en Gut Pathogens).

3. **Artículo 3:** Camargo A., Guerrero-Araya E, Castañeda S, Vega L, Cardenas-Alvarez MX, Rodríguez C, Paredes-Sabja D, Ramírez JD, Muñoz M. Intra-species diversity of *Clostridium perfringens*: A diverse genetic repertoire reveals its pathogenic potential. Front Microbiol. 2022 Jul 22; 13:952081. doi: 10.3389/fmicb.2022.952081. PMID: 35935202; PMCID: PMC9354469.

4. **Artículo 4:** Camargo A., Bohorquez L., Cáceres T., Ferrebuz-Cardozo A, Díaz J, Castellanos-Rozo J, Diaz J., Kiu R., Hall L. J., Rámirez J. D. and Muñoz M. Insights into *Clostridium perfringens* Dispersal Hotspots, Toxins, and Virulence Factors through Integrated Genomic and Phenotypic Profiling. (**EN CONSTRUCCIÓN**)

5. **Artículo 5:** Camargo A, Rámirez J. D., Kiu R., Hall L.J., Muñoz M. Unveiling the pathogenic mechanisms of *Clostridium perfringens* toxins and virulence factors. Emerg Microbes Infect. 2024 Apr 9:2341968. doi: 10.1080/22221751.2024.2341968. Epub ahead of print. PMID: 38590276.

6. **Artículo 6:** Herrera G, Vega L, Camargo A, Patarroyo MA, Ramírez JD, Muñoz M. Acquisition site-based remodelling of *Clostridium perfringens*- and Clostridioides difficile-related gut microbiota. Comp Immunol Microbiol Infect Dis. 2023 Nov; 102:102074. doi: 10.1016/j.cimid.2023.102074. Epub 2023 Oct 10. PMID: 37832162.

PRESENTACIÓN EN EVENTOS CIENTÍFICOS:

Julio/2022 Seattle, EE. UU.	**THE 16TH BIENNIAL CONGRESS OF THE ANAEROBE SOCIETY OF THE AMERICAS** **Tipo de evento:** Congreso **Ámbito:** Internacional **Tipo de producto:** Póster **Nombre del producto:** Intra-species diversity of *Clostridium perfringens*: A diverse genetic repertoire reveals its pathogenic potential
Diciembre/2022 Bogotá, Colombia	**XVIII CONGRESO COLOMBIANO DE PARASITOLOGÍA Y MEDICINA TROPICAL** **Tipo de evento:** Congreso **Ámbito:** Nacional **Tipo de producto:** Ponencia oral **Nombre del producto:** Detección de *Clostridium perfringens* en animales domésticos y de granja en el altiplano central de Colombia: Implicaciones para la salud intestinal y la transmisión zoonótica

PASANTÍA INTERNACIONAL

Norwich, Inglaterra Enero – Junio de 2023	**Tipo de producto:** Pasantía doctoral **Ámbito:** Internacional **Lugar:** Early life microbiota-host interactions laboratory, Quadram Institute Biosciences. **Líder:** Lindsay Hall

CURSOS

Noviembre 2020 Curso virtual	Curso **Introducción a la filogenómica de bacterias** 9 horas BioSciences App
Noviembre 2020 Curso virtual	Curso **Metagenómica** 9 horas BioSciences App
Septiembre 2021 Curso virtual	Curso **Ciencia de datos en R** 16 horas Biofreelancer

Marzo 2024 Curso presencial Bogotá, Colombia.	Curso **Clinical Research During Outbreaks – CREDO** 24 horas Universidad de Oxford – ISARIC- UCTS USabana

BECAS

Enero 2023 Bogotá/Colombia	**Beca para el fortalecimiento académico - Pasantía Doctoral** Estudiantes doctorales Universidad del Rosario
Tunja/Colombia	**Beca para cursar estudios doctorales** Facultad de Ciencias de la Salud Universidad de Boyacá

ORIENTACIÓN DE TRABAJOS DE GRADO

Tesis de pregrado 2023 Universidad del Rosario	**Tipo de producto:** Tesis de pregrado en Biología. **Estudiante:** Emanuella de la Cruz **Facultad:** Facultad de Ciencias Naturales **Nombre de la tesis:** Caracterización de *Clostridium paraputrificum* y *Clostridium tertium* en muestras de heces de animales de Boyacá y Cundinamarca - Colombia **Estado:** Aprobado y finalizado
Tesis de pregrado 2023 Universidad de Boyacá	**Tipo de producto:** Tesis de pregrado en Bacteriología y Laboratorio Clínico. **Estudiante:** María Alejandra Díaz **Facultad:** Facultad de Ciencias de la Salud **Nombre de la tesis:** Calidad microbiológica de productos cárnicos crudos frescos en el municipio de Gámeza, departamento de Boyacá, Colombia **Estado:** Aprobado y finalizado
Trabajo de grado 2023 Universidad Pedagógica y Tecnológica de Colombia - UPTC	**Tipo de producto:** Trabajo de grado en Medicina Veterinaria y Zootecnia. **Estudiante:** Valentina Rodríguez Montaña **Facultad:** **Nombre del trabajo:** Detección de *C. perfringens* circulante en animales domésticos del Departamento de Boyacá **Estado:** Aprobado y finalizado
Tesis de pregrado 2024 Universidad de Boyacá	**Tipo de producto:** Tesis de pregrado en Bacteriología y Laboratorio Clínico. **Estudiante:** Ingrid Dayana Cano Tipazoca **Facultad:** Facultad de Ciencias de la Salud

	Nombre de la tesis: Características fenotípicas de resistencia antimicrobiana de *Clostridium perfringens* obtenidas de muestras de heces **Estado:** En curso
Tesis de Maestría 2024 Universidad Colegio Mayor de Cundinamarca	**Tipo de producto:** Tesis de Maestría en Microbiología **Estudiante:** Dayana Sofía Torres **Facultad:** Facultad de Ciencias de la Salud, Universidad Colegio Mayor de Cundinamarca **Nombre de la tesis:** Resistencia antimicrobiana de *Clostridium perfringens* en humanos y animales del Departamento de Boyacá **Estado:** En curso

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9. CONCLUSIONES

CAPÍTULO I:

- El primer panorama epidemiológico sobre la frecuencia de detección de *C. perfringens* en varias especies animales en Colombia revela una elevada presencia de esta bacteria en animales domésticos y cerdos.
- La relación de la toxina CPB2 de *C. perfringens* con la diabetes, sugiere interacciones con el sistema inmunitario del huésped. Factores de riesgo como la inmunidad comprometida, la hiperglucemia, resaltan la importancia de comprender esta dinámica en pacientes diabéticos.
- Comprender la epidemiología local de *C. perfringens* mediante estudios microgeográficos es crucial para anticiparse a las amenazas para la salud pública y hacerles frente con eficacia.

CAPÍTULO II:

- La representación de genomas públicos de *C. perfringens* en países en desarrollo es escasa, probablemente debido a la falta de vigilancia epidemiológica y a la limitada recopilación de datos genómicos.
- Los análisis basados en MLST y el genoma central, utilizando genomas públicos de *C. perfringens*, revelan una estrecha relación entre aislamientos de diferentes hospederos. Estos hallazgos respaldan la marcada asociación de *C. perfringens* con las ETA y sugieren su potencial zoonótico.
- La identificación de toxinotipos de *C. perfringens* poco comunes en humanos, como el tipo G asociado con enfermedades en aves, junto con la presencia de aislamientos de tipo D y E vinculados con enfermedades en rumiantes, refleja la adaptabilidad de *C. perfringens* a diferentes hospedadores y resalta su papel zoonótico.
- Se requieren llevar a cabo estudios de caracterización genómica de microorganismos relevantes para la salud pública, particularmente en países en desarrollo, con el fin de comprender la diversidad genética, los patrones de dispersión y los marcadores de virulencia circulantes, que permitan instaurar medidas sanitarias y tratamientos adaptados a la realidad local.

CAPÍTULO III:

- El análisis microgeográfico de la estructura poblacional a partir de genomas colombianos de *C. perfringens*, reveló una amplia diversidad genética, sugiriendo posibles eventos de dispersión entre caninos, felinos y humanos. Estos hallazgos resaltan la necesidad de incrementar prácticas de higiene eficaces, especialmente en los cuidadores de animales domésticos con el objetivo de prevenir la transmisión a nivel comunitario.

- La presencia de alveolisina en aislamientos de *C. perfringens* de gatos asintomáticos en la región colombiana, junto con su alta capacidad de inhibición del crecimiento celular in vitro, sugiere un potencial significativo de virulencia en animales domésticos. Se requieren investigaciones adicionales para comprender mejor el papel de la alveolisina en la patogénesis de *C. perfringens*.

- Los aislamientos de *C. perfringens* portadores de la toxina PFOA, relacionada con la enterocolitis necrotizante, están presentes en individuos asintomáticos. Las pruebas fenotípicas, revelan su capacidad para generar hemólisis completa, inhibición del crecimiento celular *in vitro* y una mayor capacidad de esporulación. Esto sugiere un potencial riesgo de transmisión e infección a nivel comunitario y destaca la necesidad de comprender mejor el impacto de las toxinas accesorias a través de la caracterización fenotípica.

- Los hallazgos de MRA a aminoglucósidos, macrólidos y tetraciclinas a nivel genómico, junto con la reducida susceptibilidad fenotípica de aislamientos de *C. perfringens* a gentamicina, eritromicina, tetraciclina y metronidazol, representan una amenaza para el tratamiento adecuado de las infecciones causadas por esta bacteria. Esta capacidad para desarrollar un fenotipo resistente a múltiples fármacos de uso convencional supone un riesgo en el abordaje clínico y subraya la necesidad de realizar estudios microgeográficos para adaptar medidas terapéuticas eficientes a nivel local.

10. PERSPECTIVAS

- ✓ La mayor detección de *C. perfringens* en animales domésticos en comparación con los rumiantes resalta la importancia de factores como la dieta, la coinfección por otros patógenos y el papel de la microbiota intestinal. Por ende, los estudios futuros deberán incorporar datos acerca de hábitos alimenticios, factores demográficos y perfiles de dichas comundiades microbianas para profundizar en el entendimiento de estas complejas interacciones.

- ✓ La inclusión de un mayor número de participantes, tanto individuos de la comunidad asintomáticos como pacientes hospitalizados con síntomas gastrointestinales, de diferentes regiones de Colombia, es esencial para obtener una visión más completa y representativa de epidemiología de *C. perfringens*.

- ✓ La ampliación de investigaciones futuras incluyendo una variedad más amplia y diversa de muestras de diferentes nichos ecológicos como humanos, animales, muestras de suelo, agua y alimentos permitirá avanzar en la comprensión del potencial de transmisión de *C. perfringens.* Estos estudios, aportarán a mejorar la precisión en la detección y entendimiento de este microorganismo y descubrir métodos más eficientes y estratégicos para mitigar su creciente impacto en la salud.

- ✓ La asociación entre la presencia de aislamientos de *C. perfringens* portadores del gen de la toxina CPB2 y diabetes mellitus se convierte en un interesante campo de investigación. Profundizar en la frecuencia de detección de *C. perfringens* entre la población diabética y examinar muestras de tejido necrótico de úlceras diabéticas podría transformar el diagnóstico y tratamiento de estas afecciones, marcando un avance significativo en el manejo clínico de los pacientes afectados.

- ✓ El análisis de los datos de secuenciación de genomas completos de *C. perfringens* deberá incluir el análisis funcional de genes centrales y accesorios, para comprender mejor la plasticidad genómica de esta bacteria. Este proceso permitirá identificar genes relacionados con aspectos cruciales como el metabolismo, los mecanismos de defensa, la recombinación y los procesos de reparación, lo que a su vez enriquecerá el entendimiento sobre su elevada plasticidad genómica.

- ✓ Investigar el rol patológico de los factores de virulencia de *C. perfringens*, posiblemente a través de modelos murinos o modelos de colon humano, puede ofrecer información valiosa sobre cómo este patógeno afecta al organismo y cómo podría ser contrarrestado de manera más efectiva.

- ✓ Evaluar los niveles de expresión mediante ensayos de PCR cuantitativa en tiempo real de toxinas como CPA, CPE, CPB2 y PFOA y su asociación con el daño sobre células Caco2, podría aportar un mayor conocimiento acerca de la patogénesis de aislamientos toxigénicos de *C. perfringens*

- ✓ Las condiciones de esporulación afectan significativamente el crecimiento, la germinación y la resistencia de las esporas de *C. perfringens*. Por lo tanto, se deberá investigar los efectos de la temperatura, el pH y la actividad de desinfectantes sobre el crecimiento y la germinación de las esporas de *C. perfringens.*

- ✓ Es fundamental llevar a cabo investigaciones genómicas enfocadas en la población pediátrica, prestando especial atención a los recién nacidos prematuros con bajo peso al nacer que padecen de enterocolitis necrotizante por *C. perfringens* en las Unidades de Cuidados Intensivos. El objetivo principal podría estar enfocado a caracterizar los aislamientos microbianos implicados en la patogénesis de esta condición, con la intención de contribuir significativamente al desarrollo de tratamientos más efectivos.

11. PRODUCTOS DE LA TESIS

Los productos generados durante el desarrollo de la presente tesis doctoral se listan a continuación:

LISTA DE PUBLICACIONES:

1. **Artículo 1:** Camargo A, Páez-Triana L, Camargo D, García-Corredor D, Pulido-Medellín M, Camargo M, Ramírez J.D. and Muñoz M*. Carriage of *Clostridium perfringens* in Domestic and Farm Animals across the Central Highlands of Colombia: Implications for Gut Health and Zoonotic Transmission. Vet Res Commun. 2024.

2. **Artículo 2:** Camargo A., Bohórquez L., López D., Ferrebuz-Cardozo A., Castellanos-Rozo J., Díaz J., Rada M., Camargo M., Ramírez J. D. and Muñoz M. *Clostridium perfringens* in central Colombia: Frequency, Toxin Genes, and Risk Factors. (**SOMETIDO** en Gut Pathogens).

3. **Artículo 3:** Camargo A., Guerrero-Araya E, Castañeda S, Vega L, Cardenas-Alvarez MX, Rodríguez C, Paredes-Sabja D, Ramírez JD, Muñoz M. Intra-species diversity of *Clostridium perfringens*: A diverse genetic repertoire reveals its pathogenic potential. Front Microbiol. 2022 Jul 22; 13:952081. doi: 10.3389/fmicb.2022.952081. PMID: 35935202; PMCID: PMC9354469.

4. **Artículo 4:** Camargo A., Bohorquez L., Cáceres T., Ferrebuz-Cardozo A, Díaz J, Castellanos-Rozo J, Diaz J., Kiu R., Hall L. J., Rámirez J. D. and Muñoz M. Insights into *Clostridium perfringens* Dispersal Hotspots, Toxins, and Virulence Factors through Integrated Genomic and Phenotypic Profiling. (**EN CONSTRUCCIÓN**)

5. **Artículo 5:** Camargo A, Rámirez J. D., Kiu R., Hall L.J., Muñoz M. Unveiling the pathogenic mechanisms of *Clostridium perfringens* toxins and virulence factors. Emerg Microbes Infect. 2024 Apr 9:2341968. doi: 10.1080/22221751.2024.2341968. Epub ahead of print. PMID: 38590276.

6. **Artículo 6:** Herrera G, Vega L, Camargo A, Patarroyo MA, Ramírez JD, Muñoz M. Acquisition site-based remodelling of *Clostridium perfringens*- and Clostridioides difficile-related gut microbiota. Comp Immunol Microbiol Infect Dis. 2023 Nov; 102:102074. doi: 10.1016/j.cimid.2023.102074. Epub 2023 Oct 10. PMID: 37832162.

PRESENTACIÓN EN EVENTOS CIENTÍFICOS:

Julio/2022 Seattle, EE. UU.	**THE 16TH BIENNIAL CONGRESS OF THE ANAEROBE SOCIETY OF THE AMERICAS** **Tipo de evento:** Congreso **Ámbito:** Internacional **Tipo de producto:** Póster **Nombre del producto:** Intra-species diversity of *Clostridium perfringens*: A diverse genetic repertoire reveals its pathogenic potential
Diciembre/2022 Bogotá, Colombia	**XVIII CONGRESO COLOMBIANO DE PARASITOLOGÍA Y MEDICINA TROPICAL** **Tipo de evento:** Congreso **Ámbito:** Nacional **Tipo de producto:** Ponencia oral **Nombre del producto:** Detección de *Clostridium perfringens* en animales domésticos y de granja en el altiplano central de Colombia: Implicaciones para la salud intestinal y la transmisión zoonótica

PASANTÍA INTERNACIONAL

Norwich, Inglaterra Enero – Junio de 2023	**Tipo de producto:** Pasantía doctoral **Ámbito:** Internacional **Lugar:** Early life microbiota-host interactions laboratory, Quadram Institute Biosciences. **Líder:** Lindsay Hall

CURSOS

Noviembre 2020 Curso virtual	Curso **Introducción a la filogenómica de bacterias** 9 horas BioSciences App
Noviembre 2020 Curso virtual	Curso **Metagenómica** 9 horas BioSciences App
Septiembre 2021 Curso virtual	Curso **Ciencia de datos en R** 16 horas Biofreelancer

Marzo 2024 Curso presencial Bogotá, Colombia.	Curso **Clinical Research During Outbreaks – CREDO** 24 horas Universidad de Oxford – ISARIC- UCTS USabana

BECAS

Enero 2023 Bogotá/Colombia	**Beca para el fortalecimiento académico - Pasantía Doctoral** Estudiantes doctorales Universidad del Rosario
Tunja/Colombia	**Beca para cursar estudios doctorales** Facultad de Ciencias de la Salud Universidad de Boyacá

ORIENTACIÓN DE TRABAJOS DE GRADO

Tesis de pregrado 2023 Universidad del Rosario	**Tipo de producto:** Tesis de pregrado en Biología. **Estudiante:** Emanuella de la Cruz **Facultad:** Facultad de Ciencias Naturales **Nombre de la tesis:** Caracterización de *Clostridium paraputrificum* y *Clostridium tertium* en muestras de heces de animales de Boyacá y Cundinamarca - Colombia **Estado:** Aprobado y finalizado
Tesis de pregrado 2023 Universidad de Boyacá	**Tipo de producto:** Tesis de pregrado en Bacteriología y Laboratorio Clínico. **Estudiante:** María Alejandra Díaz **Facultad:** Facultad de Ciencias de la Salud **Nombre de la tesis:** Calidad microbiológica de productos cárnicos crudos frescos en el municipio de Gámeza, departamento de Boyacá, Colombia **Estado:** Aprobado y finalizado
Trabajo de grado 2023 Universidad Pedagógica y Tecnológica de Colombia - UPTC	**Tipo de producto:** Trabajo de grado en Medicina Veterinaria y Zootecnia. **Estudiante:** Valentina Rodríguez Montaña **Facultad:** **Nombre del trabajo:** Detección de *C. perfringens* circulante en animales domésticos del Departamento de Boyacá **Estado:** Aprobado y finalizado
Tesis de pregrado 2024 Universidad de Boyacá	**Tipo de producto:** Tesis de pregrado en Bacteriología y Laboratorio Clínico. **Estudiante:** Ingrid Dayana Cano Tipazoca **Facultad:** Facultad de Ciencias de la Salud

	Nombre de la tesis: Características fenotípicas de resistencia antimicrobiana de *Clostridium perfringens* obtenidas de muestras de heces **Estado:** En curso
Tesis de Maestría 2024 Universidad Colegio Mayor de Cundinamarca	**Tipo de producto:** Tesis de Maestría en Microbiología **Estudiante:** Dayana Sofía Torres **Facultad:** Facultad de Ciencias de la Salud, Universidad Colegio Mayor de Cundinamarca **Nombre de la tesis:** Resistencia antimicrobiana de *Clostridium perfringens* en humanos y animales del Departamento de Boyacá **Estado:** En curso

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