Interacciones parásito-hospedero-microbioma en infecciones causadas por protozoos y helmintos

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

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TABLA DE CONTENIDO

Contenidos

1. LISTA DE PUBLICACIONES

Esta tesis se estructura en siete artículos científicos generados durante la formación doctoral. Todos los artículos publicados y sometidos a publicación se encuentran anexos a este documento:

Artículo 1: **Castañeda, S**, Acosta, Claudia Patricia, Vasquez-A, Luis Reinel, Patiño, Luz H, Mejía, Rojelio, Ramírez JD. Molecular detection of intestinal parasites in a rural community of Colombia: A one health approach to explore potential environmental–zoonotic transmission. Zoonoses Public Health [Internet]. 2024 [cited 2024 Oct 23];71(6):723–735. https://doi.org/10.1111/zph. 13138.

Artículo 2: **Castañeda S**, Stensvold CR, Andersen LOBB, Acosta, Claudia Patricia, Vasquez-A, Luis Reinel, Ramírez JD. Impact of *Blastocystis* Colonization and Burden on Gut Microbiota Composition in a Non-Westernized Rural Population from Colombia. *Sometido a PLOS Neglected Tropical Diseases (Q1).*

Artículo 3: **Castañeda S,** Muñoz M, Hotez PJ, Bottazzi ME, Paniz-Mondolfi AE, Jones KM, Mejia R, Poveda C, Ramírez JD. Microbiome Alterations Driven by *Trypanosoma cruzi* Infection in Two Disjunctive Murine Models. Microbiology Spectrum [Internet]. 2023 [cited 2023 Jun 16];11(3). https://doi.org/10.1128/spectrum.00199-23

Artículo 4: **Castañeda S,** R,Poveda Charlie Suarez-Reyes, Yifan Wu, Noah Haugen, Patiño, Luz H, Weatherhead JE, Ramírez JD. Microbiota Dynamics During *Ascaris* Larval Migration: Implications for Host Microbial Communities. *Sometido a Microbial Pathogenesis (Q1).*

Artículo 5: **Castañeda S**, Adeniyi-Ipadeola G, Wu Y, Suarez-Reyes C, Jain A, Ramírez JD, Weatherhead JE. Characterizing Excretory-Secretory Products Proteome Across Larval Development Stages in *Ascaris suum*. *Sometido a Microbes and Infectio*n *(Q1)* https://doi.org/10.1101/2024.07.03.601870.

Artículo 6: **Castañeda S,** Paniz-Mondolfi A, Ramírez JD. 2024. Detangling the Crosstalk Between *Ascaris, Trichuris,* and Gut Microbiota: What´s Next? Frontiers in Cellular and Infection Microbiology [Internet]. 2022. https://doi.org/10.3389/fcimb.2022.852900.

Artículo 7: Ramírez JD, **Castañeda S,** Weatherhead JE, R,Poveda. 2024. Parasite-Microbiota Interactions: A Pathway to Innovative Interventions for Chagas Disease, Leishmaniasis, and Ascariasis**.** *Sometido a Future Microbiology (Q1).*

2. LISTA DE ABREVIATURAS

AGCC: Ácidos grasos de cadena corta

BALB/c: Cepa de ratón de laboratorio BALB/c

BLAST: Herramienta básica de alineamiento de secuencias locales

C57BL/6: Cepa de ratón de laboratorio C57BL/6

DADA2: *Divisive Amplicon Denoising Algorithm 2*

LC-MS/MS: Cromatografía líquida-espectrometría de masas en tándem

MAGs: Genomas ensamblados de metagenomas

NGS: Secuenciación de nueva generación

PCR: Reacción en cadena de la polimerasa

ES: Productos de excreción-secreción

qPCR: Reacción en cadena de la polimerasa cuantitativa

.

3. RESUMEN

El microbioma intestinal cumple un rol esencial en diversas actividades metabólicas, fisiológicas e inmunológicas en el hospedero, las cuales, en equilibrio, promueven un adecuado funcionamiento y salud intestinal, ofreciendo adicionalmente, una barrera de protección frente a la colonización e infección por agentes patógenos virales, bacterianos y eucariotas. Dentro de estos organismos, diferentes parásitos, protozoos y helmintos, tienen una importante interacción con este microbioma y se ha propuesto que esta interacción puede tener una gran relevancia con aspectos asociados a la dinámica de la infección, progresión, persistencia, heterogeneidad clínica, entre otros. Una amplia variedad de parásitos pueden ser agentes etiológicos de diversas infecciones y enfermedades tanto a nivel intestinal como sistémico y es claro que a diferencia de los principales patógenos bacterianos y virales, no existen vacunas disponibles para prevenir estas infecciones y por ello su manejo se fundamenta en aspectos de prevención y tratamiento.

Actualmente se conoce que las infecciones parasitarias tienen una interacción con el microbioma intestinal del hospedero influyendo en aspectos de salud y enfermedad, establecimiento, progresión y persistencia de la infección e incluso se ha considerado que estos cambios pueden predisponer al hospedero a diferentes enfermedades intestinales, cardiovasculares, cáncer, obesidad y algunos desórdenes del sistema nervioso central. Diversos estudios han revelado hallazgos de gran interés relacionados con, como el microbioma intestinal puede verse alterado por la presencia de parásitos, tanto protozoos como helmintos, los cuales principalmente han sido desarrollados bajo enfoques descriptivos en seres humanos y de manera complementaria, implementando modelos animales para comprender estos cambios. Sin embargo, estos estudios han usado principalmente la estrategia de metabarcoding (usando marcador 16S rRNA), las cuales brindan información asociada a la composición y estructura de la microbiota, pero que no permiten obtener información referente a predicciones funcionales que brinden un panorama general de cómo los cambios en el microbioma también pueden verse reflejados en cambios en la abundancia de ciertos genes y vías metabólicas claves en estas interacciones. Es por lo anterior, que se requieren estudios como el presente, enfocados en determinar las interacciones parásito-hospedero-microbioma a partir de la integración de una aproximación descriptiva en seres humanos y otro experimental en modelos animales utilizando complementariamente aproximaciones basadas en metabarcoding y metagenómica que permitan un visión más completa de los aspectos vinculados a esta interacción. Los cambios en la estructura y composición del microbioma intestinal en respuesta a infecciones y colonizaciones por protozoos y helmintos, tanto en seres humanos como en modelos animales facilita la correlación de estos hallazgos con distintas variables de interés que pueden representar factores de riesgo asociados a la infección, permitiendo así un mejor entendimiento de los procesos fisiopatológicos y epidemiológicos relacionados con estas infecciones. Esto adquiere mayor relevancia en países en vía de desarrollo donde la prevalencia de infecciones parasitarias es mayor y donde información relevante en el campo de la biología del parásito y de la fisiopatología de la infección y enfermedad, puede brindar un insumo esencial para el desarrollo de nuevas estrategias de manejo, prevención, profilaxis, tratamiento y

diagnóstico en el marco de las políticas de salud pública dirigidas a la mitigación de este tipo de eventos. Teniendo en cuenta lo anterior, el presente estudio tuvo como objetivo, evaluar cambios en los perfiles de microbioma en humanos y modelos animales infectados por protozoos y helmintos y sus potenciales implicaciones en las interacciones parásito-hospedero-microbioma. Como objetivos específicos, los cuales son abordados en cada uno de los capítulos que serán descritos a continuación, se establecieron, (1) Determinar la frecuencia de protozoos intestinales (*Blastocystis, Giardia, Cryptosporidium*, complejo *Entamoeba histolytica/dispar/moshkovskii*) y helmintos (*Ascaris lumbricoides, Trichuris trichiura, Uncinarias*) en una población rural del departamento del Cauca, Colombia, mediante la aplicación de técnicas moleculares (PCR - qPCR) y convencionales (microscopía). (2) Describir los cambios en la microbiota intestinal en una población rural del departamento del Cauca, Colombia con presencia de *Blastocystis* a partir de un enfoque de metabarcoding usando simultáneamente los marcadores 16S y 18S rRNA. (3) Caracterizar los cambios en el microbioma intestinal del hospedero asociados a la infección por el protozoo *Trypanosoma cruzi* en un modelo animal a partir de un enfoque metagenómico. (4) Evaluar los cambios en la microbiota del hospedero y del helminto asociados a la infección por *Ascaris suum* en un modelo animal a partir de un enfoque de metabarcoding. (5) Identificar el proteoma de los productos de excreción-secreción (ES) de diferentes etapas larvales de *Ascaris suum* (L3-huevo, L3-pulmón, L3-tráquea) a partir de un enfoque proteómico.

En el *primer capítulo*, en relación con el primer objetivo, se encontraron altas frecuencias de protozoos y helmintos intestinales en muestras de humanos, animales y agua, siendo *Blastocystis* el protozoo más frecuente. La detección en animales domésticos sugiere la posibilidad de transmisión zoonótica. Se identificaron múltiples especies de protozoos y helmintos conocidas por presentar frecuencias relevantes en humanos y animales, tales como *Blastocystis, Giardia, Cryptosporidium, Entamoeba hystolitica, Ancylostoma duodenale, Taenia solium*, y por primera vez en Colombia, se reportó la presencia de *Ancylostoma ceylanicum.* Lo anterior resalta la importancia de utilizar técnicas moleculares para la detección de estos en comunidades rurales. Los resultados indican una alta frecuencia de protozoos y helmintos intestinales y subrayan la necesidad de implementar medidas de salud pública para prevenir su transmisión, especialmente en áreas con riesgo de transmisión zoonótica y ambiental.

Respecto al segundo objetivo de este *primer capítulo*, teniendo en cuenta que se identificó que *Blastocystis* fue el protozoo más prevalente, se describieron los cambios en la microbiota intestinal de los hospederos humanos colonizados por este eucariota. Se observó que la presencia de *Blastocystis* se relacionó con una mayor diversidad de la microbiota, tanto bacteriana como eucariota. Además, se identificaron taxones diferencialmente abundantes relacionados no solo con la presencia del protozoo, sino también con su carga. Se identificó que géneros tales como *Bacteroides*, *Prevotella*, *Oscillibacter*, *Faecalibacterium* y *Alistipes* fueron diferencialmente abundantes en los individuos positivos para *Blastocystis*, encontrando adicionalmente, una relación entre la carga de *Blastocystis* y la abundancia de géneros como *Alistipes* y *Lachnospira*.

En el *segundo capítulo*, en relación con el objetivo 3, se caracterizó el efecto de la infección por *Trypanosoma cruzi* en el microbioma intestinal de un modelo murino utilizando dos cepas distintas de ratón, mediante un enfoque metagenómico. Los resultados mostraron cambios significativos en el microbioma intestinal de ambos modelos tras la infección, siendo estos cambios más pronunciados en el modelo BALB/c. Se identificaron bacterias cuya abundancia se vio alterada, incluyendo miembros de los géneros *Lactobacillus*, *Bacteroides* y *Alistipes*, entre otros. Además, los análisis de predicción funcional y ensamblaje de genomas permitieron determinar el impacto de la infección en diversos procesos metabólicos, tales como la síntesis de ácidos grasos de cadena corta, aminoácidos y procesos energéticos. Este estudio demuestra que la infección por *T. cruzi* puede alterar significativamente el microbioma intestinal, con efectos dependientes del modelo de hospedero, lo que sugiere una compleja interacción entre el parásito, el hospedero y el microbioma. Estos hallazgos podrían tener implicaciones para el desarrollo de nuevas estrategias profilácticas e incluso terapéuticas contra la enfermedad de Chagas, basadas en la modulación del microbioma.

En el *tercer capítulo*, para el cumplimiento del objetivo 4, se evaluó la microbiota asociada con diferentes etapas de desarrollo de *Ascaris* y su impacto en la microbiota del hospedero durante la migración larval en un modelo murino. Se evaluaron muestras de heces, intestino, hígado y pulmones en los días 4, 8 y 14 post-infección. El análisis de la diversidad bacteriana mediante secuenciación del 16S rRNA identificó 8,040 variantes de secuencias de amplicones (ASVs), siendo la microbiota asociada a *Ascaris* la más diversa y distinta en comparación con la del hospedero. Se encontraron géneros específicos en *Ascaris*, como *Bradyrhizobium*, *Achromobacter* y *Pseudomonas*, lo que sugiere un posible intercambio de bacterias con el hospedero durante la migración larval. Estos hallazgos proporcionan una base para investigar las dinámicas ecológicas y funcionales de las interacciones helminto-microbiota, con potenciales aplicaciones terapéuticas.

Complementariamente, para el desarrollo de objetivo 5, se realizó la identificación del proteoma de los productos excretores-secretores (ESPs) de *Ascaris suum* durante sus diferentes etapas de desarrollo larval. Los ESPs son fundamentales para la interacción del parásito con su hospedero y representan posibles blancos para el desarrollo de nuevas estrategias de control. Se identificaron un total de 1,738 proteínas en los ESPs de *A. suum*, observándose variaciones en la composición proteica a lo largo del desarrollo larval. Las proteínas identificadas participan en diversas funciones biológicas, incluyendo metabolismo, señalización celular, inmunomodulación y protección contra el sistema inmunitario del hospedero. Este análisis proporciona una visión global del proteoma de los ESPs de *A. suum* durante su desarrollo larval y podría contribuir a una mejor comprensión de la biología del parásito y de su interacción con el hospedero y su microbiota, así como al desarrollo de nuevas estrategias de control y tratamiento.

En conclusión, los resultados presentados en esta tesis doctoral subrayan la complejidad y relevancia de las interacciones entre parásitos, microbioma y huésped en diferentes escenarios biológicos y geográficos. Se evidenció una alta frecuencia de protozoos y helmintos intestinales, principalmente *Blastocystis*, ligada a condiciones deficientes de saneamiento y calidad del agua. La colonización por *Blastocystis* se asoció con una mayor diversidad bacteriana intestinal y la

presencia de taxones beneficiosos para el hospedero, lo que sugiere posibles efectos protectores en la salud intestinal. Las diferencias en la composición del microbioma entre individuos con diferentes cargas de *Blastocystis* y aquellos no colonizados subrayan una relación dosis-dependiente que, en conjunto con factores ambientales y dietéticos, puede influir en la regulación del microbioma, abriendo la posibilidad de usar *Blastocystis* como un marcador de salud intestinal en poblaciones rurales no-occidentalizadas. Los modelos murinos infectados con *Trypanosoma cruzi* evidenciaron alteraciones significativas en el microbioma y respuestas inmunológicas específicas, destacando el papel de ciertas bacterias como en la respuesta inflamatoria del hospedero y en cambios funcionales en diversas vías metabólicas como la síntesis de ácidos grasos, vitaminas y nucleótidos esenciales para el hospedero. Asimismo, el análisis de la microbiota del helminto y del hospedero durante el ciclo migratorio de *Ascaris* sugirió una potencial transferencia de bacterias desde el huésped al parásito, lo que podría influir en la supervivencia y patogenicidad del nemátodo. Estos hallazgos no solo enfatizan la necesidad de estudios longitudinales para comprender mejor las interacciones entre parásitos, microbioma y huésped, sino que también plantean nuevas perspectivas en la modulación microbiana como estrategia terapéutica para mejorar la salud de poblaciones vulnerables.

Finalmente, de manera complementaria, este proyecto doctoral buscó contribuir con las diferentes estrategias establecidas por el gobierno nacional y territorial en el marco de los lineamientos estratégicos de políticas públicas, así como también aquellas relacionadas con ciencia, tecnología e innovación. Como se ha especificado para Colombia en el *Plan Nacional de Desarrollo,* una de las metas es el fortalecimiento de las IES públicas, motivo por el que el trabajo conjunto y colaborativo llevado a cabo en el presente proyecto representa un aporte fundamental para el alcance y cumplimiento de esta meta. A nivel departamental el *Plan Estratégico de Ciencia, Tecnología e Innovación para el Departamento del Cauca (PEDCTi)* en el marco la propuesta "Visión Cauca 2020", constituye el referente sobre el cual se estructura el desarrollo de la ciencia, la tecnología y la innovación para beneficio de la sociedad en el departamento del Cauca y en general en el país. Con base en lo anterior, es claro como la contribución desde el punto de vista de la generación del conocimiento y del fortalecimiento de la educación en IES en el departamento, representa un aspecto clave en el cual el presente proyecto aporta en el avance de la innovación y desarrollo tecnológico del Cauca y en la adecuación de la sociedad a los cambios que conlleva el desarrollo científico.

Palabras clave: Microbioma, metagenómica, interacción parásito-hospedero, protozoos, helmintos, *Ascaris*, *Trypanosoma cruzi*.

4. MARCO TEÓRICO

4.1. Parasitosis por protozoos

De acuerdo con la Organización Mundial de la Salud (OMS), 1.500 millones de personas, es decir, el 24% de la población mundial presentan infecciones transmitidas por parásitos intestinales [1,2]. Estas parasitosis generalmente se encuentran asociadas a factores como la contaminación fecal del suelo, los alimentos, insuficiencia de agua potable, ausencia de saneamiento ambiental y condiciones socioeconómicas deficientes, siendo los países en vía de desarrollo los lugares en los que se reportan las mayores tasas de infección por parásitos [2,3]. Los protozoarios *Blastocystis*, *Giardia duodenalis*, *Cryptosporidium* y el complejo *Entamoeba histolytica/dispar/moshkovskii*, se caracterizan por causar importantes episodios de diarrea en niños [2,4]. El modo de transmisión de estos protozoos es a través de la vía fecal-oral después del contacto directo o indirecto con las formas infectivas [2,5,6].

Blastocystis es un género de protozoos entéricos cosmopolitas. Su prevalencia puede superar el 5% en países industrializados y alcanza hasta el 100% en países en desarrollo, como en el caso de Colombia [7,8]. Los principales síntomas clínicos relacionados con *Blastocystis* son náuseas, anorexia, flatulencia y diarrea aguda o crónica [9,10]. Así mismo, se ha establecido una asociación de *Blastocystis* con el síndrome de colon irritable y manifestaciones extra-intestinales como la urticaria [11]. Sin embargo, su rol patogénico aún es controversial ya que ha sido encontrado tanto en individuos sintomáticos como en portadores asintomáticos incluso, resultando beneficioso para el hospedero [12–17]. *Blastocystis* presenta una amplia diversidad genética, la cual ha conllevado a la subdivisión en múltiples subtipos (STs). En la actualidad se conocen al menos 40 STs [18–22]. Los ST1 al 8 infectan humanos y animales, el ST9 infecta solo humanos y los ST10 al 17 solo se han aislado en animales [10,19,22]; sin embargo, se ha demostrado que los humanos pueden estar infectados por uno o más STs de los cuales se encuentran en huéspedes no humanos, lo que establece su potencial zoonótico [22–25].

Giardia duodenalis (también conocido como *Giardia lamblia o Giardia intestinalis)* [26,27] afecta aproximadamente 280 millones de personas alrededor del mundo. En Colombia se presenta una prevalencia de *Giardia*, que oscila entre 15.4 y 61% [1,8,28]. La sintomatología producida por la infección de éste parasito es muy variable; algunos pacientes pueden presentar diarrea aguda o crónica, dolor abdominal, náuseas, vómito, deshidratación, pérdida de peso o ser asintomáticos [26,29]. Marcadores moleculares como el glutamato deshidrogenasa (*gdh*), factor de elongación alfa (efα), triosa fosfato isomerasa (*tpi*), SSU rRNA y beta giardina (*bg*) [30–32], clasifican a *Giardia* en ocho ensamblajes distintos, identificados de la A-H. Los ensamblajes A y B a su vez han sido clasificados como AI, AII, AIII, BIII y BIV e infectan principalmente a humanos [33,34].

Con relación a las especies del género *Cryptosporidium*, según datos recientes del Global Enteric Multicenter Study (GEMS) sobre la carga y la etiología de la diarrea infantil en los países en

desarrollo, estos parásitos son hoy en día una de las principales causas de diarrea moderada a grave en niños menores de 2 años [35]. Al igual que *Giardia*, esta infección fue incluida por la OMS como una enfermedad desatendida [36]. En algunas personas causa diarrea secretora y síndrome de malabsorción que puede estar acompañada de dolor abdominal, vomito, fiebre leve, malestar general, fatiga, náuseas y pérdida de apetito [6,37]. Actualmente se han identificado al menos 30 especies y 70 genotipos, de las cuales 20 han sido reportadas en humanos, siendo principalmente *Cryptosporidium hominis* y *Cryptosporidium parvum* las responsables de la mayoría de las infecciones [38,39]. Existen 10 subtipos para *C. hominis* (Ia-Ik) y 16 subtipos (IIa-Iip) para *C. parvum* [8,40]. En Colombia, se ha estimado que *Cryptosporidium* presenta una prevalencia nacional del 0.5% por microscopía, sin embargo, estudios con aproximaciones de epidemiología molecular en regiones específicas del país, como el realizado por Villamizar y cols en 2019 [38], y evidencia obtenida a partir de revisiones sistemáticas como la desarrollada por Galvan y cols en 2018 [41], han mostrado a partir de identificación por qPCR principalmente, una prevalencia entre 7,3% y 9.8%, y han permitido la caracterización de genotipos tales como *C. parvum* subtipo Iia y *C. hominis* subtipo IbA9G3R2. [1,8,38,41,42]. Adicionalmente, se ha destacado su importancia en agua y animales, donde se estima una prevalencia de 38,9% y 20,4% respectivamente, siendo esto esencial a tener en cuenta en la dinámica de transmisión [41]. Sin embargo, un aspecto relevante en la caracterización molecular y genotipificación de este protozoo está relacionado con el uso de diferentes marcadores e incluso de perfiles de MLST (Multilocus sequence typing) que puede permitir una estimación variable de la diversidad genética [40,41,43].

El género *Entamoeba* comprende varias especies, de las cuales seis pueden habitar en el intestino grueso del hombre: *E. histolytica*, *E. dispar*, *E. moshkovskii*, *E. polecki*, *E. coli* y *E. hartmannii* [44]. *Entamoeba histolytica* es considerada como patógena, causante de la amebiasis humana afectando al 10% de la población mundial y puede alcanzar hasta el 50% en zonas de centro y Sudamérica, África y Asia. En el mundo, cerca de 500 millones de infecciones se le atribuyen a *E. dispar* y para *E. moshkovskii* se ha estimado una prevalencia entre 2.2% y 61.8% [45–47]. En Colombia, la identificación molecular por qPCR ha permitido estimar una prevalencia de *E. histolytica* cercana al 1% y así mismo, otros estudios epidemiológicos han descrito especies como *E. moshkovskii* principalmente en poblaciones rurales [8,38,46–48].

Una de las grandes dificultades que se presenta en el diagnóstico de estos parásitos intestinales es la relativa y limitada especificidad y sensibilidad de la microscopía, además de la inestabilidad de algunos parásitos protozoarios que tienden a deteriorarse rápidamente fuera del huésped. Así mismo, estas determinaciones dependen de la experticia del operador y del tipo de parásito [49]; por tal motivo, es una necesidad inmediata la implementación de técnicas moleculares como la PCR y qPCR que permitan diagnosticar especies y subpoblaciones circulantes de estos parásitos en poblaciones vulnerables, con el fin de obtener mediciones precisas de la prevalencia e intensidad de las infecciones en las zonas endémicas [15,49].

4.2. Parasitosis por Helmintos

La OMS estima que la infección por helmintos afecta aproximadamente a 1500 millones de personas, principalmente en zonas tropicales y subtropicales. En América Latina se calcula que 46 millones de niños en edad escolar están en riesgo de contraer infecciones por helmintos [50].

Dentro del grupo de las helmintiasis, existe un subgrupo que se caracteriza por que su transmisión se da a través el contacto con suelo contaminado con huevos larvados o las larvas de los helmintos (geohelmintiasis). Las especies de helmintos transmitidos por el suelo que infectan al ser humano y que representan un gran interés en salud pública son *Ascaris lumbricoides, Trichuris trichiura* y las uncinarias (*Necator americanus* y *Ancylostoma duodenale*) [1,2,51]

Ascaris lumbricoides es un nemátodo que se encuentra principalmente en zonas tropicales y templadas del mundo [2,15]. En Colombia la prevalencia estimada de infección por este parásito varía de acuerdo con la población. Estudios realizados en Cauca, Cali y Turbaco determinaron prevalencias de 32%, 8.3% y 16.7% respectivamente [52,53]. La encuesta Nacional de Parasitismo encontró que la prevalencia de este parásito fue del 11.3% [1]. Morfológicamente, los huevos de diferentes tipos de helmintos son muy similares lo cual dificulta su identificación por microscopia [54].

En el caso de *Trichuris trichiura,* se estima que infecta a unos 450 millones de personas, en su mayoría niños en edad escolar [2,55,56]. Aunque muchos casos muestran solo síntomas leves o incluso son asintomáticos, la tricuriasis aún tiene importantes consecuencias para la salud (Knopp et al., 2012). De acuerdo a la Encuesta Nacional de Parasitismo, *T. trichiura*, fue el helminto más prevalente (18.4%) [1].

Las uncinarias de importancia médica que parasitan el ser humano y causan morbilidad son: *Necator americanus* y *Ancylostoma spp.* [1,2]. Los pacientes con uncinariasis suelen ser asintomáticos; sin embargo, se ha descrito que la infección crónica causada por estos parásitos puede generar anemia [58], diarrea, dolor abdominal de tipo cólico, náuseas, y eosinofilia [59]. En Colombia se estima que la prevalencia de uncinariasis es aproximadamente del 6% [1].

4.3. Importancia de las infecciones causadas por *Ascaris*

Las infecciones por *Ascaris* son una de las más frecuentes en el mundo y es considerada también como una Enfermedad Tropical Desatendida [2,50,55,60]. La prevalencia de las infecciones por helmintos supera el 10% en países en vía de desarrollo y un gran porcentaje de estas, está dado por las infecciones por *Ascaris lumbricoides*, principalmente en ciertas regiones de África y Sur América [2,55,56,60]. Se ha relacionado principalmente con desnutrición, retraso en el desarrollo por infecciones persistentes y se estima que causa cerca de 60,000 muertes anuales en población infantil principalmente [2,55,56,61,62]. Así mismo, este helminto es de gran relevancia desde la perspectiva del control y prevención. Entre otras cosas, factores como la gran resistencia de los

huevos de este parásito en el suelo húmedo, incluso por varios años, las reinfecciones pese al manejo terapéutico con antihelmínticos y la no disponibilidad de vacunas, se asocian con una alta dispersión de *Ascaris* no sólo en hospederos humanos sino también en otro tipo de hospederos [63–65]. Adicionalmente, además de la afectación generada en la salud humana por *A. lumbricoides*, existe una alta prevalencia en cerdos de la especie *Ascaris suum*, que ocasiona un proceso infeccioso muy similar al dado en el humano, generando grandes pérdidas económicas en el sector porcícola [66–71]. Lo anterior, adquiere aún mayor relevancia cuando se considera que diferentes estudios han encontrado a partir de análisis genómicos, mitocondriales y del genoma completo, que *A. suum* y *A. lumbricoides* pueden formar un complejo genético que es capaz de entrecruzarse, lo cual puede asociarse a un gran potencial de transmisión zoonótica y con ello, se destaca la necesidad de un mejor manejo de estas infecciones en porcinos bajo un enfoque de One Health para controlar de esta forma la propagación de la ascariasis humana [2,69,72,73].

4.4. Infecciones sistémicas por el protozoo *Trypanosoma cruzi*

El protozoo flagelado *T. cruzi* es el agente causal de la enfermedad de Chagas, enfermedad que afecta aproximadamente 6.3 millones de personas en el mundo [74–77]. En Colombia se estima que alrededor de 130,000 personas se encuentran infectadas con el parásito, con una incidencia de 7.69 por cada 100,000 habitantes [77,78]. Además, este parásito se encuentra asociado a diferentes manifestaciones clínicas y escenarios eco-epidemiológicos zoonóticos y enzooticos, hallándose desde el sur de Estados Unidos hasta Argentina y Chile [76,77,79,80].

T. cruzi posee un complejo ciclo de vida; se han descrito cuatro etapas morfológicas y funcionales bien diferenciadas: tripomastigotes procíclicos, tripomastigotes metacíclicos, amastigotes y epimastigotes. El ciclo de vida transcurre entre hospederos mamíferos, humanos y reservorios selváticos, donde se pueden encontrar las formas amastigotes y tripomastigotes, y entre vectores de la familia Reduviidae, quienes transmiten el parásito, en formas epimastigotes y tripomastigotes metacíclicos infectivos [76,81]. En el hospedero mamífero, se generan dos fases importantes, la primera corresponde a la infección de las células mononucleares de sangre periférica por parte de los tripomastigotes metacíclicos provenientes del vector, y la segunda, corresponde a la infección de células específicas (cardiacas, enterocitos, adipocitos, tejido nervioso), por tripomastigotes procíclicos liberados a partir de las células mononucleares, que pueden provocar importantes afectaciones a nivel intestinal y cardiaco [74,75,77,82,83].

La enfermedad de Chagas se caracteriza por dos fases, una aguda y una crónica. Se considera fase aguda a la etapa en la que aparecen o no los síntomas y en la que además, el paciente presenta tripomastigotes circulantes en sangre periférica [76,77]. En la enfermedad de Chagas, independientemente del mecanismo de transmisión, los síntomas aparentes se presentan entre los 8 y 10 días después de la invasión parasitaria o entre 20 a 40 días cuando la transmisión es por transfusión de sangre infectada. Se puede presentar fiebre prolongada, malestar general, astenia, irritabilidad, cefalea, convulsiones, hepatoesplenomegalia, edema subcutáneo local o

generalizado, miocarditis severa o se puede llegar a producir la muerte por insuficiencia cardiaca [76]. Posterior a la fase aguda, hasta un 30% de los pacientes manifiestan síntomas entre 10 a 30 años post-infección, principalmente de compromiso cardiaco, como arritmias, trastornos de la conducción AV, taquicardia, fenómenos embólicos, cardiopatía dilatada. Adicionalmente, se pueden presentar manifestaciones digestivas como megaesófago, megacolon o una combinación de las anteriores (cardiodigestivas) y en algunos casos se puede presentar encefalitis, siendo más común en pacientes inmunosuprimidos [78]. Uno de los aspectos más relevantes con la prevalencia de esta enfermedad, es que se estima que más del 90% de los individuos infectados con el parásito no son diagnosticados y que sólo el 1% reciben el tratamiento etiológico específico (con benznidazole o nifurtimox) [77].

4.5. Microbioma intestinal

El microbioma del intestino humano se refiere a un complejo sistema de múltiples microorganismos, sus productos génicos y funciones fisiológicas correspondientes, que pueden ser encontrados en el tracto gastrointestinal [84]. Dentro de esta denominación se incluyen bacterias, arqueas, virus y organismos eucariotas, que mantienen una interacción constante entre ellas y con el hospedero [85]. Se ha descrito que alrededor de 100 millones de células microbianas habitan el intestino humano, las cuales en conjunto son las encargadas de suplir diversos procesos biológicos del ser humano [86]. El desequilibrio de la microbiota intestinal ha sido asociado con diversas enfermedades, como la obesidad, la malnutrición, alergia o intolerancia a cierto tipo de alimentos, las enfermedades inflamatorias intestinales y el desarrollo de cáncer de colon, enfermedades cardiovasculares, infección por *Clostridioides difficile*, entre otras [87–92]. La diversidad y composición de la microbiota son altamente dinámicas, y depende de variables tales como el estado físico del huésped, genotipo y fenotipo inmune, así como también de factores ambientales como la dieta, el uso de antibióticos y el estilo de vida [93]. Se ha descrito que las especies pertenecientes a la familia Firmicutes y Bacteroidetes son las más comunes, abarcando más del 70-75% del total de especies. Los siguientes phyla más comunes son Actinobacteria, Proteobacteria y Verrucomicrobia. Los géneros más predominantes son *Bacteroides, Clostridium, Faecalibacterium, Eubacterium, Ruminococcus, Peptidococcus, Peptidostreptococcus* y *Bifidobacterium* [84,85,94,95].

4.6. Aproximaciones metodológicas para el estudio del microbioma: metagenómica y metabarcoding

La metagenómica y el metabarcoding constituyen enfoques metodológicos esenciales en el análisis de comunidades microbianas y eucarióticas en diversos ambientes naturales y clínicos [96–99]. Ambos métodos son ampliamente utilizados en la microbiología ambiental y médica para explorar

la diversidad biológica, aunque presentan diferencias significativas en cuanto a su teoría subyacente, alcance de la información obtenida y aplicaciones [96–99].

La metagenómica, particularmente en su modalidad de secuenciación shotgun, se refiere a la secuenciación aleatoria del ADN extraído directamente de una muestra ambiental [97,99]. Esta metodología permite la caracterización exhaustiva del contenido genético total presente en la comunidad microbiana, sin la necesidad de aislamiento y cultivo de los organismos. La secuenciación aleatoria de fragmentos genómicos facilita la identificación de los distintos taxones presentes, además de ofrecer información sobre los genes funcionales y las rutas metabólicas potencialmente activas en el ecosistema. Por lo tanto, la metagenómica, a partir de un robusto esquema de análisis bioinformático, no solo permite describir la composición taxonómica, sino también explorar la capacidad funcional y adaptativa de la microbiota y reconstruir genomas completos a partir de datos metagenómicos (MAGs) [97,99–101]. La relevancia de la metagenómica reside en su capacidad para descifrar la complejidad de los microbiomas de ambientes complejos como el intestino humano, el suelo o los ecosistemas acuáticos. Este enfoque es crucial para estudiar aspectos como la resistencia a los antimicrobianos, la interacción huésped-microbioma y la funcionalidad metabólica de comunidades microbianas, aportando una perspectiva integral que combina taxonomía con función biológica. La metagenómica ha ampliado significativamente el conocimiento sobre la ecología microbiana y la diversidad genética [97,99].

Por otro lado, el metabarcoding o metataxonómica es una técnica de secuenciación dirigida que utiliza genes marcadores filogenéticos para la identificación taxonómica de microorganismos en una muestra [97,102]. En microbiología, los genes del ARN ribosomal 16S y 18S son comúnmente utilizados para bacterias y arqueas, y para eucariotas, respectivamente, debido a la conservación de sus regiones de bajo y alto grado de variabilidad [97,103]. La amplificación por PCR de estas regiones permite su secuenciación masiva, proporcionando una caracterización taxonómica precisa de la comunidad. Sin embargo, al centrarse en secuencias genéticas específicas, el metabarcoding no proporciona información sobre el contenido funcional del genoma de los organismos detectados ni permite el ensamblaje de genomas de estos microorganismos [97,103]. La importancia del metabarcoding radica en su capacidad para realizar estudios de manera eficiente y costo-efectiva, con una menor necesidad de datos de secuenciación en comparación con la metagenómica. Se utiliza en estudios ecológicos para caracterizar la composición de comunidades microbianas y eucarióticas en distintos ambientes, siendo útil para la identificación de especies y la evaluación de biodiversidad. No obstante, su limitación principal es la falta de información sobre funciones biológicas y rutas metabólicas [97,103].

Las diferencias clave entre la metagenómica y el metabarcoding se encuentran en la naturaleza de los datos obtenidos y el alcance de su análisis. Mientras que la metagenómica proporciona una descripción holística de la comunidad microbiana al incluir tanto la identificación taxonómica como el análisis funcional a nivel genómico, el metabarcoding se limita a la identificación de organismos mediante genes marcadores sin aportar detalles sobre su capacidad funcional. La metagenómica implica un mayor coste y complejidad técnica debido a la necesidad de análisis

bioinformáticos más avanzados y a una mayor profundidad de secuenciación, en contraste con el metabarcoding, que es más accesible y ampliamente utilizado para estudios específicos de biodiversidad taxonómica. La integración de ambos enfoques permite obtener una comprensión más completa de la ecología y función de los microbiomas en diferentes contextos ambientales y clínicos [97,102,104,105].

4.7. Interacciones Protozoos Intestinales-Hospedero-Microbiota

Diferentes protozoos han sido usados como organismos modelo para estudiar las interacciones fprotozoo-hospedero-microbioma a partir de aproximaciones descriptivas en seres humanos, experimentales *in vitro* y con el uso de modelos animales [106].

En el caso de *Blastocystis*, existe evidencia obtenida a partir de estudios descriptivos en seres humanos, de que la presencia de este protozoo puede estar relacionada con una composición de microbiota "normal" o "saludable", favoreciendo un ambiente anaerobio y anti-inflamatorio, donde se destaca una mayor abundancia de taxones del género *Faecalibacterium*, importantes en el mantenimiento de la salud intestinal [16,17,107,108]. Sin embargo, estudios realizados con subtipos como el ST7, permitieron evidenciar in vitro, que este subtipo conduce a un desequilibrio de la microbiota intestinal que podría favorecer la aparición de síntomas clínicos y que puede estar asociado a la fisiopatología de la infección [109].

Para el caso de *Giardia*, se ha planteado la hipótesis de que en humanos la infección por este protozoo desencadena alteraciones permanentes de los microorganismos comensales, generando un desequilibrio en la microbiota intestinal y promoviendo la invasividad bacteriana en la mucosa intestinal [110–112]. Incluso se ha evaluado el potencial *in vitro* de ciertos taxones bacterianos para impedir el establecimiento de la infección en el caso de *Giardia*, donde se encontró que el uso de *Lactobacillus johnsonii* inhibe la adhesión de este protozoo a las células, evitando el progreso de la infección, fortaleciendo de esta manera el hecho de que el restablecimiento de la microbiota saludable a partir del uso de probióticos puede jugar un papel clave en nuevos esquemas de profilaxis y tratamiento [113].

Respecto a *Entamoeba histolytica* se ha evidenciado que el impacto que puede generar a nivel de la microbiota intestinal por acción enzimática y de adhesión puede provocar cambios en ciertos grupos bacterianos que han sido incluso considerados como marcadores de infección y de predicción del desarrollo clínico de la enfermedad. En relación con esto Morton y cols en 2015 propusieron que la presencia de *Entamoeba* se correlacionaba significativamente con la composición y diversidad del microbioma y que la colonización por este protozoo se podía predecir con un 79 % de precisión en función de la composición de la microbiota intestinal de un individuo [114]. Así mismo, Gilchrist y cols en 2016 plantearon que una alta carga de parásitos junto con el aumento de la abundancia de *Prevotella copri* se asociaron clínicamente con enfermedad diarreica, representando importantes predictores del desarrollo clínico del proceso

infeccioso [115]. Estudios como estos, recalcan la importancia de enfocar investigaciones en este contexto que puedan complementar parámetros profilácticos y diagnósticos en infecciones causadas por protozoos.

Se han efectuado también análisis en relación con *Cryptosporidium,* donde se ha mostrado que puede provocar un desequilibrio en la microbiota, principalmente a partir de la alteración de bacterias como *Escherichia coli, Bacillus spp. y Clostridium spp*, disminuyendo concomitantemente las concentraciones fecales de indol, lo cual puede estar relacionado con la acción de este metabolito sobre los tejidos del huésped para mejorar la respuesta innata aumentando la integridad epitelial y/o estimulando las vías antiinflamatoria. Estos datos indican que algunas bacterias, pueden influir en la capacidad de *Cryptosporidium* para establecer una infección y así mismo que los niveles de indol preexistentes en el intestino junto con la carga parasitaria y el estado inmunitario del hospedero pueden ser factores importantes que determinan el resultado de la exposición a *Cryptosporidium* [116,117] .

Es claro como los estudios que han buscado evaluar interacciones protozoo-hospedero-microbiota han destacado la contribución del microbioma intestinal a la variación clínica en las infecciones por protozoos parasitarios. Así mismo, los hallazgos han facilitado la comprensión parcial de estas interacciones y de ciertos mecanismos involucrados, entre los que pueden nombrarse: (I) alteración de la virulencia del parásito mediados por comunidades bacterianas específicas; (II) inducción de cambios y alteraciones que provocan un desequilibrio en la composición de la microbiota intestinal; (III) modulación de la inmunidad del huésped al protozoo parásito [118]. Lo anterior ofrece un marco de trabajo muy interesante ya que el impacto de la infección parasitaria a nivel de la microbiota intestinal puede generar firmas únicas que puede ser esenciales en el campo diagnóstico y que a su vez pueden suministrar información clave en el diseño de nuevas estrategias en el contexto clínico y de salud pública.

4.8. Interacciones Protozoo-Hospedero-Microbioma: *Trypanosoma cruzi*

En el caso de *T. cruzi* se han efectuado diferentes estudios que han permitido identificar que la composición de la microbiota intestinal puede influir en fisiopatología de las infección parasitarias modificando el equilibrio entre el huésped y sus comensales, interrumpiendo la homeostasis del ecosistema presente, además de beneficiarse del metabolismo de nutrientes y sustratos para la obtención de energía necesaria para el cumplimiento de procesos fisiológicos parasitarios involucrados en su ciclo de vida y supervivencia [119–123]. Así mismo, como en el caso de otros protozoos, se ha demostrado en modelos murinos que el mantenimiento de la homeostasis de los microorganismos comensales es fundamental en la respuesta inmune mediada en buena parte por las células dendríticas, de manera tal que la pérdida del equilibrio de estas comunidades puede influir en la susceptibilidad del hospedero a la infección [123–129].

Se han implementado aproximaciones descriptivas en seres humanos y ciertos ensayos experimentales a nivel de modelos murinos en relación con las infecciones por *Trypanosoma cruzi* y su interacción con la microbiota del hospedero. Robello y cols en 2019 encontraron que la presencia de *T. cruzi* generaba alteraciones en la microbiota intestinal en niños y que estos cambios desaparecían con el tratamiento antiparasitario, sugiriendo que el efecto de la infección sobre la microbiota intestinal podría ser más importante que el efecto del tratamiento y de cómo el restablecimiento de la microbiota estaba asociado a la resolución de la infección [130]. Estudios en modelos murinos como el realizado por McCall y cols en 2018, que incluyó estudios a nivel de microbiota y metaboloma, indicaron que la infección por este protozoo efectivamente modula el microbioma intestinal provocando cambios funcionales en el entorno químico del intestino que podrían estar afectando a las respuestas inflamatorias del huésped, lo que al final puede tener un impacto en la fisiopatología de la enfermedad de Chagas, particularmente en formas crónicas digestivas [131]. En este sentido se han evaluado los cambios en la microbiota en formas cónicas tanto indeterminadas, cardiacas y digestivas y se ha observado que la infección por *T. cruzi* da como resultado un cambio en el microbioma intestinal, provocando un desequilibrio caracterizado por menores abundancias de microorganismos asociados con la salud como *Akkermansia y Lactobacillus,* importantes en la producción ácidos grasos de cadena corta y en el mantenimiento del pH intestinal [132]. Un estudio de gran relevancia en este campo fue el realizado por Hossain y cols en 2020 en un modelo murino, donde se evaluaron aspectos relacionados con la microbiota y metaboloma a partir del uso de cepas bioluminiscentes de *T. cruzi* que además facilitaron establecer una mapeo espacial de la infección y de cómo esto se relacionaba con los cambios provocados por la presencia del parásito [123]. Allí se logró determinar que, tanto en formas agudas como crónicas, *T. cruzi* tiene un importante tránsito gastrointestinal y que afecta en términos de carga parasitaria, de microbiota y de metaboloma de manera diferencial ciertas porciones anatómicas, revelando aspectos fundamentales en la transición de formas agudas a crónicas y relacionadas con la persistencia del protozoo. Adicionalmente, se pudo observar que el esófago, el colón y el intestino grueso son regiones donde además de encontrar altas cargas parasitarias, fueron también donde se generaron mayores cambios en la composición de la microbiota y el metaboloma, lo cual resalta la importancia de estos cambios asociados a la fisiopatología de la enfermedad de Chagas, principalmente asociada a megaesófago y megacolon [123]. Se encontró que el tropismo por estas regiones puede estar vinculado con las manifestaciones crónicas de esta enfermedad y que esta persistencia se encuentra fuertemente relacionada con los cambios permanentes generados a nivel de la microbiota del hospedero en estas regiones anatómicas específicas, donde adicionalmente se encontraron alteraciones funcionales asociadas a la disminución de aminoácidos aromáticos como el triptófano (precursor de la quinurenina) y la tirosina [123]. Con base en lo anterior, los autores sugieren que los cambios en la composición de la microbiota, en conjunto con la disminución de estos metabolitos durante la infección aguda, puede responder al hecho de que los metabolitos de quinurenina tienen efectos antiparasitarios directos y contribuyen al control de la infección aguda por *T. cruzi* lo cual puede estar favoreciendo el establecimiento de la infección en esta fase inicial. Sin embargo, en la

infección crónica no se observó dicha disminución de estos aminoácidos, lo cual puede relacionarse con el hecho de que además de lo anterior, la quinurenina tiene la capacidad de activar células T reguladoras, motivo por el que en formas crónicas, esta modificación puede contribuir a la persistencia del parásito a partir de la inmunomodulación [123].

4.9. Interacciones Helmintos-Hospedero-Microbioma

Las interacciones entre los helmintos y la microbiota intestinal del hospedero puede dilucidar aspectos claves en la biología y fisiopatología de estos parásitos, razón por la cual se han buscado comprender los mecanismos que están involucrados en esta relación (Figura 1). La mayoría de estudios han estado dirigidos a la comprensión de las interacciones entre los helmintos y el sistema inmunológico del hospedero ya que se ha evidenciado la gran capacidad inmunomoduladora e inmunoreguladora que tienen estos parásitos y que les permiten infectar, diseminarse y persistir en el huésped infectado [133–139]. Sin embargo, se ha observado cómo las infecciones por helmintos ocasionan cambios en la composición de la microbiota intestinal lo cual juega un papel clave en la biología del parásito y en la dinámica de la infección [139–143]. Los cambios en la abundancia relativa de ciertos taxones bacterianos y los cambios diversidad de la microbiota intestinal frente a la presencia de helmintos, se han vinculado con efectos en el ciclo de vida del parásito, con cambios metabólicos que promueven la infección y diseminación y con alteraciones en la respuesta inmune del hospedero que pueden estar relacionados con la persistencia de este tipo de infecciones [64,66,112,144–158]. La comprensión de los mecanismos a partir de los cuales la presencia de estos parásitos influye en los cambios en la microbiota intestinal del hospedero son objeto de gran interés, ya que abren un camino muy relevante en la búsqueda nuevas estrategias de control y de alternativas terapéuticas.

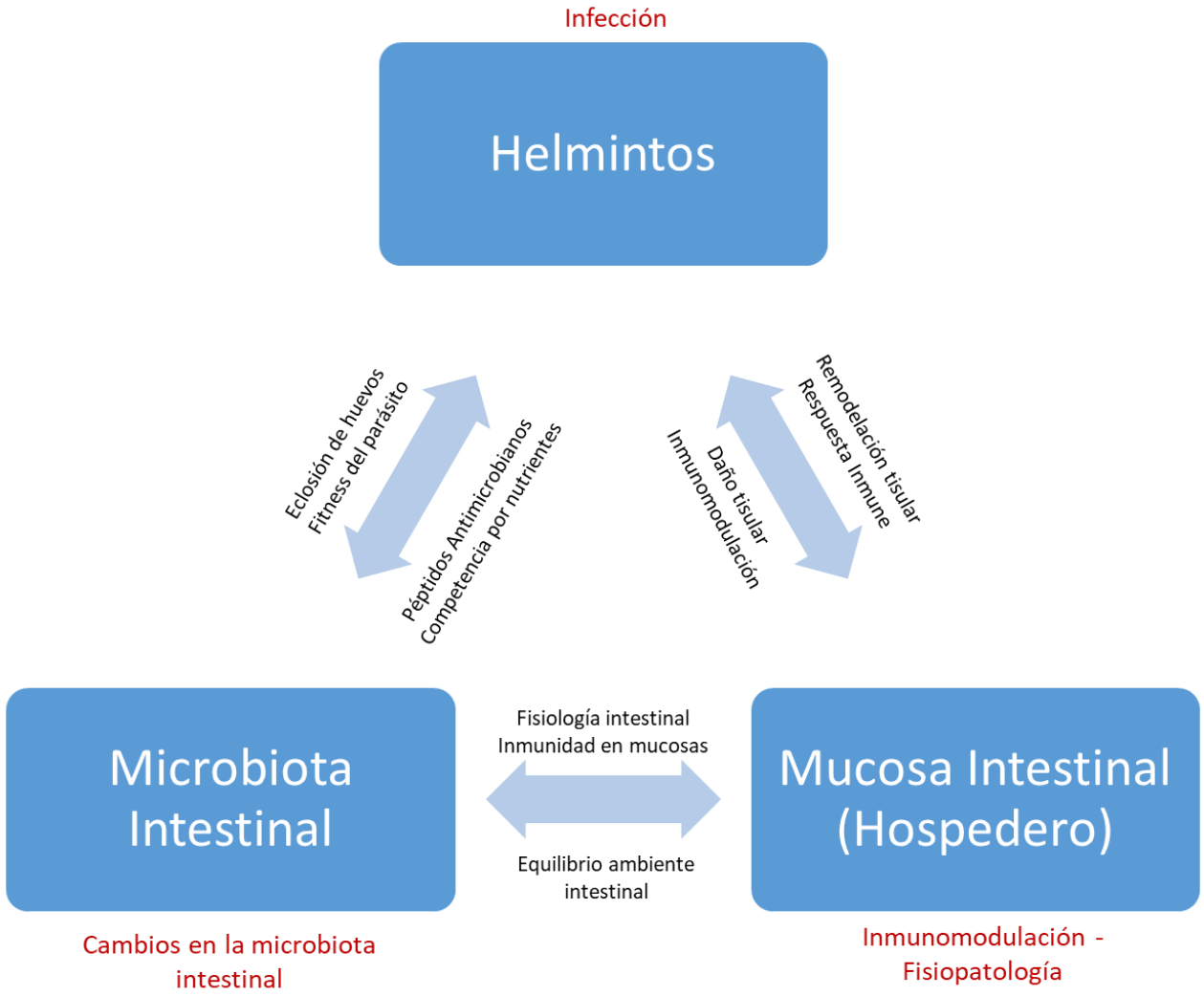

Figura 1. Modelo de influencia recíproca de interacciones huésped-parásito-microbiota. **Fuente:** Adaptado de Cortés *et al* 2018.

Los estudios de la microbiota en relación con la presencia de helmintos han mostrado resultados heterogéneos, principalmente en los estudios descriptivos realizados en humanos, esto debido a que el contexto geográfico, las condiciones socioeconómicas y de higiene, la alimentación, entre otros factores, pueden influir en la composición de la microbiota y en la respuesta a la infección [139,159,160]. Por lo anterior, se ha optado por complementar estos hallazgos a partir de la utilización de modelos animales, principalmente cerdos y ratones, que permitan reflejar las infecciones en seres humanos y de esta manera poder tener un mayor control del proceso infeccioso y de aquellas variables que influyen en la composición de la microbiota intestinal del huésped [129,152,161–164].

Los estudios realizados en torno a las interacciones *Ascaris*-Microbiota-Hospedero han sido también desarrollados a partir de diseños descriptivos en poblaciones humanas y con base en modelos animales. Una particularidad en el caso de *Ascaris* es que, durante su ciclo de vida, posterior a la infección dada por la ingestión de huevos que eclosionan dentro de las 3 horas siguientes a la ingesta, las larvas (L3) tienen la capacidad de invadir el tejido mucoso de tracto gastrointestinal donde pueden realizar posteriormente una migración hacia tejidos hepáticos y pulmonares [165,166]. En el pulmón, la infección desencadena una inflamación caracterizada por la infiltración de eosinófilos generando un complejo de síntomas denominado síndrome de Loeffler en pacientes humanos [166]. Estas características en la migración de ciertos estadios del parásito se han vinculado con posibles mecanismos de alteración de la composición y estructura de la microbiota intestinal ya que, por ejemplo, se puede promover la translocación de bacterias sobre las barreras en el intestino y, poco después, también a nivel de la mucosa pulmonar [167].

En un estudio realizado en población ecuatoriana por Cooper y cols, los autores presentan la hipótesis de que la infección por *Ascaris lumbricoides* genera un impacto a nivel de la microbiota intestinal del hospedero [155]. Allí se plantea que la infección única por *Ascaris lumbricodes* o en coinfección con otros helmintos, puede generar un perfil de microbiota alterado caracterizado por abundancias relativas más bajas de ciertos taxones, predominantemente de la clase Clostridia y más altas de bacterias del género *Streptococcus*. Así mismo, sugieren que la presencia de este helminto se relaciona con una menor diversidad en la microbiota del huésped [155]. Lo anterior fue explorado y corroborado en un modelo animal porcino donde se utilizó el helminto *Ascaris suum* [168]. En dicho estudio, se demostró que la infección redujo significativamente la diversidad de la microbiota intestinal de los cerdos y que este efecto fue independiente de la carga del parásito. Así mismo, se logró determinar una abundancia relativa diferencial asociada a la infección de al menos 49 géneros bacterianos, dentro de los que se destacan *Prevotella* y *Faecalibacterium* [168]. Adicionalmente, se observó la presencia de cambios en el perfil metabólico de los cerdos infectados por el helminto, observando una disminución en el metabolismo de carbohidratos y de aminoácidos [161]. Como en otros casos, existen resultados heterogéneos entre algunos estudios. Así por ejemplo, a pesar de que Wang y cols [168] identificaron una mayor diversidad en la microbiota intestinal de los porcinos infectados por *Ascaris suum*, un estudio previamente realizado por Williams y cols, determinó que la infección por *Ascaris* modifica la microbiota intestinal del huésped, pero en este caso sugiriendo un aumento en la diversidad de esta con la concomitante reducción en la abundancia relativa de los géneros *Lactobacillus*, *Ruminococcus* and *Catenibacterium* [70].

Lo anterior resalta la importancia de realizar tanto estudios en poblaciones con condiciones específicas a partir de diseños epidemiológicos robustos y como aquellos basados en modelos animales en los cuales se puedan controlar la mayor cantidad de variables asociadas a la infección y que puedan impactar en cambios en la microbiota intestinal del hospedero y así mismo, la necesidad de establecer protocolos experimentales y de análisis bioinformáticos reproducibles que permitan validar los resultados obtenidos en estas investigaciones.

Uno de los aspectos que también ha sido considerado en las interacciones *Ascaris*-microbiota-hospedero, es el hecho de como la infección por este parásito puede generar una susceptibilidad a la infección por otros agentes patógenos, principalmente de tipo bacteriano. En modelos porcinos se ha evidenciado como la infección por *Ascaris suum* [168] y por *Trichuris suis* [146] se relacionan con una mayor abundancia relativa del género *Campylobacter*, lo cual sugiere que las infecciones por estos helmintos pueden aumentar el riesgo de infecciones secundarias causadas por bacterias, razón por la que este representa un campo de estudio fundamental en el aspecto clínico y de salud pública [168].

4.10. Interacciones *Ascaris*-Hospedero-Microbioma

Se han propuesto diversos mecanismos potencialmente involucrados en la interacción de *Ascaris* con la microbiota del hospedero [143,169]. En las infecciones por *Ascaris* se han identificado productos secretados por este helminto que promueven la modulación de la respuesta inmune y que pueden estar relacionados con las interacciones indirectas con la microbiota intestinal del hospedero. Se han identificado productos E/S, vesículas extracelulares y otros metabolitos que pueden interactuar de maneras diferentes con la microbiota del huésped y que pueden jugar un papel clave en la biología del parásito y en la fisiopatología de la infección [71]. Se ha destacado además, la relevancia que puede tener la microbiota propia del helminto en esta interacción, siendo uno de los campos hasta ahora menos estudiado [157,170,171]. Se ha confirmado en este sentido que la presencia del helminto *Ascaris suum* tiene la capacidad de inducir en el hospedero la producción de péptidos antimicrobianos de tipo ASABF (*Ascaris suum* antibacterial factor) [66,172] y de péptidos microbianos de la familia de la cecropinas (P1 a P4) que han demostrado tener un efecto bactericida contra una amplia gama de microbios, tales como bacterias grampositivas (*Staphylococcus aureus , Bacillus subtilis y Micrococcus luteus*) y gramnegativas (*Pseudomonas aeruginosa , Salmonella typhimurium , Serratia marcescens* y *Esherichia coli*) [173]. Lo anterior resalta la capacidad de *Ascaris* de producir metabolitos que pueden ejercer un efecto sobre la microbiota intestinal del hospedero, siendo en la actualidad esta producción de diferentes metabolitos, uno de los mecanismos más estudiados en esta y otras infecciones por helmintos.

Las características de la infección por *Ascaris* que incluyen la migración de los diferentes estadios del parásito, la modulación de la respuesta inmune y en este caso particular, los cambios que puede tener sobre la microbiota del huésped, en conjunto conforman una compleja red de mecanismos que le permiten el parásito establecer la infección y sobrevivir en el hospedero. Estudios en seres humanos y en modelos animales han permitido identificar como *Ascaris* tiene la capacidad de generar cambios en la estructura y composición de la microbiota del huésped por medio de la interacción directa o a partir de producción de metabolitos y diferentes productos de E/S, como los péptidos antimicrobianos, que pueden reestructurar las poblaciones bacterianas predominantes. Lo anterior, tiene un efecto negativo sobre bacterias anaerobias estrictas esenciales para el adecuado

funcionamiento intestinal como *Faecalibacterium* [168] y *Ruminococcus* [70], con el concomitante aumento de anaerobios facultativos como *Streptococcus* [155], lo cual en conjunto con la inmunomodulación generada por este helminto, le permite propiciar un ambiente metabólico adecuado para su supervivencia y reproducción. De esta manera, es evidente que las alteraciones y el efecto a nivel de la microbiota del hospedero tienen un papel fundamental en la biología y fisiopatología del parásito y abre un campo muy interesante de estudio en la búsqueda de alternativas terapéuticas de las infecciones por *Ascaris* a partir del restablecimiento de la microbiota normal del huésped. Así mismo, es importante destacar que la capacidad inmunomoduladora que presenta este helminto y el impacto sobre la microbiota del huésped puede generar una mayor susceptibilidad de sufrir infecciones o coinfecciones por otros agentes bacterianos que pueden ser potencialmente perjudiciales para el hospedero siendo esto de gran relevancia desde el punto de vista clínico y de salud pública.

4.11. Selección de Modelos Biológicos para el Estudio de las Interacciones Parásito-Hospedero-Microbioma

La elección de parásitos intestinales (particularmente *Blastocystis)*, *Trypanosoma cruzi* y *Ascaris suum* como modelos biológicos para el estudio de las interacciones parásito-hospedero-microbioma se fundamenta en su relevancia epidemiológica, diversidad biológica y capacidad para modelar interacciones complejas [2,61,77]. El enfoque integrador que representan estos organismos abarca protozoos intestinales, sistémicos y helmintos, ofreciendo una visión comprensiva de cómo los parásitos pueden modular la composición y función del microbioma en diversos contextos de infección.

Blastocystis, un protozoo intestinal altamente prevalente en humanos, históricamente se ha asociado con alteraciones gastrointestinales. Sin embargo, estudios recientes sugieren que podría tener un papel beneficioso en la promoción de la salud intestinal y cardiometabólica del hospedador [9,16,174]. Este organismo modula de manera favorable la composición del microbioma intestinal, lo que lo convierte en un modelo ideal para explorar cómo las interacciones con la microbiota influyen en la fisiología y funciones metabólicas del hospedador [175]. La capacidad de *Blastocystis* para interactuar con bacterias comensales, patógenas y otros eucariotas, sugiere un papel complejo en la regulación de la homeostasis intestinal, la fermentación de fibra dietética y la producción de metabolitos clave [16,107,176]. Además, su alta prevalencia en diversas poblaciones lo sitúa como un indicador relevante en el estudio de la diversidad y funcionalidad del microbioma intestinal [8,20,38].

Por otro lado, *Trypanosoma cruzi*, el agente etiológico de la enfermedad de Chagas, presenta un ciclo de vida complejo que involucra múltiples tejidos del hospedador, incluyendo el tracto gastrointestinal, lo que le confiere la capacidad de provocar alteraciones sistémicas y localizadas tanto metabólicas como microbiológicas que van más allá de la respuesta inmune [77,123,177]. El

desarrollo y uso de modelos murinos ha proporcionado información clave sobre las alteraciones en la composición y función del microbioma asociadas con la infección, revelando cambios en la regulación del metabolismo de lípidos y carbohidratos [123,129]. Estos estudios no solo mejoran la comprensión de la patogénesis y fisiopatología de la enfermedad, sino que también ayudan a identificar potenciales estrategias profilácticas y terapéuticas. La manipulación del microbioma durante la infección por *T. cruzi* podría tener implicaciones significativas en la modulación de la respuesta del hospedador y en la mejora de la calidad de vida de los pacientes con Chagas [177].

En el caso de *Ascaris*, un helminto con una alta prevalencia en humanos, especialmente en zonas con condiciones socioeconómicas desfavorables, se convierte en un modelo crucial para investigar tanto la respuesta del hospedador como la interacción con la microbiota del propio parásito [2,164,178]. Las infecciones por *Ascaris* inducen cambios significativos en la microbiota intestinal, afectando la fermentación de carbohidratos y la producción de ácidos grasos de cadena corta, los cuales son esenciales en la regulación del metabolismo energético y la homeostasis intestinal [112,170,179]. Adicionalmente, la microbiota asociada al helminto podría desempeñar un papel relevante en la biología del parásito y en la modulación de la respuesta del hospedador, lo cual proporciona una perspectiva única sobre cómo los factores externos e internos contribuyen al resultado de la infección. Los modelos murinos han sido ampliamente utilizados para estudiar estas interacciones y entender la dinámica funcional del microbioma frente a infecciones prolongadas [143].

Estos tres modelos biológicos permiten explorar tanto características comunes como diferencias en las interacciones parásito-hospedero-microbioma. Aunque todos impactan la composición y función del microbioma, los mecanismos específicos de interacción varían según el parásito y el entorno fisiológico. La aplicación de estrategas metagenómicas, de metabarcoding y proteómica, permite identificar cambios taxonómicos y funcionales que pueden ser claves en la biología del parásito, el metabolismo del hospedador y la dinámica del microbioma [99,180,181]. La elección de estos modelos biológicos facilita el desarrollo de protocolos experimentales y bioinformáticos aplicables a otros protozoos y helmintos, lo cual fortalece el conocimiento sobre las dinámicas presentadas en estas interacciones, proporcionando información esencial para el desarrollo de alternativas profilácticas, diagnósticas y terapéuticas.

4.12. Relevancia del estudio de las interacciones parásito-hospedero-microbioma en infecciones causadas por protozoos y helmintos

Este estudio aborda una brecha esencial en el conocimiento de las interacciones tripartitas entre parásito, hospedero y microbioma intestinal en el contexto de infecciones causadas por protozoos y helmintos de alta relevancia epidemiológica, como *Blastocystis*, *Trypanosoma cruzi* y *Ascaris suum*. A pesar de la creciente evidencia sobre el impacto de estos parásitos en la composición y

funcionalidad del microbioma intestinal, la mayoría de los estudios actuales se han centrado en análisis descriptivos de comunidades bacterianas, relegando la investigación de otros componentes del microbioma como virus y eucariotas [97]. Esto ha dejado sin explorar perfiles funcionales críticos, que podrían revelar rutas metabólicas y genes esenciales para la adaptación y persistencia de estos parásitos en el hospedero [180–182]. Además, el enfoque predominante en protozoos de transmisión fecal-oral y exclusivamente intestinales ha dejado un vacío en el análisis de helmintos intestinales y protozoos con infecciones sistémicas y tránsito entérico, como *T. cruzi*. Este sesgo es notable, dado que estos parásitos pueden ejercer una influencia directa sobre la microbiota del hospedero y contribuir a cuadros clínicos graves y debilitantes, como la forma digestiva de la enfermedad de Chagas y el megacolon chagásico, cuyos mecanismos subyacentes aún no se comprenden completamente [122]. Asimismo, en organismos como los helmintos, cuya propia microbiota puede jugar un papel clave en la interacción con el hospedero durante su ciclo de vida y sus diferentes formas larvarias, los estudios siguen siendo escasos [157,183].

Ante este escenario, la presente tesis busca contribuir al conocimiento de estas interacciones a través de un enfoque innovador que integra técnicas de metagenómica, metabarcoding y proteómica, aplicadas tanto en modelos animales como en estudios descriptivos en poblaciones humanas. Este enfoque permitirá una caracterización taxonómica exhaustiva del microbioma modificado por estos parásitos, así como la identificación de cambios funcionales clave que se producen en distintas etapas de la infección. Al examinar los cambios en el microbioma del hospedero, del helminto y sus perfiles funcionales durante la infección, este estudio proporciona elementos fundamentales en la comprensión de cómo estas alteraciones pueden estar asociadas con la progresión y el desenlace clínico de las infecciones, suministrando una base para el desarrollo de biomarcadores específicos y posibles objetivos terapéuticos.

Mediante el uso de modelos animales bajo condiciones experimentales controladas y estudios en poblaciones humanas, este trabajo se posiciona como una contribución significativa para comprender el papel de las interacciones de protozoos y helmintos con el microbioma del hospedero y su impacto en la dinámica de establecimiento, persistencia y cronicidad de infecciones parasitarias. Este conocimiento resulta fundamental para la formulación de intervenciones diagnósticas, profilácticas y terapéuticas novedosas, con impacto directo en la salud pública y en el manejo clínico de estas infecciones de importancia global.

5. OBJETIVOS

5.1. Objetivo general

Evaluar los cambios en los perfiles de microbioma en humanos y modelos animales infectados por protozoos y helmintos, y sus potenciales implicaciones en las interacciones parásito-hospedero-microbioma.

5.2. Objetivos específicos

5.2.1. Determinar la frecuencia de protozoos intestinales (*Blastocystis, Giardia, Cryptosporidium*, complejo *Entamoeba histolytica/dispar/moshkovskii*) y helmintos (*Ascaris lumbricoides, Trichuris trichiura,* Uncinarias) en una población rural del departamento del Cauca, Colombia, mediante la aplicación de técnicas moleculares (PCR - qPCR) y convencionales (microscopía).

5.2.2. Describir los cambios en la microbiota intestinal en una población rural del departamento del Cauca, Colombia con presencia de *Blastocystis* a partir de un enfoque de metabarcoding usando simultáneamente los marcadores 16S y 18S rRNA.

5.2.3. Caracterizar los cambios en el microbioma intestinal del hospedero asociados a la infección por el protozoo *Trypanosoma cruzi* en un modelo animal a partir de un enfoque metagenómico.

5.2.4. Evaluar los cambios en el microbiota del hospedero y del helminto asociados a la infección por *Ascaris suum* en un modelo animal a partir de un enfoque de metabarcoding.

5.2.5. Identificar el proteoma de los productos de excreción-secreción (ES) de diferentes etapas larvales de *Ascaris suum* (L3-huevo, L3-pulmón, L3-tráquea) a partir de un enfoque proteómico.

6. INTRODUCCIÓN A LOS CAPITULOS

6.1. Capítulo 1

Objetivo 1: Determinar la frecuencia de protozoos intestinales (*Blastocystis, Giardia, Cryptosporidium*, complejo *Entamoeba histolytica/dispar/moshkovskii*) y helmintos (*Ascaris lumbricoides, Trichuris trichiura, Uncinarias*) en una población rural del departamento del Cauca, Colombia, mediante la aplicación de técnicas moleculares (PCR - qPCR) y convencionales (microscopía).

Objetivo 2: Describir los cambios en la microbiota intestinal en una población rural del departamento del Cauca, Colombia con presencia de *Blastocystis* a partir de un enfoque de metabarcoding usando simultáneamente los marcadores 16S y 18S rRNA.

Para cumplir el objetivo 1, el primer estudio se centró en evaluar la frecuencia y dinámica de transmisión de diversos protozoos y helmintos intestinales en una comunidad rural colombiana, adoptando un enfoque integral de "Una Salud". Mediante el análisis de 125 muestras fecales, provenientes de 99 individuos humanos, 24 perros domésticos y 2 fuentes de agua, se identificó una amplia gama de protozoos y helmintos intestinales.

Para la detección de estos organismos, se combinaron técnicas microscópicas tradicionales con métodos moleculares de alta sensibilidad, como la reacción en cadena de la polimerasa cuantitativa (qPCR). Además, se empleó la secuenciación de Oxford Nanopore para caracterizar los subtipos de *Blastocystis*, lo que permitió comprender mejor la diversidad genética de este protozoo y su potencial papel en la epidemiología de las infecciones. Los resultados revelaron una alta frecuencia de estos organismos en la comunidad estudiada. El más frecuente fue *Blastocystis*, con una frecuencia del 87%. Le siguieron *Giardia* (16%), *Ancylostoma duodenale* (22%), *Ancylostoma ceylanicum* (5.6%), *Entamoeba histolytica* (4.8%), *Cryptosporidium spp.* (9.6%) y *Taenia spp.* (0.8%). Es importante destacar que se observó una elevada frecuencia de coinfecciones, con el 43% de las muestras positivas para más de un tipo de parásito. La caracterización molecular de *Blastocystis* permitió identificar una diversidad de subtipos, siendo el ST2 el más frecuente. Este hallazgo sugiere la existencia de múltiples fuentes de colonización y la complejidad de las dinámicas de transmisión de este protozoo. Además, la detección de *Cryptosporidium spp.* en muestras de agua indica la importancia de las vías hídricas en la transmisión de estos protozoos.

Los resultados de este estudio subrayan la importancia de utilizar técnicas moleculares para una detección precisa y sensible de protozoos y helmintos intestinales, especialmente en comunidades con alta prevalencia. El enfoque de "Una Salud" permitió identificar la complejidad de las interacciones entre humanos, animales y el medio ambiente en la transmisión de estos organismos. Los hallazgos obtenidos tienen importantes implicaciones para la salud pública y resaltan la

necesidad de implementar programas de control y prevención más efectivos, dirigidos a mejorar las condiciones sanitarias y promover prácticas de higiene adecuadas.

Respecto al objetivo 2, teniendo en cuenta la alta frecuencia de *Blastocystis* identificada previamente, se buscó evaluar el impacto de la colonización por *Blastocystis* y su carga en la composición de la microbiota intestinal en una comunidad rural. La investigación se llevó a cabo en la comunidad de Las Guacas, Cauca, donde se recolectaron 88 muestras fecales de individuos. Para analizar la composición de la microbiota, se empleó secuenciación de los genes 16S y 18S del ARN ribosomal, lo que permitió caracterizar tanto la microbiota bacteriana como la eucariota.

Los resultados revelaron una clara asociación entre la presencia de *Blastocystis* y cambios significativos en la composición de la microbiota intestinal. Los individuos colonizados por *Blastocystis* presentaron perfiles de microbiota distintivos en comparación con aquellos que no lo estaban, lo cual se vio reflejado en el análisis de beta diversidad. A nivel bacteriano, se observó un aumento en la abundancia de géneros como *Bacteroides*, *Prevotella*, *Oscillibacter*, *Faecalibacterium* y *Alistipes* en los individuos positivos para *Blastocystis*. Además, se encontró una correlación entre la carga de *Blastocystis* y la abundancia de géneros como *Alistipes* y *Lachnospira*, mientras que géneros como *Akkermansia* se asociaron con una menor carga parasitaria. En cuanto a la microbiota eucariota, los resultados mostraron que la presencia de *Blastocystis* se relacionó con una mayor frecuencia de *Entamoeba coli*. Por el contrario, los individuos negativos para *Blastocystis* presentaron una mayor abundancia de hongos como *Candida albicans*. Estos hallazgos sugieren que *Blastocystis* puede influir en la composición no solo de la microbiota bacteriana, sino también de la eucariota, lo que podría tener implicaciones para la salud del hospedero. Para profundizar en el análisis, se emplearon modelos de aprendizaje automático, específicamente un modelo *random forest*. Estos modelos permitieron identificar a *Faecalibacterium* y *Bacteroides* como biomarcadores potenciales para predecir la colonización por *Blastocystis*. Estos resultados sugieren que la composición de la microbiota intestinal podría utilizarse como herramienta para diagnosticar y monitorear infecciones por *Blastocystis*.

En conclusión, este estudio proporciona evidencia de que la colonización por *Blastocystis* tiene un impacto significativo en la composición y estructura de la microbiota intestinal en una población rural colombiana. Estos hallazgos abren nuevas vías de investigación para comprender mejor el papel de *Blastocystis* en la salud humana y para desarrollar estrategias terapéuticas basadas en la modulación de la microbiota. Así mismo, propone estrategias metodológicas y analíticas que pueden ser implementadas en el estudio de otros protozoos intestinales.

Como productos de este capítulo se adjuntan los siguientes artículos científicos:

Artículo 1: Castañeda, S, Acosta, Claudia Patricia, Vasquez-A, Luis Reinel, Patiño, Luz H, Mejía, Rojelio, Ramírez JD. Molecular detection of intestinal parasites in a rural community of Colombia: A one health approach to explore potential environmental–zoonotic transmission. Zoonoses Public Health [Internet]. 2024 [cited 2024 Oct 23];71(6):723–735. https://doi.org/10.1111/zph. 13138.

Artículo 2: <u>Castañeda S</u>, Stensvold CR, Andersen LOBB, Acosta, Claudia Patricia, Vasquez-A, Luis Reinel, Ramírez JD. Impact of *Blastocystis* Colonization and Burden on Gut Microbiota Composition in a Non-Westernized Rural Population from Colombia. *Sometido a PLOS Neglected Tropical Diseases (Q1).*

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ORIGINAL ARTICLE

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Molecular detection of intestinal parasites in a rural community of Colombia: A one health approach to explore potential environmental–zoonotic transmission

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Abstract

Aims: Protozoan and helminth parasitic infections pose significant public health challenges, especially in developing countries with rural populations marked by suboptimal hygiene practices and socio-economic constraints. The parasites are the etiological agents of these infections and have a notably elevated global prevalence. Therefore, this study focuses on estimating the frequency and transmission dynamics of several parasitic species, including *Blastocystis*, *Giardia*, *Cryptosporidium* spp., *Entamoeba histolytica*, *Ascaris lumbricoides*, *Trichuris trichiura*, *Taenia* spp. and hookworms, within a rural community in southwest Colombia with a particular emphasis on the One Health framework, considering environmental and zoonotic transmission potentials.

Methods and Results: This study involved the analysis of 125 samples, encompassing human participants ($n=99$), their domestic pets (dogs) ($n=24$) and water sources ($n=2$). Parasite detection was carried out utilizing a combination of microscopy and molecular techniques. Furthermore, the characterization of *Blastocystis* subtypes (STs) was achieved through Oxford Nanopore sequencing of the rRNA-18S gene. The investigation also entailed the examination of potential associations between intestinal parasitism and various sociodemographic factors. Results revealed a high frequency of parasitic infections when employing molecular methods, with *Blastocystis* ($n=109/87\%$), *Giardia* ($n=20/16\%$), *Ancylostoma duodenale* ($n=28/22\%$), *Ancylostoma ceylanicum* ($n=7/5.6\%$), *E. histolytica* ($n=6/4.8\%$), *Cryptosporidium* spp. ($n=12/9.6\%$) and even *Taenia* ($n=1/0.8\%$) detected. *Cryptosporidium* spp. was also identified in water samples. Coinfections were prevalent, with 57% ($n=70$) of samples exhibiting single-parasite infections and 43% ($n=53$) showing various degrees of polyparasitism, emphasizing the complexity of transmission dynamics. *Blastocystis* subtyping, conducted via Oxford Nanopore sequencing, revealed a diversity of subtypes and coexistence patterns, with ST2 being the most prevalent.

Conclusions: This research underscores the importance of using molecular techniques for frequency estimation, particularly emphasizing the relevance of zoonotic transmission in parasitic infections. It highlights the significance of the One Health approach in comprehending the circulation of parasites among animals, humans and

environmental sources, thereby directly impacting public health and epidemiological surveillance.

KEYWORDS
intestinal parasites, One Health, qPCR, transmission dynamics

1 | INTRODUCTION

Parasitic infections attributed to protozoa and helminths exert a profound impact on public health, notably in developing countries, including rural populations, where they are intricately associated with suboptimal hygiene practices and socio-economic status (Cimino et al., 2015; Pullan et al., 2014; Sánchez et al., 2017; Villamizar et al., 2019). Prominent among these are *Giardia*, *Cryptosporidium* spp., *Entamoeba histolytica* and *Blastocystis*. Despite the potential for subclinical infections, these protozoa are frequently implicated in disease pathogenesis, with a particular emphasis on diarrheal illnesses, especially among paediatric populations (Galvan-Diaz et al., 2020; Sánchez et al., 2017; Sponseller et al., 2014; Wawrzyniak et al., 2013). Conversely, helminths encompass a diverse spectrum of parasitic organisms, such as *Ascaris lumbricoides*, *Trichuris trichiura* and hookworms (*Ancylostoma duodenale*, *Ancylostoma ceylanicum* and *Necator americanus*), spanning from asymptomatic infections to conditions characterized by diarrheal afflictions, malabsorption syndromes, malnutrition and stunted growth (Karo-Atar et al., 2023).

According to the World Health Organization (WHO), an estimated 3.5 billion individuals were afflicted by parasitic infections in previous years (Cooper et al., 2013; Specht & Keiser, 2022). These parasitic infections are commonly associated with factors such as faecal contamination of soil and food, limited access to clean water, inadequate environmental sanitation and impoverished socio-economic conditions. It has also been identified that there is a zoonotic potential involved in transmission dynamics (Lebov et al., 2017). Protozoa are renowned for their capacity to induce significant diarrheal episodes, particularly among children (Cimino et al., 2015; Villamizar et al., 2019).

Blastocystis is a globally distributed genus of enteric protozoa, with prevalences from 30% to 60% in developing countries. In Colombia, the reported prevalence stands at 52.1%, and molecular studies have even shown prevalences of 100% (Higuera et al., 2020). However, the pathogenic role of *Blastocystis* remains a topic of debate (Audebert et al., 2016; Castañeda et al., 2020; Stensvold & van der Giezen, 2018). Currently, 44 subtypes (STs) have been reported, based on nucleotide polymorphism within the small subunit of ribosomal RNA (rRNA-18S) (Higuera et al., 2023; Stensvold, 2013; Stensvold & Clark, 2016).

The most critical factor in transmission dynamics is associated with zoonotic transmission, underscoring its significance (Garcia-Montoya et al., 2023; Higuera et al., 2020; Villamizar et al., 2019). *G. duodenalis* (*Giardia lamblia* or *G. intestinalis*) afflicts an estimated 280 million individuals worldwide (Zajaczkowski et al., 2021). In Colombia, the prevalence of *Giardia* infection fluctuates between 15.4% and 61%, depending on the detection method (microscopy or qPCR) (Sánchez et al., 2017; Villamizar et al., 2019).

Cryptosporidium infection has been classified by the WHO as a neglected disease (Savioli et al., 2006). To date, a minimum of 30 species and 70 genotypes have been identified, with 20 of them documented in humans, with *C. hominis* and *C. parvum* primarily responsible for the majority of infections (Chalmers & Katzer, 2013; Ryan et al., 2014). In Colombia, studies have reported prevalence rates ranging from 7.8% to 9.8% through qPCR (Galván-Díaz, 2018; Villamizar et al., 2019). Additionally, their presence in water and animal reservoirs, with prevalence rates of 38.9% and 20.4% respectively. The genus *Entamoeba* encompasses several species, six of which can inhabit the human large intestine: *E. histolytica*, *E. dispar*, *E. moshkovskii*, *E. polecki*, *E. coli*, and *E. hartmannii*. *E. histolytica* is considered pathogenic, causing human amebiasis, affecting up to 10% of the global population. In Colombia, molecular identification has allowed for an estimation of *E. histolytica* prevalence close to 1% (Higuera et al., 2020; Villamizar et al., 2019). Molecular studies have also identified other species within the complex, specifically *E. dispar* and *E. moshkovskii* (Higuera et al., 2020; Villamizar et al., 2019).

On the other hand, the WHO estimates that helminth infections affect approximately 1.5 billion people, primarily in tropical

Impacts

- Our study revealed a high frequency of intestinal parasites in a rural population (Las Guacas), with *Blastocystis* being the most common, followed by *A. duodenale*, *Giardia* and *Cryptosporidium* spp.
- The presence of parasites in both humans and dogs, including *A. ceylanicum*, highlights the potential for animal-to-human transmission. Suboptimal hygiene, water usage and sanitation practices create favourable conditions for zoonotic transmission and parasite persistence.
- Our findings underscore the urgency of improved water sanitation infrastructure and access to clean water. A One Health approach is crucial for designing effective prevention and control strategies that address the interconnectedness of human, animal and environmental health.

and subtropical regions (Hotez et al., 2008; Silver et al., 2018). Soil-transmitted helminths include *A. lumbricoides*, *T. trichiura* and the hookworms (*N. americanus*, *A. duodenale* and *A. ceylanicum*) (Knopp et al., 2012; Papaiakovou et al., 2017; Silver et al., 2018). In Colombia, the estimated prevalence of *Ascaris* infection varies according to the population studied, reporting prevalence rates up to 32% (Papaiakovou et al., 2021; Saboyá et al., 2013) and the hookworm infection is approximately 6% (Ministerio de Salud y Protección Social, 2014).

One of the primary challenges in estimating the prevalence of these parasites lies in the inherent limitations associated with microscopy. As a result, efforts have been ongoing to adopt molecular techniques such as PCR and qPCR for identifying circulating species and subpopulations of these parasites, especially in rural communities (Higuera et al., 2020; Villamizar et al., 2019). Obtaining precise measurements of the prevalence and intensity of infection in regions endemic to these parasites is crucial. This consideration is particularly important due to the significant zoonotic potential and the dynamics of transmission involving various hosts, including animals, humans and environmental sources such as drinking water. Consequently, the objective of this study was to assess the presence of intestinal parasites by microscopy and to conduct molecular characterization of *Blastocystis*, *Giardia*, *Cryptosporidium* and *E. histolytica*, as well as helminths, namely *A. lumbricoides*, *T. solium*, *T. trichiura* and hookworms (*N. americanus*, *A. duodenale* and *A. ceylanicum*), in samples obtained from humans, their pets (dogs) and water sources within a rural community in Colombia.

2 | MATERIALS AND METHODS

2.1 | Ethical considerations, informed consent and necessary permissions

Informed written consent was obtained from every participant or their respective parent or caregiver. The study design and research protocols received approval from the Bioethics Committee of Universidad del Cauca, Colombia.

2.2 | Study population

A total of 125 samples, consisted of 99 samples from humans (including children and caregivers), 24 samples from pets (*Canis lupus familiaris*) and 2 water samples, were evaluated from a rural population in the Cauca department, Colombia, known as Las Guacas village, located in the municipality of Popayan in southwest Colombia (Figure 1a). Additionally, within the rural area, the sample collection was spatially divided into three defined subzones (identified as A, B and C) where households and the population are concentrated. These subzones represent areas of clustered housing within a village in a rural setting. Such clustering exhibits characteristics such as vehicular and pedestrian pathways, as well as the provision of basic public, social and welfare services infrastructure (Figure 1a). Stool samples were collected from children aged 1 to 15, their caregivers and their pets (dogs). Additionally, water samples were collected from the La Carnicería Creek – the water source from which the residents of this population obtain their daily water supply.

2.3 | Sample size

A non-probabilistic convenience sampling approach was employed, and stool samples were collected from children and caregivers who agreed to participate in the study and provided a faecal sample. Samples were also obtained from dogs living with the children and caregivers.

2.4 | Sociodemographic variables

At the time of obtaining informed consent, we conducted a structured survey involving 123 sample elements to gather data on various variables, including sex, age, availability of utilities, water quality, water treatment, presence and number of pets (dogs), faecal disposal practices, handwashing practices, garbage storage/disposal methods and antibiotic consumption (Table S1).

2.5 | Stool sample collection

Stool samples were collected from both dogs and humans. Each stool sample was obtained using sterile gloves and deposited in a sterile container without transport media. The faecal samples were divided into two portions. The initial portion of each sample was preserved using SAF solution for the direct examination and microscopic identification of intestinal parasites. Another fraction of the sample was dedicated to molecular processing and transported under refrigeration at temperatures between 2 and 8°C. Upon arrival at the laboratory, it was then stored in ultra-freezers at −80°C for subsequent analysis.

2.6 | Water sample collection

Water samples were collected from La Carnicería Creek, the primary water source for the residents of this population. A composite water sample was obtained, with two samples collected due to accessibility challenges. Each sample was gathered at a depth of 30 cm from the sampling point, with a volume of 500 mL. These samples were then transported at a temperature of 4°C in a polystyrene cooler and processed on the same day of collection to ensure optimal DNA extraction results.

For the extraction process, two 200-mL vacuum filtrations were performed for each sample using 0.45 μm cellulose

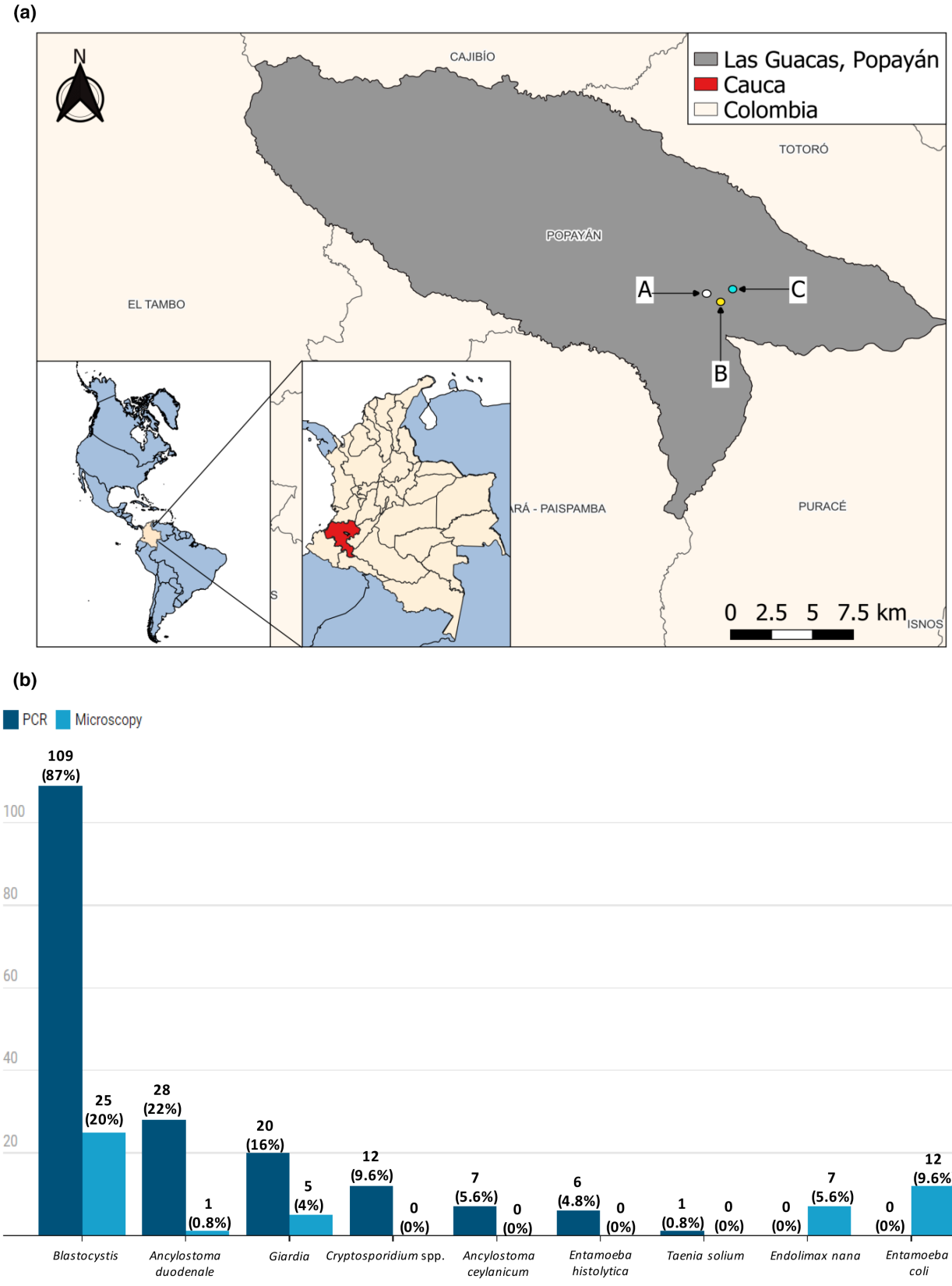

FIGURE 1 (a) Geographical sampling area corresponding to the rural region of Cauca, Colombia. (b) Frequency of intestinal parasites detected in the samples analysed by molecular techniques (qPCR) and microscopy.

nitrate membranes, followed by a refiltration through 0.2 μm membranes to capture smaller microorganisms. A standardized protocol was implemented for shotgun metagenomics. The membranes were placed in sterile 50-mL Falcon tubes, and the biomass was recovered by scraping with molecular-grade water (15 mL) twice, with each membrane being pipetted for 15 min.

After centrifugation, the pellet underwent nucleic acid extraction using a DNeasy Power Soil kit (Qiagen), with the elution solution pre-warmed to 65°C for optimal results. All steps were conducted in a laminar flow cabinet. The extracted DNA was assessed for concentration and integrity using NanoDrop and electrophoresis respectively.

2.7 | Molecular analysis

DNA extraction from stool samples was carried out using the Norgen faecal DNA extraction kit, which included an initial disruption step. Following that, a qPCR multiplex assay was performed according to the prescribed protocol and with the specified primers for the detection of *A. lumbricoides*, *Cryptosporidium* spp. (*C. parvum/C. hominis*), *A. duodenale*, *A. ceylanicum*, *N. americanus*, *Strongyloides stercoralis*, *G. lamblia*, *E. histolytica* and *T. trichiura* as previously reported (Mejia et al., 2013); the primers used are shown in Table S2. For *T. solium*, primers and probes were used from a previous study and genomic DNA as a control and standard (O'Connell et al., 2020). For *Blastocystis*, we used the primers reported by Stensvold et al. (2012) (Table S2). Given that the *Cryptosporidium*-specific PCR Multiplex method only detects *C. parvum* and *C. hominis*, we implemented a specific conventional PCR assay for *Cryptosporidium* spp. (Burnet et al., 2013). The DNA concentrations of each parasite in the samples were calculated by comparing them to a standard curve generated from 10-fold dilutions as reported elsewhere (Mejia et al., 2013).

2.8 | Molecular characterization of *Blastocystis* subtypes (STs)

Molecular characterization of *Blastocystis* was conducted on PCR-positive samples. These samples underwent PCR to generate amplicons of the complete rRNA-18S gene, approximately 1800 bp in length., using 1 μM of primers SSU-F1 (5-AAC CTG GTT GAT CCT GCC AGT AGT AGT C-3) and SSU-F1 (5-TGA TCC TTC TGC AGG TTC ACC TAC G-3) forward and reverse respectively. The experimental protocol was performed as reported elsewhere (Maloney et al., 2020).

The Oxford Nanopore Technologies (ONT) MinION platform was employed for characterization, following the protocol established by Maloney et al. (2020) and Maloney and Santin (2021). The library preparation utilized the SQK-LSK109 and SQK-LSK110 ligation sequencing kits from ONT, following the manufacturer's instructions for PCR coding amplicons (PBAC12_9112_v110_revB_10Nov2020). For barcoding each sample, the EXP-PBC001 PCR Barcoding Kit from ONT, Oxford, UK, was employed in conjunction with ligation kits. Subsequent purification steps ensured the loading of 50 or 75 fmol of the library in 12 μL into the flow cell. The sequencing runs were facilitated using MinKNOW v20.10.06 software (Maloney & Santin, 2021).

Base calling was performed using Guppy v4, followed by FASTQ read filtering to retain sequences with lengths ranging from 1600 to 2100 nucleotides. Adapters used for full-length sequencing PCR were trimmed. Off-target reads were filtered from the resulting FASTA files using the NCBI reference databases. Subtyping was accomplished through alignment, selecting the best match with full-length *Blastocystis* reference nucleotide sequences obtained from the reference database (http://entamoeba.lshtm.ac.uk/blastorefseqs.htm, accessed on 03/02/2021), in addition to other accepted full-length ST sequences present in GenBank. This alignment process utilized the CENTRIFUGE algorithm.

2.9 | Statistical analysis

Descriptive statistics were employed to summarize qualitative variables in terms of frequencies and proportions. Quantitative variables were summarized using central tendency and dispersion measures. Since qPCR identified more positive samples for each parasite and microscopy did not detect any additional positives, analyses were based on qPCR. The possible existence of associations between categorical variables was evaluated using Pearson's Chi-squared tests and Fisher's exact test. Regarding quantitative variables, a normality test (Kolmogorov–Smirnov) was initially conducted, and based on the results, non-parametric tests were applied for group comparisons. Statistical analyses were carried out using the R software. All tests of significance were two-tailed, and p-values <0.05 were considered statistically significant.

3 | RESULTS

3.1 | Sociodemographic characterization

The evaluated population consisted of 99 samples from humans, 24 samples from pets (*C. lupus familiaris*) and 2 water samples. Among the individuals assessed, the median age was 22 years (IQR: 33.5), with the majority falling within the age range of 20 to 40 years ($n=26$), followed by individuals aged 0 to 5 years ($n=21$) (Table 1). Among these individuals, 64% were women, and 36% were men. The average number of residents per household was four members. The majority of individuals surveyed (97%/$n=119$) reported using septic tanks for excreta disposal, while the remaining few (3%/$n=4$) reported using latrines.

As this is a rural community, the residents source their water from a stream, from which the water is transported. Regarding this, it was found that 42% did not carry out any type of treatment before consumption, while 58% used some method of treatment, such as boiling the water, filtering it or using chlorine compounds for treatment. Additionally, only 28% of the individuals considered the water they use to be of good quality, while the remaining individuals considered it to be of regular (57%) or even poor quality (15%) (Table 1).

TABLE 1 Characteristics of the population and statistical results of the variables that were herein analysed.

			Blastocystis			*Giardia*			*Cryptosporidium* spp.	
Group	**Variable**	**Category**	**Pos**	**Neg**	***p* value**	**Pos**	**Neg**	***p* value**	**Pos**	**Neg**
Individual human characteristics ($n=99$)	Sex	Female	57	6	0.842	7	56	0.431	1	62
		Male	33	3		6	30		3	33
	Age (Years)		21 (IQR: 32, 75)	40 (IQR: 41)	0.530	10 (IQR: 12)	24, 5 (IQR: 34, 5)	0.068	4, 5 (IQR: 16, 75)	22 (IQR: 33)
Household characteristics per survey ($n=123$)	Water quality	Treated	71	7	0.2683	11	67	0.393	8	70
		Non-treated	38	7		9	36		2	43
	Presence of Dogs	Yes	105	12	0.082	19	98	0.977	10	107
		No	4	2		1	5		0	6
Source of samples ($n=125$)	Source	Human	90	9	0.0002	13	86	0.129	4	95
		Animal	19	5		7	17		6	18
		Water	0	2		0	2		2	0

3.2 | Parasite infection and statistical analyses

Microscopic evaluation yielded 80 negative samples for all evaluated protozoa and helminths. Among the positive samples identified through microscopy, *Blastocystis* exhibited a frequency of 20.2% ($n=25$), *Endolimax nana* at 5.6% ($n=7$), *Giardia* at 4% ($n=5$) and *E. coli* at 9.6% ($n=12$). *Cryptosporidium* spp. could not be identified via microscopy (no staining was conducted) (Figure 1b). Additionally, one sample was found to contain hookworm eggs during microscopy examination (0.8%) (Table S3).

On the other hand, the frequency of different parasites by qPCR was notably higher. Thus, the frequency was for *Blastocystis* 87% ($n=109$), *A. duodenale* 22% ($n=28$), *A. ceylanicum* 5.6% ($n=7$), *Giardia* 16% ($n=20$), *E. histolytica* 4.8% ($n=6$) and *Cryptosporidium* spp. 9.6% ($n=12$), and even a specific case positive for *T. solium* 0.8% ($n=1$) was found (Figure 1b and Table S1). Specifically, among human hosts, the most frequently identified parasite was *Blastocystis* 90.1% (90/99), followed by *Giardia* 13.1% (13/99), *Ancylostoma* 9.1% (9/99), *E. histolytica* 6% (6/99), *Cryptosporidium* 4% (4/99) and *Taenia* 1% (1/99). Conversely, among dog hosts, the most common parasites identified were *Ancylostoma* 79% (19/24) and *Blastocystis* 79% (19/24), followed by *Giardia* 29% (7/24) and *Cryptosporidium* spp. 25% (6/24). In the case of water samples, only *Cryptosporidium* spp. was found in both samples (Tables 1 and S4).

Interestingly, this study is the first reported infection in Colombia with *Ancylostoma ceylanicum*, a zoonotic hookworm from wild animals including dogs, cats and rodents (Papaiakovou et al., 2017). *Cryptosporidium* was detected in both water samples analysed via qPCR. Furthermore, it is important to emphasize that all the samples that tested positive for *Cryptosporidium* were not identified by multiplex PCR, which is designed for the detection of *C. parvum* and *C. hominis*, but rather through qPCR targeting *Cryptosporidium* spp. This implies the possibility that these positive samples may correspond to other species.

The qPCR allowed the estimation of parasite DNA concentrations in fg/μL (Figure 2a). We did not find correlation between DNA concentrations of parasites evaluated. It was observed that the DNA concentrations of *Blastocystis* were higher in subzone B than in subzone A (Kruskal–Wallis, p-value = 0.0010). A similar situation was observed with *Giardia* concentrations (Kruskal–Wallis, p-value = 0.0065) (Figure 2b). Similarly, *Blastocystis* DNA concentrations were higher in human hosts than in animals (Kruskal–Wallis, p-value = 0.0075). Conversely, higher DNA concentrations were observed in animal hosts for *A. duodenale* (Kruskal–Wallis, p-value = 0.00009). Spearman correlations were conducted among the DNA concentration values of the different parasites; however, statistically significant results were not obtained.

Regarding the possible relationship between the different variables evaluated and the presence of protozoa and helminths (Table 1), no statistically significant association was found between the presence of different parasites and gender (Table 1). In the specific case of the host or sample origin, *Blastocystis* showed a higher frequency in humans than in animals (chi-square, p-value = 0.0002), while *A. duodenale* showed a higher frequency in dogs (Chi-square, p-value <0.001). Despite the significant presence of parasites in dogs, no statistically significant relationship was found between owning pets (dogs) and the presence of any of the identified parasites. Statistically significant associations were not found with other evaluated variables (Table 1).

3.3 | Coinfections and polyparasitism

Among the 125 samples evaluated, qPCR results revealed that in 6% ($n=8$) of these samples, no identification of any parasite was made. Of the samples, 57% ($n=71$) exhibited a single parasite, 28% ($n=35$) showed coinfection with two parasites, 7% ($n=9$) had three parasites and two cases, accounting for 1.6% ($n=2$), exhibited coinfection with four different parasites (Figure 3a). The most frequent coinfection was *A. duodenale–Blastocystis* ($n=17$), followed by *Giardia–Blastocystis*

	Entamoeba histolytica			*Ancylostoma* spp.			*Taenia*		
p value	Pos	Neg	*p* value	Pos	Neg	*p* value	Pos	Neg	*p* value
0.101	2	61	0.111	6	57	0.842	1	62	0.447
	4	32		3	33		0	36	
0.364	33 (IQR: 52, 75)	22 (IQR: 33)	0.572	26 (IQR: 29)	21 (IQR: 33, 75)	0.774	6 (IQR:0)	22 (IQR: 33, 75)	0.353
0.255	4	74	0.865	23	55	0.019	1	77	0.445
	2	43		5	40		0	45	
0.455	6	111	0.569	27	90	0.714	1	116	0.8201
	0	6		1	5		0	6	
<0.001	6	93	0.437	9	90	<0.001	1	98	0.876
	0	24		19	5		0	24	
	0	2		0	2		0	2	

($n=9$) (Figure 3b). Coinfections involving three parasites were mainly *A. duodenale–Giardia–Blastocystis* ($n=3$) and *E. histolytica–Giardia–Blastocystis* (Figure 3b). For the two cases of polyparasitism related to four parasites, coinfection was observed among *A. duodenale–Cryptosporidium–Giardia–Blastocystis* (Figure 3b).

3.4 | *Blastocystis* subtypes

Due to the high number of positive samples obtained for *Blastocystis*, we decided to perform subtype (ST) typing of this protozoan by complete sequencing of the rRNA-18S gene using the Oxford Nanopore platform. A total of 65.1% ($n=71$) of the samples could not be successfully typed and classified into any subtype. Among those that were successfully typed, we found unique STs and the coexistence of different STs (Figure 4). The most frequent unique subtype was ST2, accounting for 17.4% ($n=19$), followed by ST3 at 3.7% ($n=4$) and ST1 at 0.9% ($n=1$). We also identified the coexistence of STs, with the most frequent being ST2-ST3 at 6.4% ($n=7$), ST1-ST2 at 2.75% ($n=3$), ST1-ST3 at 1.83% ($n=2$), ST3-ST9 at 0.91% ($n=1$) and ST3-ST2-ST1 at 0.91% ($n=1$) (Figure 4).

4 | DISCUSSION

The rural population of Las Guacas Village, faces distinct socio-economic challenges, particularly in housing and limited access to education, healthcare, clean water and sanitation. Our study reveals water quality and sanitation issues, raising the risk of intestinal parasites (Tables 1 and S1). Most residents do not treat their water; 42% consume untreated water, posing potential risks of waterborne infections (Table 1). Inadequate excreta disposal practices can contaminate water and coexisting pets, contributing to complex transmission dynamics (Lebov et al., 2017). Parasite presence does not significantly correlate with demographics, indicating widespread infections. Further validation across various regions is necessary.

The characteristics mentioned above are closely linked to the data on intestinal parasitism that we uncovered, and they align with previous studies conducted in the region (Higuera et al., 2020; Villamizar et al., 2019). In our current study, we analysed human samples from a population consisting mainly of individuals aged 0 to 5 years and those aged 20 to 40 years, representing young children and their caregivers respectively. This demographic characteristic holds significant implications for transmission dynamics through untreated water, contaminated food and oral–faecal transmission facilitated by caregivers, particularly impacting young children who are more susceptible to infection (Pullan et al., 2014; Villamizar et al., 2019).

Additionally, the significant presence of pets (dogs) within the community has indicated that they harbour a variety of parasites commonly found in both human and animal hosts. These parasites include *A. duodenale*, *A. ceylanicum*, *Giardia*, *Cryptosporidium* spp. and *Blastocystis*. Given the prevailing suboptimal hygiene practices and patterns of water usage, this context potentially fosters favourable conditions for zoonotic transmission (Lebov et al., 2017; Walker et al., 2023) enabling the cross-species spread of these parasites between humans and animals (Ryan & Cacciò, 2013).

In this context, the frequency of intestinal parasites found in the present study through qPCR analysis was notable, with *Blastocystis* exhibiting the highest frequency at 87% ($n=109$), *A. duodenale* at 22% ($n=28$), *A. ceylanicum* at 5.6% ($n=7$), *Giardia* at 16% ($n=20$), *E. histolytica* at 4.8% ($n=6$), *Cryptosporidium* spp. at 9.6% ($n=12$) and even a specific case positive for *T. solium* at 0.8% ($n=1$) (Figure 1). It is worth noting that these frequency rates for some parasites were higher than what has been previously identified in other populations (Galvan-Diaz et al., 2020; Higuera et al., 2020; Villamizar et al., 2019). It is evident from our findings that DNA concentrations in fg/uL indicate higher burdens of *A. duodenale*, *Blastocystis* and *Giardia* compared to *E. histolytica*, which was exclusively found in

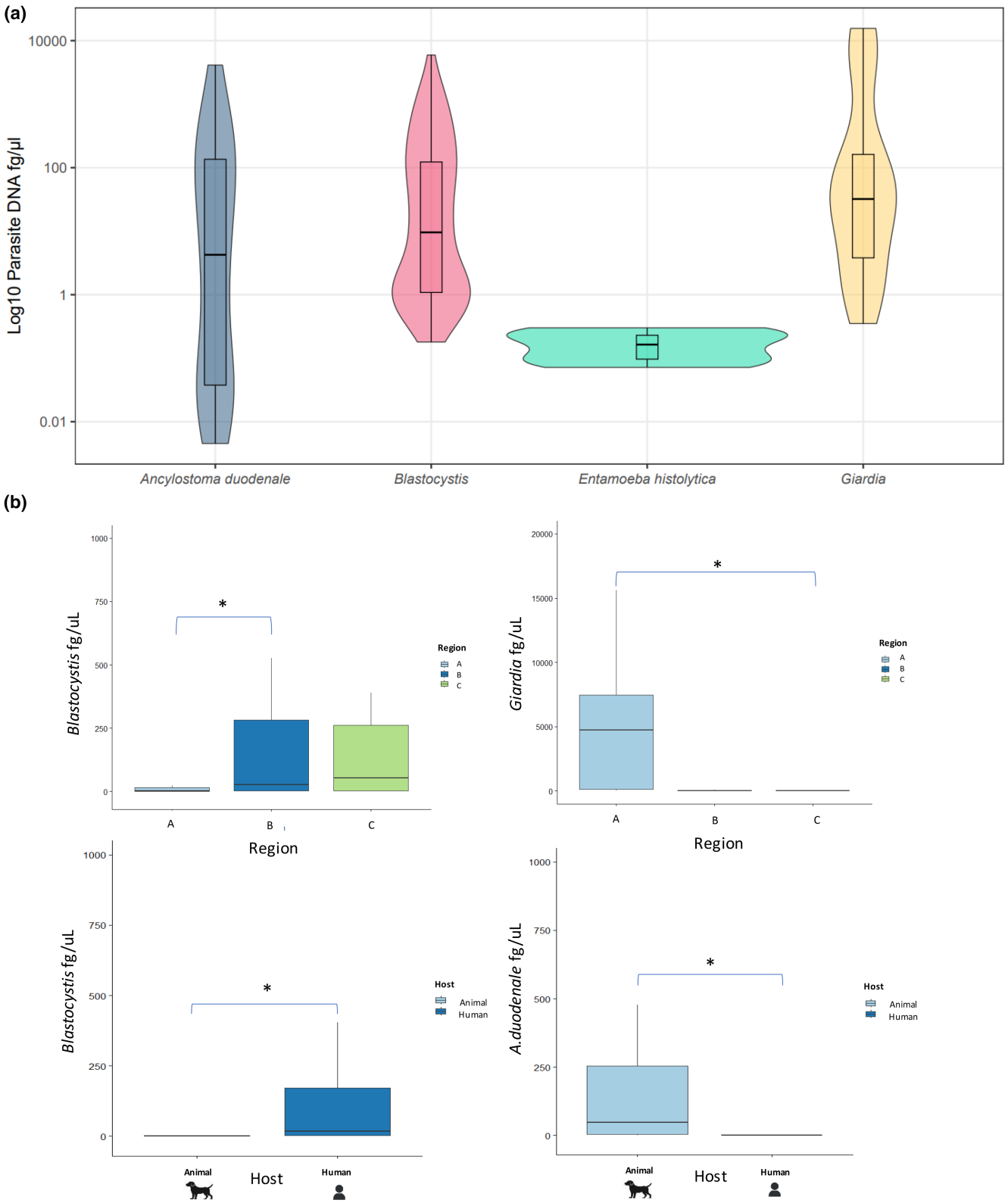

FIGURE 2 (a) Violin plot of DNA concentrations of main parasites found by qPCR. The fg/µL values obtained for each case were normalized using log10 to visually compare these concentrations. (b) DNA concentrations (fg/µL) of *Blastocystis* and *Giardia* between geographic subregions sampled (upper part). DNA concentrations (fg/µL) of *Blastocystis* and *Ancylostoma duodenale* between hosts (animal or human) (bottom). A Kruskal–Wallis non-parametric test was conducted to assess statistically significant differences in DNA concentrations of each parasite concerning the presented variables. The asterisk (*) denotes results with $p < 0.01$.

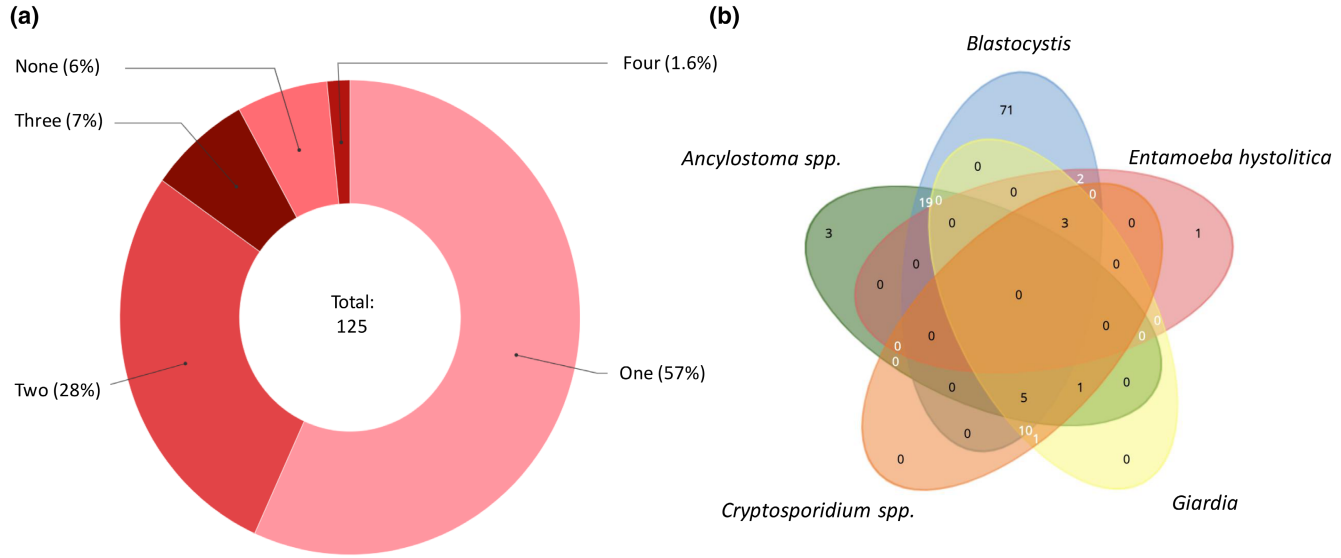

FIGURE 3 (a) Detection of polyparasitism by molecular tests (qPCR-PCR). (b) Number of parasites found in polyparasitism events. (b) Co-infections among the different protozoa and helminths studied.

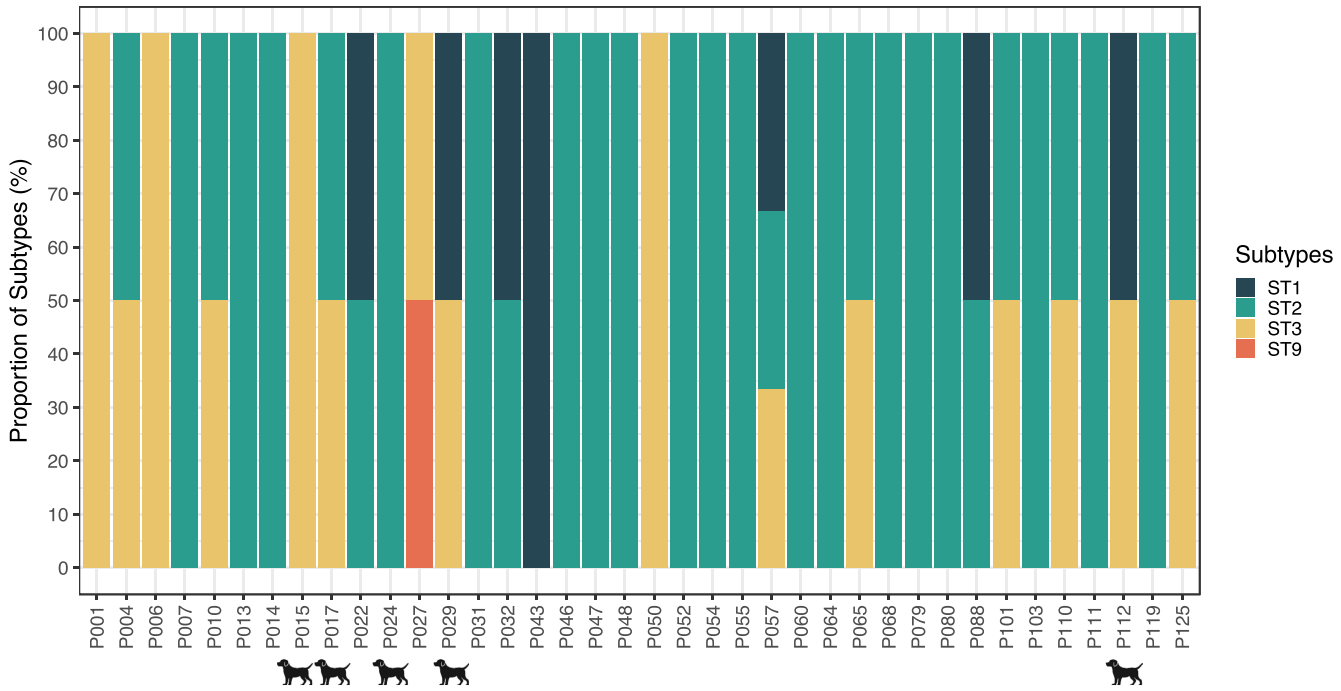

FIGURE 4 *Blastocystis* variation between STs in animal and human hosts. In this study, the detection of mixed and unique infections from different *Blastocystis* subtypes was conducted. Bar plots were constructed by host to illustrate the percentage of each ST detected by the sample. All subtypes were assigned a colour to differentiate them from each other.

human hosts (Figure 2a). This observation underlines that in rural settings, zoonotic cycles can lead to a greater number of infected hosts and consequently result in persistent infections with higher parasite concentrations. Our comparisons of DNA concentrations between different subzones and host categories revealed significant variations (Figure 2b). *A. duodenale* displayed higher concentrations in animal hosts (dogs) than in humans, while *Blastocystis* exhibited higher concentrations in humans than in animals (Figure 2b). These findings underscore the necessity of customizing treatment, prevention and diagnostic efforts for specific populations, focusing on the most affected hosts to effectively curb the transmission of these parasites (Wiśniewski et al., 2016). Additionally, this study is the first reported infection in Colombia with *A. ceylanicum*, a zoonotic hookworm from wild animals including dogs, cats and rodents

(Papaiakovou et al., 2017). These studies stress the need for research in rural areas where limited access to clean water, coupled with socio-economic conditions and poor hygiene, may increase infection and facilitate parasite spread (Higuera et al., 2020; Villamizar et al., 2019). Therefore, it is essential to bolster molecular detection strategies, such as qPCR, to obtain accurate data for informed decision-making (Table S3) (Papaiakovou et al., 2021; Villamizar et al., 2019).

The most frequently identified protozoan in humans was *Blastocystis*. The pathogenic potential of *Blastocystis* has long been a subject of debate, and its varied clinical presentations have been attributed to its extensive genetic diversity and its interactions with the host microbiota (Castañeda et al., 2020; Yason et al., 2019). However, the detection of *Blastocystis* in both humans and animals highlights its significant zoonotic potential, which may also involve other parasites known to cause illness through water and food contamination (Higuera et al., 2020; Rune et al., 2020; Villamizar et al., 2019). Considering this, we conducted subtype (ST) typing of *Blastocystis* in the positive samples. Although a substantial number of samples could not be successfully typed maybe due to the use of a longer PCR fragment that might have reduced sensitivity. The ones that were classified into STs revealed that, consistent with previous reports, the most prevalent was ST2, accounting for 17.4% ($n=19$) of cases, followed by ST3 at 3.7% ($n=4$) and ST1 at 0.9% ($n=1$) (Higuera et al., 2020; Villamizar et al., 2019). Previous molecular detection studies conducted in various populations in Colombia have identified sequence types (STs) that align with those identified in this study (Higuera et al., 2020; Villamizar et al., 2019). In the current analysis, the most prevalent was ST2. This observation is in line with the fact that approximately 90% of the isolates belonged to ST1-ST4, which are known to be responsible for the majority of human infections worldwide and are involved in zoonotic transmission cycles (Ramírez et al., 2017). The findings are notable as our study comprised mainly asymptomatic individuals without gastrointestinal symptoms, despite testing positive for *Blastocystis* and other protozoa. This aligns with the notion that ST2 is linked to asymptomatic individuals and is part of the healthy host microbiota (Castañeda et al., 2020; Stensvold & Clark, 2016; Stensvold et al., 2022).

An intriguing finding was the presence of co-infections with different STs, including ST2-ST3 at 6.4% ($n=7$), ST1-ST2 at 2.75% ($n=3$), ST1-ST3 at 1.83% ($n=2$), ST3-ST9 at 0.91% ($n=1$) and ST3-ST2-ST1 at 0.91% ($n=1$) (Figure 4). Previous studies have documented the occurrence of coinfections involving different sequence types (STs) in the Colombian population. These coinfections have primarily been associated with the presence of ST1 and ST3 (Garcia-Montoya et al., 2023). This finding is relevant for understanding the interactions among protozoans, hosts and the host microbiome, and how these infections might influence the host's response (Castañeda et al., 2020; Gentekaki et al., 2017). Additional analyses are necessary to expand the typing of a greater number of samples.

A compelling discovery pertained to the identification of *Cryptosporidium*. Initially, the multiplex qPCR, which was used to detect *C. parvum* and *C. hominis*, yielded negative results for all samples. However, upon conducting a complementary analysis employing PCR for *Cryptosporidium* spp., we identified 12 positive samples. This observation suggests that the species circulating in this region may align with those previously documented in our country, including *C. muris*, *C. andersoni*, *C. viatorum* and *C. ubiquitum* (Higuera et al., 2020). Further research is warranted to molecularly characterize these species and genotypes, thus confirming this potential correspondence. Significantly, *Cryptosporidium* spp. was the sole parasite identified in the water supply. Notably, it was detected in water from both of the filters previously described, implying that the water used and consumed by the community may potentially be contaminated by this protozoan. Furthermore, *Cryptosporidium* spp. was identified in both animal and human hosts. These findings underscore the need for a One Health approach to evaluate and address prevention measures for parasitic infections, emphasizing transmission dynamics across the environment, animals and humans and enhancing water sanitation for consumption.

Giardia was also detected in both animal and human samples, with a frequency (16%) similar to that reported in previous molecular studies (Higuera et al., 2020; Villamizar et al., 2019). Complementary investigations are essential to molecularly characterize *Giardia* assemblages and subassemblages, enabling a more comprehensive evaluation of the zoonotic potential of this protozoan (Ryan & Cacciò, 2013; Ryan et al., 2021; Wielinga et al., 2023). Conversely, positive cases of *E. histolytica* were exclusively identified in humans (4.8%). Indeed, considering that parasites such as *Giardia* and *E. histolytica* are transmitted through the oral–faecal route, it is of utmost importance to place the findings presented here in the context of programmes designed to promote the proper handling and management of food, water and waste (López et al., 2015).

In the case of helminths, *A. duodenale* was the most prevalent parasite among animal hosts, and notably, *A. ceylanicum*, a zoonotic hookworm typically associated with wild animals, was found for the first time in Colombia (Papaiakovou et al., 2017). The detection of this nematode in both animals and humans highlights the significance of this zoonotic infection in communities where transmission risk is heightened due to the presence of dogs, coupled with inadequate use of water and management of excreta (Walker et al., 2023). In Colombia, the molecular identification of helminths is limited, and reliance on microscopy has led to an underestimation of the potential circulation of this nematode (Quiroz et al., 2020). Consequently, one of the WHO's priorities has been to address the management of these nematodes through timely and appropriate chemotherapeutic treatments within the framework of One Health strategies (Lebov et al., 2017; Walker et al., 2023). Our study revealed a potentially higher burden of this nematode in dogs, as assessed by DNA concentration, compared to the human host. This indicates that dogs can potentially harbour higher parasitic burdens, thereby promoting transmission and facilitating zoonotic events (Figure 2b) (Walker et al., 2023). This is a critical step towards the development of novel strategies for controlling and preventing infections of this nature. In the case of *Ancylostoma* spp., vaccination strategies have been proposed for dogs, leading to significant reductions in infection rates

and parasite burdens (Walker et al., 2023; Wiśniewski et al., 2016). This is crucial in rural areas where freely roaming dogs contact contaminated water and excreta, facilitating transmission to other animals and humans. A comprehensive evaluation is necessary for detection and estimation of parasitic burdens in broader areas.

The frequency of confections and polyparasitism within this community was remarkable. Although no statistically significant relationships were identified between the evaluated variables and polyparasitism events, it is imperative to acknowledge that the simultaneous presence of multiple parasites in the same individual (or even in dogs where we found 1–4 parasites in the same host) can introduce complexities in understanding clinical presentations, diagnostics and even transmission dynamics (Donohue et al., 2019). The most frequently observed coinfections and co-occurrences, such as *A. duodenale–Blastocystis* and *Giardia–Blastocystis*, may hold implications for disease severity and clinical outcomes (Donohue et al., 2019). Moreover, comprehending the interactions between parasites, be it helminth–protozoa or protozoa–protozoa, in cases of intestinal polyparasitism, could potentially shed light on key aspects of parasite biology, infection, host disease pathophysiology and their interactions with the host's immune system and microbiota (Burgess et al., 2017; Donohue et al., 2019; Stensvold & van der Giezen, 2018). Hence, it is imperative to conduct studies rooted in community ecology to comprehensively grasp not only the interactions occurring at the host level but also to delve into the epidemiological and social aspects that underpin the complexities of polyparasitism.

The purpose of this research was to comprehensively analyse the molecular epidemiology of protozoa and helminths in a specific area, considering various potential sources of infection such as water, humans and dogs. By combining these samples, we aimed to compare results obtained from microscopy and molecular tests, identifying similarities and differences in parasite prevalence across different hosts and environmental sources. Additionally, we investigated coinfection events to understand potential cross-species transmission pathways. Our findings contribute significantly to the understanding of zoonotic transmission dynamics in this community and offer insights for future research and public health strategies. It is important to acknowledge certain limitations within our study. Firstly, the acquisition of a single stool sample rather than conducting serial coproscopic analyses may have limited our ability to detect low-burden parasites. Additionally, the availability of only two water samples restricts our examination of the environmental impact on transmission dynamics. Furthermore, there are limitations regarding the characterization of species, assemblages and genotypes for specific parasites. Moreover, the identification of *Blastocystis* subtypes (STs) through complete sequencing of the rRNA-18S gene was not feasible for a significant portion of the samples examined. Future research endeavours should prioritize molecular typing and the development of high-resolution methods for various parasites to overcome these limitations. This would involve analysing a greater number of samples involving human and several animal hosts using probabilistic calculations and including a larger number of environmental samples to confirm transmission dynamics. Addressing these limitations will lead to a more comprehensive understanding of the complex dynamics involving the environment, animal hosts and human hosts.

In summary, this study provides crucial insights into the molecular epidemiology of intestinal protozoa and helminths in a rural Colombian community, emphasizing the need for integrated control measures, including improved water and sanitation practices, health education and targeted treatment strategies. The findings underscore the importance of a One Health approach to understanding and addressing zoonotic transmission dynamics in both human and animal populations.

AUTHOR CONTRIBUTIONS

SC, CP, JDR and LRV conceived the study. SC, LHP and RM developed the experiments and analysed the data. All authors read and approved the final version of the manuscript.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest related to the research, authorship or publication of this article.

DATA AVAILABILITY STATEMENT

The data that supports the findings of this study are available in the supplementary material of this article.

DECLARATION OF GENERATIVE AI AND AI-ASSISTED TECHNOLOGIES IN THE WRITING PROCESS'

During the preparation of this work, the author(s) used ChatGPT in order to improve readability and language. After using this tool/service, the author(s) reviewed and edited the content as needed and take(s) full responsibility for the content of the publication.

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Impact of *Blastocystis* Colonization and Burden on Gut Microbiota Composition in a Non-Westernized Rural Population from Colombia

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Abstract

Blastocystis, a common intestinal protozoan in humans and animals, has long been debated regarding its role in human health. Recent studies suggest a connection between *Blastocystis* colonization, healthier lifestyles, and beneficial modulation of gut microbiota. Nevertheless, studies concerning the relationship between *Blastocystis* colonization, its burden, and gut microbiota composition—encompassing both bacterial and eukaryotic communities—in individuals from rural populations are limited. Therefore, this study examines the impact of *Blastocystis* colonization and its burden on gut microbiota composition in a rural community, which provides a distinct microbial profile and higher protozoan prevalence. A total of 88 human samples were collected from the rural population of Las Guacas village, located in the Cauca department, southwest Colombia. We utilized 16S and 18S rDNA sequencing to analyze both bacterial and eukaryotic microbiota, comparing *Blastocystis*-positive and -negative individuals, as well as groups with varying *Blastocystis* burdens (low, medium, high), to identify distinct microbiota profiles and differentially abundant taxa linked to each condition. The analysis revealed significant differences between *Blastocystis*-positive and -negative individuals. In terms of

bacterial composition and structure, *Blastocystis*-positive individuals exhibited distinct microbiota profiles, as shown by beta diversity analysis. Characteristic taxa included *Bacteroides*, *Prevotella*, *Oscillibacter*, *Faecalibacterium*, and *Alistipes*. Higher *Blastocystis* burdens were associated with an increased abundance of taxa like *Alistipes* and *Lachnospira*, while lower burdens correlated with beneficial bacteria such as *Akkermansia*. Regarding eukaryotic composition, beta diversity analysis revealed distinct profiles associated with *Blastocystis* colonization. Differentially abundant taxa, including *Entamoeba coli*, were more prevalent in *Blastocystis*-positive individuals, while *Blastocystis*-negative individuals exhibited a higher abundance of opportunistic fungi, such as *Candida albicans*. Machine learning models, including random forest classifiers, supported these findings, identifying specific microbial taxa like *Faecalibacterium* and *Bacteroides* as predictors of *Blastocystis* colonization. These findings suggest that *Blastocystis* may modulate gut microbiota, contributing to microbial balance providing new insights into the ecological implications of *Blastocystis* in rural populations.

Introduction

Blastocystis is an ubiquitous intestinal protozoan, commonly detected in both humans and animals worldwide [1,2] . Despite its frequent occurrence in human stool samples, its role in human health has long been debated. Traditionally regarded as a pathogen, emerging evidence suggests a more complex relationship, with studies indicating that *Blastocystis* may act as a commensal organism, or even provide benefits to gut health [3–8]. Recent research has linked *Blastocystis* to improvements in dietary habits, serving as a biomarker of healthier lifestyles, and positively influencing individualized host responses to diet and disease susceptibility [7]. This protozoan's association with the gut microbiota is particularly significant, as it seems to coincide with favorable shifts in microbial diversity and composition, shaped by a range of host factors, including immune status, lifestyle, and geographic location [5–9]. However, most studies on the impact of *Blastocystis* on microbiota ecology have been conducted in Westernized populations, which possess distinct socio-economic, demographic, dietary, and lifestyle conditions [3,8]. These factors shape a characteristic microbiota profile that differs from non-Westernized populations, particularly in rural areas [10,11]. Given these differences, rural populations may exhibit unique

microbiota profiles that could potentially alter the interaction dynamics between *Blastocystis* and the host microbiota, highlighting the need for further investigation in these contexts.

Rural communities from non-westernized offer a unique and underexplored context for investigating the intricate interactions between *Blastocystis* and the gut microbiota. It has been observed that modern Westernized populations differ from traditional and agrarian populations in terms of gut microbiota composition [11]. Non-Western individuals tend to harbor a more diverse community of protists, which contributes to the overall diversity of the human gut. This distinction underscores the importance of studying non-Westernized populations to better understand the full spectrum of gut microbial diversity and the ecological roles of protists like *Blastocystis* [11]. Additionally, protozoan colonization, including that of *Blastocystis*, is more prevalent in these settings, likely influenced by factors such as limited access to healthcare, clean water, and consumption of diets rich in unprocessed foods [12–15]. Despite this, there remains a dearth of comprehensive studies examining *Blastocystis* and its microbiota interactions in rural populations, with most existing research focusing on specific subgroups, such as school-aged children, and primarily analyzing bacterial components using 16S rRNA sequencing [5,16,17]. The role of eukaryotic microorganisms, which may hold the key to understanding broader microbial interactions, has largely been overlooked in these studies.

The ability of *Blastocystis* to alter the structure and composition of the gut microbiota has been well documented [4–8]. However, a more nuanced approach is necessary, considering not only its presence but also its burden and the potential dose-dependent effects on microbial ecosystems. Studies on *Giardia* have shown that its presence induces changes in the host microbiota. Notably, shifts in microbial diversity and the abundance of specific taxa are linked to varying levels of *Giardia* burden [18–20]. For example, Mejia (2020) found that children with *Giardia* DNA concentrations greater than 1 fg/µl exhibited significantly lower microbial diversity and a higher abundance of *Prevotella* compared to uninfected children [18]. This suggests that the burden of protozoa may be a critical factor influencing microbiota structure and composition. This is particularly relevant in rural populations from non-Westernized regions, where the prevalence and burden of protozoa like *Blastocystis* are significantly higher compared to urban populations. Contributing factors include deficiencies in access to clean water, waste management, and sanitary conditions, as well as challenges in accessing healthcare services [12,13,15,21]. Understanding the

impact of *Blastocystis* burden on both bacterial and eukaryotic components of the microbiota could offer insights into how this protozoan shapes microbial communities and influences host health.

This study aims to address a key gap by investigating the relationship between *Blastocystis* colonization, its burden, and gut microbiota composition—including both bacterial and eukaryotic communities—in individuals from a non-Westernized rural community in Colombia, South America. Specifically, we seek to determine whether *Blastocystis* colonization is associated with shifts in microbial diversity and the presence of key taxa that could impact host health. By integrating analyses of both bacterial and eukaryotic components.

Methods

Ethical considerations, informed consent, and necessary permissions

Informed written consent was obtained from every participant or their respective parent or caregiver. The study design and research protocols received approval from the Bioethics Committee of Universidad del Cauca, Colombia.

Study Population

A total of 88 human samples were evaluated from a rural population in the Cauca department, Colombia, known as Las Guacas village, located in the municipality of Popayan in southwest Colombia (Fig. S1) [15].

Sample Size

A non-probabilistic convenience sampling approach was employed, and stool samples were collected from children and caregivers who agreed to participate in the study and provided a fecal sample.

Stool Sample Collection

Each stool sample was obtained using sterile gloves, deposited in a sterile container, and transported under refrigeration at temperatures between 2-8°C. Upon arrival at the laboratory, it was then stored in ultra-freezers at -80°C for subsequent analysis. DNA extraction from stool

samples was carried out using the Norgen fecal DNA extraction kit, which included an initial disruption step.

Microbiome sequencing and analysis

Primer design

The 16S rDNA gene was targeted for amplification, using a modified version of the published universal prokaryotic primers 341F/806R, targeting the V3-V4 hyper-variable regions [22,23]. The forward primer had three additional nucleotides attached in the 5' end (ACTCCTAYGGGRBGCASCAG, 341F3) and the reverse primer had five additional nucleotides attached in the 5' end (AGCGTGGACTACNNGGGTATCTAAT, 806R5). Changes were made due to compliance with our standard PCR program (annealing temperature).

The 18S rDNA gene was selected as target gene to ensure an as broad spectrum of eukaryotic species (parasites and fungi) to be amplified from as few primer sets as possible, when assuming that the 18S rDNA sequence would be the most inter-species conserved gene [23]. 18S rDNA sequences were aligned for each species or genus and a consensus sequence was generated. The consensus sequences for each species were used for phylogenetic analysis in order to group the parasites according to their 18S rDNA sequence similarity rather than taxonomy. The consensus sequence from all species within each group was then aligned and primers were designed, to amplify all species within each group. Additionally, a consensus sequence from human 18S rDNA was aligned with each group, in order to design primers that would preferentially amplify non-human 18S rDNA. Three different primer sets were chosen, G3F1/G3R1 (GCCAGCAGCCGCGGTAATTC / ACATTCTTGGCAAATGCTTTCGCAG), G4F3/G4R3 (CAGCCGCGGTAATTCCAGCTC / GGTGGTGCCCTTCCGTCAAT) and G6F1/G6R1 (TGGAGGGCAAGTCTGGTGCC / ACGGTATCTGATCGTCTTCGATCCC). G3 and G6 primers are targeting the hyper-variable regions V3-V4 and G4 is targeting the hypervariable region V3-V5 of the 18S rDNA gene. Each primer set was aligned using BLAST to the NCBI database, using NCBI's Primer-Blast, with standard settings (excluding predicted Refseq transcripts and uncultured / environmental samples).

Library preparation and sequencing

Purified genomic DNA from each sample was initially amplified using the 16S and three different 18S primers. For each primer, the rDNA was amplified using a short PCR setup; Initial denaturation at 95°C for 3 min, 20 cycles of 95°C (16S: for 30 sec; 18S: for 1 min), 60°C for 1 min, and 72°C for 30 sec, and a final elongation at 72°C (16S: for 7 min; 18S: for 4 min). PCR was performed in a 25µl volume, using the PrimeSTAR® HS DNA Polymerase premix (Takara Bio, Shiga, Japan) with 0.4µM of each primer and 2µl template. This PCR is referred to as PCR1 or amplification PCR. The products from PCR1 were prepared for sequencing by a second PCR (PCR2 or adaptor PCR) using the same PCR program. PCR2 attaches an adaptor A, an index i5, and a forward sequencing primer site (FSP) in the 5' end of the amplicons and an adaptor B, an index i7, and a reverse sequencing primer site (RSP) to the 3' end of the amplicons. Hence, four different PCR amplicon products were generated for each sample. DNA was quantified using the Quant-IT^{TM} dsDNA High Sensitive Assay Kit (Thermo Fisher Scientific), and PCR2 products were pooled in equimolar amounts between samples into the pooled amplicon library (PAL). Undesirable DNA amplicons were removed from the PAL by Agencourt AMPure XP bead purification in a two-step process. Firstly, DNA fragments below 300nt were removed by a 10µL PAL to 24µL AMPure beads ratio, following the manufactures protocol and eluted in 40µL TE buffer. Secondly, large DNA fragments above 1kbp were removed by 10µL AM1 to 16µL AMPure beads ratio, as previously described. The resulting AMPure beads purified PAL was denoted bPAL. The bPAL was diluted to its final concentration of 7.5pM DNA with a 0.001 N NaOH concentration, used for sequencing on the Illumina MiSeq desktop sequencer (Illumina Inc., San Diego, CA 29122, USA). The library was sequenced with the 500-cycle MiSeq Reagent Kit V2 in a 2x250nt setup (Illumina Inc., San Diego, CA 29122, USA).

Data analysis

The data analysis was performed using BION, a semi-commercial software package that utilizes a k-mer-based approach for species-level annotation [23]. BION can handle non-overlapping paired end reads and custom reference databases. It accepts raw sequences and includes several processing steps: de-multiplexing, primer extraction, sampling, sequence and quality-based trimming and filtering, de-replication, clustering, chimera checking, reference data similarity assessment, and taxonomic mapping and formatting.

Prokaryotic sequences were mapped against the RDP reference database (v11.04), while eukaryotic sequences were mapped using the SILVA reference database (v128). For eukaryotes, an in-house improved taxonomy definition was applied, aligning with prokaryotic taxonomy levels (phylum, class, order, family, genus, species, and sequence levels) [8,23–25]. Taxonomy profiling was conducted with an 85% k-mer similarity cut-off at the species level, corresponding to approximately 98% sequence similarity. At the genus level, the cut-off was set at 60% k-mer similarity, equivalent to around 95% sequence similarity. Query sequences with k-mer similarity below 60% were excluded from further analysis. Mapped query sequences were clustered at 98% k-mer similarity, providing a resolution higher than the basecalling error rate.

Microbial diversity analysis was performed using the R Phyloseq package [26]. This package was employed for calculating diversity and abundance indices and generating the corresponding graphs. Linear discriminant analysis (LDA) effect size (LEfSe) analysis and DESeq2, were employed to identify taxa with differential abundance [27]. The threshold of the logarithmic LDA score was 4.0 with a maximum kw value of 0.01 for LEfSe analysis. For DEseq2, a pCutoff of 0.05 and an FCcutoff of 1.0 were used.

Blastocystis Positive and Negative Classification and Burden levels

To assess *Blastocystis* colonization status (positive or negative) and categorize colonization burden, we analyzed sequencing data generated using Illumina technology, which targeted the G3, G4, and G6 regions of the 18S rDNA gene, following previously established protocols [8,25,28]. Colonization status was determined based on read counts obtained from the target regions. We first evaluated the distribution of read counts across all samples to establish a threshold for distinguishing *Blastocystis* presence from absence. An empirical cutoff of 100 reads was used to classify samples as either *Blastocystis*-negative (≤100 reads) or *Blastocystis*-positive (>100 reads), based on an analysis of read count distribution and alignment with recommendations from the literature. This cutoff was chosen to minimize false positives while maintaining sensitivity to detect true colonization. For *Blastocystis*-positive samples, we categorized the burden into three levels (low, medium, or high) using a modified quartile-based approach. Specifically, individuals in the 0-25th percentile were classified into the "Low" burden group, those in the 25th-75th percentile were assigned to the "Middle" burden group, and individuals with read counts exceeding

the 75th percentile were placed in the "High" burden group. This classification was applied to ensure a statistically meaningful distribution across groups, allowing for comparisons that account for variability in read counts. The corresponding read count ranges were defined as follows: Negative (0-100 reads), Low (110-694 reads), Middle (694-6118 reads), and High (more than 6118 reads). To reduce skewness and improve the normality of the read count data, a natural logarithmic transformation (ln) was applied to the positive read counts. This transformation helped to compress the range of values and diminish the influence of extreme outliers, making the data more suitable for parametric statistical analyses. The log-transformed burden levels were as follows: Negative (0-4.61), Low (4.62-6.52), Middle (6.53-8.76), and High (8.77 and above). By employing this classification framework and normalization procedure, we provided a robust statistical basis for analyzing *Blastocystis* colonization and burden across the study population.

Statistical analysis

The quantitative variables were summarized in terms of medians and interquartile range, and the qualitative variables were summarized in frequencies and proportions. The number of reads in each sample was normalized using the average sequence depth. As indicators for the comparisons between groups, relative abundance, and alpha diversity metrics, observed richness, Shannon, and Fisher index, were used [29]. The alpha diversity indices were compared between groups using the non-parametric Mann-Whitney-Wilcoxon and Kruskal Wallis test. To explore the differences in the general composition of the microbial community between groups (beta diversity), the Bray-Curtis taxonomic distances were calculated. Using the *Phyloseq* package, a principal coordinates analysis diagram was produced, in which the Bray-Curtis dictations were used between the samples to visualize the behavior of the groups. Permutational analysis of variance (PERMANOVA) test for multivariate effect was done using the adonis function from the vegan package [30], stratified by groups.. Statistical analyses were carried out using the R software [31]. For all continuous values, normality hypotheses were evaluated using the Kolmogorov-Smirnov test. All tests of significance were two-tailed, and P-values < 0.05 were considered statistically significant.

Machine learning model

We employed a machine learning-based classification approach to predict the variable of interest from microbiota composition. Specifically, a random forest classifier, a supervised method designed to optimize classification accuracy, was utilized [32,33]. The dataset was analyzed in R using ggplot2, phyloseq, randomForest, and pROC packages. Samples were filtered to include only those with *Blastocystis* groups status (Positive or Negative), and OTUs from the *Blastocystidae* family were removed. Rare OTUs were pruned based on a relative abundance threshold of 0.01%. The random forest model, consisting of 100 trees, was trained to classify *Blastocystis* status, and the 20 most important OTUs were identified using the Mean Decrease in Gini Index. Model performance was evaluated through a confusion matrix, precision, recall, F1 score, specificity, and ROC curve with AUC values. Visualization of OTU was performed using ggplot2.

Results

General microbiota profile

The composition of intestinal microbiota among participants with and without *Blastocystis* was evaluated using together 16S and 18S rDNA (Fig. 1-3, Fig. S2-S5). The general profile shows that Firmicutes was the dominant Phyla, followed by Bacteroidetes, Proteobacteria, Actinobacteria, and Ascomycota (Fig S1a). Within these phyla, the most abundant bacterial families' taxa were Lachnospiraceae and Rimunococcaceae, and for eukaryotes, were Saccharomycetaceae and Entamoebida (Fig S1 c, d).

Bacterial Microbiota Composition

Alpha and beta diversity analyses revealed potential differences in bacterial composition between Blastocystis-positive and Blastocystis-negative individuals.

To evaluate changes in bacterial alpha diversity, we calculated observed richness, Pielou's evenness, Shannon, and Simpson indices (Fig. 1a). While the overall composition of the intestinal bacterial community did not differ significantly between groups, there was a trend toward higher observed richness in the *Blastocystis*-positive group. No significant differences in diversity metrics

were observed in relation to varying levels of *Blastocystis* burden. However, beta diversity analysis revealed significant distinctions (PERMANOVA: adonis2, $P = 0.0001$) between *Blastocystis*-positive and -negative individuals, indicating the existence of two distinct microbiota profiles based on *Blastocystis* presence. Notably, in *Blastocystis*-positive individuals, taxa such as *Bacteroides uniformis*, *Oscillibacter* sp., and *Prevotella copri* were more abundant, while *Eubacterium rectale* and *Blautia luti* were more prevalent in *Blastocystis*-negative individuals (Fig. 1b).

Microbiota Bacterial Composition: Relative Abundances and Differential Taxa

To understand the global composition of the microbiota in relation to *Blastocystis* status, we analyzed relative abundances and differential taxa. Both *Blastocystis*-positive and -negative groups were predominantly characterized by the families Lachnospiraceae, Ruminococcaceae, Clostridiaceae, Prevotellaceae, and Bacteroidaceae. However, the *Blastocystis*-positive group exhibited a subtle shift, with a reduction in the relative abundance of Lachnospiraceae and an increase in Ruminococcaceae and Bacteroidaceae, suggesting specific microbial alterations associated with *Blastocystis* colonization (Fig. S3a). When examining relative abundances based on *Blastocystis* burden levels, these patterns became more pronounced in the high-burden group, where the abundance of Ruminococcaceae and Bacteroidaceae was notably higher compared to other groups (Fig 3a).

We employed Linear Discriminant Analysis Effect Size (LEfSe) and DESeq2 to analyze the taxa differentially abundant among the comparison groups. Our comparison between *Blastocystis*-positive and -negative groups revealed that the *Blastocystis*-positive group exhibited higher abundances of genera such as *Bacteroides*, *Faecalibacterium*, and *Alistipes*. In contrast, the *Blastocystis*-negative group showed a greater prominence of genera like *Blautia*, *Staphylococcus*, *Enterococcus*, and *Klebsiella* (Fig. 1 c, d, and Fig. S3b). Further analysis of *Blastocystis* burden groups (high, medium, and low) identified distinct taxa associated with each burden level. High burden levels were linked to genera such as *Alistipes*, *Lachnospira*, and *Arcanobacterium*; medium burden levels were associated with *Acetivibrio* and *Peptoniphilus*; and low burden levels correlated with *Bacteroides*, *Phascolarctobacterium*, *Paraprevotella*, and *Akkermansia* (Fig. 3c). These findings suggest that specific alterations in the microbiota may be linked not only to the presence

of *Blastocystis* but also to the burden of *Blastocystis*. This implies that variations in *Blastocystis* burden could have a relevant impact on the dynamics underlying changes in the composition and structure of the host microbiota.

Eukaryotic Microbiota Composition

Beta diversity analysis of the eukaryotic microbiota also showed potential differences between Blastocystis-positive and -negative groups.

Although alpha diversity indices did not show significant differences between *Blastocystis*-positive and -negative groups, our analysis revealed that among *Blastocystis* burden groups, higher burden levels were associated with reduced eukaryotic diversity compared to the *Blastocystis*-negative group (Mann-Whitney-Wilcoxon test, $P < 0.05$) (Fig. 2a). This suggests that the presence of *Blastocystis*, as an eukaryote, may diminish the abundance of other eukaryotic organisms, particularly fungi, in the host microbiota. Beta diversity analysis further confirmed significant differences in eukaryotic microbiota composition between *Blastocystis*-positive and -negative individuals (PERMANOVA: adonis2, $P = 0.0001$), indicating distinct eukaryotic profiles associated with *Blastocystis* presence. Specifically, *Entamoeba coli* was commonly found in *Blastocystis*-positive individuals, whereas the fungi *Saccharomyces cerevisiae* and *Candida albicans* were more characteristic of the *Blastocystis*-negative group (Fig. 2b).

Eukaryotic Microbiota Composition: Relative Abundances and Differential Taxa

In terms of eukaryotic microbiota, both groups were predominantly characterized by the families Saccharomycetaceae, Blastocystidae, Entamoebida, Malasseziaceae, and Trichomycetes. In the *Blastocystis*-positive group, we observed a notable reduction in the relative abundance of Saccharomycetaceae and an increase in Entamoebida (Fig. S4a). Regarding *Blastocystis* burden groups, we observed that higher *Blastocystis* burdens were associated with a reduction in the relative abundance of other eukaryotes, particularly fungi from the families *Saccharomycetaceae* and *Malasseziaceae*. Notably, the family *Entamoebida* consistently maintained a higher relative abundance across all burden levels in *Blastocystis*-positive individuals (Fig. 3b).

To identify differentially abundant eukaryotic taxa for each group, we employed LEfSe and DESeq2. In comparing *Blastocystis*-positive and -negative groups, we found that the *Blastocystis*-positive group exhibited a differential abundance of genera such as *Barnettozyma*, *Entamoeba*, and *Endolimax*, while the *Blastocystis*-negative group was characterized by higher abundances of *Candida* and *Malassezia* (Fig. 2 c,d and Fig. S3b). Additionally, analysis of *Blastocystis* burden groups revealed that high burden levels were associated with genera such as *Issatchenkia*, *Pleurostomophora*, and *Citeromyces*, whereas low burden levels were associated with genera like *Adelina* and *Cyniclomyces* (Fig. 3d).

Microbiota composition as a predictor of *Blastocystis* presence

The random forest classifier was trained to predict *Blastocystis* colonization status based on microbiota composition (Fig. S6). The model achieved an out-of-bag (OOB) error rate of 23.86%, indicating the proportion of samples misclassified during the internal validation process. The confusion matrix reveals that the model correctly classified 41 of 49 negative samples (class error = 16.3%) and 26 of 39 positive samples (class error = 33.3%). When evaluating the model on the test data, it achieved a perfect accuracy of 1.0, with a 95% confidence interval of (0.9589, 1). Additionally, the P-value for accuracy being greater than the No Information Rate (NIR) was highly significant ($P < 2.2e-16$), indicating that the model's performance was far superior to random chance. These results suggest that the random forest model was highly effective in classifying *Blastocystis* colonization status based on microbiota composition, although there was some degree of misclassification in the training process, particularly among positive samples.

The random forest model identified the top 20 taxa that were most important for classifying *Blastocystis* colonization based on the Mean Decrease in Gini Index, which reflects each taxa contribution to the accuracy of the model. A bar plot of the Mean Decrease in Gini values revealed that taxa predictors varied significantly in their importance (Fig. S6). The taxa with the highest Mean Decrease in Gini were the most influential in differentiating between positive and negative *Blastocystis* colonization. This visualization highlights the specific taxa that play a key role in driving the model's predictive performance, indicating potential microbial markers associated with *Blastocystis* status.

Discussion

Our findings suggest that *Blastocystis* colonization is associated with significant shifts in both the composition and structure of the gut microbiota, which may correlate with improvements in host health observed in non-westernized rural populations in previous studies [7]. Piperni et al. (2024) reported that the changes in the gut microbiome related to *Blastocystis* vary across different geographical regions and lifestyles, highlighting a strong connection with healthier, plant-based diets common in these populations [7]. Additionally, their study indicated that *Blastocystis* was associated with improved cardiometabolic profiles, and its presence increased following a diet-improvement intervention program [7]. These findings suggest that *Blastocystis* may have a profound impact on the host—not only in terms of microbiome composition and structure but also regarding metabolic and immunological processes that contribute to improved health conditions [7]. These results are particularly relevant for non-westernized rural populations, such as the Colombian cohort used in the present study, where environmental exposures, dietary practices, and lifestyle factors differ significantly from those in urban settings [8,10,11]. Such differences may profoundly influence microbiota diversity and parasite prevalence. In this context, we found that the presence of *Blastocystis* was related to increased bacterial diversity, which aligns with previous research [7,8]. This suggests that *Blastocystis* may play a role in promoting a more diverse and stable microbial community. Greater microbial richness is often associated with improved health outcomes, as a more diverse microbiota provides functional redundancy, enhancing the gut's resilience to disruptions such as infections or dietary changes [34–36].

Beta diversity analysis revealed that the bacterial microbiota exhibited a distinct profile concerning *Blastocystis* presence, suggesting that this protozoan may modulate both the structure and composition of the gut microbiota (Fig. 1). The analysis also showed that among *Blastocystis*-positive individuals, certain bacterial taxa, such as *Bacteroides uniformis*, *Oscillibacter* sp., and *Prevotella copri*, were more prevalent, consistent with findings from previous studies. *B. uniformis* is well-known for its role in carbohydrate metabolism and energy extraction from plant-based diets, contributing to nutrient absorption and gut barrier integrity [37–39]. In this context, *B. uniformis* has been shown to improve whole-body glucose disposal, increase butyrate production, and restore the balance of intraepithelial lymphocytes and type-3 innate lymphoid cells in the intestinal epithelium. These functions enhance both the metabolic and immunological properties

of the gut barrier, which is particularly beneficial in non-westernized rural populations with higher fiber intake [37]. *Oscillibacter* has been associated with anti-inflammatory effects, which may help protect against gut inflammation and promote overall well-being. In addition to these properties, *Oscillibacter* has demonstrated potential benefits for lipid homeostasis and cardiovascular function. By contributing to the regulation of lipid metabolism, it may play a key role in maintaining metabolic balance, further supporting its protective effects on the host's overall condition [8,40]. Furthermore, *Prevotella copri*, although associated with inflammation in certain contexts, is a key indicator of diets rich in fiber and plant-based foods [11]. *P. copri* is also recognized as an important SCFA-producing bacterium, which plays a crucial role in maintaining proper metabolic functions in the gut and supporting energy processes in enterocytes [41,42]. The increased abundance of SCFA-producing taxa in *Blastocystis*-positive individuals suggests that *Blastocystis* may enhance these beneficial microbial functions, contributing to gut health.

These findings underscore the complex relationship between *Blastocystis* colonization, diet, and gut bacterial microbiota, highlighting how the presence of *Blastocystis* may contribute to microbiota diversity by promoting taxa linked to improved metabolic and immune functions. This interaction could have positive health implications, particularly for individuals consuming fiber-rich diets, as the promoted taxa may enhance nutrient absorption, immune responses, and gut homeostasis. These effects are especially relevant in non-westernized rural populations, where diet and lifestyle play a significant role in shaping microbiota composition [7,8,37,39,42–44].

Furthermore, *Blastocystis* burden levels appeared to be related to specific microbial taxa, suggesting a potential dose-dependent relationship between *Blastocystis* burden and its impact on the microbiota (Fig. 3). In examining *Blastocystis* burden groups (high, medium, and low), higher *Blastocystis* burdens were related to taxa such as *Alistipes* and *Lachnospira*, both known for their anti-inflammatory properties and role in maintaining gut homeostasis [45–47]. *Alistipes*, in particular, has been linked to a reduced risk of inflammation and metabolic disorders, suggesting that higher *Blastocystis* levels, along with other host conditions, may confer protective effects against inflammatory processes [46]. In contrast, lower *Blastocystis* burdens were associated with taxa such as *Akkermansia*, a bacterium recognized for maintaining the gut mucus layer and improving metabolic health. These findings suggest that the interaction between *Blastocystis* and the microbiota is complex and potentially modulated by the protozoan’s abundance in the gut [48].

To accurately assess these relationships, it is essential to develop study designs and protocols based on longitudinal cohorts. Such approaches would allow for a more dynamic evaluation of *Blastocystis* colonization over time and its potential impact on gut microbiota composition. Moreover, studies should not only focus on *Blastocystis* but also consider other common parasites and luminal protozoa that may co-exist in individuals. These co-occurring organisms could interact in agonistic or synergistic ways, influencing the observed microbiota changes. Investigating these interactions would provide more precise assessments of *Blastocystis* burden and yield deeper insights into the causal mechanisms and dynamics underlying its presence, as well as its broader impact on the host's microbiota structure and composition.

We aimed to explore the eukaryotic microbiota concerning *Blastocystis* presence (Fig. 2). Our findings revealed that *Blastocystis* colonization was associated with relevant changes in the eukaryotic microbiota. In the *Blastocystis*-positive group, we observed an increased abundance of *Entamoeba coli*, a commensal protozoan often considered benign [3]. This contrasts with *Blastocystis*-negative individuals, who were more likely to harbor *Saccharomyces cerevisiae* and *Candida albicans* (Fig. 2). There are a limited number of studies that involve the analysis of eukaryotic microbiota; however, changes in fungal communities have been reported in conditions such as irritable bowel syndrome (IBS), characterized by an enrichment of *Saccharomycetes* and a decrease in alpha diversity, which is consistent with our findings [49]. Furthermore, while *Candida albicans* is a normal component of the gut microbiota in healthy individuals, it has been associated with mucosal diseases and can translocate into the bloodstream, potentially leading to invasive, life-threatening infections, particularly in immunocompromised patients [50,51]. Additionally, *C. albicans* has been observed to contribute to the development of colitis in mouse models and shows increased abundance in patients with Crohn’s disease, although the pathogenic mechanisms remain not fully understood [52]. These findings suggest a potentially beneficial and complex interaction between *Blastocystis* and other eukaryotes; however, the level and type of interaction among *Blastocystis*, other microbial eukaryotes (including fungi), and the host microbiome remain unclear. Future studies should focus on exploring the eukaryotic component of the gut microbiome as a whole, rather than targeting individual microbial species, to better elucidate the role of eukaryotic microbiota within the gut ecosystem.

Further analysis regarding *Blastocystis* burdens groups revealed that the burden of *Blastocystis* may be related to the abundance of specific eukaryotic taxa (Fig. 3). Higher *Blastocystis* burdens were linked with genera such as *Issatchenkia*, *Pleurostomophora*, and *Citeromyces*. These genera, likely stemming from the consumption of fermented foods and vegetables, may indicate a response to or modulation by elevated *Blastocystis* levels [49,53,54]. These genera, while not extensively studied, could contribute to the gut's eukaryotic diversity by promoting an anaerobic environment and potentially interacting with other microbial components to maintain balance. However, their impact on these interactions requires further analysis to determine whether these changes are beneficial or detrimental to the host. The observed differences in eukaryotic taxa associated with *Blastocystis* presence and burden underscore the protozoan's potential role in shaping the eukaryotic microbiota [53,54]. Metagenomic and metabolomic approaches, particularly in longitudinal prospective studies, are crucial for characterizing all microbiota components and predicting functional changes. These methods allow for a comprehensive understanding of the mechanisms and direction of changes mediated by *Blastocystis* presence and burden. By integrating these approaches, it will be possible to establish causal links, identify metabolic pathways affected by *Blastocystis*, and elucidate how these shifts impact host health, potentially revealing key insights into the protozoan's role in modulating the gut ecosystem.

The random forest classifier's ability to predict *Blastocystis* colonization status based on microbiota composition highlights its potential as a promising approach [55]. The model demonstrates superior performance compared to random chance, validating its utility in distinguishing between *Blastocystis*-positive and -negative individuals. The classifier's performance, while showing some misclassification in training—particularly among positive samples—suggests that the model effectively identifies key microbial indicators associated with *Blastocystis* colonization. The model identified several taxa as critical for classifying *Blastocystis* status (Fig. S3). Notably, and in line with the results presented earlier, *Faecalibacterium prausnitzii* and *Bacteroides ovatus* were among the top taxa with high Mean Decrease in Gini values. *Faecalibacterium prausnitzii* is well known for its anti-inflammatory properties and its critical role in maintaining gut health, which may correspond with the observed microbiota stability in the presence of *Blastocystis*. The prominence of these taxa suggests that *Blastocystis* colonization could be linked to the promotion of beneficial microbial communities that contribute to gut homeostasis.[56–58]. Similarly, *Bacteroides ovatus* is involved in the breakdown of complex

carbohydrates, contributing to a more balanced and diverse microbial environment. Likewise, *Bacteroides thetaiotaomicron* and *Ruminococcus bromii*, both significant in the classifier, are involved in complex carbohydrate digestion and the production of short-chain fatty acids, which are crucial for gut health and homeostasis [5,8,56–61]. *Entamoeba coli*, another important taxon discussed before, is a commensal eukaryote often found alongside *Blastocystis*. Its presence may indicate a balanced eukaryotic community that can coexist with *Blastocystis* without causing dysbiosis [3]. These findings suggest that the classifier could be a valuable tool for predicting *Blastocystis* colonization by analyzing the relative abundances of specific taxa. For instance, a high presence of *Faecalibacterium prausnitzii* or *Bacteroides ovatus* might indicate a higher likelihood of *Blastocystis* colonization. This predictive capability could provide key insights into microbial interactions in future research, potentially aiding in the development of targeted interventions or treatments aimed at maintaining or improving gut health. By leveraging these microbial signatures, we may better understand the functional dynamics of *Blastocystis* within the gut ecosystem.

It is important to acknowledge the limitations of our study. First, the sample size was relatively small, and we were unable to collect detailed information regarding key variables, such as diet. The hypothetical role of *Blastocystis* suggested through our analysis necessitates further investigation using longitudinal studies that incorporate metagenomic, metabolomic, and immunological approaches, alongside comprehensive data on dietary habits, lifestyle factors, and comorbidities. Such information is crucial for making accurate inferences about *Blastocystis*-host interactions, particularly considering the co-occurrence of other eukaryotes that may influence the observed changes [11,62]. Further research is needed to elucidate the mechanisms underlying the interactions between *Blastocystis* and the host microbiota. While our study employed a metabarcoding approach to characterize both bacterial and eukaryotic communities, this method does not enable predictions of metabolic and functional changes that may elucidate the dynamics involved in these interactions. Larger sample sizes and prospective longitudinal studies are essential to determine the directionality and causality of these relationships. Although we identified several relevant taxa, particularly within fungal communities, a deeper understanding of the significance of these specific taxa in the microbiota is necessary to clarify their potential positive impacts on host health. This comprehensive approach will enhance our ability to interpret the role of *Blastocystis* and its possible effects on the gut ecosystem.

Our findings have significant implications for understanding the role of *Blastocystis* in gut health. Rather than being a pathogen, *Blastocystis* may act as a commensal organism that supports a diverse and functionally rich gut microbiota [5–8]. The presence of *Blastocystis* was related to taxa that promote nutrient absorption, immune regulation, and protection against pathogens, all of which are critical for maintaining overall health. In non-westernized rural populations, where diet and environmental factors play a significant role in shaping the microbiota, *Blastocystis* may enhance the gut's ability to adapt to these influences, contributing to a healthier microbiome [7]. The potential for *Blastocystis* to modulate the microbiota beneficially also opens the door to new therapeutic approaches. By promoting the growth of beneficial bacteria and maintaining a balanced eukaryotic community, *Blastocystis* may serve as a marker of gut health and could be used as a target for microbiota-based therapies [7]. Future research should focus on exploring the mechanisms by which *Blastocystis* interacts with the microbiota and the specific conditions under which it exerts its beneficial effects.

In conclusion, our study suggests that *Blastocystis* colonization is linked with a more diverse and functionally rich gut microbiota, challenging the traditional view of *Blastocystis* as a pathogen. The presence of *Blastocystis* was linked to taxa that play critical roles in gut health, particularly in non-westernized rural populations where diet and environmental exposures differ from urban settings. By fostering a diverse microbial community and promoting beneficial taxa, *Blastocystis* may act as a commensal organism that supports gut health and resilience. Further research is needed to fully understand the role of *Blastocystis* in gut microbiota dynamics and its potential implications for human health.

Figure Legends

Figure 1. Bacterial profile 16S rRNA. (a) Diversity metrics—observed richness, Pielou, Shannon, and Simpson indices—compared between *Blastocystis*-positive and -negative groups. Statistical analyses were performed using the Kruskal-Wallis (KW) test, followed by a post-hoc Mann-Whitney-Wilcoxon (MWW) test for multiple comparisons. The plots show interquartile ranges (IQR; boxes), medians (lines within the boxes), and the lowest and highest values within 1.5 times the IQR from the first and third quartiles (whiskers). (b) Principal coordinates analysis (PCoA) ordination of Bray-Curtis distances of microbial communities, comparing *Blastocystis*-positive

and -negative groups. Permutational analysis of variance (PERMANOVA) was performed using the adonis function from the vegan package. *** p-values <0.001. (c) Boxplot of differentially abundant taxa across *Blastocystis*-positive and -negative groups, evaluating distribution patterns. The threshold for the logarithmic LDA score was 2.0, with a maximum Kruskal-Wallis (KW)/Wilcoxon Test value of 0.01. (d) Volcano plots showing differentially abundant taxa between *Blastocystis*-positive and -negative groups, based on DESeq2 analysis.

Figure 2. Eukaryotic profile 18S rRNA. (a) Diversity metrics—observed richness, Pielou, Shannon, and Simpson indices—compared between *Blastocystis*-positive and -negative groups. Statistical analyses were performed using the Kruskal-Wallis (KW) test, followed by a post-hoc Mann-Whitney-Wilcoxon (MWW) test for multiple comparisons. The plots show interquartile ranges (IQR; boxes), medians (lines within the boxes), and the lowest and highest values within 1.5 times the IQR from the first and third quartiles (whiskers). (b) Principal coordinates analysis (PCoA) ordination of Bray-Curtis distances of microbial communities, comparing *Blastocystis*-positive and -negative groups. Permutational analysis of variance (PERMANOVA) was performed using the adonis function from the vegan package. *** p-values <0.001. (c) Boxplot of differentially abundant taxa across *Blastocystis*-positive and -negative groups, evaluating distribution patterns. The threshold for the logarithmic LDA score was 2.0, with a maximum Kruskal-Wallis (KW)/Wilcoxon Test value of 0.01. (d) Volcano plots showing differentially abundant taxa between *Blastocystis*-positive and -negative groups, based on DESeq2 analysis.

Figure 3. Microbiota profile across burden groups of *Blastocystis*-positive samples. Bar charts display the relative abundance of the top taxa at the family level between burden groups of *Blastocystis*-positive samples, based on (a) 16S rRNA data and (b) 18S rRNA data. Linear discriminant analysis effect size (LEfSe) identifying taxa enriched in burden groups of *Blastocystis*-positive samples, corresponding to (c) 16S rRNA data and (d) 18S rRNA data. The threshold for the logarithmic LDA score was 2.0, with a maximum Kruskal-Wallis (KW)/Wilcoxon Test value of 0.01.

Figure S1. Geographical sampling area corresponding to the rural region of Cauca, Colombia.

Figure S2. (a) Proportion of samples by total reads showing abundance by phylum. (b) Diversity metrics—richness, Shannon, and Simpson indices—compared between *Blastocystis*-positive and -negative groups. (c) Bar chart displaying the relative abundance of the top taxa at the family level

for individual samples in *Blastocystis*-positive and -negative groups. (d) Bar chart showing the relative abundance of the top taxa at the family level aggregated by group.

Figure S3. (a) Bar chart showing the relative abundance of the top taxa at the family level between *Blastocystis*-positive and -negative groups. (b) Linear discriminant analysis effect size (LEfSe) identifying taxa enriched in *Blastocystis*-positive and -negative groups, with a bar plot showing enriched taxa at the genus level.

Figure S4. (a) Bar chart showing the relative abundance of the top taxa at the family level between *Blastocystis*-positive and -negative groups. (b) Linear discriminant analysis effect size (LEfSe) identifying taxa enriched in *Blastocystis*-positive and -negative groups, with a bar plot showing enriched taxa at the genus level.

Figure S5. Diversity metrics—observed richness, Pielou, Shannon, and Simpson indices—are compared between burden groups of *Blastocystis*-positive samples, corresponding to (a) 16S rRNA data and (b) 18S rRNA data. Statistical analyses were performed using the Kruskal-Wallis (KW) test, followed by a post-hoc Mann-Whitney-Wilcoxon (MWW) test for multiple comparisons. The plots show interquartile ranges (IQR; boxes), medians (lines within the boxes), and the lowest and highest values within 1.5 times the IQR from the first and third quartiles (whiskers).

Figure S6. A random forest model was trained to classify *Blastocystis* colonization status. The figure highlights the 20 most important taxa identified based on the Mean Decrease in Gini Index, reflecting their contribution to the model's predictive accuracy.

Table S1. Summary of sequence results and classification in *Blastocystis*-positive and -negative groups, as well as across different burden groups. The table provides an overview of total reads, and classification accuracy for each group.

Data availability

The code for generating the figures and the analysis is available at https://github.com/scastanedag/Blastocystis_burdens.git.

Declaration of Competing Interest

The authors declare that no competing interests exist.

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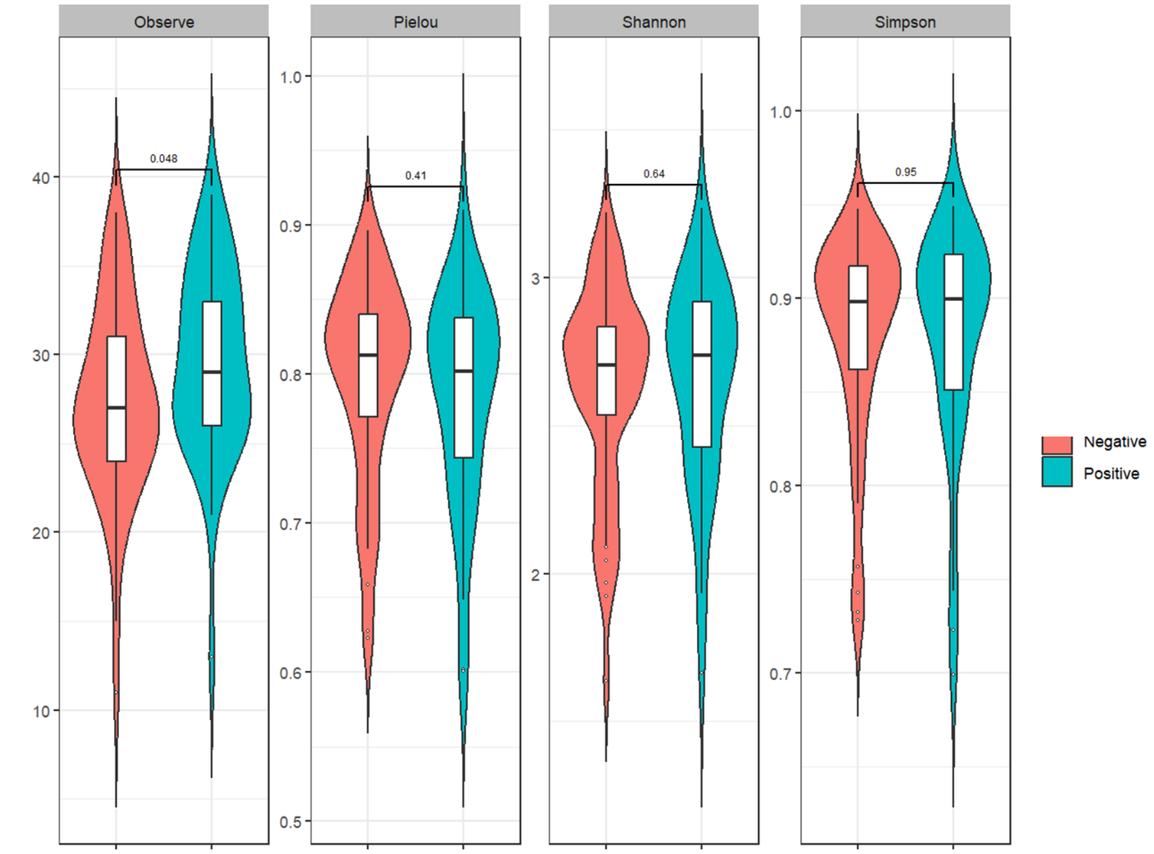
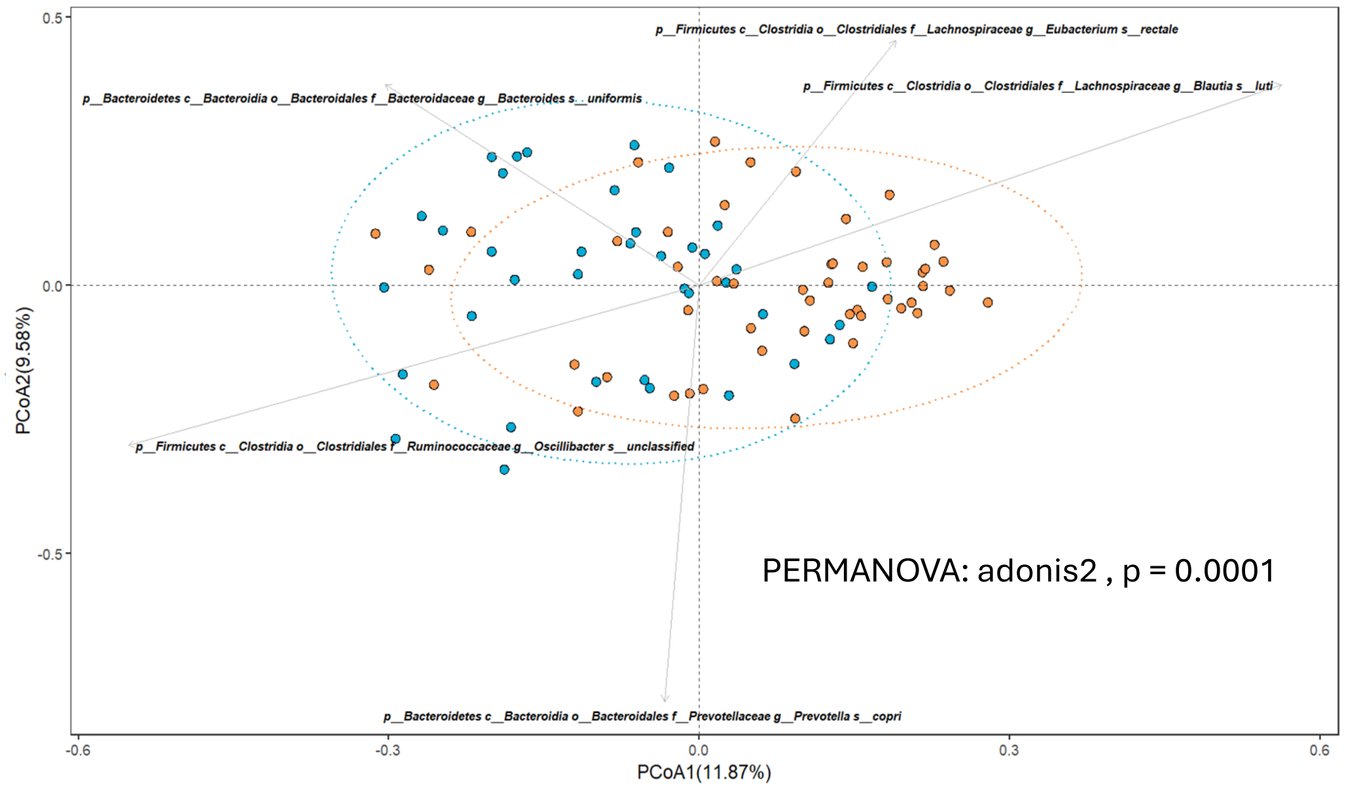
793

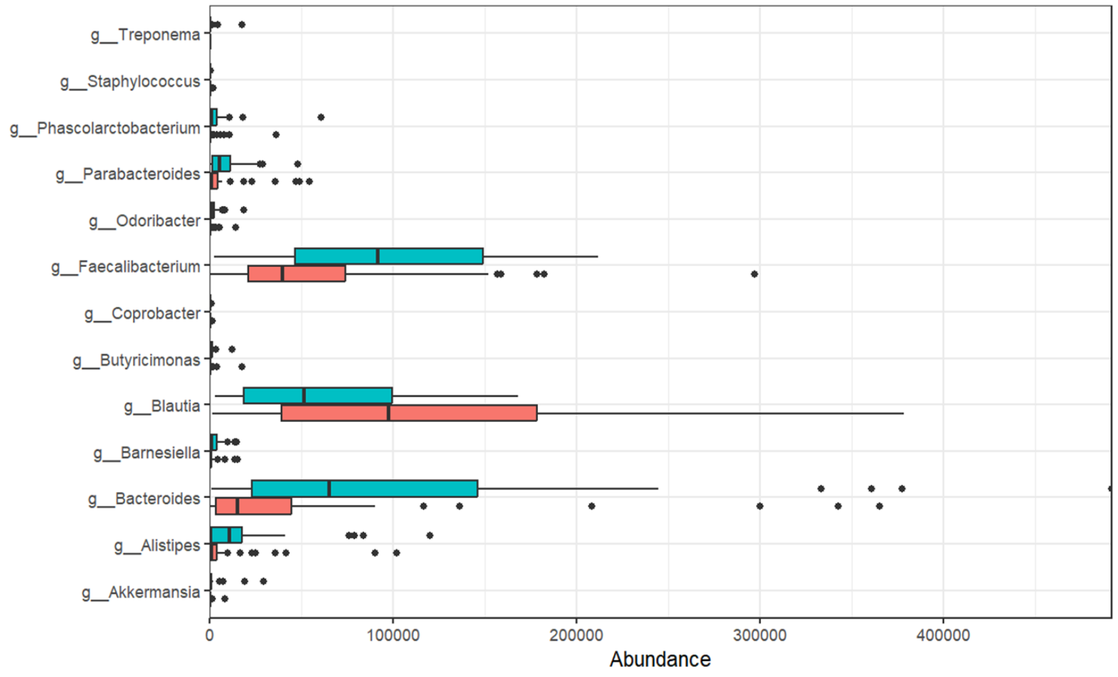
a.


Observe
Pielou
Shannon
Simpson
0.048
0.41
0.64
0.95
Negative
Positive

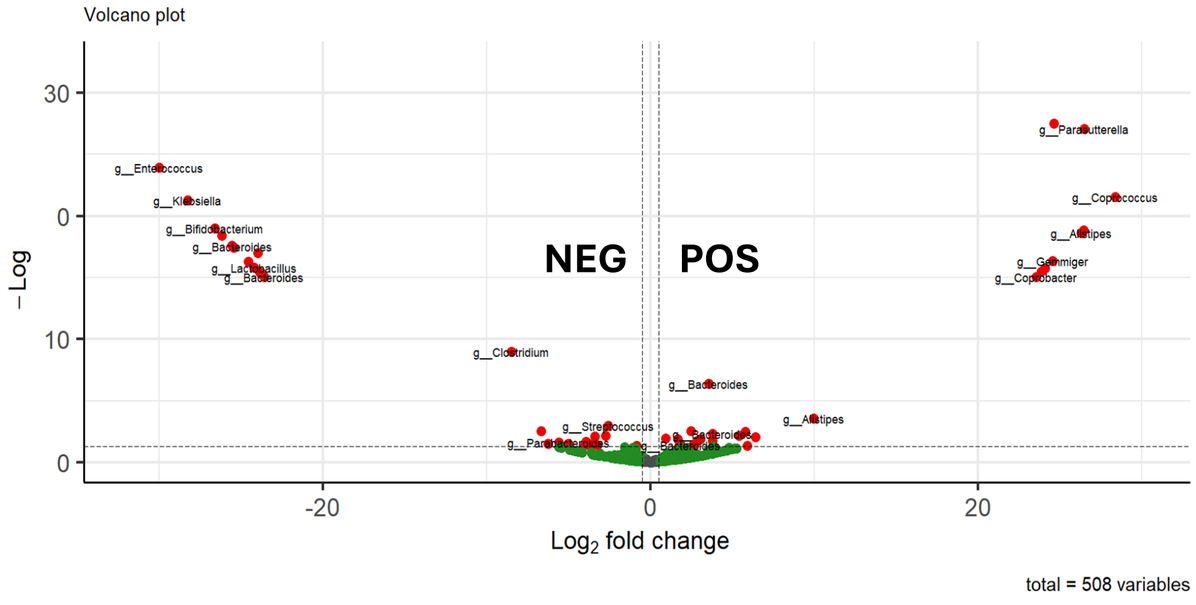
b.


p__Firmicutes c__Clostridia o__Clostridiales f__Lachnospiraceae g__Eubacterium s__rectale
p__Firmicutes c__Clostridia o__Clostridiales f__Lachnospiraceae g__Blautia s__luti
p__Bacteroidetes c__Bacteroidia o__Bacteroidales f__Bacteroidaceae g__Bacteroides s__uniformis
p__Firmicutes c__Clostridia o__Clostridiales f__Ruminococcaceae g__Oscillibacter s__unclassified
p__Bacteroidetes c__Bacteroidia o__Bacteroidales f__Prevotellaceae g__Prevotella s__copri
PERMANOVA: adonis2 , p = 0.0001
PCoA2(9.58%)
PCoA1(11.87%)

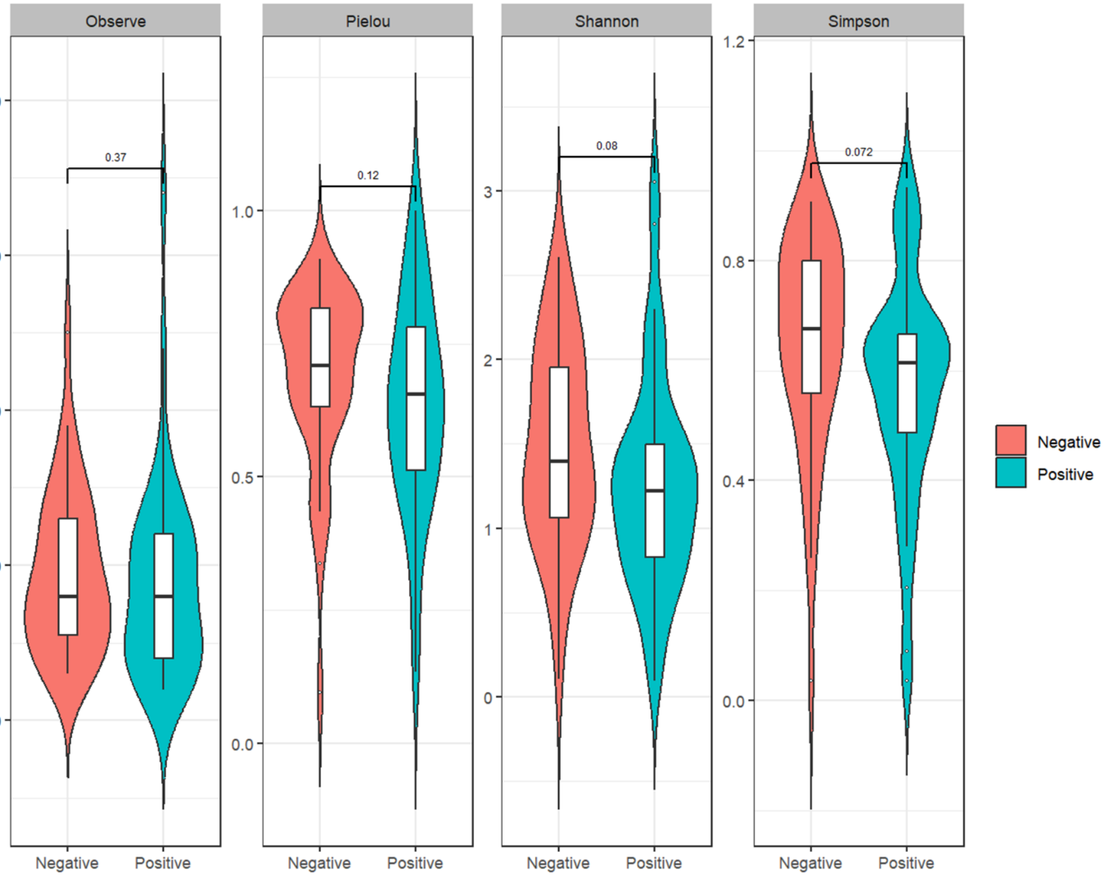
c.


g__Treponema
g__Staphylococcus
g__Phascolarctobacterium
g__Parabacteroides
g__Odoribacter
g__Faecalibacterium
g__Coprobacter
g__Butyricimonas
g__Blautia
g__Barnesiella
g__Bacteroides
g__Alistipes
g__Akkermansia
Abundance
Negative
Positive

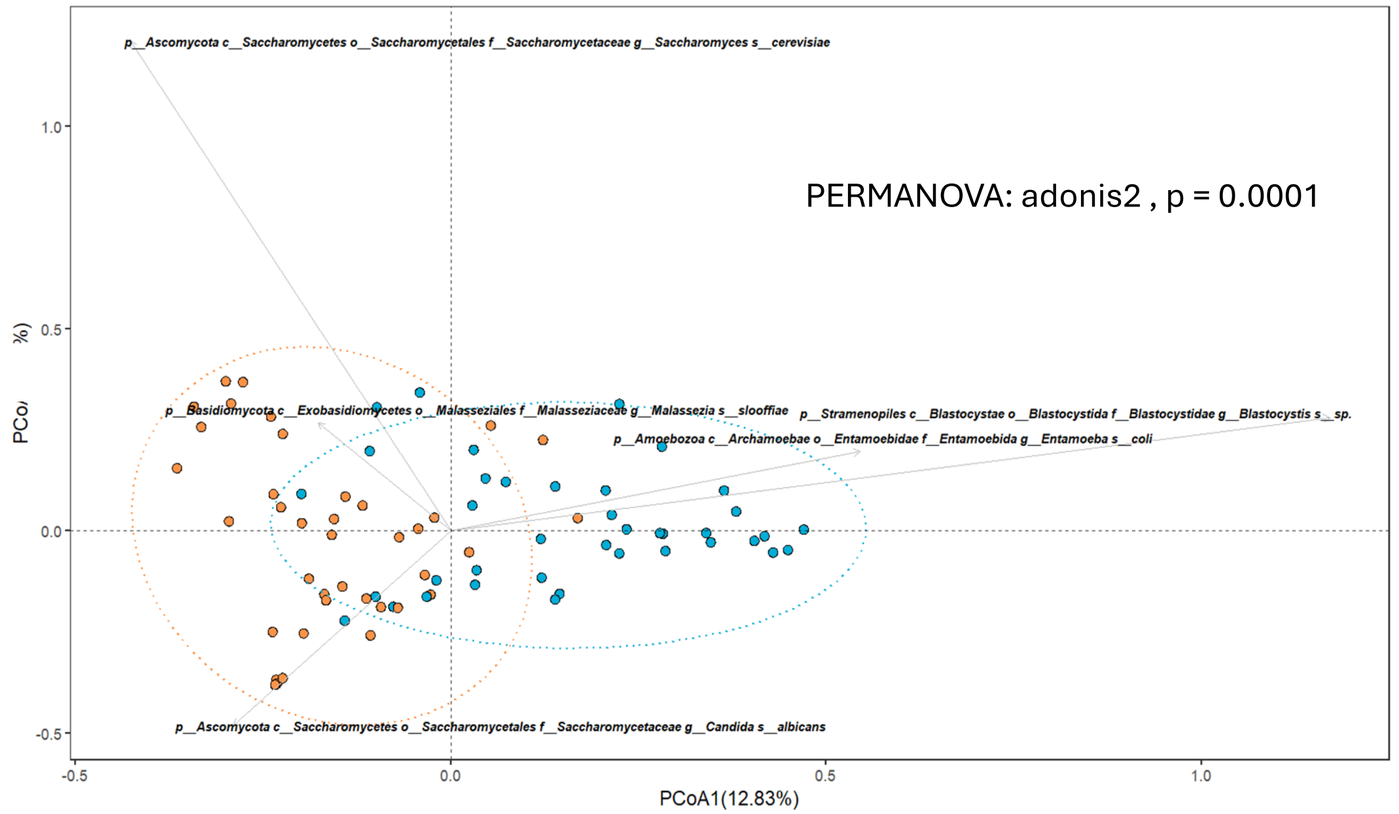
d.


Volcano plot
NEG
POS
g__Parasutterella
g__Enterococcus
g__Klebsiella
g__Bifidobacterium
g__Bacteroides
g__Lactobacillus
g__Coprococcus
g__Alistipes
g__Gemmiger
g__Coprobacter
g__Clostridium
g__Streptococcus
g__Parabacteroides
Log2 fold change
total = 508 variables

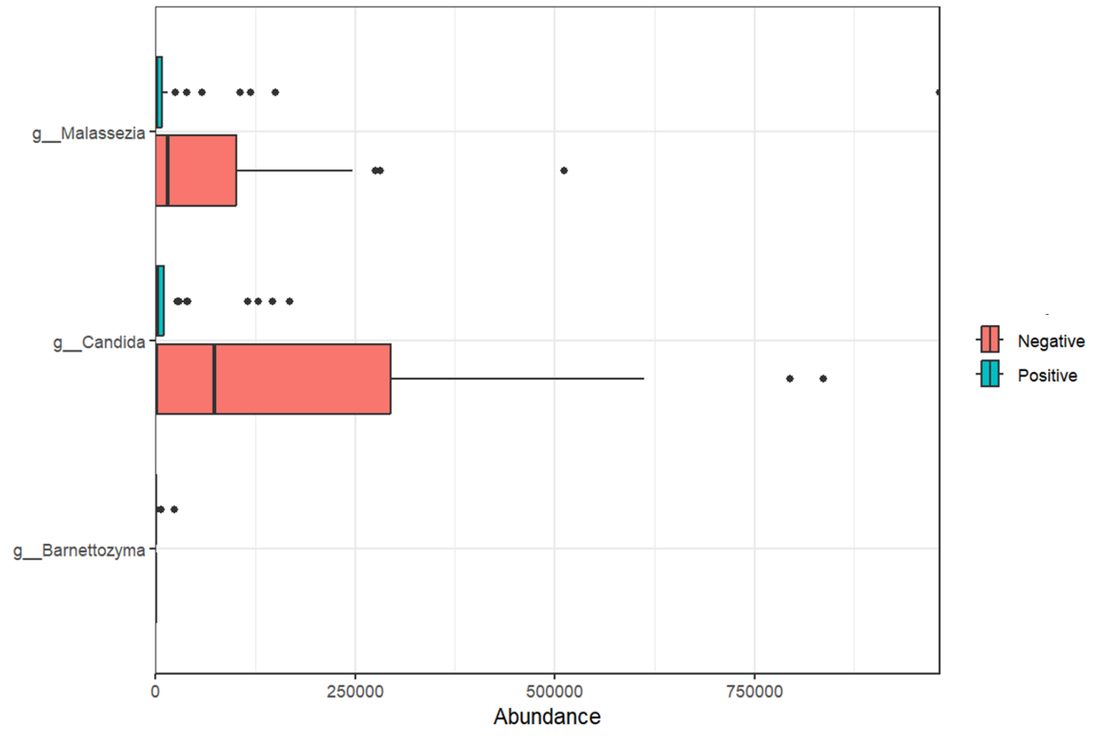
a.


Observe
Pielou
Shannon
Simpson
0.37
0.12
0.08
0.072
Negative
Positive

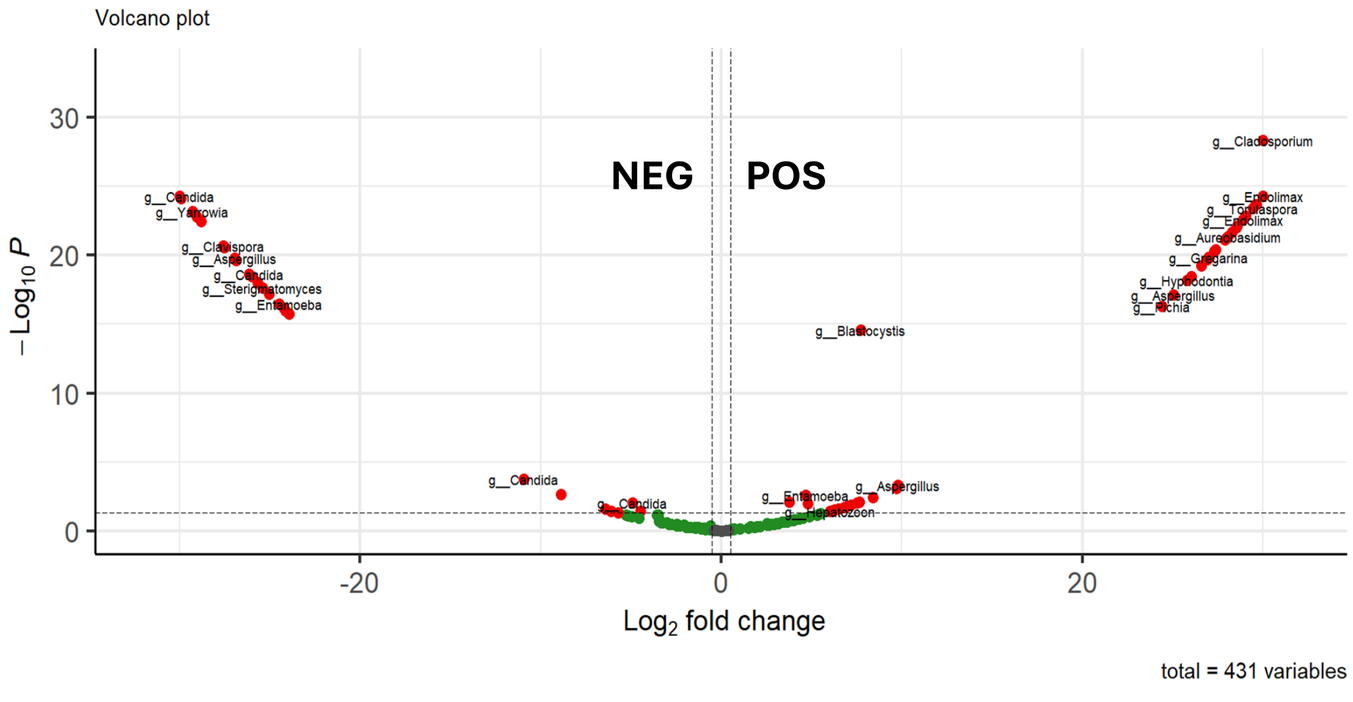
b.


p__Ascomycota c__Saccharomycetes o__Saccharomycetales f__Saccharomycetaceae g__Saccharomyces s__cerevisiae
PERMANOVA: adonis2 , p = 0.0001
p__Basidiomycota c__Exobasidiomycetes o__Malasseziales f__Malasseziaceae g__Malassezia s__slooffiae
p__Stramenopiles c__Blastocystae o__Blastocystida f__Blastocystidae g__Blastocystis s__sp.
p__Amoebozoa c__Archamoebae o__Entamoebidae f__Entamoeba g__Entamoeba s__coli
p__Ascomycota c__Saccharomycetes o__Saccharomycetales f__Saccharomycetaceae g__Candida s__albicans
PCoA1(12.83%)

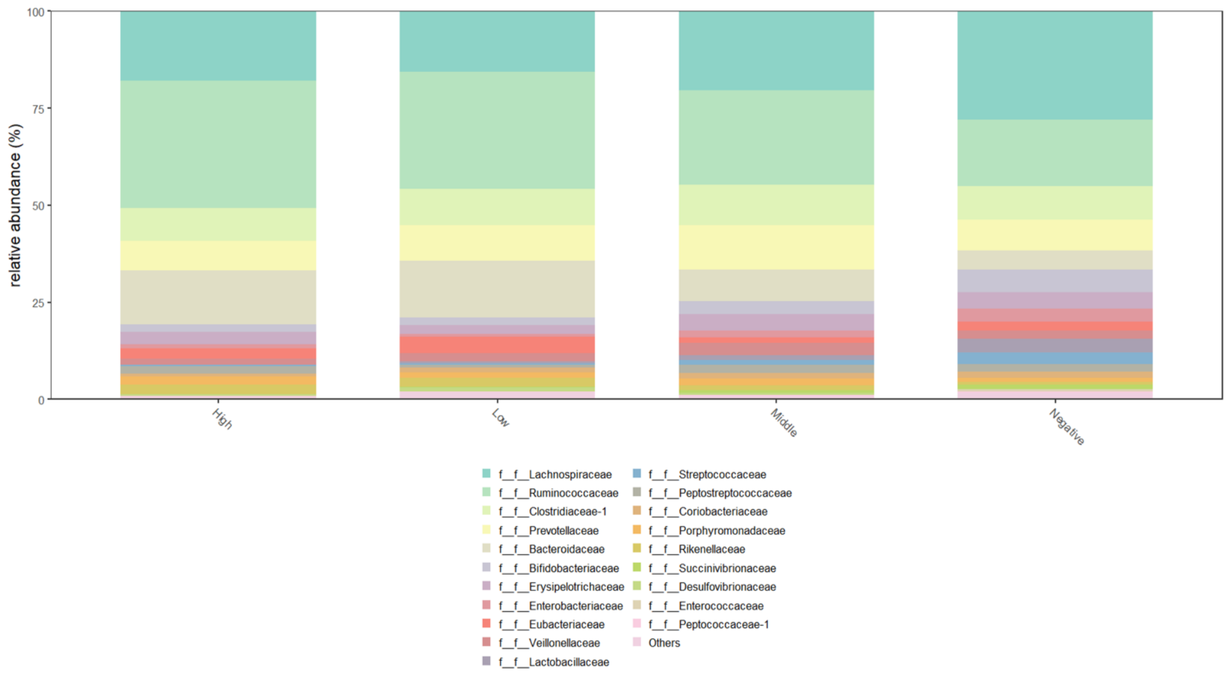
c.


g__Malassezia
g__Candida
g__Barnettozyma
Abundance
Negative
Positive

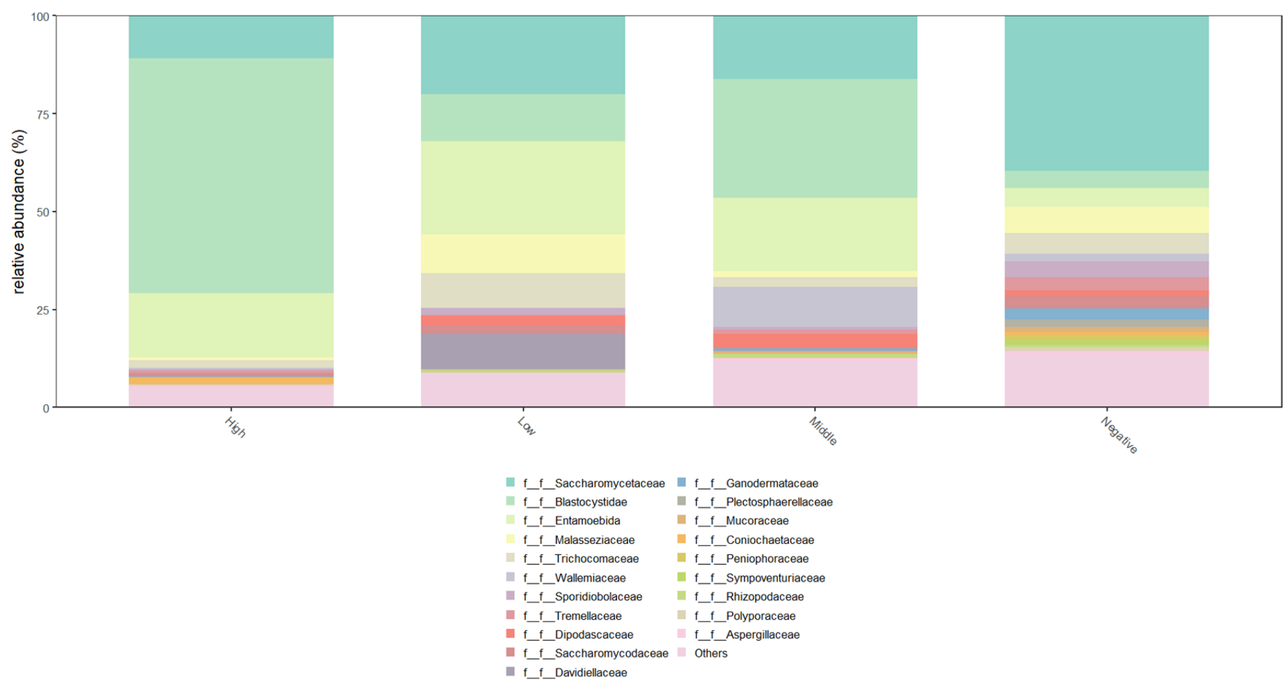
d.


Volcano plot
NEG
POS
g__Cladosporium
g__Candida
g__Yarrowia
g__Cladispora
g__Aspergillus
g__Candida
g__Sterigmatomyces
g__Entamoeba
g__Endolimax
g__Torulaspora
g__Endolimax
g__Aureobasidium
g__Gregarina
g__Hyphodontia
g__Aspergillus
g__Fichia
g__Blastocystis
g__Candida
g__Candida
g__Entamoeba
g__Aspergillus
g__Haptozoon
Log2 fold change
total = 431 variables

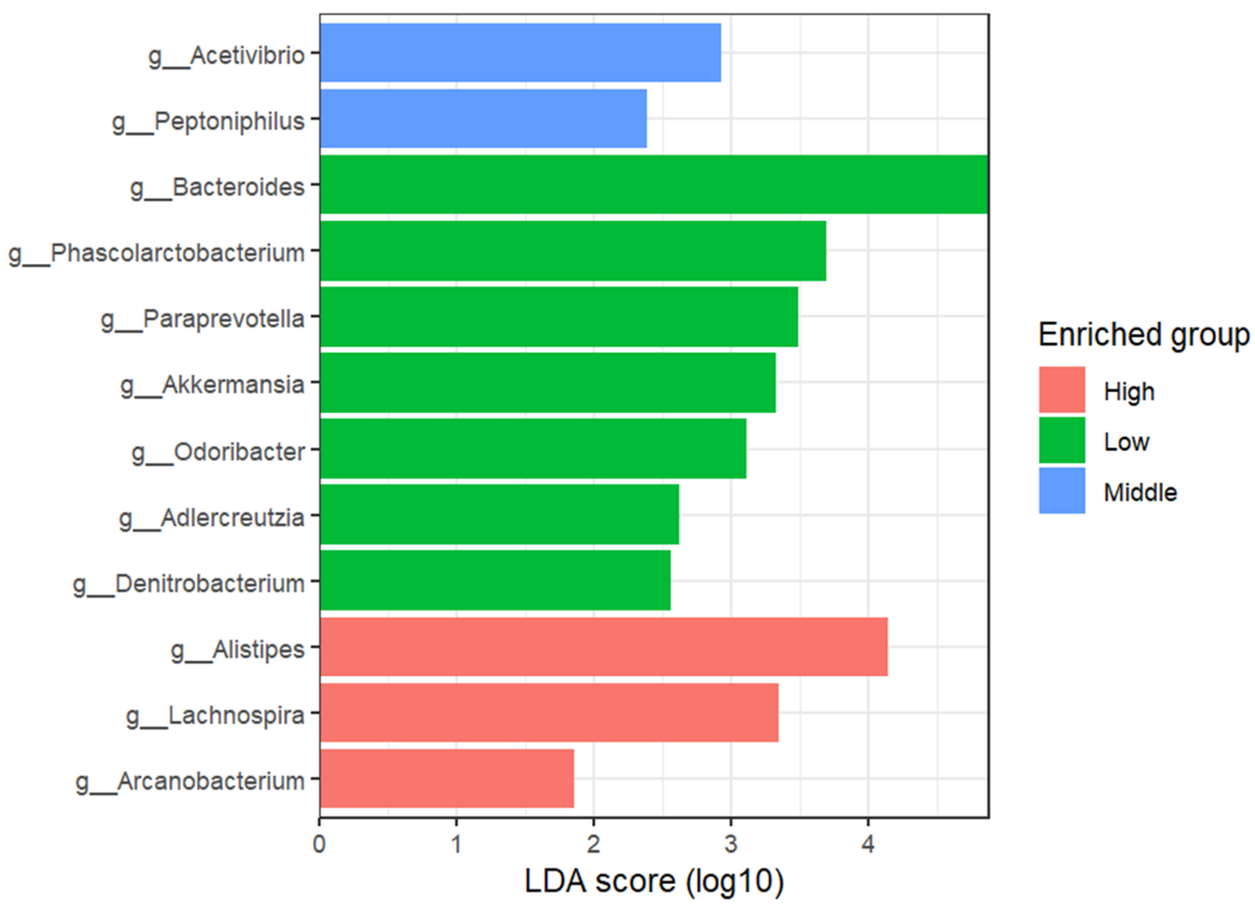
a.


relative abundance (%)
100
75
50
25
0
High
Low
Middle
Negative
f__f__Lachnospiraceae
f__f__Ruminococcaceae
f__f__Clostridiaceae-1
f__f__Prevotellaceae
f__f__Bacteroidaceae
f__f__Bifidobacteriaceae
f__f__Erysipelotrichaceae
f__f__Enterobacteriaceae
f__f__Eubacteriaceae
f__f__Veillonellaceae
f__f__Lactobacillaceae
f__f__Streptococcaceae
f__f__Peptostreptococcaceae
f__f__Coriobacteriaceae
f__f__Porphyromonadaceae
f__f__Rikenellaceae
f__f__Succinivibrionaceae
f__f__Desulfovibrionaceae
f__f__Enterococcaceae
f__f__Peptococcaceae-1
Others

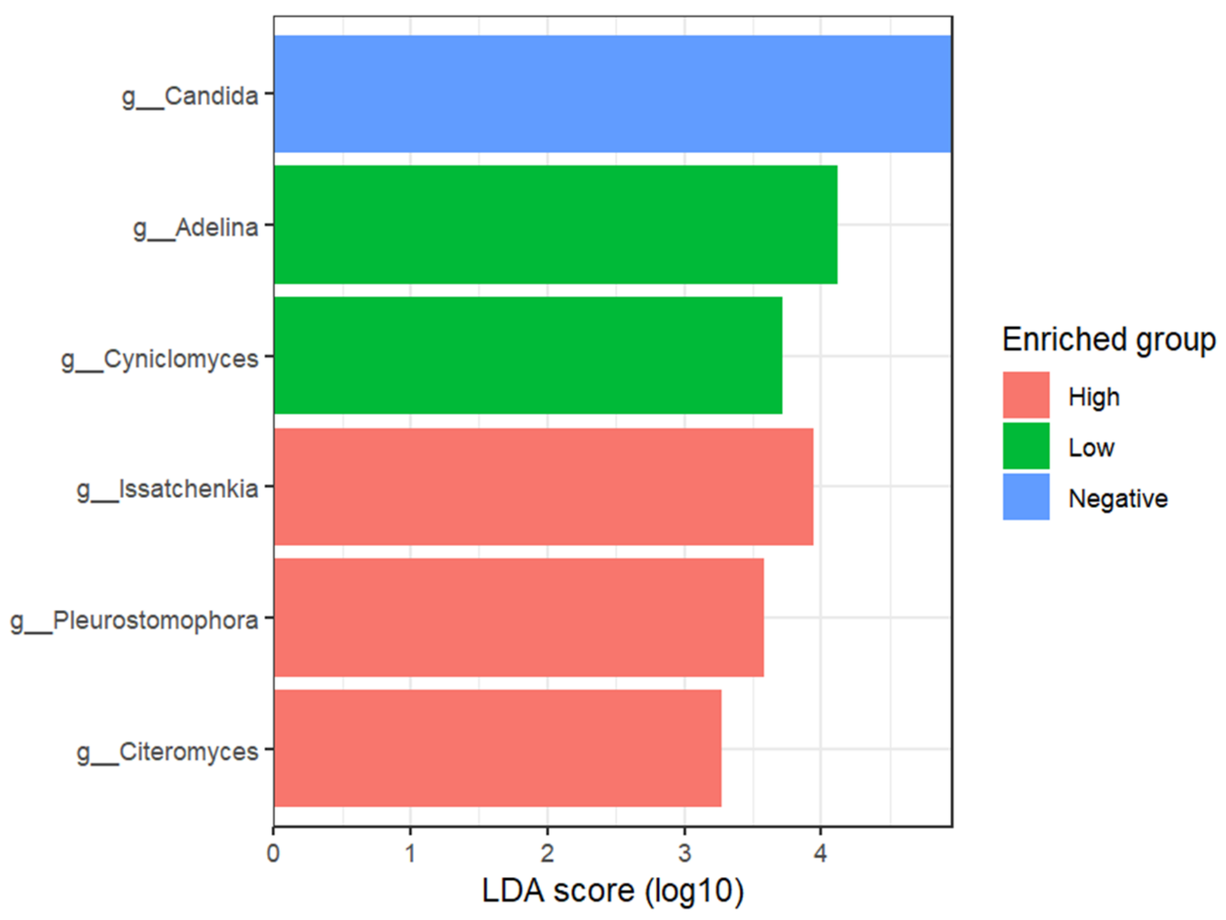
b.

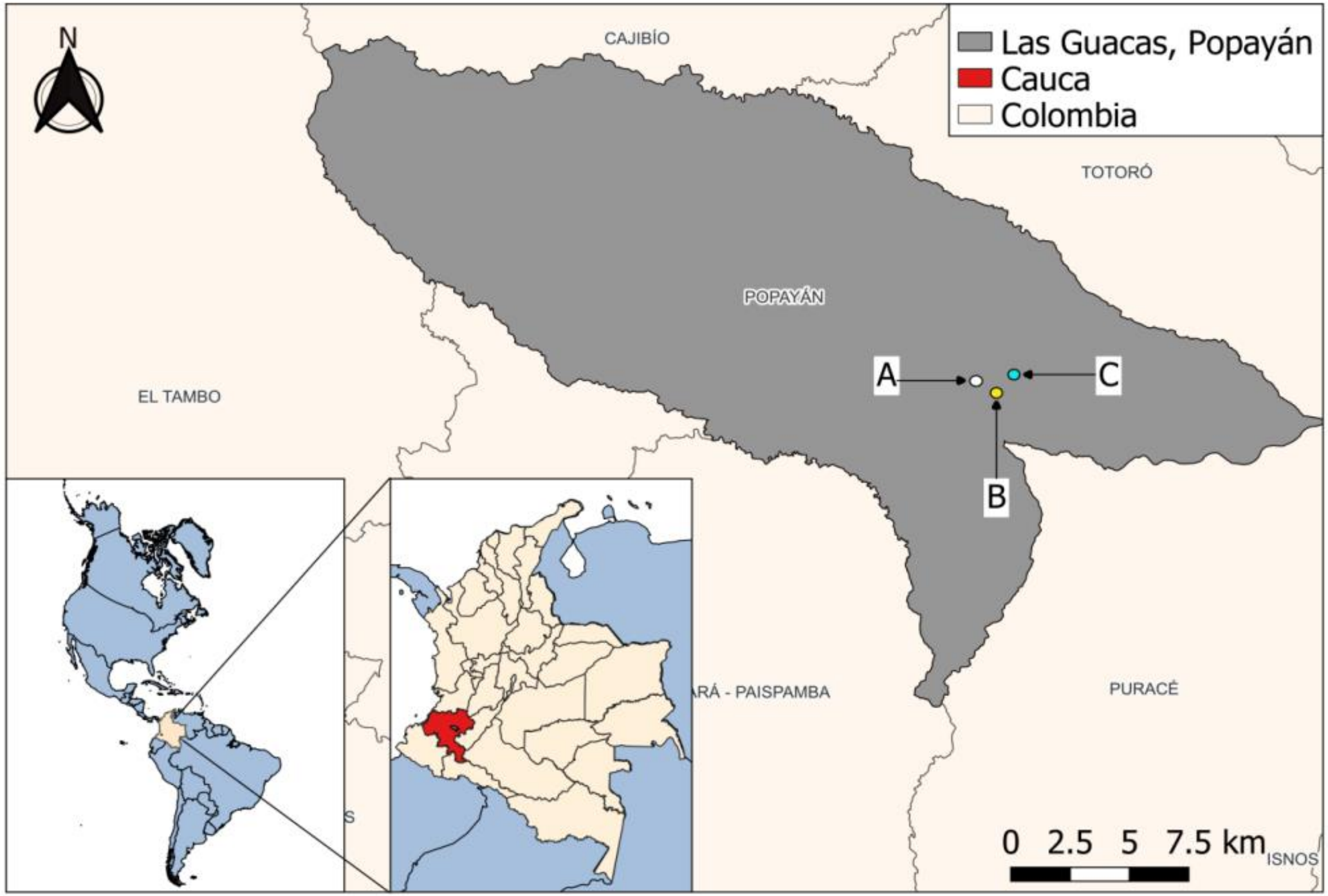

relative abundance (%)
100
75
50
25
0
High
Low
Middle
Negative
f__f__Saccharomycetaceae
f__f__Blastocystidae
f__f__Entamoebida
f__f__Malasseziaceae
f__f__Trichocomaceae
f__f__Wallemiaceae
f__f__Sporidiobolaceae
f__f__Tremellaceae
f__f__Dipodascaceae
f__f__Saccharomycodaceae
f__f__Davidiellaceae
f__f__Ganodermataceae
f__f__Plectosphaerellaceae
f__f__Mucoraceae
f__f__Coniochaetaceae
f__f__Peniophoraceae
f__f__Sympoventuriaceae
f__f__Rhizopodaceae
f__f__Polyporaceae
f__f__Aspergillaceae
Others

c.

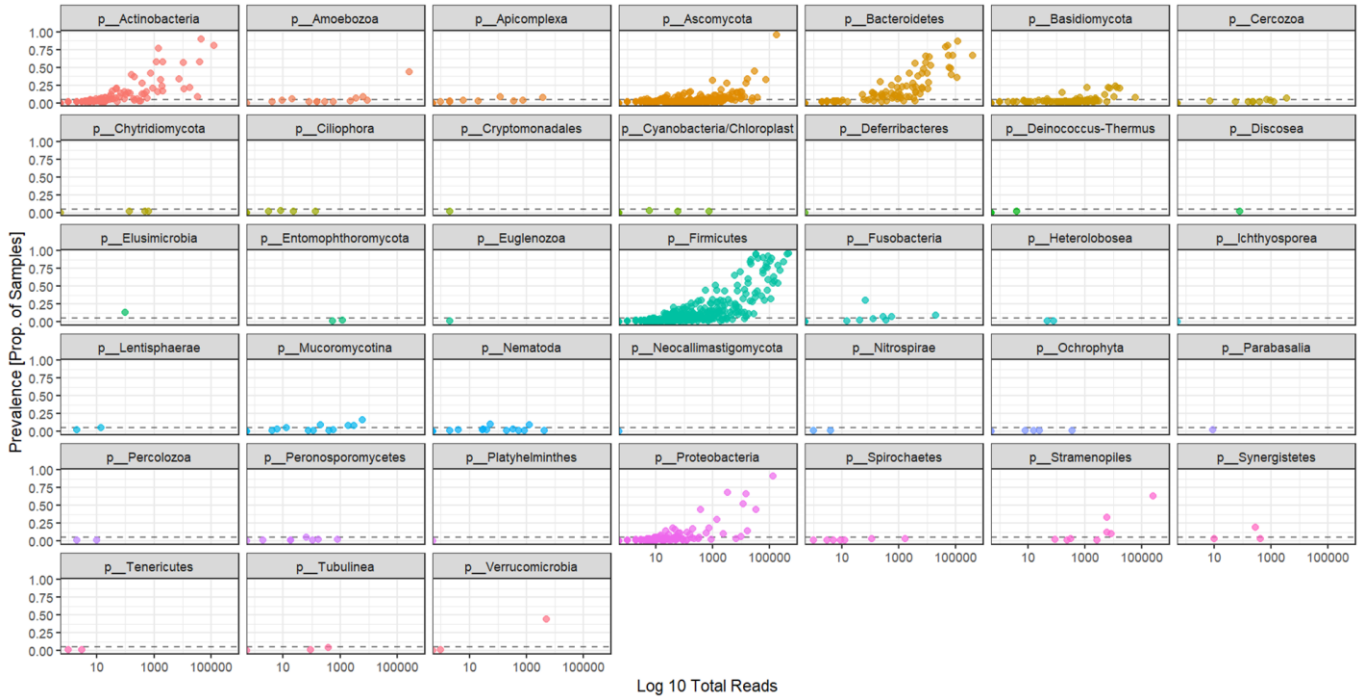

g__Acetivibrio
g__Peptoniphilus
g__Bacteroides
g__Phascolarctobacterium
g__Paraprevotella
g__Akkermansia
g__Odoribacter
g__Adlercreutzia
g__Denitrobacterium
g__Alistipes
g__Lachnospira
g__Arcanobacterium
0
1
2
3
4
LDA score (log10)
Enriched group
High
Low
Middle

d.

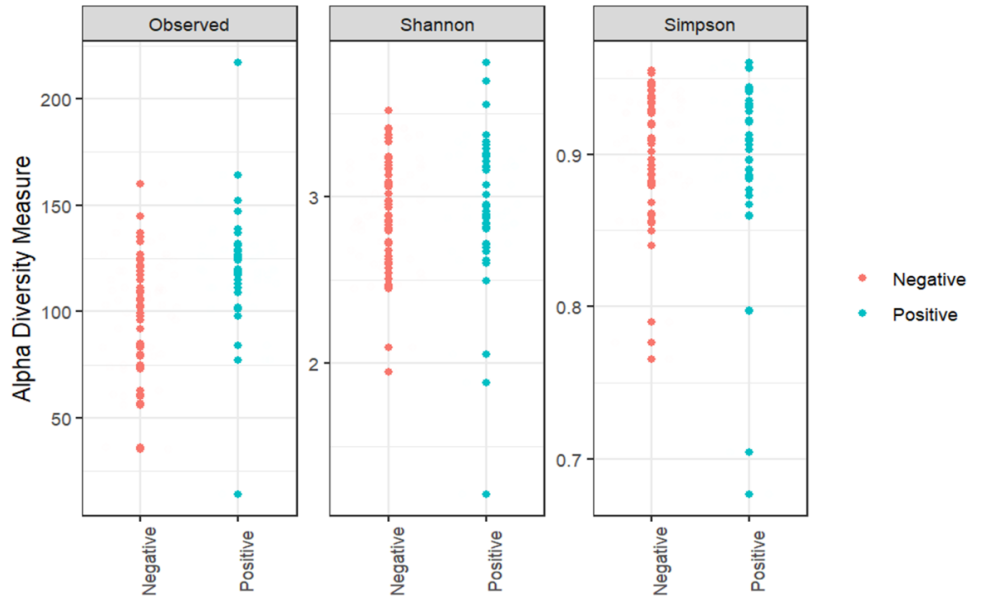

g__Candida
g__Adelina
g__Cyniclomyces
g__Issatchenkia
g__Pleurostomophora
g__Citeromyces
0
1
2
3
4
LDA score (log10)
Enriched group
High
Low
Negative


N
Las Guacas, Popayán
Cauca
Colombia
CAJIBÍO
TOTORÓ
POPAYÁN
EL TAMBO
A
B
C
PURACÉ
0 2.5 5 7.5 km

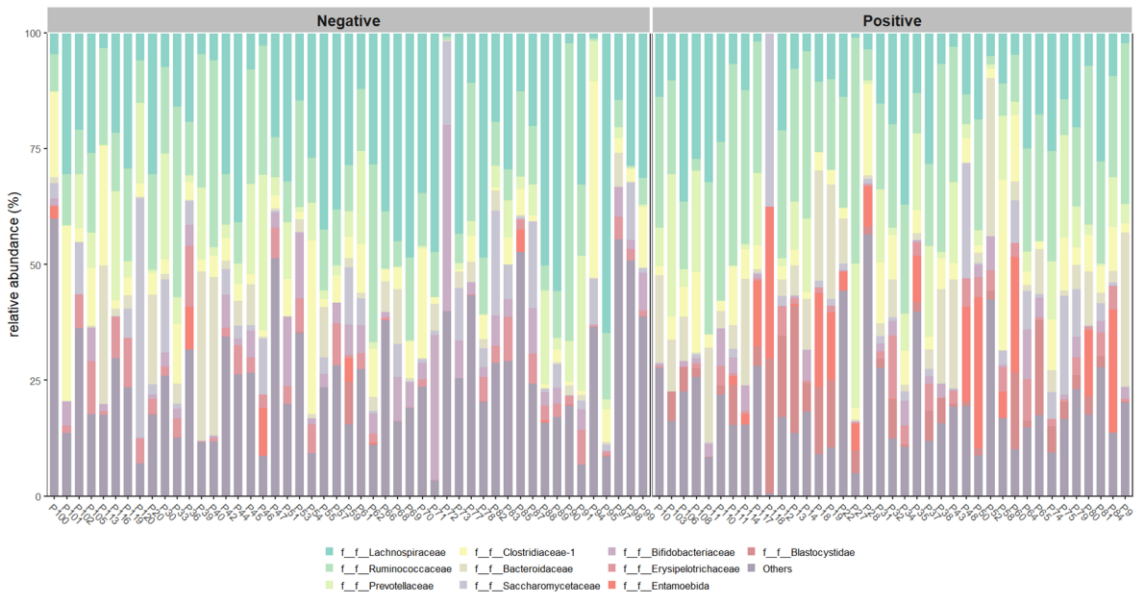
a.


p__Actinobacteria
p__Amoebozoa
p__Apicomplexa
p__Ascomycota
p__Bacteroidetes
p__Basidiomycota
p__Cercozoa
p__Chytridiomycota
p__Ciliophora
p__Cryptomonadales
p__Cyanobacteria/Chloroplast
p__Deferribacteres
p__Deinococcus-Thermus
p__Discosea
p__Elusimicrobia
p__Entomophthoromycota
p__Euglenozoa
p__Firmicutes
p__Fusobacteria
p__Heterolobosea
p__Ichthyosporea
p__Lentisphaerae
p__Mucoromycotina
p__Nematoda
p__Neocallimastigomycota
p__Nitrospirae
p__Ochrophyta
p__Parabasalia
p__Percolozoa
p__Peronosporomycetes
p__Platyhelminthes
p__Proteobacteria
p__Spirochaetes
p__Stramenopiles
p__Synergistetes
p__Tenericutes
p__Tubulinea
p__Verrucomicrobia
Prevalence [Prop. of Samples]
Log 10 Total Reads

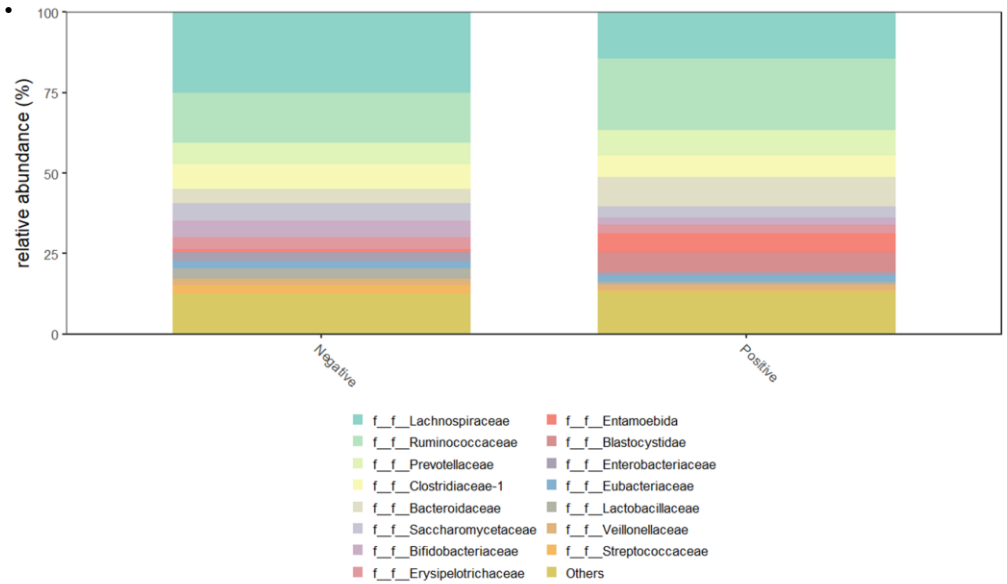
b.


Observed
Shannon
Simpson
Alpha Diversity Measure
Negative
Positive

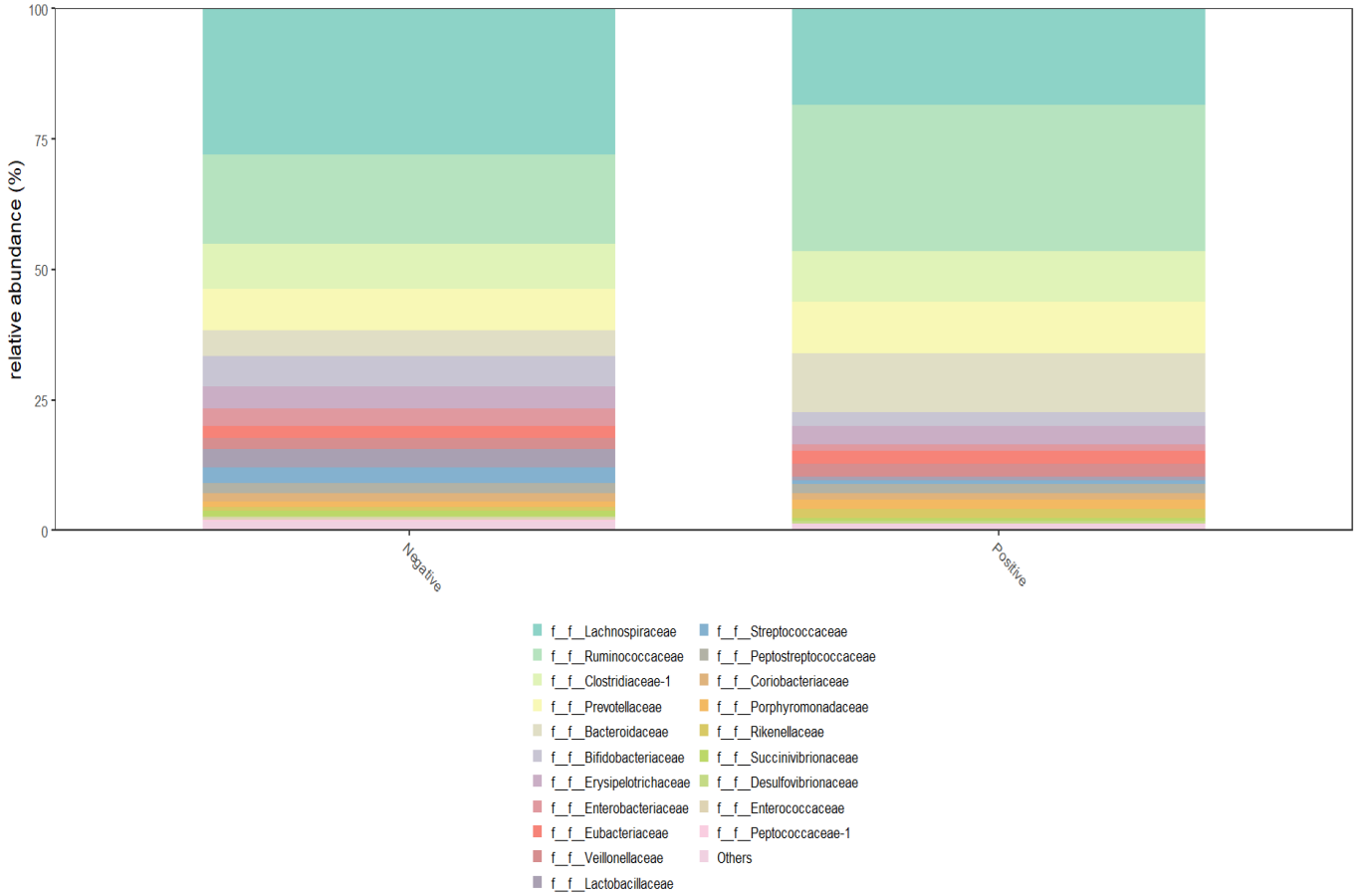
c.


Negative
Positive
relative abundance (%)
f__f__Lachnospiraceae
f__f__Ruminococcaceae
f__f__Prevotellaceae
f__f__Clostridiaceae-1
f__f__Bacteroidaceae
f__f__Saccharomycetaceae
f__f__Bifidobacteriaceae
f__f__Erysipelotrichaceae
f__f__Entamoebida
f__f__Blastocystidae
Others

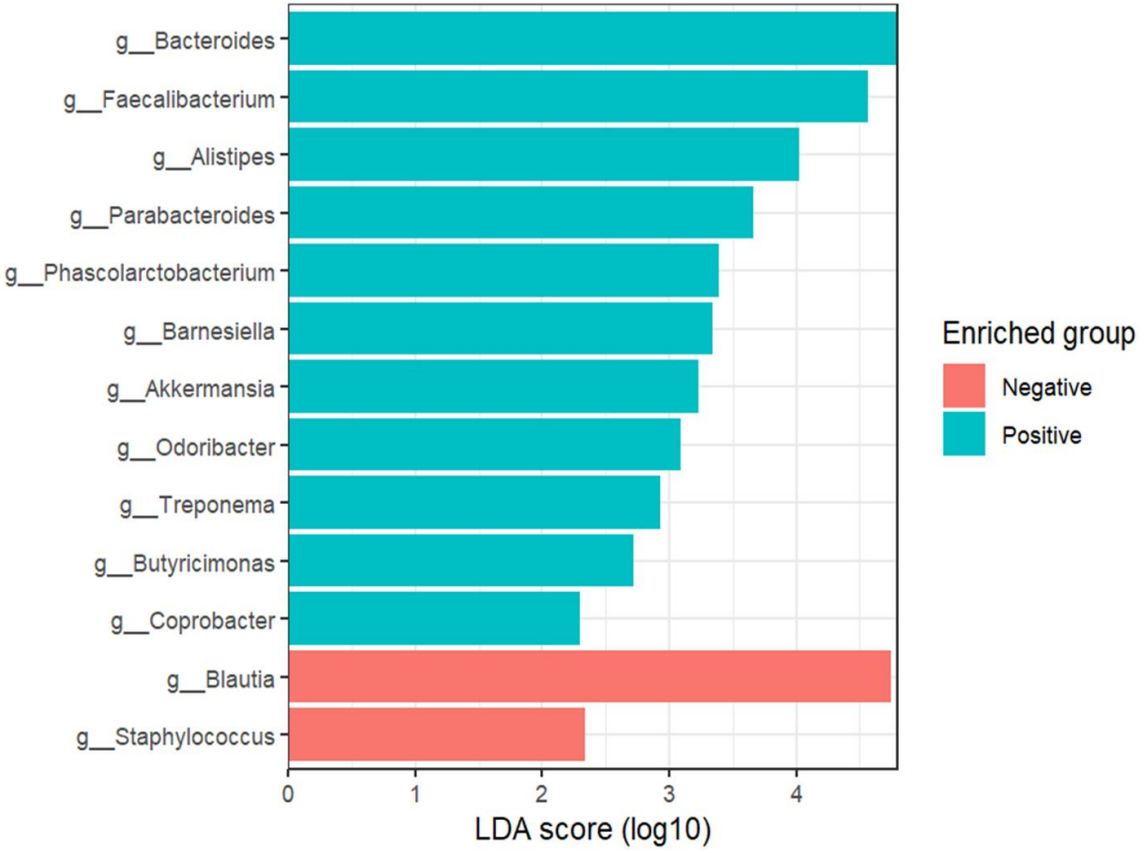
d.


relative abundance (%)
Negative
Positive
f__f__Lachnospiraceae
f__f__Ruminococcaceae
f__f__Prevotellaceae
f__f__Clostridiaceae-1
f__f__Bacteroidaceae
f__f__Saccharomycetaceae
f__f__Bifidobacteriaceae
f__f__Erysipelotrichaceae
f__f__Entamoebida
f__f__Blastocystidae
f__f__Enterobacteriaceae
f__f__Eubacteriaceae
f__f__Lactobacillaceae
f__f__Veillonellaceae
f__f__Streptococcaceae
Others

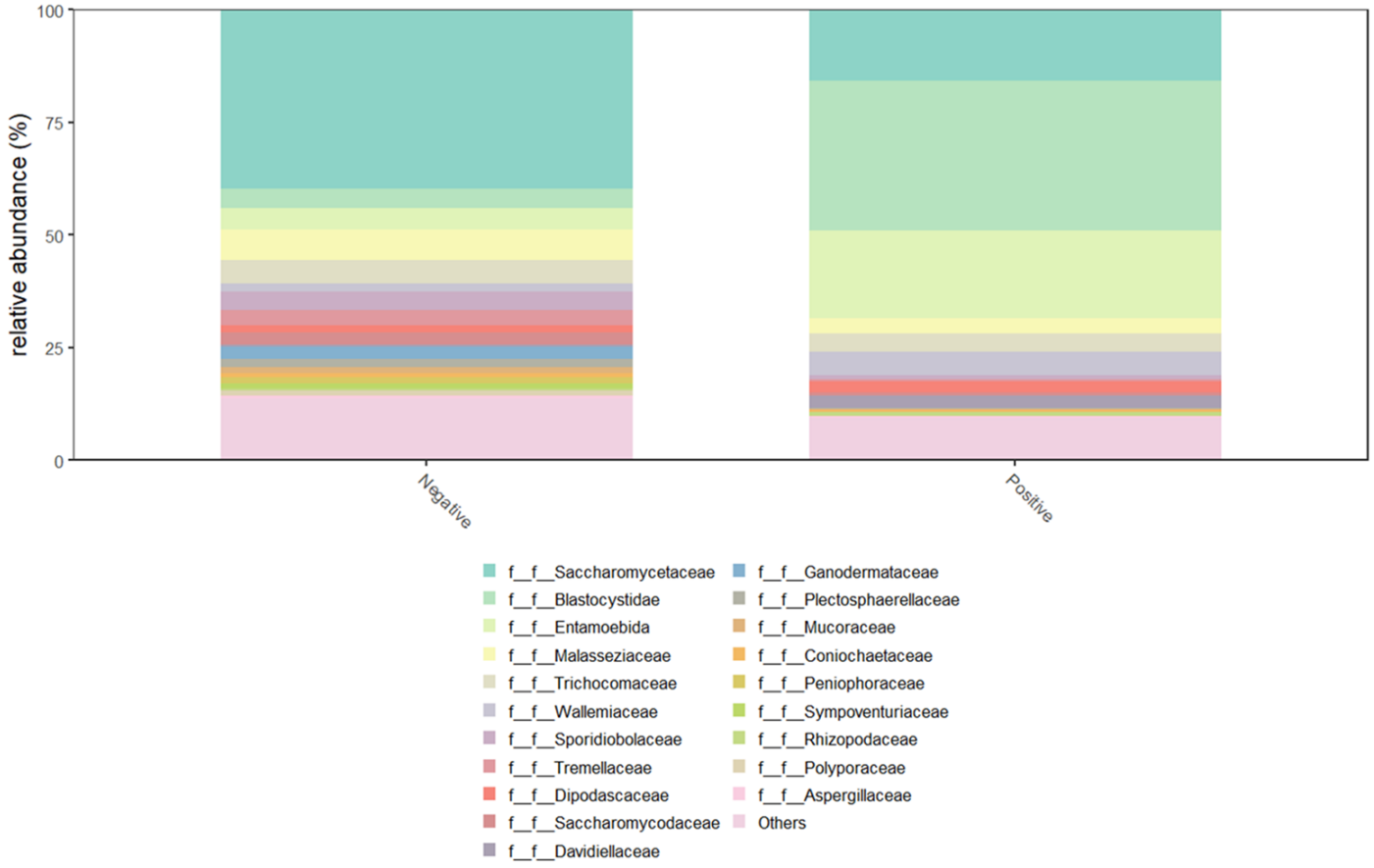
a.


100
75
50
25
0
relative abundance (%)
Negative
Positive
f__f__Lachnospiraceae
f__f__Ruminococcaceae
f__f__Clostridiaceae-1
f__f__Prevotellaceae
f__f__Bacteroidaceae
f__f__Bifidobacteriaceae
f__f__Erysipelotrichaceae
f__f__Enterobacteriaceae
f__f__Eubacteriaceae
f__f__Veillonellaceae
f__f__Lactobacillaceae
f__f__Streptococcaceae
f__f__Peptostreptococcaceae
f__f__Coriobacteriaceae
f__f__Porphyromonadaceae
f__f__Rikenellaceae
f__f__Succinivibrionaceae
f__f__Desulfovibrionaceae
f__f__Enterococcaceae
f__f__Peptococcaceae-1
Others

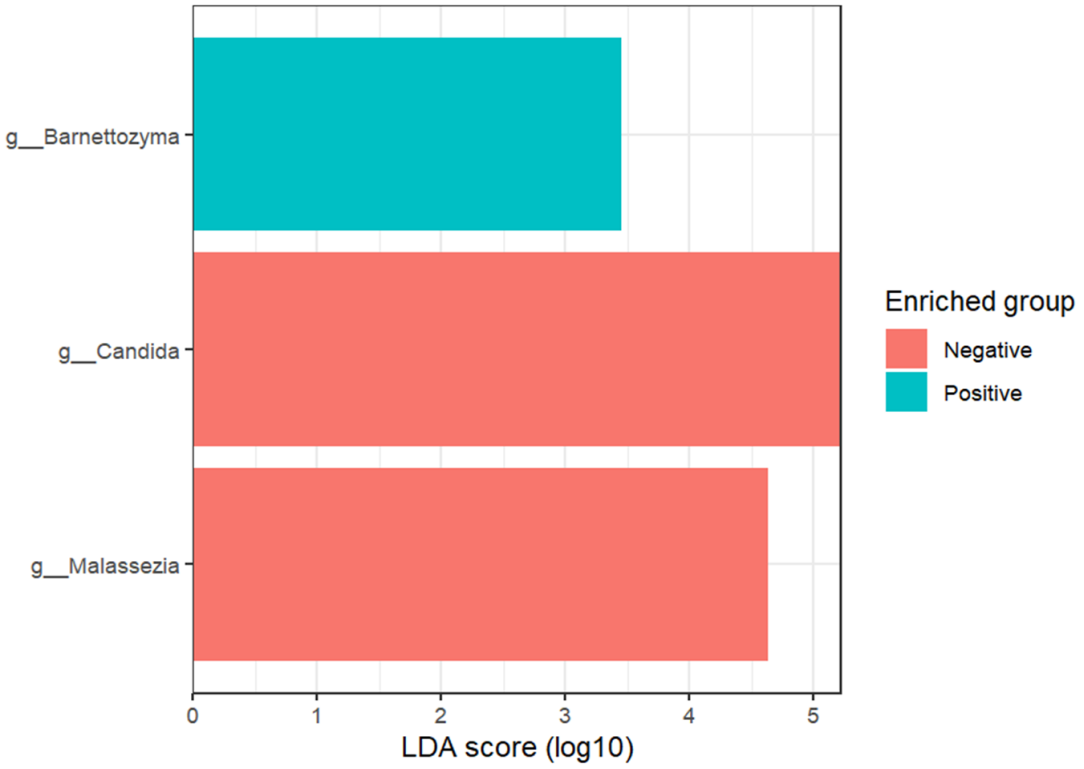
b.


g__Bacteroides
g__Faecalibacterium
g__Alistipes
g__Parabacteroides
g__Phascolarctobacterium
g__Barnesiella
g__Akkermansia
g__Odoribacter
g__Treponema
g__Butyricimonas
g__Coprobacter
g__Blautia
g__Staphylococcus
0
1
2
3
4
LDA score (log10)
Enriched group
Negative
Positive

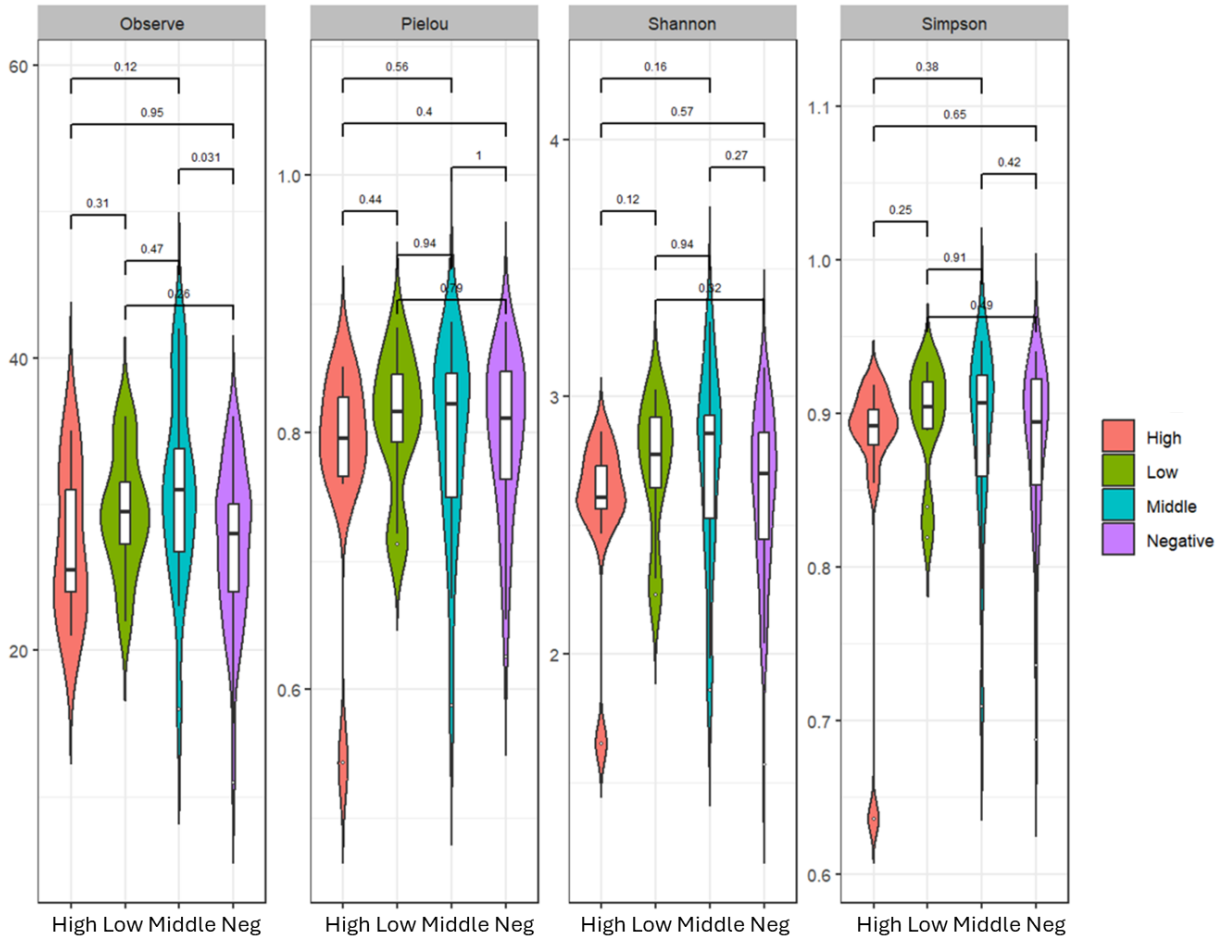
a.


100
75
50
25
0
relative abundance (%)
Negative
Positive
f__f__Saccharomycetaceae
f__f__Blastocystidae
f__f__Entamoebida
f__f__Malasseziaceae
f__f__Trichocomaceae
f__f__Wallemiaceae
f__f__Sporidiobolaceae
f__f__Tremellaceae
f__f__Dipodascaceae
f__f__Saccharomycodaceae
f__f__Davidiellaceae
f__f__Ganodermataceae
f__f__Plectosphaerellaceae
f__f__Mucoraceae
f__f__Coniochaetaceae
f__f__Peniophoraceae
f__f__Sympoventuriaceae
f__f__Rhizopodaceae
f__f__Polyporaceae
f__f__Aspergillaceae
Others

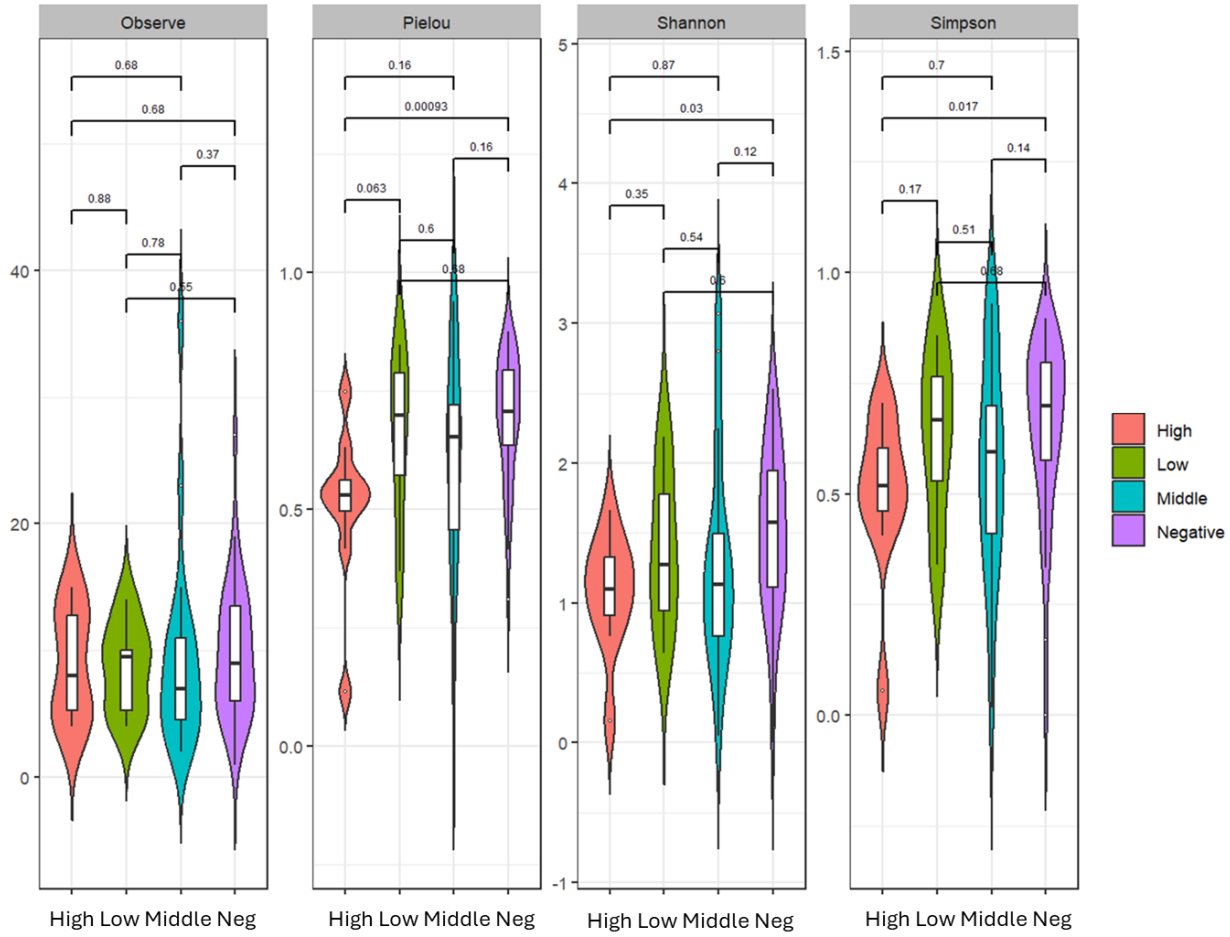
b.

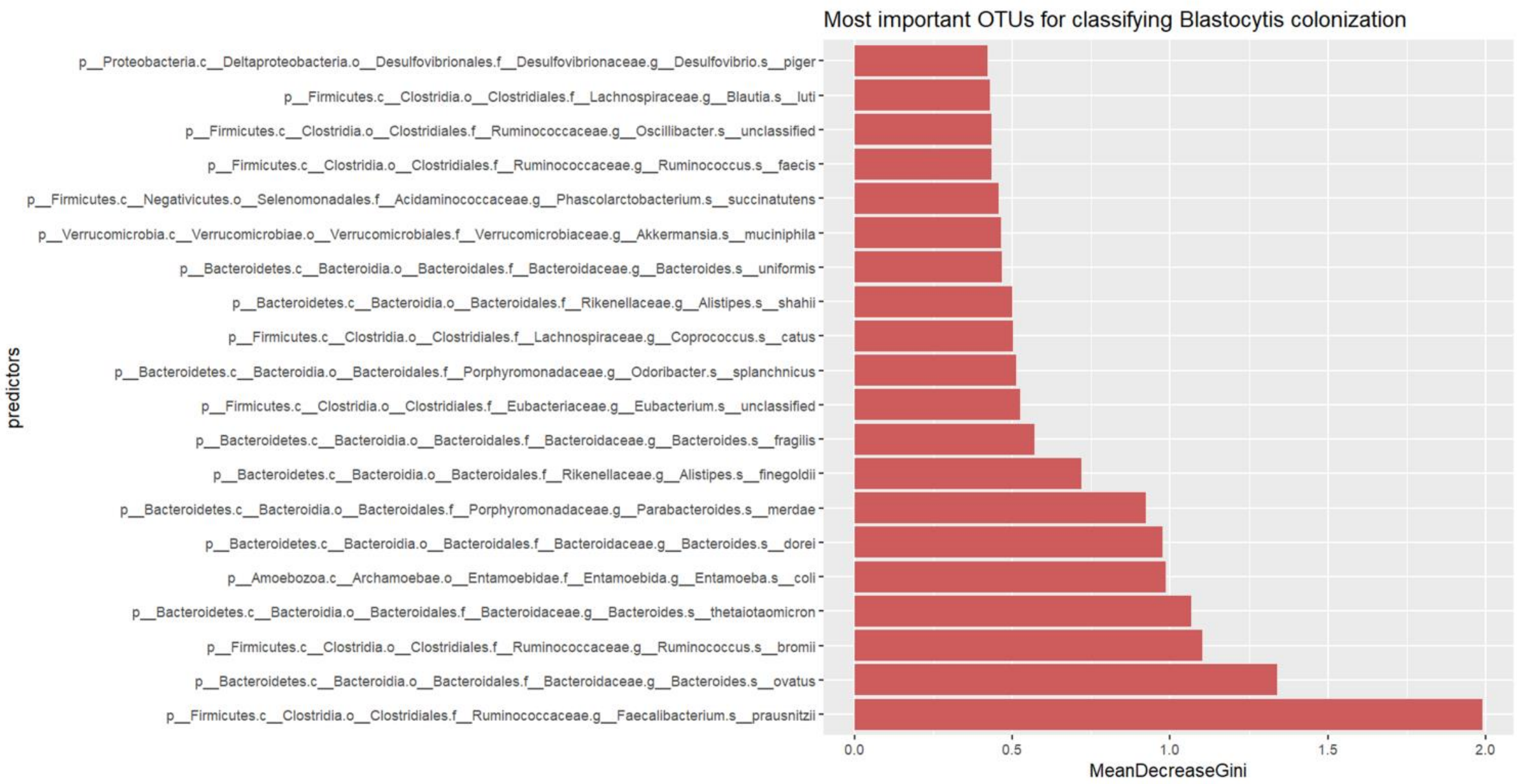

g__Barnettozyma
g__Candida
g__Malassezia
0
1
2
3
4
5
LDA score (log10)
Enriched group
Negative
Positive

a.


Observe
Pielou
Shannon
Simpson
High Low Middle Neg
High
Low
Middle
Negative

b.


Observe
Pielou
Shannon
Simpson
High Low Middle Neg
High
Low
Middle
Negative


Most important OTUs for classifying Blastocytis colonization
predictors
p__Proteobacteria.c__Deltaproteobacteria.o__Desulfovibrionales.f__Desulfovibrionaceae.g__Desulfovibrio.s__piger
p__Firmicutes.c__Clostridia.o__Clostridiales.f__Lachnospiraceae.g__Blautia.s__luti
p__Firmicutes.c__Clostridia.o__Clostridiales.f__Ruminococcaceae.g__Oscillibacter.s__unclassified
p__Firmicutes.c__Clostridia.o__Clostridiales.f__Ruminococcaceae.g__Ruminococcus.s__faecis
p__Firmicutes.c__Negativicutes.o__Selenomonadales.f__Acidaminococcaceae.g__Phascolarctobacterium.s__succinatutens
p__Verrucomicrobia.c__Verrucomicrobiae.o__Verrucomicrobiales.f__Verrucomicrobiaceae.g__Akkermansia.s__muciniphila
p__Bacteroidetes.c__Bacteroidia.o__Bacteroidales.f__Bacteroidaceae.g__Bacteroides.s__uniformis
p__Bacteroidetes.c__Bacteroidia.o__Bacteroidales.f__Rikenellaceae.g__Alistipes.s__shahii
p__Firmicutes.c__Clostridia.o__Clostridiales.f__Lachnospiraceae.g__Coprococcus.s__catus
p__Bacteroidetes.c__Bacteroidia.o__Bacteroidales.f__Porphyromonadaceae.g__Odoribacter.s__splanchnicus
p__Firmicutes.c__Clostridia.o__Clostridiales.f__Eubacteriaceae.g__Eubacterium.s__unclassified
p__Bacteroidetes.c__Bacteroidia.o__Bacteroidales.f__Bacteroidaceae.g__Bacteroides.s__fragilis
p__Bacteroidetes.c__Bacteroidia.o__Bacteroidales.f__Rikenellaceae.g__Alistipes.s__finegoldii
p__Bacteroidetes.c__Bacteroidia.o__Bacteroidales.f__Porphyromonadaceae.g__Parabacteroides.s__merdae
p__Bacteroidetes.c__Bacteroidia.o__Bacteroidales.f__Bacteroidaceae.g__Bacteroides.s__dorei
p__Amoebozoa.c__Archamoebae.o__Entamoebidae.f__Entamoebida.g__Entamoeba.s__coli
p__Bacteroidetes.c__Bacteroidia.o__Bacteroidales.f__Bacteroidaceae.g__Bacteroides.s__thetaiotaomicron
p__Firmicutes.c__Clostridia.o__Clostridiales.f__Ruminococcaceae.g__Ruminococcus.s__bromii
p__Bacteroidetes.c__Bacteroidia.o__Bacteroidales.f__Bacteroidaceae.g__Bacteroides.s__ovatus
p__Firmicutes.c__Clostridia.o__Clostridiales.f__Ruminococcaceae.g__Faecalibacterium.s__prausnitzii
0.0
0.5
1.0
1.5
2.0
MeanDecreaseGini

sample	*Blastocystis*_ Illumina	Blastocystis_reads	na lo	Burden_Illumina
P10	Positive	155	5.0499	Low
P100	Negative	90	4.5109	Negative
P101	Negative	2	1.0986	Negative
P102	Negative	0	0	Negative
P103	Positive	5538	8.6196	Middle
P105	Negative	2	1.0986	Negative
P106	Positive	5799	8.6656	Middle
P108	Positive	1354	7.2116	Middle
P11	Positive	151	5.0239	Low
P110	Positive	860	6.7581	Middle
P111	Positive	7239	8.8874	High
P113	Negative	3	1.3863	Negative
P114	Positive	205	5.3279	Low
P116	Negative	1	0.6931	Negative
P117	Positive	681	6.525	Low
P118	Positive	707	6.5624	Middle
P119	Negative	2	1.0986	Negative
P12	Positive	29299	10.2853	High
P120	Negative	4	1.6094	Negative
P13	Positive	40782	10.616	High
P14	Positive	6438	8.7701	High
P18	Positive	33422	10.417	High
P19	Positive	30841	10.3366	High
P20	Negative	88	4.4886	Negative
P22	Positive	327	5.793	Low
P27	Positive	1837	7.5164	Middle
P28	Positive	1540	7.3402	Middle
P30	Negative	19	2.9957	Negative
P31	Positive	1262	7.1412	Middle
P32	Positive	5228	8.562	Middle
P33	Negative	45	3.8286	Negative
P34	Positive	502	6.2206	Low
P35	Positive	3076	8.0317	Middle
P36	Negative	11	2.4849	Negative
P37	Positive	8088	8.9983	High
P38	Positive	4503	8.4127	Middle
P39	Negative	9	2.3026	Negative
P40	Negative	30	3.434	Negative
P42	Negative	3	1.3863	Negative
P43	Positive	481	6.1779	Low
P44	Negative	3	1.3863	Negative
P45	Negative	3	1.3863	Negative
P46	Negative	83	4.4308	Negative

P47	Negative	11	2.4849	Negative
P48	Positive	1771	7.4799	Middle
P50	Positive	2251	7.7196	Middle
P51	Negative	2	1.0986	Negative
P52	Positive	1112	7.0148	Middle
P53	Negative	21	3.091	Negative
P54	Negative	1	0.6931	Negative
P55	Negative	3	1.3863	Negative
P57	Negative	38	3.6636	Negative
P58	Positive	116	4.7622	Low
P59	Negative	24	3.2189	Negative
P60	Positive	23421	10.0614	High
P61	Negative	14	2.7081	Negative
P62	Negative	13	2.6391	Negative
P64	Positive	1246	7.1285	Middle
P65	Positive	27841	10.2343	High
P66	Negative	24	3.2189	Negative
P68	Negative	0	0	Negative
P69	Negative	0	0	Negative
P70	Negative	0	0	Negative
P71	Negative	0	0	Negative
P72	Negative	0	0	Negative
P73	Negative	0	0	Negative
P74	Positive	6441	8.7706	High
P75	Positive	4885	8.4941	Middle
P77	Negative	24	3.2189	Negative
P78	Negative	1	0.6931	Negative
P79	Positive	3321	8.1083	Middle
P80	Positive	3390	8.1289	Middle
P81	Positive	3258	8.0892	Middle
P82	Negative	4	1.6094	Negative
P83	Negative	0	0	Negative
P84	Positive	143	4.9698	Low
P85	Negative	0	0	Negative
P87	Negative	3	1.3863	Negative
P88	Negative	0	0	Negative
P89	Negative	0	0	Negative
P9	Positive	110	4.7095	Low
P90	Negative	100	4.6151	Negative
P91	Negative	0	0	Negative
P94	Negative	0	0	Negative
P95	Negative	6	1.9459	Negative
P97	Negative	13	2.6391	Negative
P98	Negative	1	0.6931	Negative

P99	Negative	68	4.2341	Negative

6.2. Capítulo 2

Objetivo 3: Caracterizar los cambios en el microbioma intestinal del hospedero asociados a la infección por el protozoo *Trypanosoma cruzi* en un modelo animal a partir de un enfoque metagenómico.

Para cumplir con el objetivo 3, en este estudio se propuso evaluar el impacto de la infección por *T. cruzi* en la composición y función del microbioma intestinal en un modelo murino. Para evaluar esta interacción, se utilizó un modelo murino en el que se infectaron ratones de las cepas BALB/c y C57BL/6 con la cepa Tulahuen TcVI de *Trypanosoma cruzi*. Posterior a la infección, se recolectaron muestras fecales para analizar la composición del microbioma intestinal mediante una aproximación metagenómica. Además, se realizaron análisis metagenómicos para reconstruir genomas bacterianos y determinar las funciones metabólicas de las comunidades bacterianas.

Los resultados revelaron alteraciones significativas en la composición y función del microbioma intestinal en los ratones infectados con *T. cruzi*. Se observó una alteración en la estructura de la comunidad microbiana, con una disminución en la abundancia de géneros como *Bacteroides thetaiotaomicron*, *Faecalibaculum rodentium* y *Lactobacillus johnsonii* , que son importantes productores de ácidos grasos de cadena corta (AGCC) y que desempeñan un papel clave en el mantenimiento de la integridad de la barrera intestinal. Por el contrario, se observó un aumento en la abundancia de géneros como *Akkermansia muciniphila* y *Staphylococcus xylosus*, que se han asociado con procesos inflamatorios y desregulación inmunitaria. Los análisis metagenómicos de predicción funcional revelaron una disminución en la abundancia de genes implicados la síntesis de lípidos y de aminoácidos, lo que sugiere una alteración en las capacidades metabólicas de la microbiota intestinal en los animales infectados. Adicionalmente, se reconstruyeron genomas ensamblados a partir de datos metagenómicos (MAGs) de alta calidad de *L. johnsonii, A. muciniphila* entre otras especies, confirmando, cambios funcionales asociados a vías metabólicas que se ven directamente afectadas por la pérdida de abundancia de taxones bacterianos específicos.

Estos hallazgos sugieren que la infección por *T. cruzi* induce un desequilibrio en el microbioma intestinal, que puede contribuir a la patogénesis de la enfermedad de Chagas. Los mecanismos por los cuales *T. cruzi* altera el microbioma intestinal aún no están completamente esclarecidos, pero se han propuesto varios mecanismos, como la competencia por nutrientes, la producción de metabolitos tóxicos y la modulación de la respuesta inmunitaria del hospedero. La comprensión de las interacciones entre el parásito, el hospedero y el microbioma intestinal abre nuevas perspectivas para el desarrollo de estrategias terapéuticas basadas en la modulación de esta microbiota intestinal. Futuras investigaciones deben centrarse en identificar los mecanismos moleculares que subyacen a la disbiosis intestinal inducida por *T. cruzi* y en evaluar el potencial terapéutico de los probióticos, prebióticos y otros tratamientos dirigidos a la microbiota intestinal.

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

Artículo 3: <u>Castañeda S</u>, Muñoz M, Hotez PJ, Bottazzi ME, Paniz-Mondolfi AE, Jones KM, Mejia R, Poveda C, Ramírez JD. Microbiome Alterations Driven by *Trypanosoma cruzi* Infection in Two Disjunctive Murine Models. Microbiology Spectrum [Internet]. 2023 [cited 2023 Jun 16];11(3). https://doi.org/10.1128/spectrum.00199-23

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RESEARCH ARTICLE

Microbiome Alterations Driven by *Trypanosoma cruzi* Infection in Two Disjunctive Murine Models

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ABSTRACT Alterations caused by *Trypanosoma cruzi* in the composition of gut microbiome may play a vital role in the host-parasite interactions that shapes physiology and immune responses against infection. Thus, a better understanding of this parasite-host-microbiome interaction may yield relevant information in the comprehension of the pathophysiology of the disease and the development of new prophylactic and therapeutic alternatives. Therefore, we implemented a murine model with two mice strains (BALB/c and C57BL/6) to evaluate the impact of *Trypanosoma cruzi* (Tulahuen strain) infection on the gut microbiome utilizing cytokine profiling and shotgun metagenomics. Higher parasite burdens were observed in cardiac and intestinal tissues, including changes in anti-inflammatory (interleukin-4 [IL-4] and IL-10) and proinflammatory (gamma interferon, tumor necrosis factor alpha, and IL-6) cytokines. Bacterial species such as *Bacteroides thetaiotaomicron*, *Faecalibaculum rodentium*, and *Lactobacillus johnsonii* showed a decrease in relative abundance, while *Akkermansia muciniphila* and *Staphylococcus xylosus* increased. Likewise, as infection progressed, there was a decrease in gene abundances related to metabolic processes such as lipid synthesis (including short-chain fatty acids) and amino acid synthesis (including branched-chain amino acids). High-quality metagenomic assembled genomes of *L. johnsonii* and *A. muciniphila* among other species were reconstructed, confirming, functional changes associated with metabolic pathways that are directly affected by the loss of abundance of specific bacterial taxa.

IMPORTANCE Chagas disease (CD) is caused by the protozoan *Trypanosoma cruzi*, presenting acute and chronic phases where cardiomyopathy, megaesophagus, and/or megacolon stand out. During the course of its life cycle, the parasite has an important gastrointestinal tract transit that leads to severe forms of CD. The intestinal microbiome plays an essential role in the immunological, physiological, and metabolic homeostasis of the host. Therefore, parasite-host-intestinal microbiome interactions may provide information on certain biological and pathophysiological aspects related to CD. The present study proposes a comprehensive evaluation of the potential effects of this interaction based on metagenomic and immunological data from two mice models with different genetic, immunological, and microbiome backgrounds. Our findings suggest that there are alterations in the immune and microbiome profiles that affect several metabolic pathways that can potentially promote the infection's establishment, progression, and persistence. In addition, this information may prove essential in the research of new prophylactic and therapeutic alternatives for CD.

KEYWORDS Chagas disease, MAG, microbiome, *Trypanosoma cruzi*

Editor Jian Li, Hubei University of Medicine

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The flagellate protozoan *Trypanosoma cruzi* is the causative agent of Chagas disease, a neglected tropical disease endemic in Latin America that affects eight million people worldwide, causing about 12,500 deaths per year (1–4). Chagas disease has two clinical phases: acute and chronic. The acute phase can be asymptomatic or symptomatic with circulating trypomastigotes in peripheral blood. Decades later, 30 to 40% of patients develop a chronic phase with cardiovascular symptoms, while 10 to 15% present gastrointestinal symptoms (megaesophagus and megacolon) or remain asymptomatic (2).

Different studies have identified that the host gut microbiota composition can influence Chagas disease's physiopathology. Robello et al. found in 2019 that the presence of *T. cruzi* produced alterations in the intestinal microbiota in children, producing an increased fecal abundance of *Streptococcus*, *Blautia*, *Butyrivibrio*, and *Roseburia* and a lower fecal abundance of *Bacteroides* (5). These changes have also been observed in murine models using bioluminescent strains of *T. cruzi*, highlighting a significant intestinal transit that impacts host gut microbiome. In this context, McCall et al., in 2018, observed functional changes in the intestinal chemical environment linked to conjugated linoleic acid derivatives obtained from specific members of the *Ruminococcaceae* and *Lachnospiraceae* families, thus impacting the pathophysiology of Chagas disease, particularly in chronic digestive stages. Likewise, Hossain et al. in 2020 demonstrated that high parasite burdens in the esophagus, colon, and intestine modify the composition of the microbiota (beta diversity) and metabolome (metabolites such as kynurenine and long-chain acylcarnitines). Nevertheless, an essential constraint of these studies is the use of meta-taxonomic strategies through the 16S-rRNA marker with a limited resolution at the genus level. Likewise, using murine models with different genetic and immunological backgrounds can condition the microbiome's composition, leading to results that are not comparable between different studies (6–8).

These findings demonstrate the importance of the interaction of *T. cruzi* with the intestinal microbiome during infection. Also, how its proper understanding may reveal potential physiological and pathophysiological mechanisms associated with Chagas disease. Therefore, we performed a metagenomic analysis in two different mice models (BALB/c and C57BL/6) to evaluate *T. cruzi* infection and its impact on the microbiome. We identified taxa related to infection and functional changes associated with genes and pathways that could explain the possible metabolic alterations related to the infectious process and its interaction with the host. In addition, we were able for the first time to reconstruct MAGs from several bacterial species.

RESULTS

Parasitemia. At the time of euthanasia, by splenocyte cell counting, we found that both BALB/c and C57BL/6 *T. cruzi*-infected mice had splenomegaly. Although blood parasite burden results showed that BALB/c and C57BL/6 had a different progression in accordance with the infection, both contained detectable parasite burdens from day two and a first peak of infection at 10 DPI (Fig. 1a). At 16 days postinfection (dpi), parasite burdens in the heart and intestine showed greater amounts of the parasite in BALB/c mice than in C57BL/6 mice; nevertheless, no statistically significant differences were observed (Fig. 1b).

Immunological profile. We found, as expected, a proinflammatory profile for both C57BL/6 and BALB/c animals infected characterized by cytokines such as gamma interferon (IFN-γ), tumor necrosis factor alpha (TNF-α), and interleukin-6 (IL-6). Furthermore, anti-inflammatory cytokines such as IL-10 were higher in infected mice, mainly in BALB/c mice (Fig. 1c).

To identify the specific response patterns of each mouse strain, the values of the different cytokines were normalized, considering the reference values presented by noninfected mice. Therefore, the comparison between mice strains shows that BALB/c mice have higher values of the proinflammatory cytokines (IFN-γ and IL-6) and the anti-inflammatory cytokines (IL-10 and IL-4) in response to infection than C57BL/6 mice (Welch two-sample *t* test, $P < 0.05$) (see Fig. S1 in the supplemental material).

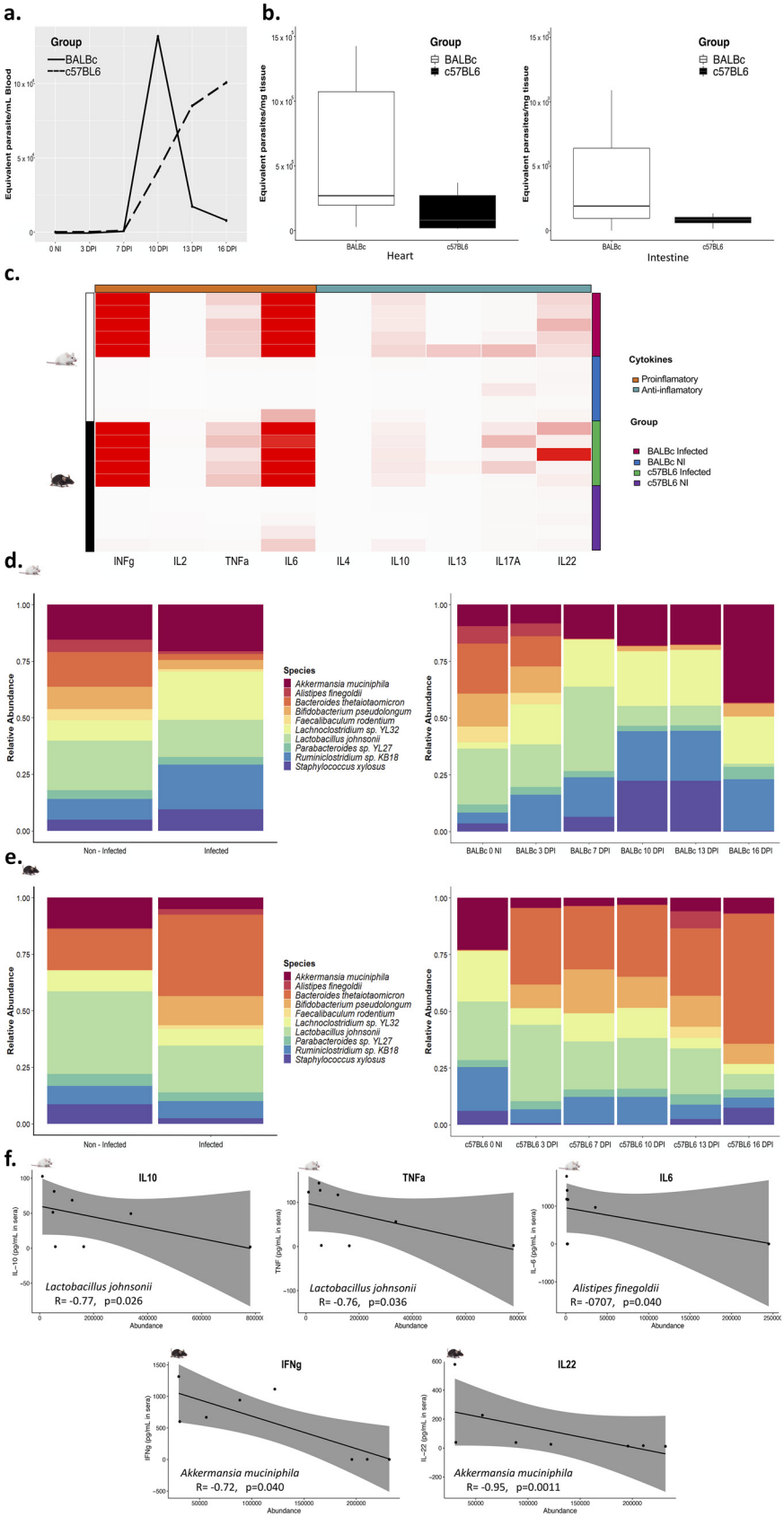

FIG 1 (a) Parasite burdens from total DNA were calculated by real-time PCR, and a standard curve was used to calculate the parasite equivalents/mL in blood. A solid line represents the parasite

(Continued on next page)

Characterization of the gut microbiome. After quality preprocessing and removal of host reads, approximately 14 million reads per sample were obtained for both mice strains. Of the percentage that could be taxonomically classified, more than 99% corresponded to bacteria and less than 1% to viruses. No reads corresponding to eukaryotes were identified. Metagenomic analysis showed the taxonomic identification of 4,782 bacterial species corresponding to BALB/c mice and 4,565 species to C57BL/6 mice. The predominant phyla were *Bacillota* (*Firmicutes*), *Bacteroidota* (*Bacteroidetes*), *Pseudomonadota* (*Proteobacteria*), *Actinomycetota* (*Actinobacteria*), *Verrucomicrobia*, and *Fusobacteria*. However, the mouse strains showed differences concerning the relative abundances of the different phyla (see https://github.com/gimur/Trypanosoma_cruzi-host-microbiome.git, files summary_taxonomy_BALBc_github.tsv and summary_taxonomy_BL6_github.tsv). The 10 species with the highest relative abundance in BALBc and C57BL/6 are shown in Fig. 1d and e (left panel), showing changes between the noninfected and infected groups.

Changes in the abundance of the different taxa in BALB/c mice were observed between the noninfected and the infected groups as the infection progressed (Fig. 1d, right panel). A loss in the relative abundance of specific bacterial species such as *Lactobacillus johnsonii*, *Bacteroides thetaiotaomicron*, *Bifidobacterium pseudolongum*, and *Faecalibaculum rodentium* at different time points was observed. In contrast, there was an increase in the relative abundance of other bacterial taxa, such as *Akkermansia muciniphila* and *Staphylococcus xylosus* (Fig. 1d, right panel). On the other hand, a small proportion of viruses were identified, mainly related to *Lactobacillus* phages that decrease as the infection progresses, and *Mus musculus* mobilized endogenous polytropic proviruses that increase with time (see Fig. S2a). These changes were progressive, more evident at 10 dpi when a first peak in the parasite burden was reached.

Since changes in the intestinal microbiota may contribute to the immune response, a correlation analysis was performed between serum cytokine concentrations and the abundance of the top 10 bacterial taxa described above (Fig. 1d and e). Spearman's test showed significant correlations between the abundance of some bacterial taxa and the levels of different cytokines. In BALB/c mice, *Lactobacillus johnsonii* showed a negative correlation with the anti-inflammatory cytokine IL-10 ($P = 0.026$, $R = -0.77$) and with the proinflammatory cytokine TNF-α ($P = 0.036$, $R = -0.76$). Likewise, *Alistipes finegoldii* correlated negatively with the proinflammatory cytokine IL-6 ($P = 0.040$, $R = -0.707$) (Fig. 1f).

Regarding C57BL/6 mice, the differences were not as noticeable as in the case of BALB/c mice. However, some changes were evident. As the *T. cruzi* infectious progressed, the abundance of *Bacteroides thetaiotaomicron* increased, reaching its highest proportion 16 dpi. Likewise, a reduction in the relative abundance of *L. johnsonii* and *A. muciniphila* (Fig. 1e, right panel). In C57BL/6 mice, the viruses showed no changes compared to BALB/c (see Fig. S2b). Correlation analysis between serum cytokine concentrations and changes in the abundances of some intestinal microbiota taxa were statistically significant in C57BL/6 mice. *A. muciniphila* and IFN-γ was also evident ($P = 0.040$, $R = -0.72$), and there was also a strong negative correlation with the anti-inflammatory cytokine IL-22 ($P = 0.0011$, $R = -0.95$) (Fig. 1f).

FIG 1 Legend (Continued)
burden in BALB/c infected mice ($n = 5$), and a dashed line represents the parasite burden in C57BL/6 infected mice ($n = 5$). (b) Parasite burden in cardiac tissue and intestinal tissue. A Student *t* test was used to compare burdens in cardiac tissue, and a Wilcoxon signed-rank exact test was used to compare burdens in intestinal tissue. (c) Heatmap showing the profile of anti-inflammatory and proinflammatory cytokines according to the evaluation group. The average linkage was used as the clustering method. (d) The relative abundance of the 10 most abundant bacterial species in BALB/c mice is shown for the control ($n = 5$) and infected ($n = 5$) groups (left) and each of the measurement points (right). (e) The relative abundance of the 10 most abundant bacterial species in C57BL/6 mice is shown for the control ($n = 5$) and infected ($n = 5$) groups (left) and each of the measurement points (right). NI, noninfected; DPI, days postinfection. (f) Correlation analysis between abundance values of *Lactobacillus johnsonii*, *Alistipes finegoldii*, and *Akkermansia muciniphila* and levels of cytokines IL-10, TNF-α, IL-6, IFN-γ, and IL-22. A Spearman correlation test was implemented, the R coefficient, and *P* value are indicated.

Beta diversity estimation showed no differences in both cases (infected and noninfected mice). The analysis of nonparametric multidimensional scaling (NMDS) revealed that the overall microbiome profiles did not differ in global terms, and the changes may be related to specific taxa and not to the general microbiota of the different groups (see Fig. S2c).

Functional analysis. The functional analysis performed by Humann3 was evaluated using the multivariate mixed-effects model implemented by Maaslin2 to identify statistically significant differences between infected and noninfected mice. It was found that changes in the abundance of specific genes involved several metabolic pathways between mice before and after infection. As in the case of the microbiota evaluation at the taxonomic level, in the functional analysis, the changes were more evident in BALB/c mice than in C57BL/6 mice (Fig. 2a and d). Genes that showed an adjusted *P* value of <0.05 were retained for analysis.

We identified 3,838 genes with statistically different abundances between noninfected and infected BALB/c mice. For C57BL/6, only 23 genes showed differences between noninfected and *T. cruzi*-infected mice (material available in https://github.com/gimur/Trypanosoma_cruzi-host-microbiome.git, files significant_results_Humann_BALBc.tsv and significant_results_Humann_BL6.tsv). Table 1 summarizes the major metabolic pathways, the genes involved in these pathways, the taxa associated with these genes, the false discovery rate (FDR) value, the effect size in infected mice, and the *P* and *q* statistical significance values. The genes that showed differences were related to metabolic processes such as lipid synthesis (including short-chain fatty acids [SCFAs]), branched-chain amino acid (BCAA) synthesis, purine synthesis, pyrimidine degradation, tryptophan metabolism, cobalamin metabolism, and pyruvate metabolism, among others (Fig. 2a and d). (Material is available at https://github.com/gimur/Trypanosoma_cruzi-host-microbiome.git, files PathTotal_merge_BALBc.csv and PathTotal_merge_BL6.csv).

Regarding lipid metabolism, potential infection-related bacteria such as *Bacteroides thetaiotaomicron* and *Parasutterella excrementihominis* were essential in lipid metabolism, specifically related to beta-oxidation and fatty acid synthesis. We found a reduction in the enzymes such as enoyl coenzyme A hydratase (enoyl-CoA hydratase), tiglyl-CoA hydratase, long-chain fatty acid CoA ligase, acetyl-CoA carboxylase carrier malonyltransferase, and beta-ketoacyl acyl carrier protein in the infected mice (Table 1). Interestingly, some bacterial taxa that increased with infection were associated with a higher abundance of genes that were reduced by the decrease in other taxa. Thus, for example, *Acetatifactur muris*, in infected mice, was associated with a greater abundance of genes related to long-chain fatty acid CoA ligase (Table 1).

In terms of lipid metabolism, the fatty acid synthesis super pathway showed statistically significant differences, showing a higher number of reads in noninfected mice than in infected mice (*t* test, $P = 0.026$) (Fig. 2b). This strongly correlates with specific enzymes in the functional analysis, such as butyrate kinase, which decreased with infection progress (Fig. 2c). This enzyme in noninfected BALB/c mice was mainly related to *B. thetaiotaomicron*. However, this taxon was reduced as infection progressed and other bacteria increased their relative abundance, mainly *Lachnospiraceae* and *Staphylococcus lentus* (Fig. 2c).

Regarding amino acid metabolism, both BALB/c and C57BL/6 mice showed a decrease in these metabolic pathways (Fig. 2a and d). The most relevant infection-related taxa were *B. thetaiotaomicron*, *Dubosiella newyorkensis*, *F. rodentium*, *Acutalibacter muris*, and *P. excrementihominis*. These bacteria, were significantly related to specific metabolic processes such as the synthesis of short-chain amino acids (leucine, valine, and isoleucine), ornithine, arginine, and synthesis of chorismate (a derivative of shikimic acid metabolism), known as an essential precursor of aromatic amino acids such as phenylalanine, tryptophan, and tyrosine (Fig. 2a and d). Specifically, BALB/c infected mice showed a lower abundance of genes related to aminoacyl-tRNA synthetases, tryptophan synthase, BCAA transaminase, and chorismate synthase (Table 1).

A decrease in purine synthesis was found in infected BALB/c and C57BL/6 mice (Fig. 2a and d). In particular, a lower abundance of genes such as ribonucleoside diphosphate

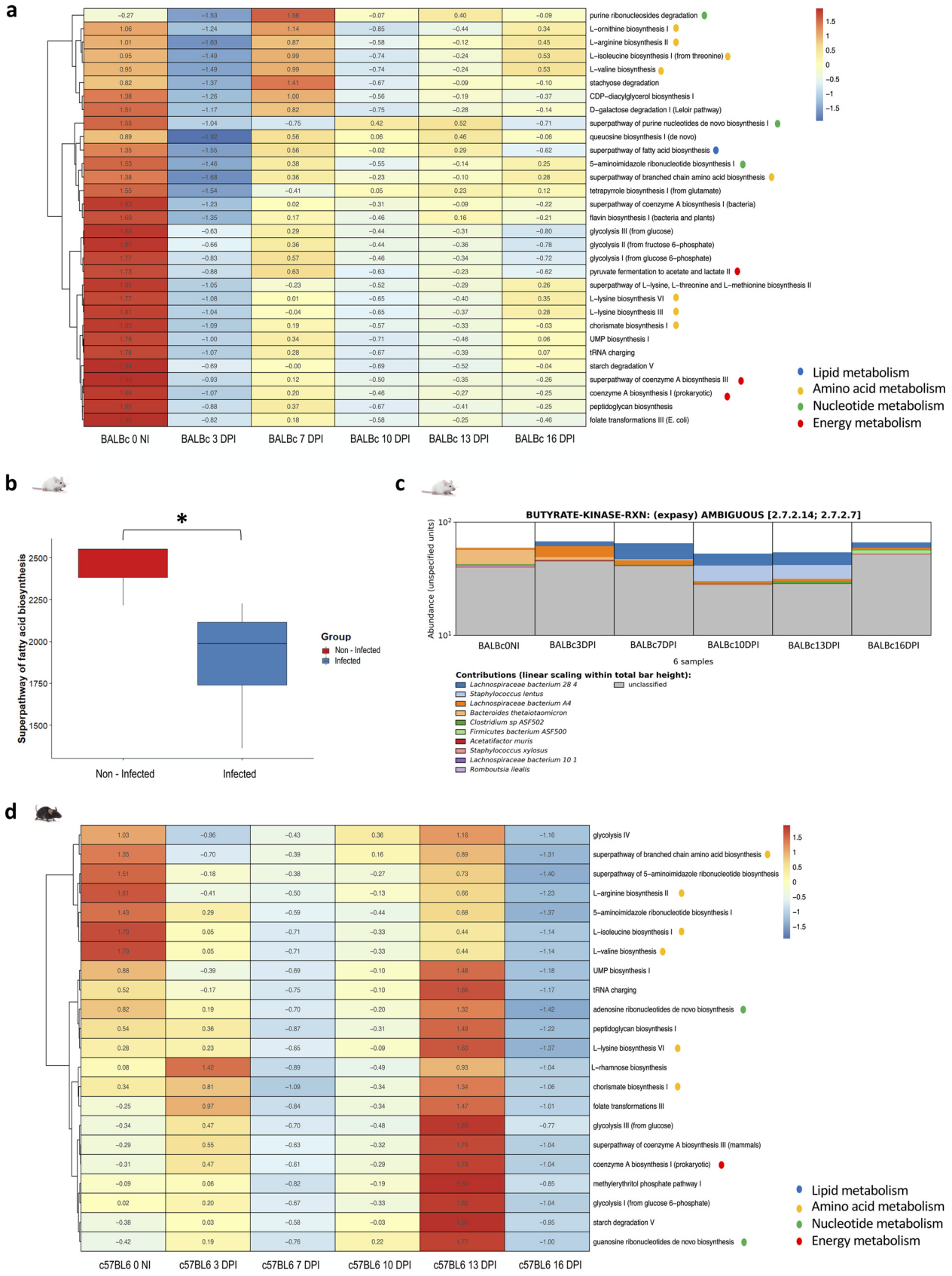

FIG 2 Functional analysis from metagenomic data. The main results were obtained by Humann3, and the R package, Maaslin2, using a multivariate mixed-effects model. (a and d) Metabolic pathways involving genes with differential abundances between infected and noninfected

(Continued on next page)

reductase, hypoxanthine phosphoribosyltransferase, and purine nucleoside phosphorylase related to *B. thetaiotaomicron*, *P. excrementihominis*, and *F. rodentium* was observed (Table 1). In infected BALB/c mice, the abundance of genes associated with the *A. muris*-related ribonucleoside triphosphate reductase enzyme was found.

As for oxidative metabolism, the CoA synthesis pathway changes were evidenced in both BALB/c and C57BL/6 mice (Fig. 2a and d). In BALB/c infected mice, we observed a decrease in this pathway as *T. cruzi* infection progressed. Enzymes belonging to the tricarboxylic acid cycle were in lower abundance in infected mice, such as succinate CoA ligase ADP-forming and dephospho-CoA kinase (Table 1).

Changes in glycolysis and fermentation pathways were observed in BALB/c and C57BL/6 mice (Fig. 2a and d). In BALB/c infected mice, a decrease in the pyruvate fermentation pathway was observed as the infection progressed. In this context, a lower abundance of the genes related to the pyruvate kinase fundamental in glucose metabolism converting phosphoenolpyruvate to pyruvate was observed (Table 1).

Metagenome-assembled genomes and pangenomic analysis. From the metagenomic data for the BALB/c mice, we reconstructed eight high-quality draft metagenome-assembled genomes (MAGs). The quality statistics were evaluated with CheckM, considering completeness (>90%) and contamination (<5%) (see Table S1). The taxonomic assignment was performed with GTDB-Tk through ANI and pplacer analysis recovering genomes from *L. johnsonii*, *A. muciniphila*, *S. lentus*, *A. muris*, *F. rodentium*, *A. finegoldii*, and *S. xylosus* (Fig. 3). The MAG for *B. thetaiotaomicron* corresponded to a medium-quality draft since it showed contamination of 6.6% (see Table S1). Visual reconstruction of MAGs was performed in the process, including a comparative analysis against a reference genome to each case, genome annotation with Prokka, GC percentage, and GC skew (Fig. 3).

Pangenomic analysis of MAGs was made with Panaroo (see Table S2), where *A. muciniphila* showed the lowest value of the core genome with 9.6% (n = 743/7,645) (see Fig. S3a), *A. finegoldii* presented a core genome of 11.7%, *L. johnsonii* a core genome of 33.4%, *S. xylosus* a core genome of 42%, and *F. rodentium* a core genome of 59.2%, the highest such value (see Fig. S4). Aligning the extracted amino acid sequences of gene clusters of MAGs with high-quality genomes available in PATRIC indicated a close genetic congruence between each reconstructed MAGs and corresponding downloaded genomes, mainly with isolates from mice (see Fig. S3 and S4).

Functional analysis from MAGs. To evaluate the coding potential of the obtained MAGs, COGs were analyzed for the global data set corresponding to reconstructed MAGs. The overall results show that the highest number of genes are related to carbohydrate metabolism and transport, followed by cell wall structure, biogenesis, outer membranes, and processes associated with cell division, such as transcription, translation, and replication (Fig. 4a).

Comparative analysis concerning each of the MAGs obtained showed that *B. thetaiotaomicron* plays a key role in several essential metabolic processes in host physiology (Fig. 4b). Thus, genes of COGs linked to coenzyme metabolism, carbohydrate metabolism, energy metabolism, amino acid, lipid, and nucleotide metabolism were more abundant in *B. thetaiotaomicron*. The functional pathways (KEGG modules) in the MAGs were reconstructed from Koafm KEGG analysis through the KEGG mapper. Complete modules (functional units of genes) were found in all MAGs mainly related to essential processes such as carbohydrate metabolism, lipid metabolism, nucleotide metabolism, and amino acid metabolism.

FIG 2 Legend (Continued)
mice for BALB/c (a) and C57BL/6 (d) mice. Heatmaps were created with MicrobioSee using Ward's linkage method for hierarchical cluster analysis. The metabolic pathways to which the different processes belong are represented by different colored dots indicated in the legend. (b) Abundance of reads related to the superpathway of fatty acid biosynthesis as observed for noninfected (NI) and infected BALB/c mice. A Student t test was used to compare these groups. (c) Bar plot obtained from HUMAnN 3.0 for butyrate kinase that was modified in terms of abundance of reads during *T. cruzi* infection. The figure includes the regrouping of bacterial species and the classification of the samples by evaluation point. *, $P < 0.05$. BALB/c infected mice, n = 5; BALB/c noninfected mice, n = 5; C57BL/6 infected mice, n = 5; C57BL/6 noninfected mice, n = 5.

TABLE 1 Functional analysis for gene and metabolic pathway prediction in infected and noninfected mice[a]

Metabolic pathway	Gene	Associated bacterium	FDR	Effect size in infected BALB/c mice	*P*	*q*
Lipid metabolism[b]	Enoyl-CoA hydratase	*Bacteroides thetaiotaomicron*	5.743E–02	−4.6	0.000691712	0.005358871
	Tiglyl-CoA hydratase	*Bacteroides thetaiotaomicron*	5.359E–03	−4.64	0.000691712	0.005358871
	Long-chain fatty acid CoA ligase	*Bacteroides thetaiotaomicron*	1.835E–0	−3.09	0.003273173	0.018350702
	Acetyl-CoA carboxylase	*Bacteroides thetaiotaomicron*	1.463E–02	−3.39	0.002282365	0.014634670
	Acyl carrier malonyltransferase	*Bacteroides thetaiotaomicron*	1.110E–02	−3.76	0.001535573	0.011100064
	Beta ketoacyl acyl carrier protein	*Parasutterella excrementihominis*	1.182E–02	−3.6	0.001679678	0.011815909
	Long-chain fatty acid CoA ligase	*Acetatifactur muris*	2.075E–02	2.1	0.004208897	0.020745468
Amino acid metabolism[c]	Isoleucine tRNA ligase	*Bacteroides thetaiotaomicron*	1.419E–0	−3.43	0.002190388	0.014189590
	Leucine tRNA ligase	*Bacteroides thetaiotaomicron*	1.566E–02	−3.3	0.002510489	0.015657878
	Tryptophan tRNA ligase	*Dubosiella newyorkensis*	1.557E–02	−3.31	0.002489752	0.015570720
	Tryptophan synthase	*Bacteroides thetaiotaomicron*	9.214E–03	−4.01	0.001215979	0.009213813
		Faecalibaculum rodentium	1.951E–02	−2.9	0.003771024	0.019505084
	Branched-chain amino acid transaminase	*Acutalibacter muris*	1.950E–02	−2.96	0.003768102	0.019500525
	Chorismate synthase	*Parasutterella excrementihominis*	2.021E–02	−2.92	0.003967455	0.020212716
Nucleotide metabolism	Ribonucleoside diphosphate reductase	*Parasutterella excrementihominis*	6.064E–03	−4.48	0.000787853	0.006064403
	Hypoxanthine phosphoribosyltransferase	*Faecalibaculum rodentium*	6.568E–03	−4.39	0.000856150	0.006567651
	Purine nucleoside phosphorylase	*Bacteroides thetaiotaomicron*	9.383E–03	−3.98	0.001243739	0.009382987
Biosynthetic pathway to cobalamin	Precorrin 2 dehydrogenases	*Faecalibaculum rodentium*	2.315E–02	−2.73	0.005085668	0.023150662
Oxidative metabolism and CoA synthesis pathway	Succinate CoA ligase ADP forming	*Bacteroides thetaiotaomicron*	1.430E–02	−3.42	0.002212495	0.014301774
	Dephospho-CoA kinase	*Dubosiella newyorkensis*	1.779E–02	−3.14	0.003048271	0.017789402
Glycolysis and fermentation pathways	Pyruvate kinase	*Bacteroides thetaiotaomicron*	1.743E–02	−3.16	0.002959204	0.017426635
		Dubosiella newyorkensis	2.144E–02	−2.82	0.004491714	0.021443220

[a]Identification of genes and associated taxa was performed using the Humann3 tool, and statistical evaluation to determine statistically significant changes was performed with Maaslin2 from a multivariate mixed-effects model. The table shows the metabolic pathway, the genes involved in this pathway, the taxa associated with these genes, the false discovery rate (FDR), the effect size in infected mice, and the *P* and *q* statistical significance values. CoA, coenzyme A.

[b]That is, beta-oxidation and fatty acid synthesis and metabolism.

[c]That is, the synthesis of SCFAs (leucine, valine, and isoleucine), the synthesis of ornithine, the synthesis of arginine, and the synthesis of chorismate (a derivative of shikimic acid metabolism and precursor of aromatic amino acids such as phenylalanine, tryptophan, and tyrosine).

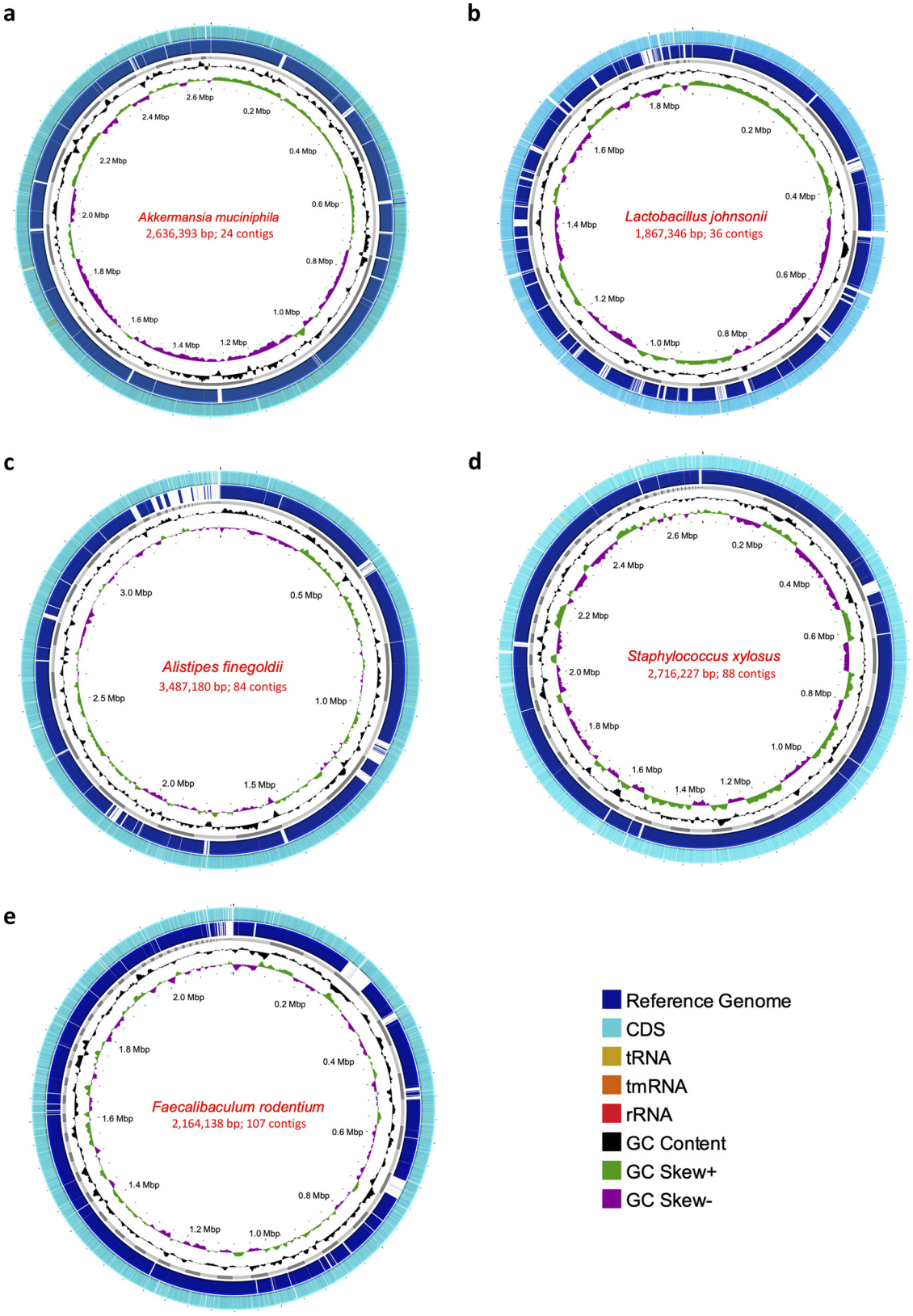

FIG 3 (a to e) Genomes (MAGs) reconstructed for *Akkermansia muciniphila* (a), *Lactobacillus johnsonii* (b), *Alistipes finegoldii* (c), *Staphylococcus xylosus* (d), and *Faecalibaculum rodentium* (e). In each of the MAGs, the size in base pairs and the resulting number of contigs of each genome obtained can be seen. For each case, the innermost ring represents the CG skew (green and purple). Subsequently, the %GC value is found (black). The next ring shows the resulting assembled genome (MAG) contigs (gray). Also, the

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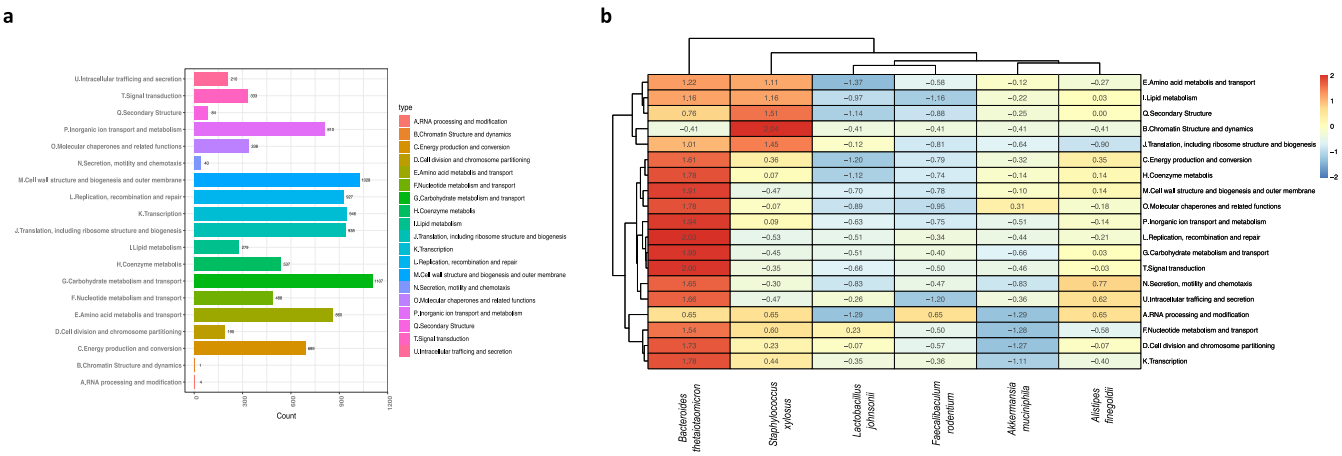

FIG 4 Functional analysis from MAGs. Ortholog assignment was performed using EggNOG-mapper. The results obtained here were used for clustering according to COGs of proteins in the complete set of reconstructed genomes (n = 6) (a) and in the different species obtained (b).

Despite sharing the modules corresponding to common metabolic pathways among these microorganisms, specific differences were evident in some submodules between species associated with noninfected BALB/c mice such as *B. thetaiotaomicron* (see Fig. S5a) and species related to infected BALB/c and C57BL/6 mice such as *S. xylosus* (see Fig. S5b). It was found that in amino acid biosynthesis, *B. thetaiotaomicron* presents complete pathways for the synthesis of histidine mediated by the pentose pathway in its nonoxidative phase. Also, for the biosynthesis of the aromatic amino acid tryptophan from chorismate and lysine from aspartate. In terms of the biosynthesis of BCAAs, the functional analysis of the *B. thetaiotaomicron* genome shows the presence of the complete pathway involved in this process (see Fig. S5a). In contrast, *S. xylosus*, showed an incomplete pathway associated with histidine synthesis, where enzymes such as phosphoribosylformimino-5-aminoimidazole carboxamide were absent. Regarding the synthesis of aromatic amino acids, specifically tryptophan, *S. xylosus* presents an incomplete pathway where it lacks the enzyme anthranilate synthase, fundamental in the final step from anthranilate to indole glycerol phosphate, the precursor of tryptophan. As far as lysine synthesis is concerned, it was observed that *S. xylosus* lacks enzymes such as diaminopimelate epimerase, N-acetyldiaminopimelate deacetylase, and L,L-diaminopimelate aminotransferase, necessary for the passage from tetrahydrodipicolinate to lysine. In the specific case of BCAAs, *S. xylosus* lacks the enzyme belonging to this pathway, specifically citramalate synthase, necessary to obtain 2-methylmalate from pyruvate (see Fig. S5b).

DISCUSSION

We identified that both mouse models showed detectable parasitemia as early as 2 dpi and burdens in both heart and intestine that were higher in BALB/c mice in both cases (Fig. 1a and b). In fact, involvement in both models was evidenced by infected mice developing splenomegaly. This is consistent with previous studies using chemiluminescent strains (6). *T. cruzi* generates high burdens in different portions of the gastrointestinal tract that are relevant during the acute phase, mainly at the level of the large intestine, and that can even be linked to the persistence of the parasite toward chronic digestive forms (6). As expected, the analysis of the cytokines in sera showed a proinflammatory immune response on the C57BL/6 led by IFN-γ, TNF-α, and IL-6 (Fig. 1c). In addition, the BALB/c mice revealed higher levels of anti-inflammatory cytokines such

FIG 3 Legend (Continued)
reference genome for each genome (dark blue), which was aligned by BLAST and the white areas represent regions where no identity with the assembled MAG was found. Finally, in the outer ring, there are the coding regions obtained by Prokka, where coding regions (CDS) (light blue), tRNA (light brown), tmRNA (orange), and rRNA (red) can be identified.

as IL-10 and IL-4 than C57BL/6 mice. This highlights the importance of this intestinal transit and how it may be related to changes in the intestinal microbiome of the infected host, a transit that deserves further investigation (6).

We observed that the structure and composition of the gut microbiome obtained through the metagenomic analyses showed the same taxa with the highest relative abundances for both animal models. These corresponded to *L. johnsonii*, *A. muciniphila*, *B. thetaiotaomicron*, *B. pseudolongum*, *A. finegoldii*, *F. rodentium*, and *S. xylosus*. However, these taxa showed changes in their relative abundance during the *T. cruzi* infection, becoming more prominent in BALB/c mice (Fig. 1d). Changes in the intestinal microbiome have previously been associated with *T. cruzi* infection, even in forms of digestive Chagas disease such as megacolon. Reduced relative abundances of *Bacteroides*, *Akkermansia*, and *Lactobacillus* have been reported (5, 6, 9, 10). This is consistent with our findings, demonstrating that this parasite could directly affect even other taxa that may be key in the interaction with this protozoan and that may have a direct impact on immunological and metabolic changes in the intestinal environment associated with infection.

As a result, these changes in the microbiota can potentially be associated with the immune response. Certain bacteria found in the present study, mainly strict anaerobes, are essential for the maintenance of intestinal health and proper immunoregulation (6, 9–12). This was evident with the correlation analysis between the immune response and microbiome structure, identifying a negative correlation between the reduction of *L. johnsonii* abundance and IL-10 in BALB/c mice (Fig. 1f). Regarding C57BL/6 mice, we found that the decrease of *A. muciniphila* correlated negatively with IL-22 and IFN-γ, explaining to some extent how *Akkermansia* may or may not favor inflammatory processes (13). Bacteria such as *L. johnsonii* and *B. thetaiotaomicron* are known due to their essential activity in the maintenance of the intestinal barrier, the inhibition of pathogen colonization, the reduction of inflammation through the regulation of the immune response, and in pivotal metabolic processes such as short-chain fatty acid (SCFA) biosynthesis (9, 13–22). *T. cruzi* infection directly affects the conditions of the intestinal environment causing a reduction of these populations and concomitantly, allowing the increase of taxa such as those found here. *S. xylosus* and pathobionts reported previously, such as *E. faecalis*, *P. mirabilis*, and *E. coli*, that are facultative anaerobes with the property of growing in this environment and associated with several inflammatory processes (23, 24). These changes favor an aerobic and inflammatory environment that metabolically may benefit the parasite. It is essential to evaluate how these taxa can represent an advantage in the infection's establishment and progression, and whether the microbiome's restructuring and reestablishment can potentially be a key factor in the elimination of the parasite and control of *T. cruzi* infection.

We corroborated the metabolic changes using functional prediction analysis from metagenomic data. The previously described disruption of the relative abundance of bacterial taxa was associated with a decrease in several metabolic processes (Fig. 2). Furthermore, BALB/c mice present a reduction in the biosynthesis of SCFAs (Fig. 2b), correlating with the taxa affected by infection and the immune response profile. An essential function of the biosynthesis of SCFAs involves the maintenance of cell differentiation and proliferation. The potential reduction of this metabolite (SCFA) may prevent proper tissue regeneration in a proinflammatory environment, thus promoting damage to the gut that allows for more efficient colonization by pathogens (25, 26). This suggests that changes in this critical metabolic pathway may favor the establishment and persistence of *T. cruzi* infection and be linked to the pathophysiology of the disease. However, further investigation is required to fulfill this premise.

The reconstruction of genomes from metagenomes (MAGs) supported some of the previously described findings of taxa that can potentially play a vital role in the infectious process (Fig. 3). Pangenome analysis of these MAGs revealed congruence of the reconstructed genomes with previously assembled and reported genomes. In most

cases, they were found to be related to isolates obtained from mice (see Fig. S3 and S4). The analysis of the metabolic pathways performed on MAGs shows how *B. thetaiotaomicron* plays a fundamental role in essential metabolic processes for host physiology, such as amino acid metabolism and transport, and lipid metabolism, among others (16, 27, 28) (Fig. 4b). The observations presented above strengthen the hypothesis that the decrease of the relative abundance of this taxon may be directly implicated in the decrease of these metabolic capacities of the microbiome in *T. cruzi* infection. This highlights the importance of this species within the intestinal microbiome and its potential relevance in the *T. cruzi*-host-microbe interaction. On the other hand, the analysis of the MAG of a bacterium that increased with the progress of the infection such as *S. xylosus* showed that this taxon presents incomplete metabolic pathways suggesting that these bacteria are unable to supply certain biological processes (see Fig. S5). It is necessary thus to direct new studies to corroborate how bacteria such as *B. thetaiotaomicron* can directly modulate *T. cruzi* infection and how their postinfection reestablishment, for example, can favor the response to the parasite, as well as generate less tissue damage through appropriate regulation of the immune response.

It was evidenced that during the *T. cruzi* infection, there was a modification of the gut microbiome structure in which several taxa of interest related to the infection, such as *B. thetaiotaomicron* may play a key role in the interaction with the protozoan. However, with this approach, it is not possible to establish the causality and directionality of the effects evidenced at the level of the immune system and the microbiome. Regarding the above, it can be suggested that *T. cruzi* infection may have indirect effects on the host microbiome related to (i) the inflammatory processes of the immune response against the protozoan and (ii) with the anatomical, histological, and physiological changes associated with the pathophysiology of the disease, as in the case of megacolon, and additionally, direct effects derived directly from the presence and action of the parasite at the intestinal level.

Considering this, infection by *T. cruzi* and its interaction at the gut level with the host could potentially be related to several factors: (i) the generation of modifications in immune profiles that promote an increase in oxidative stress and a proinflammatory environment that favors the parasite and promote an alteration in the structure of the intestinal microbiota; (ii) the changes in this microbiota structure are directly related to functional and metabolic changes (microbiome) involving, for example, the synthesis of SCFAs, having an important relevance in the modulation of the immune response and contributing to the proinflammatory state of the infection that *T. cruzi* is able to evade; (iii) and, finally, *T. cruzi* infection may probably be generating changes in the microbiome that functionally involve biosynthesis pathways of different amino acids and nucleotide metabolism, which are potentially related to their utilization by different stages of this protozoan and are of great relevance in the life cycle of this parasite.

Conclusions. The changes in the intestinal microbiome identified in this study suggest that there are alterations in the immune profiles and in several essential metabolic pathways that favor the immunological and biochemical processes associated with the infection. These results provide valuable insights needed for the research on new prophylactic and therapeutic alternatives in Chagas disease. Previous studies have shown that the use of probiotics based on different species of *Lactobacillus* and *Bifidobacterium* (important in the present study) in infections by *Giardia*, *Cryptosporidium*, *Eimeria*, and even in systemic infections such as malaria, can favor the resolution of the infection by reducing parasitic burdens, interrupting the advance of the parasite's life cycle, among others (29). Therefore, it is a potential area for further research and biotechnological improvement of novel therapeutic strategies for Chagas disease.

It is important to highlight, however, that a limitation of metagenomic studies is that functional analyses are based on the prediction of the genes and metabolic pathways. Therefore, it is necessary to implement transcriptomic and metabolomic studies that can directly corroborate these results and allow the identification of potential biomarkers of infection. Furthermore, the present analysis refers to the acute phase of infection; then, it is essential to also perform this evaluation in the chronic phase and

thus compare the changes caused by this parasite at different times of infection and disease. In addition, it is relevant to establish the causality and directionality of the changes generated by the infection and thus determine whether the immunological effects cause changes in the microbiome or whether, on the contrary, changes in the microbiome promote alterations in the immune response profiles. Nevertheless, our study to the best of our knowledge is the first to report MAGs in hosts during an infection process with *T. cruzi*. Finally, this study provides a relevant overview of parasite-microbiome interactions that deserves attention and should be prioritized in the future to unveil prospective therapeutic alternatives for Chagas disease.

MATERIALS AND METHODS

Mice and parasites. Female C57BL/6 and BALB/c mice from the same vendor (Jackson Laboratories), aged 5 to 8 weeks, were used. Animal experiments were performed in full compliance with the *Guide for the Care and Use of Laboratory Animals*, 8th edition (30), under a protocol approved by Baylor College of Medicine's Institutional Animal Care and Use Committee (IACUC), assurance number and D16-00475. The *Trypanosoma cruzi* strain Tulahuen (ATCC 30208), a discrete typing unit (DTU) VI, was used for infection experiments. Trypomastigotes were obtained from the blood of severe combined immune-deficient (SCID) mice. Animals were injected intraperitoneally with the parasite, and trypomastigotes were isolated from blood at day 16 postinfection. All mice were maintained in the same housing conditions and on the same diet at the Baylor College of Medicine facility.

***T. cruzi* infection.** Groups of five both BALB/c and C57BL/6 mice were kept noninfected in order to allow comparisons of the conditions evaluated throughout the follow-up. In addition, groups of five mice, BALB/c and C57BL/6, were infected with 500 blood-stage trypomastigotes of *T. cruzi* by intraperitoneal injection. Beginning at 3 dpi, blood was collected twice weekly throughout the study by tail vein microsampling to monitor parasitemia by quantitative PCR (qPCR) and stool for metagenome analysis. At 16 dpi, mice were euthanized, and then whole blood was collected by cardiac puncture. Lastly, different tissues (heart, and intestine) were collected and frozen at −80°C.

Sample collection. Fecal samples were collected (in a pool corresponding to each group) on day 0 (prior infection) and at 3, 7, 10, 13, and 16 dpi. In addition, at these time points, blood samples were taken (per mouse) for the evaluation of parasitemia by qPCR twice a week. On day 16, mice were sacrificed, and parasitemia was evaluated in cardiac and intestinal tissue.

Parasite burdens. To evaluate the parasite burden from blood, gut, and cardiac tissue, total DNA was isolated using a PDQeX prepGEM Universal kit (MicroGEM). Parasite burdens from total DNA were calculated by real-time PCR using the TaqMan system amplifying the satellite region of *T. cruzi* nuclear DNA (primers 5′-ASTCGGCTGATCGTTTTCGA-3′ and 5′-AATTCCTCCAAGCAGCGGATA-3′ and probe 5′-6-FAM-CACACACTGGACACCAA-MGB-3′), where FAM is 6-carboxyfluorescein, and MGB is minor groove binder (Life Technologies) (31, 32). To normalize the data, we used the glyceraldehyde-3-phosphate dehydrogenase (GAPDH; primers 5′-CAATGTGTCCGTCGTGGATCT-3′ and 5′-GTCCTCAGTGTAGCCCAAGATG-3′) and probe 5′-6-FAM-CGTGCCGCCTGGAGAAACCTGCC-MGB-3′ (Life Technologies) (33). The qPCRs were carried out with 4 ng of DNA from blood and 20 ng of DNA from cardiac tissue and intestine; a standard curve was used to calculate the parasite equivalents in blood, gut, and cardiac tissue (34).

Cytokine evaluation. The levels of IL-2, IL-4, IL-6, IL-10, IL-13, IL17A, IL-22, IFN-γ, and TNF-α were measured in sera from noninfected and infected mice; a Luminex-based assay was used, as previously described (35). The readout was performed using a MagPix Luminex instrument. Cytokine concentrations in the supernatant were calculated based on a standard curve, and for each sample, duplicate wells were averaged, and further analysis was done.

DNA extraction for metagenome analysis. All stool samples from animals infected and noninfected were collected and immediately stored on ice. Subsequently, DNA was extracted using MP FastDNA spin kits for soil (MP Biomedicals, Irvine, CA) (36, 37). Extracted DNA was resuspended in 50 μL of pyrogen-free water and stored at −20°C. DNA samples were shipped to Novogene for 150-bp PE sequencing by Illumina NovaSeq 6,000 to obtain 4 Gb of raw data per sample.

Bioinformatics analysis. (i) Sequence processing, taxonomic assignment, and functional profiling. From the raw reads, a quality assessment was performed by FastQC (38). Quality and adapter trimming was then conducted by Trimmomatic (39) using the parameters ILLUMINACLIP: TruSeq3-PE.fa:2:30:10:2: keepBothReads MINLEN:150 AVGQUAL:20 TRAILING:20. Mapping to the *Mus musculus* genome (GRCm39) was performed using the Bowtie2 tool (40) to remove reads corresponding to the host.

The Centrifuge tool was used in taxonomic assignment from the cleaned reads (41). The obtained outputs were transformed to Kraken-Report format with the Centrifuge-kreport function. Relative abundance values for each taxon identified at each time of infection were obtained from the Centrifuge report for the corresponding analysis and visualized by Pavian (42). In addition, a beta-diversity analysis was performed to compare the microbiota composition between noninfected and infected mice using NMDS.

Simultaneously, Humann3 (HMP Unified Metabolic Analysis Network) (43) was used for functional profiling, including identifying changes in gene abundance and metabolic pathways. Since this tool receives only one fastq file as input, forward and reverse reads from each sample were concatenated to generate the file. A normalization was made with RPK values to relative abundance values or "copies per million" (cpm) units through the humann_renorm_tablescript function to facilitate comparisons between samples with different

sequencing depths. The default "units" of the HUMAnN microbial function are the UniRef gene families used to calculate reaction and pathway abundances. From the abundance of gene families, reconstruction of the abundance of other functional categories was performed using the humann_regroup_tablescript function. Gene family abundance values normalized by cpm to the MetaCyc reaction abundances (RXN), included with the default Humann3 facility, were regrouped. The humann_rename_table function was used to generate a readable output, and then the sample outputs were joined with the humann_join_tables function. Finally, a stratified table was obtained with humann_split_stratified_table. To evaluate the results of Humann3, the R package, Maaslin2 (44) was used to determine the association between measurement times, condition (infected – noninfected), and functional characteristics of the microbiome using a multivariate mixed-effect model. Heatmaps were created with MicrobioSee using Ward's linkage method for hierarchical cluster analysis.

(ii) MAGs, pangenome reconstruction, and functional analysis. From the clean reads, the assembly process was performed using Spades, with the –meta parameter (45) and Megahit (46). MetaQuast was used to determine these assemblies' quality statistics and to select the best assembly (47). To estimate the coverage of a bowtie2 and thus proceed to binning, reads were mapped for each sample against the corresponding assemblage. The best-quality assemblies with contigs longer than 1,500 nucleotides were binned into MAGs the using MetaBat, Maxbin, and Concoct tools, followed by refinement with DASTool (48). The quality of these final bins was evaluated with CheckM (48–51). Taxonomic assignment of these good-quality bins was performed with Genome Taxonomy Database GTDB-Tk (52). Bins with completeness >90% and contamination <5% that were taxonomically assigned were treated as MAGs for further analysis. MAGs for *Akkermansia muciniphila*, *Lactobacillus johnsonii*, *Alistipes finegoldii*, *Staphylococcus xylosus*, *Faecalibaculum rodentium*, and *Bacteroides thetaiotaomicron* were obtained. The MAGs were annotated using Prokka (53). The process was implemented for visual genome reconstruction. In this case, a BLAST alignment was performed with the default parameters with the reference genome corresponding to each previously defined species, and further downloaded from the NCBI: NZ_AP021898.1, *Akkermansia muciniphila*; NZ_CP062068.1, *Lactobacillus johnsonii*; NZ_JADMWR010000010.1, *Alistipes finegoldii*; NZ_CP008724.1, *Staphylococcus xylosus*; NZ_CP011391.1, *Faecalibaculum rodentium*; and NZ_CP040530.1, *Bacteroides thetaiotaomicron*. The .gff annotation files obtained for each case were also included in this reconstruction, where the GC percentage and the GC Skew with the default parameters were determined.

The .gff files obtained from the annotation also were used to conduct a pangenome analysis using Panaroo tool (54), considering an identity of 99% and their presence in the compared genomes of 99% (–clean-mode strict -a core –aligner mafft –core_threshold 0.99). For this purpose, the complete and good-quality genomes available in PATRIC (Pathosystems Resource Integration Center) were downloaded: 96 genomes of *Akkermansia muciniphila*, 15 of *Lactobacillus johnsonii*, 18 of *Alistipes finegoldii*, 20 of *Staphylococcus xylosus*, and 4 of *Faecalibaculum rodentium*. The results were used to generate phylogenetic trees in ITol (55), based on alignments obtained from the core genome using Panaroo and the .newick files generated from these alignments. For the graphical representation of the core genome was used Phandango (56).

The Kyoto Encyclopedia of Genes and Genomes (KEGG) was used to identify the metabolic capacities present in MAGs (57, 58). KofamKOALA was used to assign K numbers to the sequence data by HMMER/HMMSEARCH against Kofam (a customized HMM database of KEGG Orthologs [KOs]). The outputs were used for visual reconstructions of metabolic pathways using the KEGG Mapper. Complementarily, an evaluation was performed for annotation through ortholog assignment using EggNOG-mapper (59). Finally, the results were used for clustering according to Clusters of Orthologous Groups (COGs) of proteins (60).

Statistical analysis. Statistical analyses were carried out using R software (61). For continuous values (parasite burdens, relative abundance, cytokine levels, and abundance of gene reads), normality hypotheses were evaluated using the Kolmogorov-Smirnov and Shapiro-Wilk tests. A comparison of the values of numerical variables (parasite burdens, relative abundances, cytokine levels, and abundance of gene reads) was performed between study groups (noninfected and infected mice) and between time points of infection. For this purpose, parametric tests (Student *t* test for comparisons between two groups) and nonparametric tests (Wilcoxon signed-exact-ranks test for comparisons between two groups and Kruskal-Wallis test for comparisons between multiple groups with the corresponding Dunn's test for *post hoc* multiple comparisons adjusted with the Bonferroni method) were used. Likewise, to evaluate the correlation between the numerical variables corresponding to abundances of the different taxa and the pg/mL serum levels of the cytokines, Spearman's nonparametric test was used. All significance tests, whether parametric or nonparametric, were two tailed, and *P* values of <0.05 were considered statistically significant.

Ethics approval and consent to participate. Animal experiments were performed in full compliance with the *Guide for the Care and Use of Laboratory Animals*, 8th edition (30), under a protocol approved by Baylor College of Medicine's IACUC, under assurance number D16-00475.

Data availability. Sequence data supporting the conclusions of this article are available in the SRA database under the BioProject PRJNA915351. Code for generating the figures and the analysis is available at https://github.com/gimur/Trypanosoma_cruzi-host-microbiome.git.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 1.3 MB.

SUPPLEMENTAL FILE 2, XLSX file, 0.01 MB.

SUPPLEMENTAL FILE 3, XLSX file, 0.01 MB.

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We declare that we have no competing interests.

S.C., C.P., and J.D.R. designed the study. S.C., R.M., and C.P. performed the experiments. S.C. and M.M. analyzed the data. P.J.H., M.E.B., A.E.P.-M., and R.M.J. provided recommendations to the results and analysis. S.C., C.P., and J.D.R. drafted the manuscript. All authors read and approved the final version of the manuscript.

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6.3. Capítulo 3

Objetivo 4: Evaluar los cambios en la microbiota del hospedero y del helminto asociados a la infección por *Ascaris suum* en un modelo animal a partir de un enfoque de metabarcoding.

Objetivo 5: Identificar el proteoma de los productos de excreción-secreción (ES) de diferentes etapas larvales de *Ascaris suum* (L3-huevo, L3-pulmón, L3-tráquea) a partir de un enfoque proteómico.

Para cumplir con el objetivo 4, el estudio se centró en caracterizar la microbiota asociada a diversas etapas de desarrollo de *Ascaris* y su impacto en la microbiota del hospedero durante la migración larval en un modelo murino. Se infectaron quince ratones con 2500 huevos embrionados de *A. suum*. A los 4, 8 y 14 días post-infección (DPI), se realizó la eutanasia de cinco ratones infectados con *Ascaris* y ratones control de la misma edad. Se recolectaron muestras de heces, intestino, hígado y pulmones. Las larvas se aislaron de los huevos embrionados in vitro, así como del hígado a los 4 DPI y del pulmón a los 8 DPI. Utilizando la secuenciación del 16S rRNA, analizamos la diversidad bacteriana en muestras de diferentes etapas de *Ascaris* y en tejidos del hospedero. Nuestros resultados revelaron un total de 8,040 variantes de secuencias de amplicones (ASVs), siendo las muestras de *Ascaris* las que mostraron la mayor diversidad. Es notable que la microbiota asociada a *Ascaris* difería significativamente de la del hospedero, observándose una mayor diversidad en el parásito.

El análisis de abundancia diferencial identificó patrones taxonómicos distintos, destacando géneros específicos como *Bradyrhizobium*, *Achromobacter* y *Pseudomonas* en *Ascaris*. Nuestros hallazgos sugieren que *Ascaris* alberga una microbiota única que podría intercambiar bacterias con el hospedero durante la migración larval. Estas perspectivas abren camino para investigaciones adicionales sobre la dinámica ecológica y funcional de las interacciones helminto-microbiota, lo que podría informar estrategias terapéuticas novedosas dirigidas a estas relaciones microbianas para mitigar las infecciones por helmintos y mejorar los resultados de salud en los hospederoes.

Los resultados obtenidos indican que *Ascaris* puede modular activamente su microbiota, creando un microambiente favorable para su supervivencia y desarrollo dentro del hospedero. Además, el estudio sugiere que existe un intercambio dinámico de microorganismos entre el parásito y el hospedero durante el ciclo de vida de *Ascaris*. Este intercambio podría tener implicaciones importantes para la salud del hospedero, ya que la microbiota asociada a *Ascaris* podría influir en la respuesta inmunitaria y en su susceptibilidad a otras infecciones.

Para el objetivo 5, se llevó a cabo un análisis con el fin de caracterizar el proteoma de los productos de excreción-secreción (ES) de diferentes etapas larvarias de *Ascaris suum* (L3-huevo, L3-pulmón, L3-tráquea) que puedan resultar relevantes en la interacción parásito-hospedero-microbioma y que puedan resultar como potenciales objetivos de intervención que reduzcan la morbilidad global inducida por *Ascaris*. Se aislaron larvas específicas por etapa, que fueron cultivadas in vitro y de las cuales se recolectaron los productos ES.

Las larvas L3 se aislaron de huevos embrionados (L3-huevo), del pulmón de ratones Balb/c infectados con huevos de *Ascaris suum* a los 8 días post-infección (L3-pulmón), y de la tráquea de ratones Balb/c infectados a los 12 días post-infección (L3-tráquea). Los productos ES se obtuvieron mediante el cultivo de larvas. Se realizó un análisis proteómico utilizando cromatografía líquida-espectrometría de masas en tándem (LC-MS/MS) y herramientas bioinformáticas como MaxQuant, Perseus y Andromeda, siguiendo un protocolo detallado disponible en GitHub. Este análisis incluyó la identificación de péptidos, puntuación y cuantificación contra una base de datos específica del organismo, junto con controles de calidad, evaluación de correlación y determinación de abundancia diferencial mediante el algoritmo Amica.

En total, se identificaron 58 proteínas únicas en los productos ES. De estas, 14 proteínas fueron comunes a todas las etapas, mientras que otras fueron específicas de cada etapa. El análisis de componentes principales reveló perfiles proteicos distintos para cada etapa, sugiriendo que los proteomas son cualitativamente diferentes. El análisis de ontología genética indicó un enriquecimiento GO específico por etapa en diversas clases de proteínas, como las proteínas nucleares en los productos ES de L3-huevo y las enzimas metabólicas en los productos ES de L3-pulmón y L3-tráquea. Este estudio pone de manifiesto las diferencias específicas por etapa en la composición de los productos ES de *Ascaris*. Es esencial investigar más a fondo los roles funcionales de estas proteínas y sus interacciones con las células del hospedero para desarrollar estrategias terapéuticas y diagnósticas innovadoras contra la ascariasis.

Como producto de este capítulo se adjuntan los siguientes artículos científicos:

Artículo 4: Castañeda S, R,Poveda Charlie Suarez-Reyes, Yifan Wu, Noah Haugen, Patiño, Luz H, Weatherhead JE, Ramírez JD. Microbiota Dynamics During *Ascaris* Larval Migration: Implications for Host Microbial Communities. *Sometido a Microbial Pathogenesis (Q1)*.

Artículo 5: Castañeda S, Adeniyi-Ipadeola G, Wu Y, Suarez-Reyes C, Jain A, Ramírez JD, Weatherhead JE. Characterizing Excretory-Secretory Products Proteome Across Larval Development Stages in *Ascaris suum*. *Sometido a Microbes and Infection (Q1)* https://doi.org/10.1101/2024.07.03.601870.

Artículo 6: Castañeda S, Paniz-Mondolfi A, Ramírez JD. 2024. Detangling the Crosstalk Between *Ascaris*, *Trichuris*, and Gut Microbiota: What´s Next? Frontiers in Cellular and Infection Microbiology [Internet]. 2022. https://doi.org/10.3389/fcimb.2022.852900.

Artículo 7: Ramírez JD, **Castañeda S,** Weatherhead JE, R,Poveda. 2024. Parasite-Microbiota Interactions: A Pathway to Innovative Interventions for Chagas Disease, Leishmaniasis, and Ascariasis**.** *Sometido a Future Microbiology (Q1).*

Microbiota Dynamics During *Ascaris* Larval Migration: Implications for Host Microbial Communities

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Abstract

The complex interactions between parasites, their hosts, and associated microbiota hold significant implications for host health and disease outcomes. Helminths like *Ascaris lumbricoides* and *Ascaris suum* can significantly alter the host's intestinal microbiota, affecting both parasite biology and host pathology. Despite extensive research on host-microbiota changes due to helminth infections, the study of helminth-associated microbiota remains limited. This study aims to characterize the microbiota associated with various developmental stages of *Ascaris* and its impact on the host's microbiota during larval migration in a murine model. Fifteen mice were infected with 2500 embryonated *A. suum* eggs via oral gavage. Five *Ascaris*-infected mice and age-matched naïve mice were euthanized at 4-, 8-, and 14-days post-infection (DPI). Stool, intestine, liver, and lung samples were collected. Larvae were isolated from embryonated eggs *in vitro*, from the liver at 4 DPI, and the lung at 8 DPI. Utilizing 16S rRNA sequencing, we analyzed bacterial diversity in samples from different *Ascaris* stages and host tissues. Our results revealed a total of 8,040 amplicon sequence variants (ASVs) with *Ascaris* samples displaying the highest diversity. Notably, *Ascaris*-associated microbiota differed significantly from that of the host, with higher diversity observed in the parasite. Differential abundance analysis identified distinct taxonomic patterns, highlighting specific genera such as *Bradyrhizobium*, *Achromobacter*, and *Pseudomonas* in *Ascaris*. Our findings suggest that *Ascaris* harbors a unique microbiota that potentially exchanges bacteria with the host during larval migration. These insights pave the way for further research into the ecological and functional dynamics of helminth-microbiota interactions, which may inform novel therapeutic strategies targeting these microbial relationships to mitigate helminth infections and improve host health outcomes.

Key Words

Ascaris, microbiome, parasite-host interactions

1. Introduction

The interactions between parasites, their hosts, and their associated microbiota form a complex and dynamic system that have significant implications for host health and disease outcomes [1–3]. Helminths, such as *Ascaris lumbricoides* and *Ascaris suum*, are of particular interest due to their widespread prevalence and the considerable burden they impose on human and animal populations [4–9]. Evidence suggests that these helminths can alter the composition and structure of the host's intestinal microbiota, potentially playing a critical role in the parasite's biology and pathophysiology [10–18].

Studies evaluating the host-helminth-intestinal microbiota relationship have found that alterations in bacterial taxa abundance and diversity of the host intestinal microbiota in the presence of helminths can significantly influence the parasite's life cycle, promoting infectiveness, dissemination, and modulation of the host immune response [2,13,15,19–25]. However, contrasting findings from descriptive studies on human populations reveal that helminth infections do not consistently impact the host microbiota, suggesting that different methodological approaches may lead to heterogeneous results [14,21,26]. Researchers have developed and implemented murine and porcine models of *Ascaris* infection to address these inconsistencies and control potential confounding variables. These models aim to model the infection process in humans, allowing for a controlled study of parasite-host-microbiota interactions. Through these models, researchers can evaluate the changes in microbiota composition and structure caused by helminths and examine the dynamic interactions between helminth infections and host viscera microbiota [27–32].

Ascaris infection involves complex biological characteristics including an essential extra-intestinal migration, during which infective larvae (L3) invade gastrointestinal mucosal tissue and migrate through the liver and lungs before returning to the intestines to develop into adults worms [12,33–35]. This larval migration likely alters the composition and structure of host microbiota in extra-intestinal viscera like the liver and lung. However, no studies to date have explored alterations of host microbiota during the larval migratory phase. While minimal data exists regarding the impact of *Ascaris* on extra-intestinal viscera, adult *Ascaris* worms in the intestines have been found to reshape the host intestinal microbiota structure. Adult worms in the intestines cause a decrease in essential anaerobic bacteria like *Faecalibacterium* and *Ruminococcus*, and an increase in facultative anaerobes like *Streptococcus* creating a favorable metabolic environment for long-term parasite survival in the intestines [2,13,15,19–25]. While *Ascaris* infection has been shown to alter host microbiota, data on these complex interactions has not been consistent. Using an *Ascaris suum* infected pig model, Wang et al. (2019) [13] reported reduced alpha diversity in intestinal tissue while Williams et al. (2017), also in pigs [36], observed increased alpha diversity but decreased relative abundance of *Lactobacillus*, *Ruminococcus*, and *Catenibacterium* genera.

Despite numerous studies exploring changes in host microbiota due to helminth infections, research on helminth-associated microbiota remains limited [37–39]. Investigating the helminth's own microbiota is crucial, as it defines stage-specific transient microbiota and their interactions with the host, uncovering mutualistic relationships between specific bacterial groups and the parasite essential for its biology, infectivity, and dissemination [39,40]. For example, *Wolbachia* bacteria found in Onchocercidae nematodes, causing lymphatic filariasis and onchocerciasis, provide vital metabolites in a symbiotic relationship crucial for the parasite [41]. Despite these insights, few studies have explored *Ascaris*-microbiota composition and its role in parasite-host interactions. Midha et al. (2020) conducted a study analyzing the intestinal microbiome of adult *Ascaris suum* in pigs, comparing it with the host's gut microbiome during infection [37]. Using 16S rRNA sequencing, they observed lower diversity in the parasite's gut compared to the host's, with reduced diversity specifically at the infection site in the jejunum. The host's microbiome at this site influenced the parasite's microbiome, although distinct microbial signatures differentiated the nematodes from their hosts. This study provided the first insights into *Ascaris*-associated microbiota dynamics during host interaction [37].

Prior research underscores the complexity of the Ascaris-host-microbiota interaction and highlights the need for more robust research on the host and parasite microbiota and how they influence each other over time. Here, we characterize the microbiota associated with different *Ascaris* developmental stages and examining the influence of the *Ascaris* microbiota on host microbiota during larval migration through the intestine, liver, and lungs in a murine model. Employing metabarcoding via 16S rRNA gene sequencing, we aim to elucidate dynamic changes in microbiota composition within the parasite and the host, enhancing our understanding of helminth-microbiota's ecological and functional dynamics. Exploring these interactions may uncover new strategies for managing *Ascaris* infections and mitigating their impacts on host health.

2. Methods

2.1. Mice and parasites

8-week-old female BALB/c mice were obtained from Jackson Laboratories. All animal experiments were carried out following the guidelines outlined in the 8th edition of the Guide for the Care and Use of Laboratory Animals under the approval protocol by Baylor College of Medicine's Institutional Animal Care and Use Committee (IACUC), AN-9173. Embryonated *Ascaris suum* eggs for the experiments were obtained from adult female worms collected from *Ascaris* infected pigs maintained at Baylor College of Medicine under IACUC AN-8544. All the mice were maintained in the same housing conditions and on the same diet at the Baylor College of Medicine facility.

2.2. Hatching Ascaris suum larvae (L3) in vitro

To determine the larvae' inherited microbiome, embryonated eggs were hatched *in vitro*. Eggs were placed in H_2SO_4 solution and centrifuged. The pellet was resuspended in a sodium hypochlorite solution and incubated for a 2-hour. After incubation, eggs were collected by centrifugation and washed three times with ultrapure water. Eggs were resuspended in Hanks Balanced Salt Solution (HBSS) with a pH of 2.0, adjusted using hydrogen chloride, and incubated for 30 minutes at 37°C with 5% CO_2. Then, eggs were centrifuged and resuspened in HBSS with a pH of 7.0 to neutralize the eggs for 30 minutes. Finally, eggs were centrifuged and resuspended in culture media (RPMI-1640 + 44% penicillin-streptomycin), placed in a 6-well plate, and incubated for 96 hours. Egg hatching was monitored using light microscopy. Once the larvae hatched, the larva was pelleted by centrifuge and were stored at -80°C.

2.3. Ascaris suum infection

15 Balb/c mice were infected with embryonated 2500 *A.suum* eggs in PBS via oral gavage and 15 Balb/c mice were sham infected with PBS via oral gavage. A group of 5 *Ascaris*-infected mice and 5 age-matched naïve mice were euthanized at 4-, 8-, and 14-days post-infection (DPI). Stool, duodenum, liver, and lung were collected and frozen at –80°C. Larvae were isolated from host tissue from the liver at 4 DPI and the lung at 8 DPI.

2.4. Ascaris suum larvae (L3) isolation from tissues

Liver tissue was collected at 4 DPI, and lung tissue was collected at 8 DPI and macerated with 1X PBS. The macerated mixture, tissue+PBS, was transferred into a Baermann apparatus equipped with a conical tube and incubated at 37°C with 5% CO_2 for 4 hours. After incubation, samples were filtered to let larvae settle at the bottom. The supernatant was removed, and the pellet was washed with ultra-pure water three times by centrifugation at 800g for 10 minutes at 4°C. After the final wash, the supernatant was removed, and the isolated larvae from lungs or livers were stored at -80°C.

2.5. DNA extraction for microbiota analysis

Larvae, tissues, and stool were homogenized mechanically, disrupting them with beads and shaking, using the MP FastPrep-24 homogenizer (MP Biomedicals, Eschwege, Germany). DNA was isolated with a specific kit selected to ensure the extraction of high-quality DNA for further analysis. DNA from stool samples was isolated using the stool nucleic acid isolation kit (Norgen Biotek Corp., Thorold, Canada). DNA from 25-35 mg of lung and liver was obtained using DNEasy (Qiagen, Germany). Finally, larvae isolations from invitro hatching, lungs, and livers were isolated with the DNeasy PowerSoil Pro Kits (Qiagen, Germany). Each isolation was

performed by following the manufacturer's protocol. All extracted DNA was resuspended in 100 µL of pyrogen-free water, quantified in a Nanodrop (Thermo Scientific), and kept at -80°C.

2.6. 16S rRNA-amplicon-based sequencing and microbiome analysis

All samples ($n = 49$) were subjected to amplicon sequencing of the V4 hypervariable region of 16S rRNA gene using the Illumina NovaSeq platform, paired-end, PE 150pb. The bioinformatics pipeline began with the extraction of barcodes and primers from the resulting paired-end sequences using the Quantitative Insights Into Microbial Ecology (QIIME) analysis program [42]. Using DADA2, the quality profile of the reads was evaluated to select the length with quality greater than 30 and, thus, minimize erroneous reads [43]. Normalization and rarefaction curves were implemented to prepare and normalize the data and to confirm that the sequencing depth was sufficient to capture the diversity of the samples analyzed. DADA2 was also employed to determine the central sample inference algorithm of the reads for inferring amplicon sequence variants (ASVs). This process eliminated duplications by combining all identical sequencing reads into single sequences, and chimeras were removed before taxonomic allocation. Finally, the taxonomic assignment was performed using the sequences of the Silva v138 formatted reference database [44,45].

The analysis of microbiota diversity, including the calculation of diversity indices and relative abundances, as well as the construction of corresponding graphs, was carried out using the R *Phyloseq* and *Vegan* packages [46]. Additionally, linear discriminant analysis (LDA) effect size (LEfSe) analysis and DESeq2, were employed to identify taxa with differential abundance [47]. The threshold of the logarithmic LDA score was 4.0 with a maximum kw value of 0.01 for LEfSe analysis. For DEseq2, a pCutoff of 0.05 and an FCcutoff of 1.0 were used.

2.7. Statistical analysis

The number of reads in each sample was normalized using the average sequence depth. Relative abundance and alpha diversity metrics, including observed richness, Shannon, and Simpson indices, were used to compare groups [48]. Depending on the number of comparison groups, alpha diversity indices were compared using non-parametric Mann-Whitney-Wilcoxon and Kruskal-Wallis tests. To explore differences in the general composition of the microbial community (beta diversity) concerning groups, Bray-Curtis taxonomic distances were calculated. A principal coordinates analysis (PCoA) diagram was also generated, where Bray-Curtis distances between samples were used to visualize group comportment. Permutational multivariate analysis of variance using distance matrices (PERMANOVA) was conducted to test for multivariate effects, employing the adonis function from the vegan package[49]. Statistical analyses were performed using R software[50]. All significance tests were two-tailed, and P-values < 0.05 were considered statistically significant.

3. Results

3.1. Sequencing results

We obtained a total of 9,573,891 raw reads from all samples, with a mean of 195,385 reads per sample. After DADA2 filtration, denoising, merging, and chimeric removal, we retained 7,944,034 reads, representing 83% of the total, with a mean of 162,123 reads per sample (Table S1). The DADA2 pipeline enabled us to identify 8,040 amplicon sequence variants (ASVs). The general profile, encompassing all samples, unveiled the prevalence of bacteria from the phyla Bacteroidota, Firmicutes, Proteobacteria, and Actinobacteria (Fig. 1a).

3.2. Alpha Diversity Analysis Reveals Elevated Microbial Diversity in Ascaris Larvae-Associated Microbiota

We conducted alpha diversity analysis using observed richness, the Shannon index, and the Simpson index to elucidate overall composition and diversity differences among sample groups—specifically *Ascaris* stages, feces, intestine, liver, and lung. *Ascaris* samples showcased the highest diversity, followed by feces and intestine, exhibiting similar values. As anticipated, the liver and lung displayed the lowest diversity, attributable to their limited bacterial burden (Fig. 1b).

We compared the diversity between *Ascaris* infected and non-infected mice and evaluated each tissue group based on infection status (Fig. 1 c-g). Overall, we did not observe significant differences in diversity in varying tissues between infected and non-infected mice. When comparing tissue samples to *Ascaris* larva, the feces and intestine sample groups had no significant differences in diversity related to infection status or between the tissue groups and the *Ascaris* larvae stages (Fig. 1c, d). In contrast, the liver and lung host tissue samples exhibited statistically significant differences in diversity compared to *Ascaris* larvae. In both cases, *Ascaris* larvae displayed higher diversity than each independent host tissue group (Kruskal Wallis test, p-value < 0.01) (Fig. 1e, f). Moving forward, to analyze diversity specifically in different *Ascaris* stages, we compared the Shannon index among *in vitro* larvae, larvae 4 day post-infection (DPI) (liver), and larvae 8 DPI (lung). Although we did not detect statistically significant differences, it is noteworthy that diversity increased with the progression of the life cycle from day 4 to day 8 post infection (Fig. 1g, S1).

To complement the previous evaluation, we aimed to compare the diversity between the host and *Ascaris* at various days post-infection (naïve, 4 DPI, 8 DPI, and 14 DPI). For this purpose, we evaluated diversity by examining each sample group (feces, intestine, liver, lung) and each larval stage (larva *in vitro*, larvae in the liver 4 DPI, and larvae in the lung 8 DPI). Although we did not detect statistically significant differences, it is remarkable that diversity in the liver and lung increased after larval passage through these organs on days 4 DPI and 8 DPI, respectively. This

suggests a potential microbiota exchange from the *Ascaris* larvae to the host, considering our previous demonstration of higher diversity in *Ascaris*-associated microbiota (Fig. S1 a,b).

3.3. Beta Diversity Analysis Indicates Distinct Microbiota Composition in Ascaris Compared to Host

We have observed that the *Ascaris*-associated microbiota exhibited higher diversity values regardless of the larval stages, and even when compared with the intestine and feces, these values were quite similar. The migration of larvae from the intestine to the liver and lung likely contributes to the increase in *Ascaris* diversity. Additionally, it is plausible that *Ascaris* shares bacteria with the host at the liver and lung levels, further contributing to the increased diversity in these organs. Given these findings, we aimed to determine the similarity between the microbiota of *Ascaris* and the host tissue groups. We first performed rarefaction on the samples to control for potential bias to achieve this objective. Then, we conducted a permutational analysis of variance (PERMANOVA) based on Bray-Curtis dissimilarities (Fig S2a). Subsequently, we performed Principal Coordinates Analysis (PCoA) to visualize clusters corresponding to sample groups: *Ascaris*, feces, intestine, liver, and lung (Fig. 2a).

We discovered that despite transversing the tissue durations during the life cycle, the *Ascaris* microbiota differs significantly from that of the host, regardless of the organ tissue (Fig. 2a). Additionally, we observed that the microbiota of the liver and lung form a distinct cluster that is more similar to the intestines than to the feces. Interestingly, the intestinal microbiota differed significantly from the bacterial composition of feces (PERMANOVA: adonis2 p= 0.001). Complementary beta diversity analyses were conducted to identify compositional differences between groups of infected and non-infected mice and *Ascaris* larvae. We found that the microbiota composition were similar between *Ascaris* infected and non-infected mice, suggesting that *Ascaris* infection may not be associated with global changes in the microbiota structure of the host. However, once again, we observed that the host microbiota differed substantially from the *Ascaris*-associated microbiota (PERMANOVA: adonis2 p= 0.003) (Fig. S2b).

3.4. Microbiota Composition: Unique and Shared Taxa between Ascaris and Host

Understanding that the global composition of microbiota differs between the host and *Ascaris* larvae, we determined a relative abundance profile of tissue groups by DPI to identify potential differences. We observed that the overall microbiota of *Ascaris* stages was primarily characterized by the Proteobacteria phylum, followed by Firmicutes and Bacteroidota. Intriguingly, the microbiota of the lung and liver were quite similar at the phylum level, showing a high relative abundance of Proteobacteria and Bacteroidota phyla. The intestine was mainly composed of Proteobacteria, Firmicutes, and Bacteroidota, while the feces primarily consisted of Firmicutes, Bacteroidota, and Verrucomicrobia (Fig. 2b).

Considering these characteristics, we performed a specific analysis of each tissue group by DPI and of each *Ascaris* larval stage at several taxonomical levels (Fig. 2 b, c, Fig. S2 c,d). The *Ascaris* larva in vitro showed that the most abundant genera were *Delftia*, *Sphingomonas*, *Achromobacter*, *Chryseobacterium*, and *Pseudomonas*, all of which belong to the Proteobacteria phylum. Interestingly, larval migration is associated with changes in the microbial composition. We observed in larvae isolated from the liver at 4 DPI and larvae isolated from the lung at 8 DPI had an increase in the relative abundance of taxa such as *Bradyrhizobium*, but also of *Lactobacillus*, and the *Lachnospiraceae NK4136 group*, which are likely derived from the host (Fig. 2c). These findings again support the early finding of increased alpha diversity in the *Ascaris* larvae as the larval life cycle progresses. Conversely, the lungs and liver tissue microbiota did not demonstrate any changes in the bacterial composition during the larval life cycle progression. Throughout the study, both organs were mainly constituted by the genus *Delftia* and *Chryseobacterium* (Fig. 2c).

In the non-infected intestine, we observed a relative abundance of taxa such as the *Muribaculaceae family*, *Akkermansia*, and *Lactobacillus* at 4 DPI and 8 DPI, which partially reduced by 14 DPI. Conversely, in the infected intestine, we observed an increase in the relative abundance of *Delftia* and *Chryseobacterium* at 4 DPI and 8 DPI after infection, likely originating from *Ascaris* larvae (Fig. 2c). In both non-infected and infected feces, we observed higher relative abundances of taxa such as the *Muribaculaceae family*, *Lachnospiraceae NK4A136*, *Akkermansia*, *Lactobacillus*, *Alistipes*, and the *Oscillospiraceae family*, which are commonly found in host feces (Fig. 2c).

Considering these results, we propose that despite beta diversity analysis showing that *Ascaris* possesses a distinct overall microbiota compared to the host, certain taxa are shared between them. We evaluated the microbiota common to *Ascaris* stages, *Ascaris* infected host tissues and non-infected host tissues to establish core microbiota for the helminth and the host (Fig. 2d). We identified 273 taxa comprising the *Ascaris*-core microbiota, 111 taxa in *Ascaris* infected mice, and 178 taxa in non-infected mice. Seventy-seven taxa were shared across these three groups. Regarding condition-specific Amplicon Sequence Variants (ASVs), 147 taxa were unique to *Ascaris*, 7 were exclusive to infected hosts, and 41 were found only in non-infected hosts. Interestingly, 8 ASVs were exclusively shared between *Ascaris* and infected hosts (Fig. 2d). These 8 ASVs, identified at the genus level and characterized at species level individually using BLAST, included *Achromobacter xylosoxidans*, *Pseudomonas fluorescens* , *Blautia wexlerae*, *Blautia coccoides* and *Lachnospiraceae* NK4A136 (Fig 2 c,d). These bacteria may play a significant role in the infection process and could have unique behaviors that facilitate colonization, life cycle progression, and infection, representing a notable aspect of the parasite-host-microbiota interaction.

3.5. ***Differential Abundance Analysis: Revealing Microbial Profiles Across Ascaris and its host***

To identify microbiota members exhibiting differential abundance across sample groups—*Ascaris* larvae, Feces, Intestine, Liver, and Lung—we employed a linear discriminant analysis effect size

(LEFSe) and DEseq2. Our analysis unveiled distinct taxa: in *Ascaris*, the genera *Bradyrhizobium*, *Achromobacter*, and *Pseudomonas* exhibited notable differential abundance. Conversely, the genus *Delftia* and the family Comamonadaceae displayed distinct differential abundance in the liver. Likewise, the genus *Chryseobacterium* emerged as a differentially abundant taxon in the lung. The intestine exhibited differential abundance in the Lactobacillaceae family. Finally, differentially abundant taxa in feces included *Muribaculaceae*, *Lachnospiraceae NK4A136 group*, and the family Oscillospiraceae (Fig 3a, Fig S3a). We extended this approach to discern taxa exhibiting differential abundance between infected and non-infected hosts and *Ascaris* larvae. We found that in infected hosts, the genera *Alistipes*, *Eisenbergiella*, and *Faecalibacterium* were abundant, while the genus *Delftia* was characteristic of non-infected hosts. In the case of *Ascaris*, consistent with previous findings, the genera *Achromobacter*, *Pseudomonas*, *Sphingomonas*, *Bradyrhizobium*, *Rhodococcus*, and *Blautia* were differentially abundant. (Fig. 3c,d, S3b-d).

When scrutinizing the pattern of differentially abundant taxa across sample groups, notable similarities appeared particularly between the liver and lung, delineating a distinct cluster characteristic of these organs, predominantly composed of Proteobacteria, notably genus *Delftia* and *Chryseobacterium*. This shared taxonomic composition partially extended to the intestine, where taxa associated with Firmicutes and Bacteroidota were also prominent, reflective of typical intestinal microbial profiles. Likewise, this intestinal pattern shared similarities with the differentially abundant taxa observed in fecal samples, where taxa such as *Lachnospiraceae NK4A136* and *Akkermansia* were notably abundant. Conversely, *Ascaris* larvae displayed a unique profile of differentially abundant taxa, exhibiting partial overlap with the host, particularly in terms of Proteobacteria. Additionally, as previously noted, representatives of Firmicutes and Bacteroidota were also discernible in the *Ascaris* larvae groups (Fig 3b).

4. Discussion

To date, parasite-host-microbiota interactions focus on how the parasite influences the microbiota within the host organism [1,51–54]. Various methodological approaches have been employed to identify host microbiota changes induced by *Ascaris, specifically adult worms in the intestines,* often yielding contradictory results [10,55–57]. It is well-established that *Ascaris* can modulate the immune response in the intestines allowing for survival of up to 2 years within the host intestines [58]. However, there is limited information about the *Ascaris larvae*-associated microbiota and its potential relationship with the host, particularly during the essential larval migratory cycle [37]. This relationship could be crucial for understanding the infection's success and the parasite's biological cycle within the host [40,59,60]. Given this context, our study focused on characterizing the *Ascaris*-associated microbiota at different developmental stages and examining its impact on the host's microbiota during the larval migration phase through the intestine, liver, and lung in a murine model.

Regarding the potential microbiota changes produced in the host, we did not find alpha diversity differences between infected and non-infected mice with any tissue group (Fig 1c-g). Previous studies have shown discordant results regarding the impact of *Ascaris* on the structure and composition of the host microbiota in the intestines. For example, Wang et al. (2019) and Midha (2022) demonstrated that infection with *Ascaris* significantly reduced the alpha diversity of the intestinal microbiota in pigs, and this effect was independent of the parasite burden [13,37]. Conversely, studies like those conducted by Stracke et al. (2021) have shown that *Ascaris* infection does not affect alpha diversity values [61]. These discrepancies likely arise from differences in experimental models, approaches, and other methodological factors such as sample size, which can introduce variability. For instance, the differences in host species (pigs vs. mice), the nature of the microbiota in these hosts, and the specifics of experimental conditions (e.g., diet, housing conditions, infection load) could all contribute to the observed variations in results [27,29,30]. Moreover, the stages of infection and the timing of sample collection can significantly influence microbiota diversity. Addressing these variations necessitates longitudinal studies and suitable animal models. Developing robust animal models that can be followed over time will allow for more standardized results that could be extrapolated to other hosts, such as humans.

Our study found higher alpha diversity in *Ascaris* larvae-associated microbiota compared to the host tissues (intestine, liver, lung) (Fig. S1), suggesting a diverse microbial community within the larvae. Diversity within *Ascaris* the *Ascaris* larvae increased during the larval migration cycle, indicating progressive microbiota accumulation across different host tissues. This aligns with lower diversity observed in vitro, recently hatched *Ascaris* larvae compared to more mature developmental staged larvae in the liver and lung. While helminth microbiota may originate through vertical transmission, horizontal acquisition from the environment, both outside and inside of the host, is likely [64]. Our findings suggest that specific bacterial populations are acquired during *Ascaris* larval migration through the host likely via horizontal acquisition. Further research is needed to explore these mechanisms and determine the integration of acquired bacteria into the *Ascaris* larvae, and ultimately the adult worm, microbiota.

The higher diversity observed in *Ascaris*-larvae associated microbiota compared to host tissues could have several implications (Fig 1 b-e, Fig S1). A more diverse microbial community within the parasite may offer several advantages, including increased resilience to environmental changes, a heightened ability to exploit host resources, and the production of metabolites crucial for immune evasion in the host. This diverse microbiota might also modulate the host immune response, aiding the parasite in evading immune detection and establishing chronic infection in the intestines following the larval migration cycle [37,40]. Confirming these findings requires designing studies to unravel helminths' microbiota acquisition and transmission mechanisms.

Beta diversity analysis revealed that the microbiota composition of *Ascaris* is markedly different from that of the host (Fig 2a, S2b). Recent studies have corroborated these findings, indicating that helminths possess a distinct microbiota compared to their hosts, sharing only a limited number of taxa. For instance, a recent study demonstrated that the gut microbial structure of adult *A.*

lumbricoides significantly differs from that of the human hosts, exhibiting clear separation in beta diversity analysis [62]. Similarly, in a pig model, adult *Ascaris*-microbiota composition across various intestinal tract segments detected both shared and differential bacteria between the *Ascaris* microbiome and host microbiome [37]. These findings underscore that *Ascaris* harbors its distinct microbiota and influences the microbial ecosystem within its host and vice versa. The unique microbiota within *Ascaris* may facilitate the parasite's survival and adaptation by providing essential functions not available from the host's microbiota alone [37,60,62].

The unique microbial communities within *Ascaris* larvae likely play a role in modulating the host's immune responses, facilitating the establishment and persistence of the parasite. . We performed taxonomic characterization of Ascaris stages and host tissues to elucidate unique microbial communities, yielding insightful findings. *Ascaris* microbiota was predominantly Proteobacteria, followed by Firmicutes and Bacteroidota, consistent with studies on *C. elegans* showing Gamma-proteobacteria and Bacteroidetes dominance [63,64]. Proteobacteria's prominence highlights its metabolic versatility and immunomodulatory potential, crucial for nutrient acquisition and energy metabolism in *Ascaris* within the host [24,63,64]. Lung and liver microbiota exhibited similar phylum-level compositions, rich in Proteobacteria and Bacteroidota, suggesting comparable niches for bacterial communities. In contrast, the intestine contained diverse Proteobacteria, Firmicutes, and Bacteroidota, while feces were mainly Firmicutes, Bacteroidota, and Verrucomicrobia. These unique compositions underscore *Ascaris*' distinct bacterial environment and emphasize the need for organ-level studies to understand parasite-host-microbiota interactions fully. Further studies on host organ level and *Ascaris* developmental stage microbiota are critical to understanding parasite-host-microbiota interactions and could inform the development of novel strategies to prevent helminth infections.

At the genus level, we observed significant changes during *Ascaris* larval migratory cycle. *In vitro*, the most abundant genera in *Ascaris* larvae were *Delftia*, *Sphingomonas*, *Achromobacter*, *Chryseobacterium*, and *Pseudomonas*, all of which belong to the Proteobacteria phylum. The initial microbial community in the early-stage larvae may be derived from vertical acquisition or the external environment [60]. However, larvae isolated from the liver at 4 DPI and larvae isolated from the lung at 8 DPI, had increased relative abundance of other taxa, such as *Bradyrhizobium*, *Lactobacillus*, and the *Lachnospiraceae NK4A136 group*, with the latter two likely derived from the host [60]. This shift in *Ascaris* microbiota after infection aligns with models such as *Heligmosomoides polygyrus*, where worm-associated microbes were similar to those in the infected host ileum and dominated by Lactobacillaceae, while infective larvae-associated microbes were unique and dominated by Pseudomonadaceae. This supports likely horizontal bacterial acquisition by *Ascaris* from the host [15,60,65,66], incorporating and modifying their microbiota to gain advantageous microbial populations. Studies that elucidate whether the acquired bacteria are transient or integrated into *Ascaris*' own microbiota longitudinally are essential.

The presence of genera such as *Delftia* and *Chryseobacterium* within host tissues, particularly the liver and lung, underscores their significant roles in modulating host immune responses and

shaping tissue microenvironments. These findings align with previous studies characterizing the lung microbiota in mice, where the *Delftia* genus was identified as one of the most prevalent taxa in this model [67]. Moreover, these bacteria are known for interacting with immune cells and fine-tuning inflammatory processes, which may contribute to parasite persistence and pathogenesis [68]. However, further research is warranted to elucidate the functional implications of these microbiota changes and their specific roles in *Ascaris* biology and host health. Understanding the mechanisms by which these bacteria influence host-parasite interactions could provide valuable insights into novel therapeutic strategies for combating parasitic infections.

A core microbiota analysis unveiled eight ASVs shared between *Ascaris* larvae and infected host tissues. Four of these ASVs were identified at the genus level, and further characterized at the species level using BLAST: *Achromobacter xylosoxidans*, *Pseudomonas fluorescens*, *Blautia wexlerae*, *Blautia coccoides*, and *Lachnospiraceae NK4A136*. These common taxa might be essential in *Ascaris* pathogenicity, required for promoting infection, supporting the organism during the essential but metabolically taxing larval migratory cycle, and facilitating parasite establishment and survival [37,60]. The potential transfer of microbiota from *Ascaris* to the host during migration, particularly genera like *Lactobacillus* and *Lachnospiraceae*, raises intriguing questions about their impact on parasite and host cross-talk. These bacteria could influence gut barrier function, nutrient absorption, or host susceptibility to other pathogens [69–71]. *Pseudomonas* specifically has been demonstrated in *C. elegans* models to have the ability to protect the worm against infection with *B. thuringiensis* through production of massetolide E and limiting worm-induced damage in the host [63]. *Pseudomonas*, *Achromobacter*, and *Blautia* can collectively create an anti-inflammatory environment that contributes to immunomodulation. Additionally, *Blautia*'s production of metabolites, including those derived from tryptophan, may play a pivotal role in the pathogenicity of *Ascaris* [63,72]. These findings propose novel insights into the function of the native and acquired microbiota in helminths, highlighting the role of microbes in supporting the worms' development and survival in the host. Shared ASVs suggest that certain bacterial taxa may be integral to the parasite’s life cycle and pathogenicity. These findings underscore the need for further research to elucidate the specific mechanisms by which these bacteria influence both the parasite and the host. Moreover, they represent a crucial step in identifying potential taxa necessary for the larval migratory cycle and long-term survival of the worms in the host intestines, thereby paving the way for developing microbiota-modulation strategies in helminth infections.

The results of the differential abundance analysis revealed distinct taxonomic patterns across different sample groups, shedding light on the microbial dynamics within the *Ascaris*-infected host. Specific genera such as *Bradyrhizobium*, *Achromobacter*, and *Pseudomonas* exhibited notable differential abundance in *Ascaris* larvae. Notably, distinct clusters of taxa, predominantly Proteobacteria like *Delftia* and *Chryseobacterium*, were observed in the host organs, particularly the liver and lung, delineating organ-specific microbial profiles. Interestingly, similarities were also observed between the intestine and fecal samples, indicating shared taxa associated with

Firmicutes and Bacteroidota, typical of intestinal microbial communities [73]. However, the helminth exhibited a unique taxonomic profile, with partial overlap with the host microbiota, especially in terms of Proteobacteria, and representatives of Firmicutes and Bacteroidota. These findings underscore the complex interplay between *Ascaris* and the host microbiota, highlighting organ-specific microbial signatures and potential interactions that warrant further investigation. Moreover, the lack of significant differences in abundance patterns between infected and non-infected hosts and *Ascaris* suggests that the presence of the parasite may not significantly alter the overall microbial composition in the host during larval migration.

This study lays the groundwork for further exploration of *Ascaris*–host-microbiota interactions that could provide significant insight into the pathogenicity of *Ascaris*. However, this study has several limitations. First, 16S rRNA-amplicon-based sequencing was used in this study. Nevertheless, whole-genome sequencing could provide more robust taxonomic resolution and functional characterization of the microbiota. This study used a well-established mouse model; nonetheless, mice are not native hosts of ascariasis, which may reduce the applicability of these findings. Alternatively, the porcine model is a native host of ascariasis and has distinct similarities to humans, making it the optimal host for confirming our findings. This study was conducted for 14 days and highlights changes in microbiota during the acute period. However, longitudinal studies are required to gain a more in-depth analysis of the long-term consequences of the *Ascaris*-host-microbiota interaction. Lastly, supporting the microbiome analysis with metagenomics, transcriptomics, and metabolomics will offer a comprehensive view of the *Ascaris*–host-microbiota interaction to inform helminth control strategies.

5. Conclusions

In summary, this study characterized the microbiota of *Ascaris* larvae at various developmental stages in the context of host tissues during the larval migration cycle. The outcomes of this study revealed significant differences in microbiota composition and diversity amongst groups. The prevalence of Proteobacteria in the *Ascaris* microbiota emphasizes its pivotal role in parasite biology, while the varied microbiota profiles across host tissues underscore the intricate interplay between host and parasite-associated microbial communities. This research also included an evaluation of *Ascaris* larvae microbiota and its changes during the larval life cycle, as well as an evaluation of the host microbiota in extra-intestinal organs and how they change over time. These findings allow further exploration into helminth-microbiota interactions' ecological and functional dynamics. Future research should unravel the functional roles of these bacterial communities within *Ascaris*, as well as elucidating the mechanisms involved in the horizontal and vertical acquisition of certain bacterial populations.

Data availability

Sequence data supporting this article's conclusions are available in the SRA database under BioProject PRJNA1117354. The code for generating the figures and the analysis is available at https://github.com/scastanedag/Ascaris_Microbiome.git.

Declaration of Competing Interest

The authors declare that no competing interests exist.

Authors' contributions

SC, JW and JDR conceived the study; CP, CSR, YW, NH performed the experiments; SC and LHP analyzed the data. SC, JW and JDR drafted the manuscript. All authors approved the final version of the manuscript.

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Ethics approval

All animal experiments were carried out following the guidelines outlined in the 8th edition of the Guide for the Care and Use of Laboratory Animals under the approval protocol by Baylor College of Medicine's Institutional Animal Care and Use Committee (IACUC), AN-9173.

Figure Legends

Figure 1. (a) Proportion of samples by total reads to show abundance by phylum. Main phyla are highlighted with dashed red lines. **(b)** Diversity metrics—richness, Shannon, and Simpson indices—were observed by sample groups (*Ascaris*, feces, intestine, liver, lung). **(c)** Boxplot of the

Shannon index comparing *Ascaris* diversity against infected and non-infected host feces, **(d)** intestine, **(e)** liver, and **(f)** lung. **(g)** Shannon index comparison between *Ascaris* larval stages evaluated. Statistical analyses were performed using the Kruskal-Wallis (KW) test and a multiple comparison post hoc Mann-Whitney-Wilcoxon (MWW) test. Plotted are interquartile ranges (IQRs; boxes), medians (lines in the boxes), and the lowest and highest values within 1.5 times IQR from the first and third quartiles (whiskers above and below the boxes). * p-values <0.05, ** p-values <0.01, ns: not significant

Figure 2. (a) Principal coordinates analysis (PCoA) ordination of Bray-Curtis distances of the microbial communities by sample groups. Permutational analysis of variance (PERMANOVA) test for multivariate effect was done using the adonis function from the vegan package. *** p-values <0.001. **(b)** Bar chart of the relative abundance of top taxa at the phylum level between sample groups. **(c)** Bar chart of the relative abundance of top taxa at the genus level between samples by day of infection (DPI: Days post-infection). **(d)** Venn diagram of core microbiota in *Ascaris* and infected and non-infected hosts. The arrows from panel (c) to panel (d) include ASVs characterized at the species level that are part of shared taxa between *Ascaris* and infected mice. *Blautia* is also part of these ASVs, although it is not included in the top genus taxa in the bar plot.

Figure 3. Linear discriminant analysis (LDA) effect size (LEfSe) analysis is used to identify taxa enriched in different sample groups. **(a)** Bar plot of taxa enriched at various taxonomic levels. **(b)** Heatmap of differentially abundant taxa across sample groups to evaluate distribution and patterns. The threshold for the logarithmic LDA score was 4.0, and the maximum Kruskal-Wallis (KW) value was 0.01. Volcano plots of differentially abundant taxa across sample groups to evaluate distribution and patterns concerning *Ascaris* and infection status based on DESeq2 analysis, comparing (a) host infected mice and *Ascaris*, and (b) host non-infected mice and *Ascaris*.

Supplementary Material

Table S1. Summary of the preprocessing of sequencing data, which included quality assessment, trimming, and other steps performed using DADA2.

Figure S1. (a) Diversity Shannon index by sample day of infection and larvae stage in liver **(b)** lung **(c)** intestine, **(d)** and feces. Plotted are interquartile ranges (IQRs; boxes), medians (lines in the boxes), and the lowest and highest values within 1.5 times IQR from the first and third quartiles (whiskers above and below the boxes).

Figure S2. (a). The rarefaction curve of the samples was evaluated. The dotted line shows the approximate number of reads at which the sequences increase and then plateau. **(b)** Principal coordinates analysis (PCoA) ordination of Bray-Curtis distances of the microbial communities by infection status. Permutational analysis of variance (PERMANOVA) test for multivariate effect was done using the adonis function from the vegan package. ** p-values <0.003. **(c)** Bar chart of

the relative abundance of top taxa at the class and **(d)** family level between samples by day of infection (DPI: Days post infection).

Figure S3. Linear discriminant analysis (LDA) effect size (LEfSe) analysis is used to identify taxa enriched in different sample groups. **(a)** Cladogram of taxa enriched at various taxonomic levels. **(b)** Bar plot of taxa enriched at various taxonomic levels regarding *Ascaris* and infection status. **(c)** Heatmap of differentially abundant taxa across sample groups to evaluate distribution and patterns concerning *Ascaris* and infection status. (e) Boxplot of differentially abundant taxa across sample groups to evaluate abundance involving *Ascaris* and infection status. The threshold for the logarithmic LDA score was 4.0, and the maximum Kruskal-Wallis (KW) value was 0.01.

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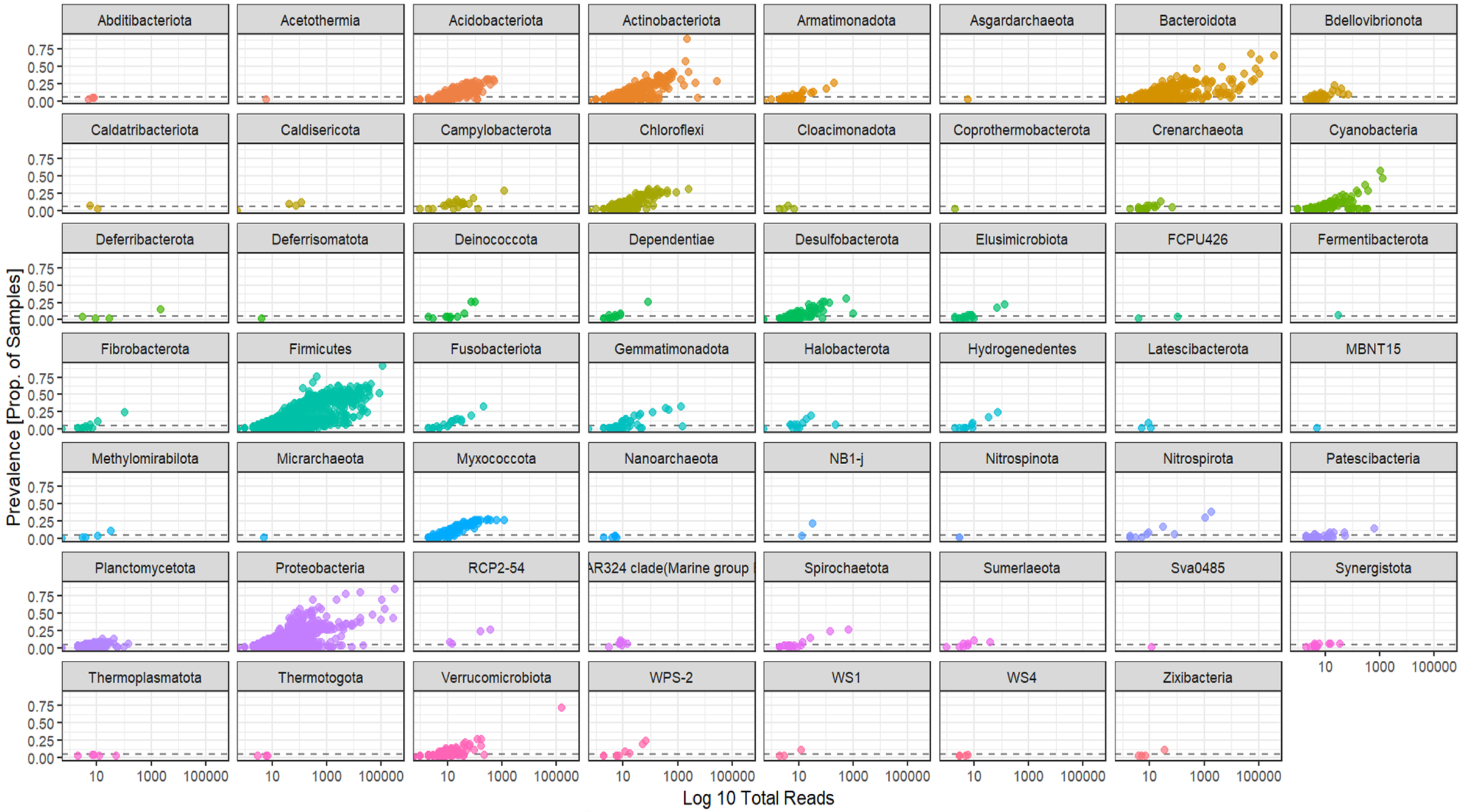
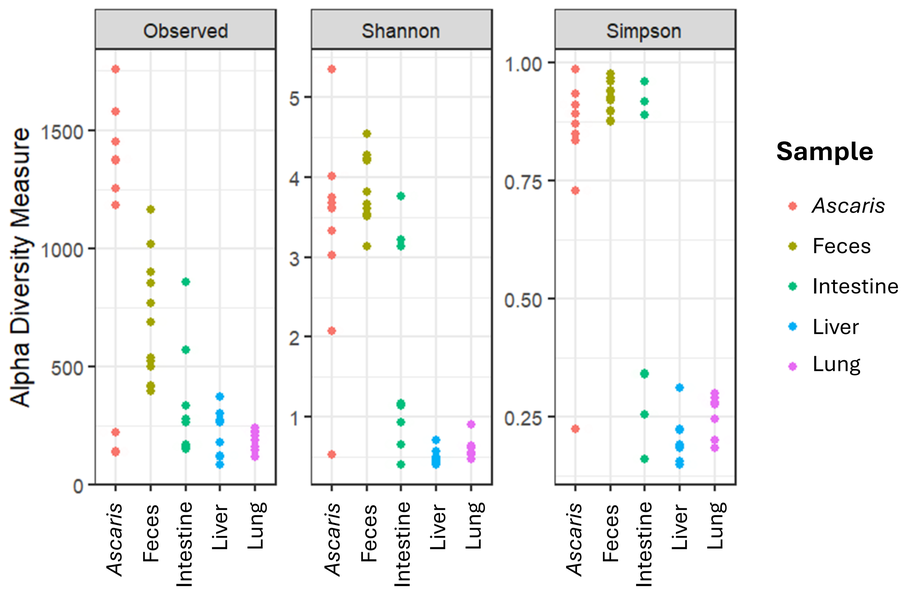
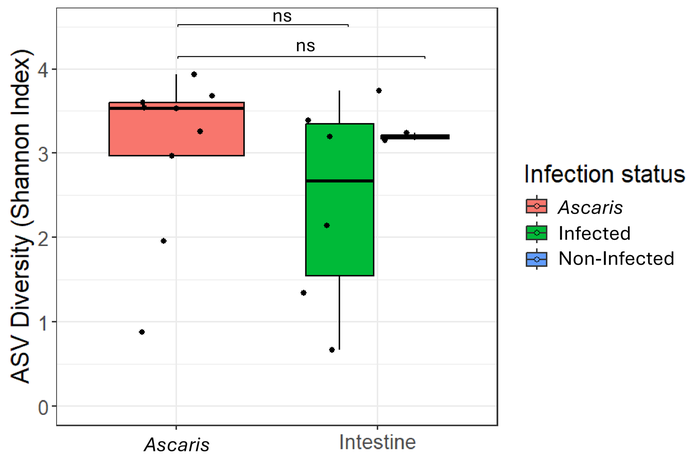
a.


Abditibacteriota
Acetothermia
Acidobacteriota
Actinobacteriota
Armatimonadota
Asgardarchaeota
Bacteroidota
Bdellovibrionota
Caldatribacteriota
Caldisericota
Campylobacterota
Chloroflexi
Cloacimonadota
Coprothermobacterota
Crenarchaeota
Cyanobacteria
Deferribacterota
Deferrisomatota
Deinococcota
Dependentiae
Desulfobacterota
Elusimicrobiota
FCPU426
Fermentibacterota
Fibrobacterota
Firmicutes
Fusobacteriota
Gemmatimonadota
Halobacterota
Hydrogenedentes
Latescibacterota
MBNT15
Methylomirabilota
Micrarchaeota
Myxococcota
Nanoarchaeota
NB1-j
Nitrospinota
Nitrospirota
Patescibacteria
Planctomycetota
Proteobacteria
RCP2-54
AR324 clade(Marine group
Spirochaetota
Sumerlaeota
Sva0485
Synergistota
Thermoplasmatota
Thermotogota
Verrucomicrobiota
WPS-2
WS1
WS4
Zixibacteria
Prevalence [Prop. of Samples]
0.00
0.25
0.50
0.75
10
1000
100000
Log 10 Total Reads

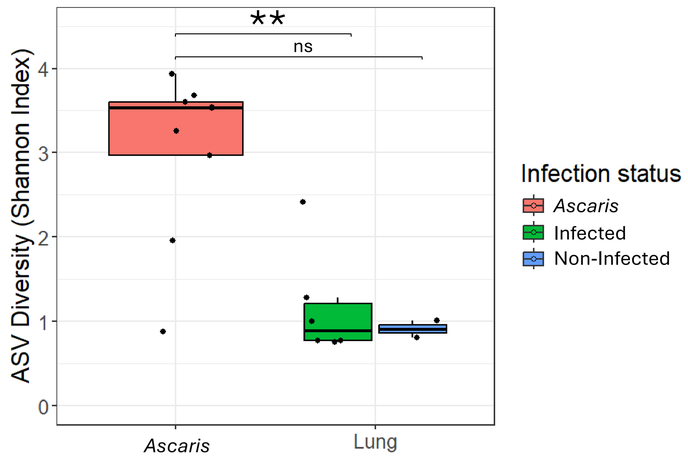
b.


Observed
Shannon
Simpson
Alpha Diversity Measure
Ascaris
Feces
Intestine
Liver
Lung
Sample

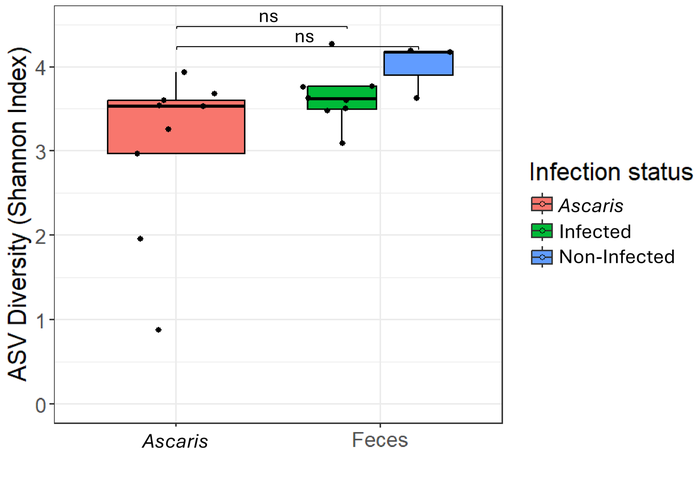
d.


ns
ns
ASV Diversity (Shannon Index)
Ascaris
Intestine
Infection status
Ascaris
Infected
Non-Infected

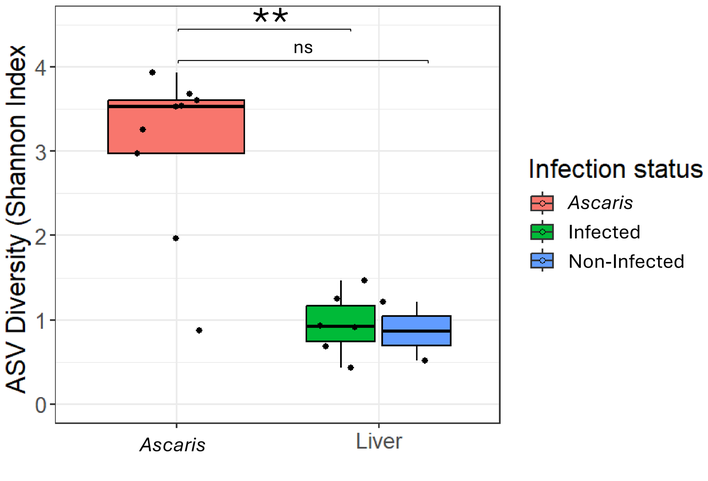
f.


**
ns
ASV Diversity (Shannon Index)
Ascaris
Lung
Infection status
Ascaris
Infected
Non-Infected

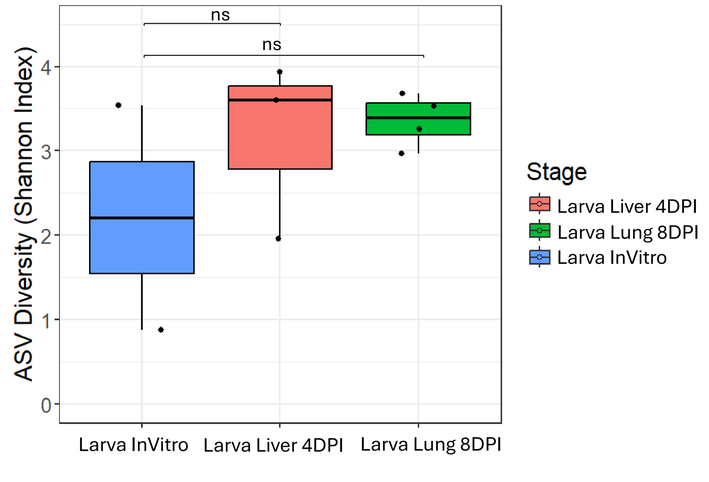
c.

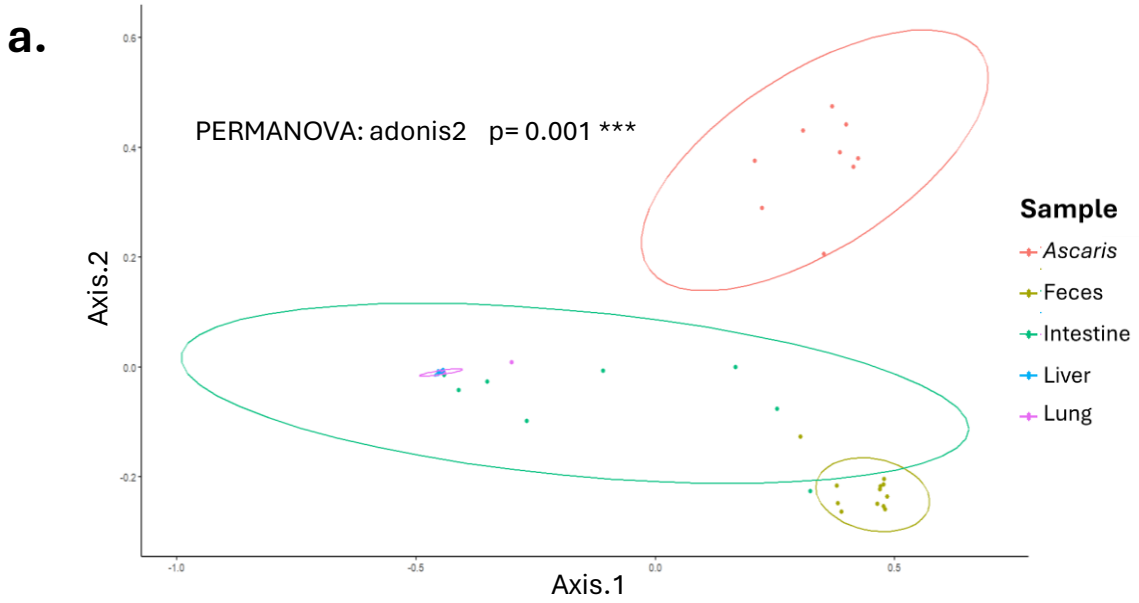

ns
ns
ASV Diversity (Shannon Index)
Ascaris
Feces
Infection status
Ascaris
Infected
Non-Infected

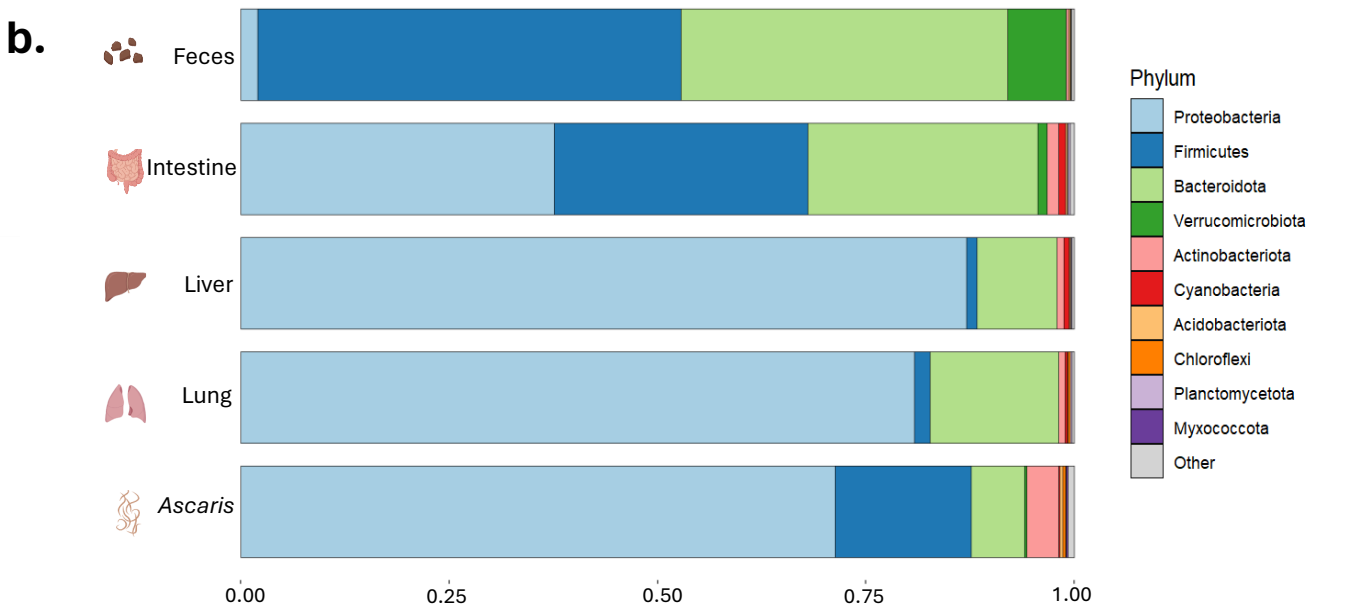
e.

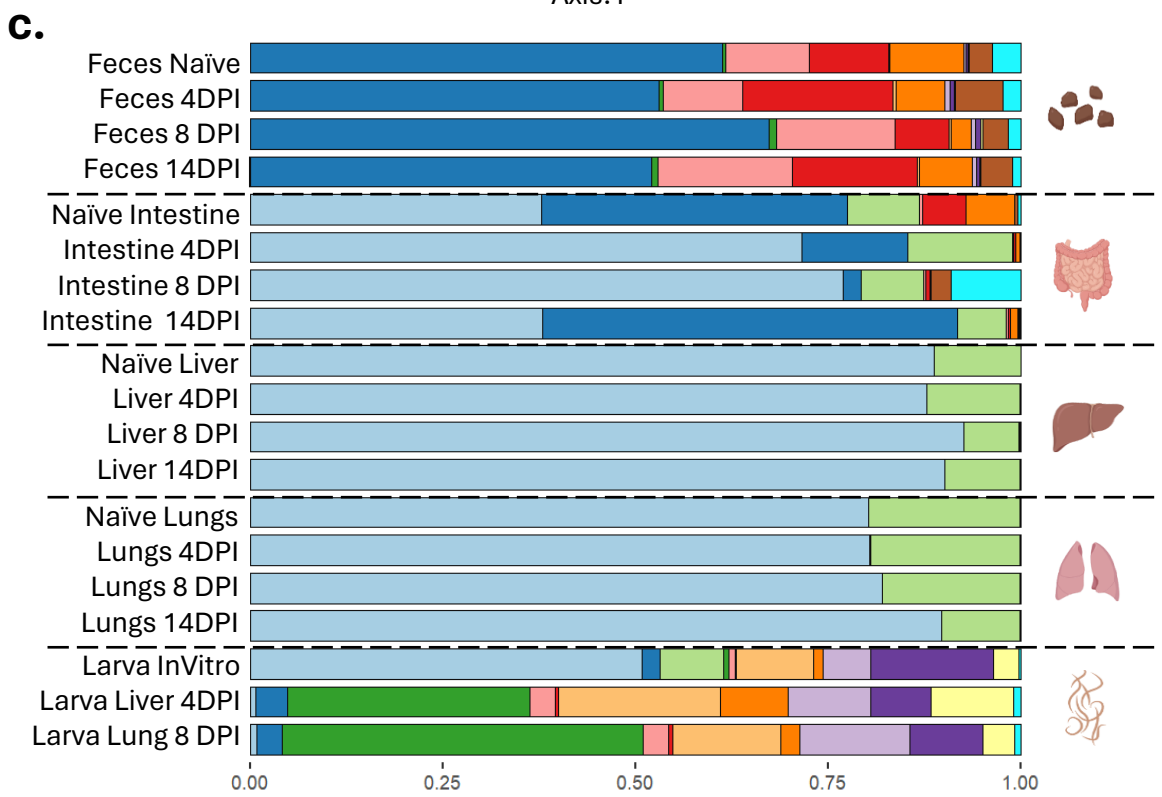

**
ns
ASV Diversity (Shannon Index
Ascaris
Liver
Infection status
Ascaris
Infected
Non-Infected

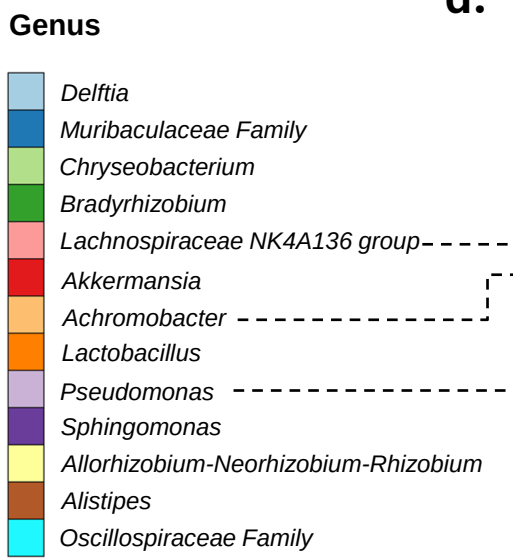
g.

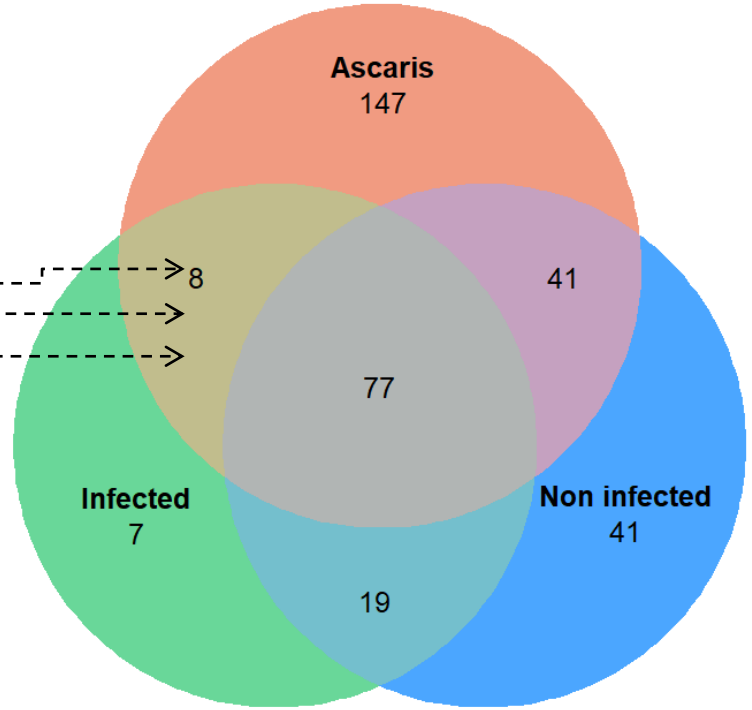

ns
ns
ASV Diversity (Shannon Index)
Larva InVitro
Larva Liver 4DPI
Larva Lung 8DPI
Stage
Larva Liver 4DPI
Larva Lung 8DPI
Larva InVitro

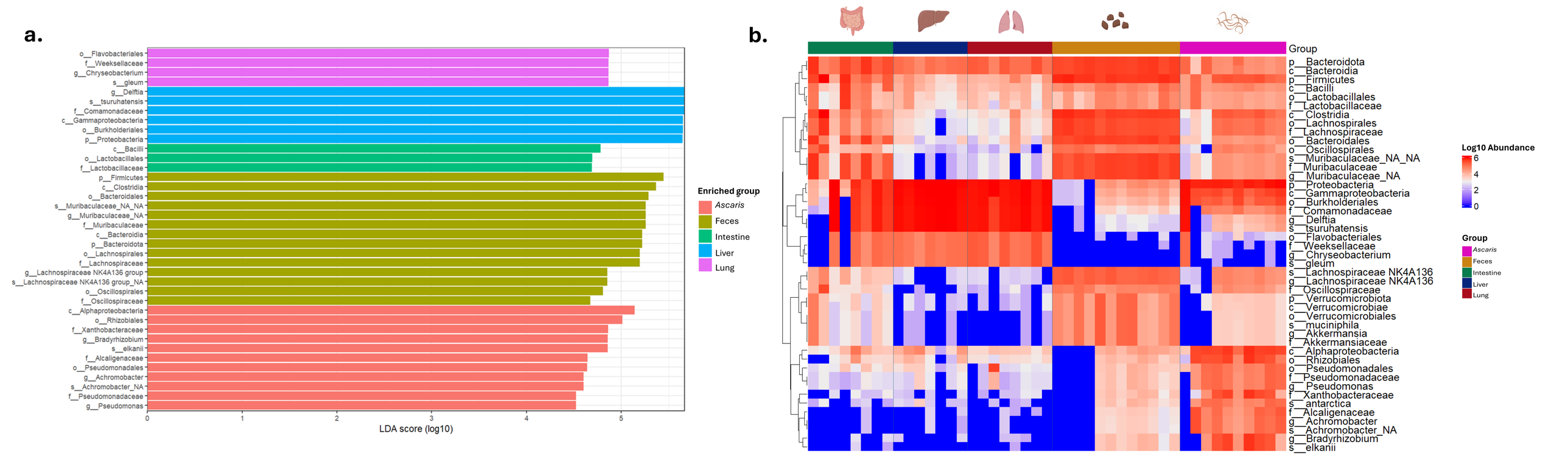

a.
PERMANOVA: adonis2 p= 0.001 ***
Axis.2
Axis.1
Sample
Ascaris
Feces
Intestine
Liver
Lung

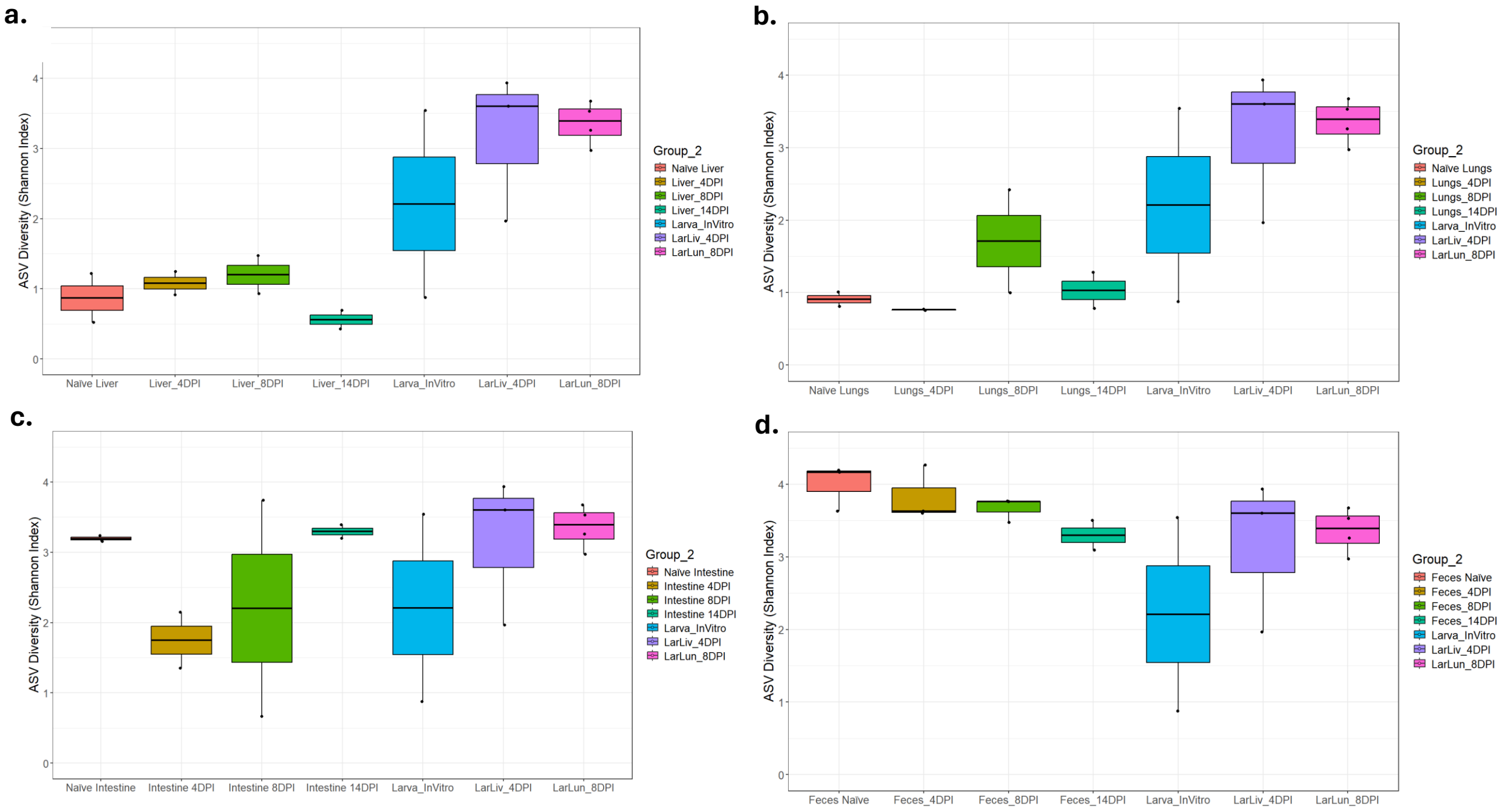

b.
Feces
Intestine
Liver
Lung
Ascaris
0.00
0.25
0.50
0.75
1.00
Phylum
Proteobacteria
Firmicutes
Bacteroidota
Verrucomicrobiota
Actinobacteriota
Cyanobacteria
Acidobacteriota
Chloroflexi
Planctomycetota
Myxococcota
Other

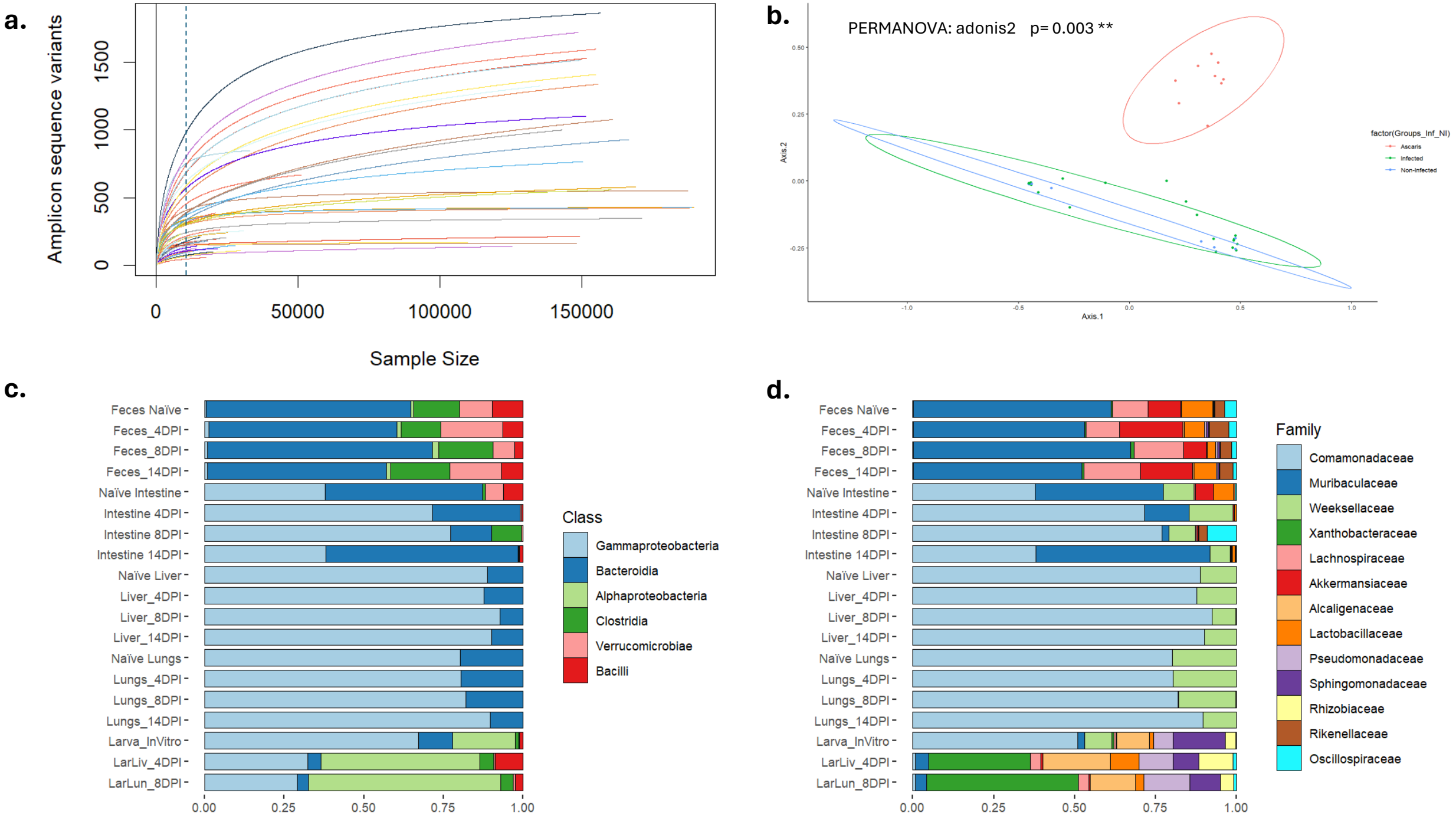

c.
Feces Naïve
Feces 4DPI
Feces 8 DPI
Feces 14DPI
Naïve Intestine
Intestine 4DPI
Intestine 8 DPI
Intestine 14DPI
Naïve Liver
Liver 4DPI
Liver 8 DPI
Liver 14DPI
Naïve Lungs
Lungs 4DPI
Lungs 8 DPI
Lungs 14DPI
Larva InVitro
Larva Liver 4DPI
Larva Lung 8 DPI
0.00
0.25
0.50
0.75
1.00

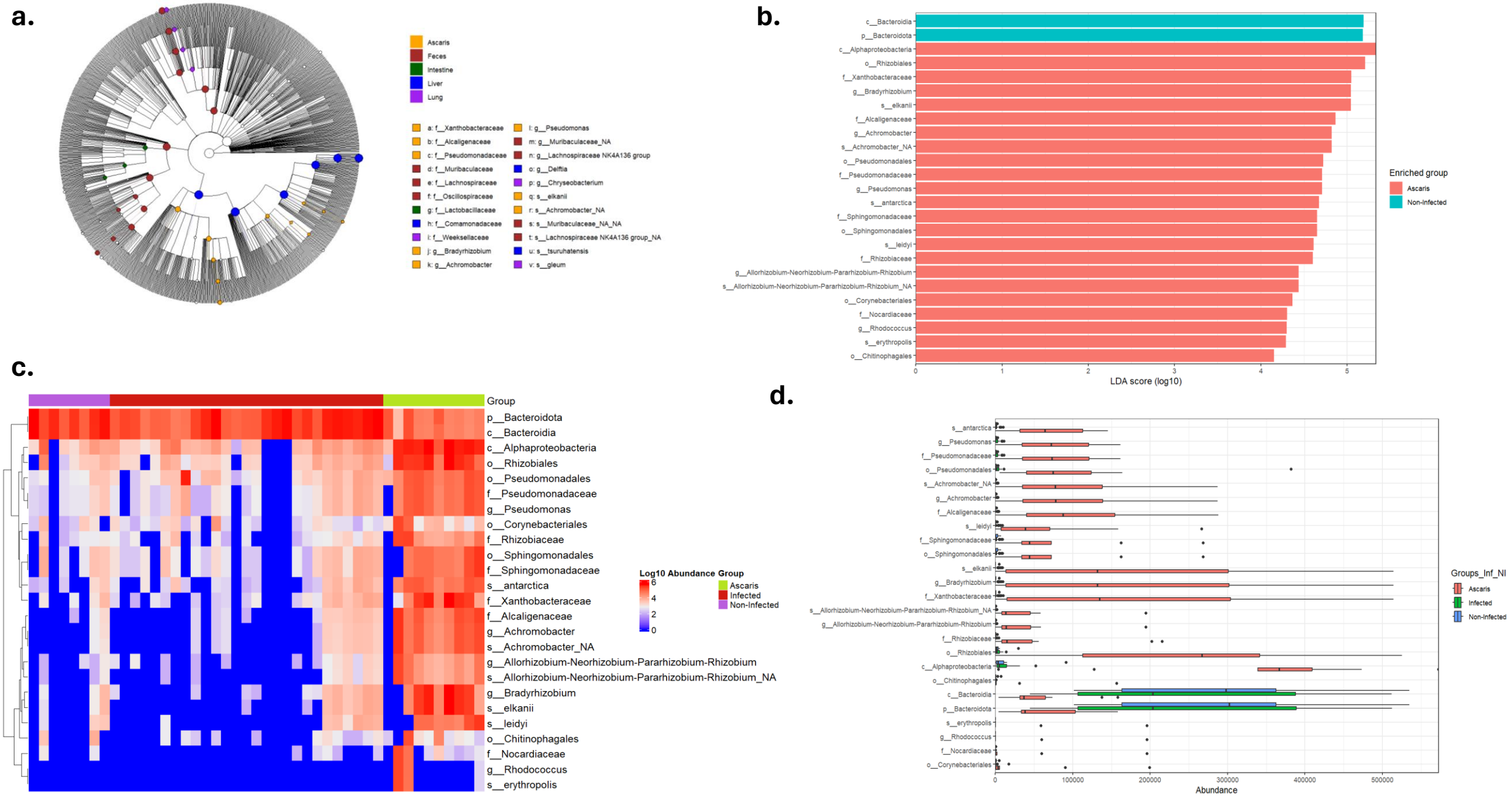

Genus
Delftia
Muribaculaceae Family
Chryseobacterium
Bradyrhizobium
Lachnospiraceae NK4A136 group
Akkermansia
Achromobacter
Lactobacillus
Pseudomonas
Sphingomonas
Allorhizobium-Neorhizobium-Rhizobium
Alistipes
Oscillospiraceae Family


d.
Ascaris
147
8
41
77
Infected
7
Non infected
41
19


a.
o__Flavobacteriales
f__Weeksellaceae
g__Chryseobacterium
s__gleum
g__Delftia
s__tsuruhatensis
f__Comamonadaceae
c__Gammaproteobacteria
o__Burkholderiales
p__Proteobacteria
c__Bacilli
o__Lactobacillales
f__Lactobacillaceae
p__Firmicutes
c__Clostridia
o__Bacteroidales
s__Muribaculaceae_NA_NA
g__Muribaculaceae_NA
f__Muribaculaceae
c__Bacteroidia
p__Bacteroidota
o__Lachnospirales
f__Lachnospiraceae
g__Lachnospiraceae NK4A136 group
s__Lachnospiraceae NK4A136 group_NA
o__Oscillospirales
f__Oscillospiraceae
c__Alphaproteobacteria
o__Rhizobiales
f__Xanthobacteraceae
g__Bradyrhizobium
s__elkanii
f__Alcaligenaceae
o__Pseudomonadales
g__Achromobacter
s__Achromobacter_NA
f__Pseudomonadaceae
g__Pseudomonas
0
1
2
3
4
5
LDA score (log10)
Enriched group
Ascaris
Feces
Intestine
Liver
Lung
b.
Group
p__Bacteroidota
c__Bacteroidia
p__Firmicutes
c__Bacilli
o__Lactobacillales
f__Lactobacillaceae
c__Clostridia
o__Lachnospirales
f__Lachnospiraceae
o__Bacteroidales
o__Oscillospirales
s__Muribaculaceae_NA_NA
f__Muribaculaceae
g__Muribaculaceae_NA
p__Proteobacteria
c__Gammaproteobacteria
o__Burkholderiales
f__Comamonadaceae
g__Delftia
s__tsuruhatensis
o__Flavobacteriales
f__Weeksellaceae
g__Chryseobacterium
s__gleum
s__Lachnospiraceae NK4A136
g__Lachnospiraceae NK4A136
f__Oscillospiraceae
p__Verrucomicrobiota
c__Verrucomicrobiae
o__Verrucomicrobiales
s__muciniphila
g__Akkermansia
f__Akkermansiaceae
c__Alphaproteobacteria
o__Rhizobiales
o__Pseudomonadales
f__Pseudomonadaceae
g__Pseudomonas
f__Xanthobacteraceae
s__antarctica
f__Alcaligenaceae
g__Achromobacter
s__Achromobacter_NA
g__Bradyrhizobium
s__elkanii
Log10 Abundance
6
4
2
0
Group
Ascaris
Feces
Intestine
Liver
Lung


a.
b.
c.
d.
ASV Diversity (Shannon Index)
Group_2
Naïve Liver
Liver_4DPI
Liver_8DPI
Liver_14DPI
Larva_InVitro
LarLiv_4DPI
LarLun_8DPI
Naïve Lungs
Lungs_4DPI
Lungs_8DPI
Lungs_14DPI
Naïve Intestine
Intestine 4DPI
Intestine 8DPI
Intestine 14DPI
Feces Naïve
Feces_4DPI
Feces_8DPI
Feces_14DPI


a.
Amplicon sequence variants
0
500
1000
1500
0
50000
100000
150000
Sample Size
b.
PERMANOVA: adonis2 p= 0.003 **
Axis.2
Axis.1
factor(Groups_Inf_NI)
Ascaris
Infected
Non-Infected
c.
Feces Naïve
Feces_4DPI
Feces_8DPI
Feces_14DPI
Naïve Intestine
Intestine 4DPI
Intestine 8DPI
Intestine 14DPI
Naïve Liver
Liver_4DPI
Liver_8DPI
Liver_14DPI
Naïve Lungs
Lungs_4DPI
Lungs_8DPI
Lungs_14DPI
Larva_InVitro
LarLiv_4DPI
LarLun_8DPI
0.00
0.25
0.50
0.75
1.00
Class
Gammaproteobacteria
Bacteroidia
Alphaproteobacteria
Clostridia
Verrucomicrobiae
Bacilli
d.
Feces Naïve
Feces_4DPI
Feces_8DPI
Feces_14DPI
Naïve Intestine
Intestine 4DPI
Intestine 8DPI
Intestine 14DPI
Naïve Liver
Liver_4DPI
Liver_8DPI
Liver_14DPI
Naïve Lungs
Lungs_4DPI
Lungs_8DPI
Lungs_14DPI
Larva_InVitro
LarLiv_4DPI
LarLun_8DPI
0.00
0.25
0.50
0.75
1.00
Family
Comamonadaceae
Muribaculaceae
Weeksellaceae
Xanthobacteraceae
Lachnospiraceae
Akkermansiaceae
Alcaligenaceae
Lactobacillaceae
Pseudomonadaceae
Sphingomonadaceae
Rhizobiaceae
Rikenellaceae
Oscillospiraceae


a.
Ascaris
Feces
Intestine
Liver
Lung
a: f__Xanthobacteraceae
b: f__Alcaligenaceae
c: f__Pseudomonadaceae
d: f__Muribaculaceae
e: f__Lachnospiraceae
f: f__Oscillospiraceae
g: f__Lactobacillaceae
h: f__Comamonadaceae
i: f__Weeksellaceae
j: g__Bradyrhizobium
k: g__Achromobacter
l: g__Pseudomonas
m: g__Muribaculaceae_NA
n: g__Lachnospiraceae NK4A136 group
o: g__Delftia
p: g__Chryseobacterium
q: s__elkanii
r: s__Achromobacter_NA
s: s__Muribaculaceae_NA_NA
t: s__Lachnospiraceae NK4A136 group_NA
u: s__tsuruhatensis
v: s__gleum
b.
c__Bacteroidia
p__Bacteroidota
c__Alphaproteobacteria
o__Rhizobiales
f__Xanthobacteraceae
g__Bradyrhizobium
s__elkanii
f__Alcaligenaceae
g__Achromobacter
s__Achromobacter_NA
o__Pseudomonadales
f__Pseudomonadaceae
g__Pseudomonas
s__antarctica
f__Sphingomonadaceae
o__Sphingomonadales
s__leidyi
f__Rhizobiaceae
g__Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium
s__Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium_NA
o__Corynebacteriales
f__Nocardiaceae
g__Rhodococcus
s__erythropolis
o__Chitinophagales
0
1
2
3
4
5
LDA score (log10)
Enriched group
Ascaris
Non-Infected
c.
Group
p__Bacteroidota
c__Bacteroidia
c__Alphaproteobacteria
o__Rhizobiales
o__Pseudomonadales
f__Pseudomonadaceae
g__Pseudomonas
o__Corynebacteriales
f__Rhizobiaceae
o__Sphingomonadales
f__Sphingomonadaceae
s__antarctica
f__Xanthobacteraceae
f__Alcaligenaceae
g__Achromobacter
s__Achromobacter_NA
g__Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium
s__Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium_NA
g__Bradyrhizobium
s__elkanii
s__leidyi
o__Chitinophagales
f__Nocardiaceae
g__Rhodococcus
s__erythropolis
Log10 Abundance
6
4
2
0
Group
Ascaris
Infected
Non-Infected
d.
s__antarctica
g__Pseudomonas
f__Pseudomonadaceae
o__Pseudomonadales
s__Achromobacter_NA
g__Achromobacter
f__Alcaligenaceae
s__leidyi
f__Sphingomonadaceae
o__Sphingomonadales
s__elkanii
g__Bradyrhizobium
f__Xanthobacteraceae
s__Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium_NA
g__Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium
f__Rhizobiaceae
o__Rhizobiales
c__Alphaproteobacteria
o__Chitinophagales
c__Bacteroidia
p__Bacteroidota
s__erythropolis
g__Rhodococcus
f__Nocardiaceae
o__Corynebacteriales
0
100000
200000
300000
400000
500000
Abundance
Groups_Inf_NI
Ascaris
Infected
Non-Infected

	input	filtered	denoisedF	denoisedR	merged	nonchim
B1	208401	176629	176358	176334	175237	172842
B10	162311	152782	152550	152569	151120	145113
B11	210233	198233	198046	198058	196453	181178
B12	103352	97557	97462	97461	96536	90756
B13	205338	154422	154315	154263	152925	151468
B14	205757	193886	193758	193772	192238	186185
B15	163505	153978	153591	153504	151874	147984
B16	204509	191614	191447	191463	190110	182380
B17	166512	157738	157437	157339	155598	150331
B18	120337	112905	112703	112696	111578	104144
B19	204196	117196	117022	117050	116365	114806
B2	203965	192333	191135	191202	187203	179359
B20	312938	294399	293417	293340	289095	278456
B21	141283	132785	132678	132647	131572	127715
B22	203826	191612	191524	191529	190110	184284
B23	197683	186507	186279	186276	184610	176793
B24	210850	198778	198622	198579	196973	189002
B25	205690	196739	196338	196138	194615	189293
B26	204456	195514	195141	195036	193562	188627
B27	204111	194806	194518	194503	193155	187876
B28	206656	197098	196465	196276	193936	187351
B29	206418	193268	193172	193165	191695	187969
B3	108050	101853	101627	101615	100490	96885
B30	208537	136502	136402	136310	135331	133997
B31	205680	195039	194818	194683	191813	189738
B32	164545	155310	155234	155238	154193	147759
B4	193912	183213	183128	183120	181795	172704
B5	218772	205259	205138	205138	203424	190365
B6	156937	147951	147692	147749	146270	142484
B7	189508	178298	178082	178052	176340	169869
B8	188411	177194	176890	176834	175183	166262
B9	206231	193665	193098	193054	190718	183181
Feces_1	203331	194285	193305	192949	187844	166657
Feces_1	203749	194767	192630	192059	181061	143646
Feces_4	216809	205942	204633	204161	197048	169056
Feces_4	203424	193351	191529	190972	182313	150789
Feces_8	204225	194955	193188	192723	184741	156447
Feces_8	210816	201487	199967	199596	191878	161127
Feces_I	203676	193529	191432	190664	181664	152265
Feces_I	206132	196203	195026	194424	187656	160356
LarLiv_4	205162	179476	177669	177285	169995	152787
LarLiv_4	206295	180099	178861	178310	171603	153604
LarLiv_4	204036	188767	185903	185387	176158	158673

LarLun_	204983	182557	181645	181336	176294	160462
LarLun_	202088	176575	175287	174930	168529	150847
LarLun_	213737	192562	190990	190828	182825	157450
LarvaIn	181497	156028	155410	155119	150032	137700
PreInfF	205797	195628	193906	193344	185897	159957
PreInfF	205224	194969	193547	193038	185714	155055

Characterizing Excretory-Secretory Products Proteome Across Larval Development Stages in *Ascaris suum*

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Abstract

Introduction: *Ascaris lumbricoides* and *Ascaris suum* are parasitic nematodes that primarily infest the small intestines of humans and pigs, respectively. Ascariasis poses a significant threat to human health and swine health. Understanding *Ascaris* larval development is crucial for developing novel therapeutic interventions that will prevent ascariasis in both humans and pigs. This study aimed to characterize the excretory-secretory (ES) proteome of different *Ascaris suum* larval stages (L3-egg, L3-lung, L3-trachea) to identify potential targets for intervention to prevent *Ascaris*-induced global morbidity. **Methods:** Stage-specific larvae were isolated, cultured in vitro and ES-product was collected. Third-stage

Ascaris larvae (L3) were isolated from embryonated eggs (L3-egg), isolated from the lungs of Balb/c mice infected with *Ascaris suum* eggs at day 8 post infection (L3-lungs) and isolated from the trachea of Balb/c mice infected with *Ascaris suum* eggs at day 12 post infection (L3-trachea). ES products were obtained by culturing larvae. Proteomic analysis was conducted using liquid chromatography-tandem mass spectrometry (LC-MS/MS) and bioinformatic tools including MaxQuant, Perseus, and Andromeda, following a detailed protocol available on GitHub. The analysis encompassed peptide identification, scoring, and quantification against an organism-specific database, with subsequent quality control, correlation assessment, and differential abundance determination using the Amica algorithm. **Results:** A total of 58 unique proteins were identified in the ES products. Fourteen proteins were common across all stages, while others were stage-specific. Principal component analysis revealed distinct protein profiles for each stage, suggesting qualitatively different proteomes. Gene ontology analysis indicated stage-specific GO enrichment of specific protein classes, such as nuclear proteins in L3-egg ES products and metabolic enzymes in L3-lung and L3-trachea ES products. **Discussion:** This study revealed stage-specific differences in the composition of *Ascaris* ES products. Further investigation into the functional roles of these proteins and their interactions with host cells is crucial for developing novel therapeutic and diagnostic strategies against ascariasis.

Introduction

Ascaris lumbricoides is a highly prevalent parasite globally [1]. According to the latest prevalence estimates there are approximately 800 million cases of ascariasis globally [2, 3]. Ascariasis disproportionately affects young children, with pre-school and school-aged children harboring the greatest worm burden [4–7]. In endemic regions, the use of anthelmintic preventive chemotherapy, through mass drug administration programs, aims to reduce worm burden and disease prevalence [2, 8,

9]. However, due to the ubiquitous contamination of *Ascaris* eggs in the environment, children endure recurrent infections throughout childhood [2, 9–12]. High worm burden and frequent re-infection of ascariasis results in a significant level of global morbidity, leading to nearly 754,000 Disability Adjusted Life Years (DALYs) [9, 13]. As a result of *Ascaris*-induced long-term morbidity, there is an urgent need to develop novel therapeutic interventions, like vaccines, to prevent ascariasis. However, developing therapeutic interventions that prevent a high burden of disease and reinfection will require an in-depth understanding of how *Ascaris* larvae develop into adult worms and how they interact with the host immune response [14]. Similarly, *Ascaris suum* presents significant health and economic challenges to the swine industry due to its widespread infection among pigs worldwide [1, 15]. *A. suum* can affect pigs of all age groups, but its prevalence within specific age categories, particularly young pigs, is often influenced by housing conditions and management practices. Overall ascariasis in pig populations leads to significant economic losses due to reduced fee conversion efficiency and increased condemnation resulting in production losses [16, 17].

Ascaris lumbricoides and *Ascaris suum* are nearly identical morphologically and genetically and routinely cause cross-infection between hosts [1, 18, 19]. Both *Ascaris* spp. larvae undergo an essential migratory phase that promotes development from larvae to mature adult worm [20]. What distinguishes the life cycle of *Ascaris* spp. from other soil-transmitted helminths is its hepato-tracheal migration. Following ingestion of embryonated *Ascaris* eggs from the environment, third-stage larvae (L3) emerge from the eggs in the intestines. L3 migrate from the intestines to the liver, then proceeds to the lungs via the circulatory system. Within the lung parenchyma, L3 larvae develop into mature L3 as they are ascending the trachea and swallowed back into the intestines. In the intestines, L3 mature first into L4 larvae and than into adult worms [20, 21].

Throughout its migration, *Ascaris* spp larvae release a wide range of molecules termed excretory-secretory (ES) products. These products encompass antimicrobial peptides, proteins, lipids, metabolites,

among others, and are integral in shaping the intricate interactions between the host and the parasite. They influence key aspects such as worm survival, migration, and immune modulation, ultimately aiding in the successful establishment and persistence of the parasite within the host [22–25].

With significant advancements in mass spectrometry and genomic technologies, numerous challenges and constraints in the proteomic analysis of parasite ES proteins have been effectively addressed. Consequently, ES proteomes for parasitic nematodes such as *Ancylostoma caninum*, *Brugia malayi*, *Haemonchus contortus*, *Teladorsagia circumcincta*, and *Trichinella spiralis* have been successfully characterized [26–32]. Unraveling the composition and functional significance of ES products across *Ascaris* life cycle presents a compelling avenue to advance our understanding of parasite biology and potential targets for intervention strategies.

Given the close relation between *A. suum* and *A. lumbricoides*, and the availability of the *A. suum* genome, we undertook the characterization of the protein composition of ES in three distinct larval stages of *Ascaris* (L3-egg, L3-lung, and L3-trachea) using tandem mass spectrometry annotated with the *A. suum* genome in a mouse model. Identifying highly abundant and conserved, stage-specific proteins within the *Ascaris* ES proteome presents a promising avenue for discovering novel targets that could be utilized in the development of preventive and therapeutic interventions against *Ascaris* infection.

Methods

Stage-specific larval isolation

Hatching of third-stage larvae (L3 egg stage): Embryonated eggs in H_2SO_4 solution were centrifuged, the supernatant was removed, and the pellet was re-suspended in sodium hypochlorite solution and incubated for 2 hours. After incubation, the eggs were centrifuged with subsequent removal of the supernatant. The eggs were washed with ultra-pure water 3 times and re-suspended in Hanks Balanced

Salt Solution (HBSS) with pH 2.0 using hydrogen chloride to achieve the desired pH and incubated for 30 minutes in 37°C + 5% CO_2. The eggs were centrifuged, the supernatant was removed while the pellet was re-suspended in 10 mL of HBSS solution pH 7.0 to neutralize eggs for 30 minutes. The eggs were centrifuged, and the egg pellet was re-suspended in culture media (RPMI-1640) with penicillin-streptomycin (4% pen/strep). The egg solution was placed in 6-well culture plate (4mL per well) and incubated over 96 hours. Egg hatching was monitored using light microscopy. Once the larvae hatched, new media was placed in the culture plates and incubated overnight at 37°C +5% for 48 hours. The experiment was completed in triplicate.

Collection of third stage larvae from the lungs (L3-lung): 5 Balb/c mice were infected with 2500 *Ascaris suum* eggs in a total volume of 100 µL distilled water by oral gavage. After 8 days of infection, mice were euthanized by intraperitoneal injection of ketamine (up to 150 mg/kg) and xylazine (up to 15 mg/kg). After euthanasia, the lungs were removed, macerated in a petri dish, placed in a modified Baermann apparatus in warm PBS and incubated at 37°C +5% CO_2 for 4 hours. The fluid in the Baermann apparatus was transferred to a 50 mL conical tube and centrifuged. The supernatant was removed, and the pellet was washed with ultra-pure water three times. After the final wash and removal of supernatant, the larvae were transferred into a 6-well culture plate with 4 mL of culture media to each well. The culture plates were incubated at 37°C +5% CO_2 for 48 hours. The experiment was completed in triplicate.

Collection of fourth-stage larvae from the trachea (L3 trachea): 5 Balb/c mice were infected as described above. On post-infection day 12, the mice were euthanized, and a tracheostomy was created by placing a 20-gauge angiocatheter into the trachea. The angiocatheter was flushed with 0.8 mL of PBS three times, all returned fluid was collected and passed through a 40µm cell strainer. The 40µm cell strainers were washed using 4mL of PBS to release the larvae into a 6 well cell culture plate. Culture plates were rested for 5 minutes to allow larvae to settle to bottom of the culture plate with gravity. PBS was removed from the wells. The larvae were then washed with 4mL distilled water a total of three times. After completion

of the wash, 4 mL of culture media was added to each well and the 6-well culture plates were incubated at 37°C +5% CO_2 for 48 hours. The experiment was completed in triplicate.

Preparation and proteomic analysis of ES products

After 48 hours the supernatant was pooled, filtered through a 0.45 µm filter (PALL Corporation), frozen in liquid nitrogen and stored until use as ES product. This process was repeated in three separate experiments. Using mass spectrometry at the Baylor College of Medicine Mass Spectrometry Proteomics core a total of 40ml volume of ES product from each developmental stage was lyophilized and trypsin digested using S-Trap™ (Protifi, NY) according to manufacturer's protocol. Digested peptides were cleaned using a C18 disk plug (3M Empore C18) and dried in a speed vac. The peptide concentration was normalized to a total of 1 ug prior to the mass spectrometry run. Liquid chromatography-tandem mass spectrometric (LC-MS/MS) analysis was carried out using a nano-LC 1200 system (Thermo Fisher Scientific, San Jose, CA) coupled to Orbitrap Fusion™ Lumos ETD mass spectrometer (Thermo Fisher Scientific, San Jose, CA). Peptides were loaded on a C-18 trap-column (Reprosil-Pur Basic C18, 2cm X 100µm, ID 1.9 µm, Dr. Maisch GmbH, Germany) and eluted using a 75min gradient of 5-28% acetonitrile/0.1% formic acid at a flow rate of 750nl/min on a C-18 analytical column (Reprosil-Pur Basic C18, 5cm x 150µm, ID 1.9 µm, Dr. Maisch GmbH, Germany). The peptides were directly electro-sprayed into the mass spectrometer operated in a data-dependent mode with 'top 30' method. The full MS scan was acquired in Orbitrap in the range of 300-1400m/z at 120,000 resolution followed by MS2 in Ion Trap (HCD 28% collision energy) with 15sec dynamic exclusion time.

Bioinformatic analysis of ES products

The analytical framework employed MaxQuant, Perseus, and Andromeda, as detailed at https://github.com/FredHutch/maxquant-pipeline. This protocol encompassed the identification of

peptides, determination of mass, and intensity of peptide peaks in mass spectrometry (MS) spectra, thereby facilitating the identification of isotopic patterns [33–35].

Search parameters were a precursor tolerance of 20 ppm, MS/MS tolerance of 0.05 Da, Dynamic modification of Oxidation and protein N-terminal Acetylation was allowed. The query of peptide and fragment masses was conducted against a database housing organism-specific sequences of *Ascaris*, obtained from UniProt (https://www.uniprot.org/uniprotkb?facets=reviewed:false&query=(taxonomy_id:6253)) PMID:21685128. Subsequently, scoring through a probability-based method termed peptide scoring was performed, with the implementation of a false discovery rate (FDR) approach to limit the occurrence of matches by chance. FDR was ascertained through statistically rigorous methodologies addressing multiple hypothesis testing. Additionally, the database search incorporated not only target sequences but also their reverse and contaminant counterparts, aiding in the establishment of a statistical cutoff for acceptable spectral matches. Peptides were accepted based on a false discovery rate (FDR) of <0.05 at both the peptide and protein levels. Proteins were quantified using the LFQ value from MaxQuant employing default settings [33–35].

Following this preprocessing, the acquired outputs are undergoing a comprehensive analysis encompassing quality control (QC), correlation assessment, density analysis, differential abundance determination, and group comparisons, facilitated by the Amica algorithm (https://github.com/tbaccata/amica) [36], using default parameters: Log2 fold change threshold 1.5; significance cutoff (which value to use) 0.05.

To perform gene ontology analysis, the software QuickGO and PROTEINSIDE was used to determine GO Biological Process and Cellular Component ontology enrichments using the *Ascaris suum* genome as a

reference (PRJNA62057). Additionally, DeepLoc was employed to identify proteins with signal peptides within the dataset, thereby identifying secreted proteins [37].

Subsequent analyses of the output obtained by MaxQuant were evaluated also by Perseus [33–35]. Finally, we implement the open-source tool Protter, for visualization of proteoforms and interactive integration of annotated and predicted sequence features [38].

Results

Protein identification

The proteins within the ES products of different *Ascaris* larval stages (L3-egg, L3-lung, L3-trachea) were characterized using LC-MS/MS. MaxQuant searches of the data identified 58 unique proteins by Gene ID in the ES products of all three larval developmental stages combined (Table 1).

Table 1. Quantification values for each protein were calculated using the LFQ values obtained from MaxQuant at each larval stage.

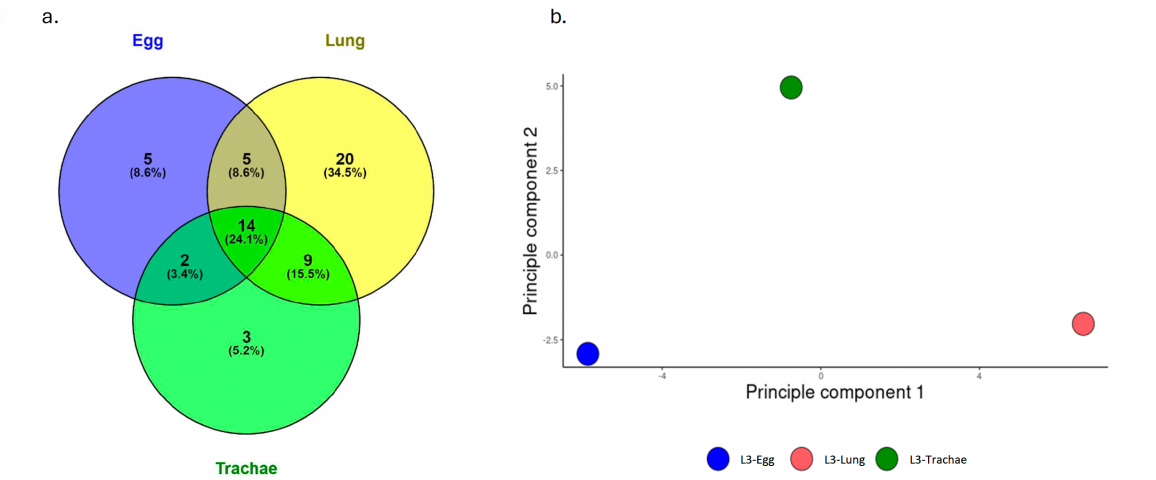
L3-egg	L3-lung	L4-Trachae	UniprotID
27.04009303	20.74246405	0	H2BIV5
25.41225377	25.84699386	0	F1KR40
22.84943946	0	0	F1LBV5
22.10939071	20.74517534	0	F1L344
21.91936237	0	0	F1KVQ9
21.57934356	0	0	F1KSS5
21.47565824	0	0	F1LBT0
21.12578173	22.23467712	0	F1L9F1
20.37517522	0	0	F1KS54
19.81000358	22.3112727	0	F1L8A5
0	22.76131667	0	A1BQ59
0	22.13276328	0	F1LEG5
0	23.73003389	0	E5D7U6
0	28.43614924	0	F1KPF6
0	24.14615969	0	F1KPQ7

0	23.15489844	0	F1KPR0
0	21.00319396	0	F1KTM8
0	22.2581922	0	F1KVW3
0	23.34516265	0	F1KW99
0	20.23420932	0	F1KY56
0	19.65308998	0	F1L0P6
0	19.73080511	0	F1L458
0	21.06375055	0	F1L4D2
0	20.71883385	0	F1L5H2
0	21.55173267	0	F1L6I9
0	22.7215273	0	F1L719
0	18.54105879	0	F1L8Z9
0	22.88675116	0	F1LA47
0	17.8615716	0	F1L9N2
0	22.31318419	0	F1LGZ3
18.28301598	24.94422663	19.19770144	F1L4H4
20.44394718	0	19.82074614	F1KY69
0	22.50671145	19.99327565	F1LEM8
0	20.26979329	20.67564353	F1L5G6
0	0	21.02093112	F1L3T3
17.82538119	0	21.86996729	F1KTX5
0	22.31418049	22.1747197	F1L5E5
0	21.38869003	22.27489111	F1KQZ7
21.14730928	26.45290204	22.28872642	F1LG13
18.99733387	26.98901884	22.59204088	F1L183
0	0	23.31049629	F1L3P9
0	20.93099138	23.87605224	Q17092
0	27.15034974	24.27717784	F1KZM0
23.36932896	26.90161692	24.360046	F1KYQ7
21.99851881	25.92036685	24.38455928	C1KG49
21.83259956	27.01669575	24.91948058	F1KXW6
0	27.67229379	24.95965144	F1L5S2
18.55730663	27.53519491	25.18744692	B5RHZ9
22.1978154	26.01624942	25.54176662	F1L1W1
20.94941923	27.96675257	25.73034492	F1L6S0
0	27.78338006	25.75692173	F1LGK7
0	28.90844769	25.920435	F1LC49
23.61851724	29.11812855	26.47830872	F1LHE9
0	0	26.52578865	F1L965
23.79530883	24.89513517	27.2341618	F1L676
24.89901388	28.13692198	27.274621	F1L3U5
25.63994896	28.31613649	28.07729697	F1LEJ7

23.37848643	29.99010154	29.48346905	F1L8W7

178

179 Specifically, 5 proteins were exclusively identified in L3-egg, while L3-lung exhibited 20 unique proteins,
180 and L3-trachea displayed 3 proteins exclusive to its stage. Additionally, 14 proteins were detected in ES
181 products conserved across all three larval development stages (Figure 1a). The protein profiles of the ES
182 products from each larval developmental stage were compared using principal component analysis (PCA)
183 to assess their relatedness. The PCA revealed three distinct proteome clusters corresponding to each
184 stage (Figure 1b).



185

186 **Figure 1.** (a) The Venn diagram illustrates the distribution of unique and shared proteins across the ES
187 product of *Ascaris* stages. (b) Principal Component Analysis (PCA) reveals that the overall composition
188 remains distinctive for each developmental stage ES product.

189 ***Comparative analysis***

190 We further compared the proteomic profiles between different larval stages using volcano plots and heat
191 maps. It is noteworthy that, although statistically significant differences were not observed among the
192 analyzed samples, distinct groups of proteins associated with L3-egg, L3-lung, and L3-Ttachea ES products
193 were identified, alongside some shared proteins (Figure 2a). The lack of difference in protein enrichment

between stages' ES products may be attributed to the similarity between the proteomes or to an insufficient number of proteins for a robust comparison due to the heterogeneity in the samples. Additionally, based on the obtained results, Spearman's correlation analysis reveals distinct correlation coefficients among the various protein groups across stages. Specifically, a correlation of 0.54 is observed between L3-lung and L3-trachea ES proteins. In contrast, the correlation between L3-egg and L3-lung ES proteins is determined to be 0.21, while the correlation between L3-egg and L3-trachea ES proteins is calculated to be 0.32 (Figure 2b).

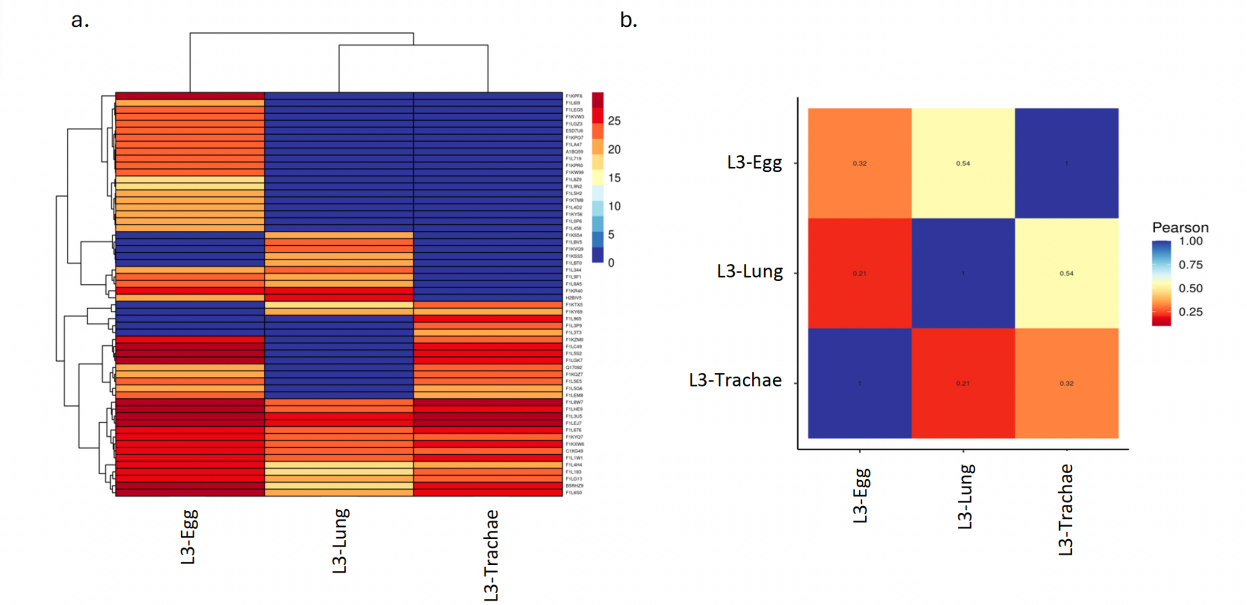


Figure 2. (a) HeatMap of protein profile identified by each developmental ES product stage of *Ascaris*. (b) Spearman's correlation analysis among the *Ascaris*-related protein groups.

Gene ontology

The Gene Ontology (GO) annotation of proteins enriched in the ES product was analyzed by molecular function, cellular component, and biological process (Table 2, Figure S1), as well as according to their property of being secreted proteins (Table S1).

Table 2. Identified protein function, implicated biological processes, and subcellular localization.

Stage	UniProt ID	Protein Name	Function	Molecular Function	Biological Process	Cellular Localization
Egg	F1LBV5	Ell-associated factor Eaf	Transcription	Transcription	regulation of DNA-templated transcription	nucleus
Egg	F1KVQ9	Protein SDA1	ribosomal large subunit biogenesis	ribosomal large subunit biogenesis	ribosomal large subunit export from nucleus	nucleus
Egg	F1KSS5	BLTP2/FMP27/Hobbit C-terminal domain-containing protein	-	-	-	-
Egg	F1LBT0	Chondroitin proteoglycan 3	-	-	-	-
Egg	F1KS54	Metalloendopeptidase	metalloendopeptidase activity	zinc ion binding	proteolysis	membrane
Egg/Lung	H2BIV5	Trehalose-6-phosphate synthase	trehalose synthesis	catalytic activity	trehalose biosynthesis	cytosol
Egg/Lung	F1KR40	Structural maintenance of chromosomes protein	Hydrolase	atp-depending activity	-	nucleus
Egg/Lung	F1L344	Pleiotropic regulator 1	mRNA splicing, via spliceosome	mRNA splicing, via spliceosome	mRNA splicing, via spliceosome	nucleus
Egg/Lung	F1L9F1	Phosphoglycerate kinase	plasma membrane	phosphoglycerate kinase activity	glycolytic process	cytosol
Egg/Lung	F1L8A5	Tubulin beta chain	structural constituent of cytoskeleton	GTPase activity	microtubule-based process	cytosol
Egg/Lung/Trachae	F1LEJ7	Polyubiquitin	peptidase activity	proteolytic activity	protein degradation	cytosol
Egg/Lung/Trachae	F1L3U5	Actin-2	protein binding	protein binding	signal transduction	cytosol
Egg/Lung/Trachae	F1L676	Triosephosphate isomerase	isomerase activity	isomerase activity	gluconeogenesis	-
Egg/Lung/Trachae	F1LHE9	Calmodulin	calcium ion binding	protein binding	calcium signaling	cytosol
Egg/Lung/Trachae	F1L8W7	Histone H2B type 1-W	nucleosome assembly	dna-binding	chromatin organization	nucleus
Egg/Lung/Trachae	F1KYQ7	Heat shock 70 kDa protein A	ATP-dependent protein folding chaperone	ATP-dependent protein folding chaperone	Stress response	membrane
Egg/Lung/Trachae	F1L1W1	Heat shock protein 70 B2	ATP-dependent protein folding chaperone	ATP-dependent protein folding chaperone	Stress response	cytosol
Egg/Lung/Trachae	C1KG49	Heat shock protein 90	ATP-dependent protein folding chaperone	ATP-dependent protein folding chaperone	Stress response	cytosol
Egg/Lung/Trachae	F1KXW6	14-3-3-like protein	-	-	-	-

Egg/Lung/Trachae	F1LG13	Peptidyl-prolyl cis-trans isomerase	endoplasmic reticulum	peptidyl-prolyl cis-trans isomerase activity	protein folding	endoplasmic reticulum
Egg/Lung/Trachae	F1L6S0	Fructose-bisphosphate aldolase	catalytic activity	fructose-bisphosphate aldolase activity	glycolytic process	cytosol
Egg/Lung/Trachae	F1L183	Heat shock 70 kDa protein C	ATP binding	ATP-dependent protein folding chaperone	Stress response	endoplasmic reticulum
Egg/Lung/Trachae	B5RHZ9	Elongation factor 1-alpha	Elongation factor	GTPase activity	Protein biosynthesis	cytosol
Egg/Lung/Trachae	F1L4H4	ATP synthase subunit beta	Translocase	ATP hydrolysis activity	ATP synthesis	membrane
Egg/Trachae	F1KY69	Moesin/ezrin/radixin homolog 1	actin binding	actin binding	actin binding	cytosol
Egg/Trachae	F1KTX5	Neurexin-2-alpha	-	-	-	membrane
Lung	A1BQ59	Translationally-controlled tumor protein homolog	-	-	-	-
Lung	F1LEG5	Protein disulfide-isomerase 2	isomerase	protein disulfide isomerase activity	protein disulfide isomerase activity	endoplasmic reticulum
Lung	E5D7U6	phosphopyruvate hydratase	phosphopyruvate hydratase activity	phosphopyruvate hydratase activity	Glycolysis	cytosol
Lung	F1KPF6	Muscle M-line assembly protein unc-89	ATP binding	protein kinase activity	Neurogenesis	membrane
Lung	F1KPQ7	227 kDa spindle- and centromere-associated protein	-	-	-	nucleus
Lung	F1KPR0	Spectrin alpha chain	calmodulin binding	Calmodulin-binding	actin filament capping	cytosol
Lung	F1KTM8	Intermediate filament protein ifa-1	-	-	-	cytosol
Lung	F1KVW3	Collagen alpha-5(VI) chain	-	-	collagen trimer	cytosol
Lung	F1KW99	H(+)-transporting two-sector ATPase	Translocase	proton-transporting ATPase activity, rotational mechanism	Hydrogen ion transport	-
Lung	F1KY56	Ras-related protein Rab-1A	GTP binding	GTPase activity	GTPase activity	-
Lung	F1L0P6	Dihydrolipoyl dehydrogenase	Oxidoreductase	Oxidoreductase	Oxidoreductase	cytosol
Lung	F1L458	Disorganized muscle protein 1	-	-	-	-
Lung	F1L4D2	Troponin T	regulation of muscle contraction	regulation of muscle contraction	regulation of muscle contraction	cytosol

Lung	F1L5H2	Fructose-bisphosphate aldolase	fructose-bisphosphate aldolase activity	fructose-bisphosphate aldolase activity	Glycolysis	cytosol
Lung	F1L6I9	Calumenin-A	calcium ion binding	calcium ion binding	calcium ion binding	cytosol
Lung	F1L719	inorganic diphosphatase	Hydrolase	magnesium ion binding	phosphate-containing compound metabolic process	cytosol
Lung	F1L8Z9	Malate dehydrogenase	Oxidoreductase	L-malate dehydrogenase activity	Tricarboxylic acid cycle	cytosol
Lung	F1LA47	Serine/threonine-protein phosphatase	Hydrolase	myosin phosphatase activity	myosin phosphatase activity	-
Lung	F1L9N2	Enoyl-CoA hydratase	catalytic activity	catalytic activity	catalytic activity	-
Lung	F1LGZ3	Peptidyl-prolyl cis-trans isomerase	Isomerase	peptidyl-prolyl cis-trans isomerase activity	protein folding	-
Lung/Trachae	F1KQZ7	Protein abnormal spindle	-	-	-	-
Lung/Trachae	F1L5G6	Tubulin alpha chain	GTP binding	structural constituent of cytoskeleton	microtubule-based process	cytosol
Lung/Trachae	F1KZM0	Myosin regulatory light chain	calcium ion binding	calcium ion binding	calcium ion binding	-
Lung/Trachae	F1LEM8	Polyadenylate-binding protein 1	RNA-binding	RNA-binding	RNA-binding	nucelus
Lung/Trachae	F1L5S2	Histone H3	DNA binding	structural constituent of chromatin	structural constituent of chromatin	nucleus
Lung/Trachae	F1LC49	Histone H4	DNA binding	structural constituent of chromatin	structural constituent of chromatin	nucelus
Lung/Trachae	F1L5E5	Venom allergen 3	-	-	-	-
Lung/Trachae	F1LGK7	Tropomyosin	-	-	-	-
Lung/Trachae	Q17092	Intermediate filament protein	Intermediate filament protein	Intermediate filament protein	Intermediate filament protein	cytosol
Trachae	F1L3P9	1-Cys peroxiredoxin	Antioxidant	Antioxidant	Antioxidant	-
Trachae	F1L3T3	26S proteasome non-ATPase regulatory subunit 3	enzyme regulator activity	enzyme regulator activity	regulation of protein catabolic process	cytosol
Trachae	F1L965	Homeobox protein lin-39	DNA binding	DNA binding	DNA binding	nucleus

Additionally, based on annotated and predicted protein sequences derived from previous analyses, we conducted a visual reconstruction of proteoforms of the main proteins identified. This aimed to represent protein sequence characteristics within the context of protein topology and experimental proteomic evidence (Figure 3). Notably, proteins identified in L3-egg ES product exhibit a pronounced association with nuclear proteins engaged in replicative and transcriptional processes, including Ell-associated factor Eaf, Protein SDA1, BLTP2/FMP27/Hobbit C-terminal domain-containing protein, Chondroitin proteoglycan 3, and Metalloendopeptidase. Conversely, proteins identified in L3-lung and L3-trachea ES products predominantly pertain to cytoplasmic proteins involved in energetic processes, such as glycolysis, and proteins related to the cytoskeleton (Figure S2, S3). For L3-lung ES products, the identified proteins include translationally-controlled tumor protein homolog, Protein disulfide-isomerase 2, phosphopyruvate hydratase, Muscle M-line assembly protein unc-89, 227 kDa spindle-and centromere-associated protein, Spectrin alpha chain, Intermediate filament protein ifa-1, Collagen alpha-5(VI) chain, H(+)-transporting two-sector ATPase, Ras-related protein Rab-1A, Dihydrolipoyl dehydrogenase, Disorganized muscle protein 1, Troponin T, Fructose-bisphosphate aldolase, Calumenin-A, inorganic diphosphatase, Malate dehydrogenase, Serine/threonine-protein phosphatase, Enoyl-CoA hydratase, and Peptidyl-prolyl cis-trans isomerase (Figure S2, S3). On the other hand, for L3-trachea ES products, proteins found include 1-Cys peroxiredoxin, 26S proteasome non-ATPase regulatory subunit 3, and Homeobox protein lin-39. These findings underscore distinct molecular emphases and functional roles across different developmental stages of *Ascaris*, shedding light on the nuanced biological processes characterizing each stage.

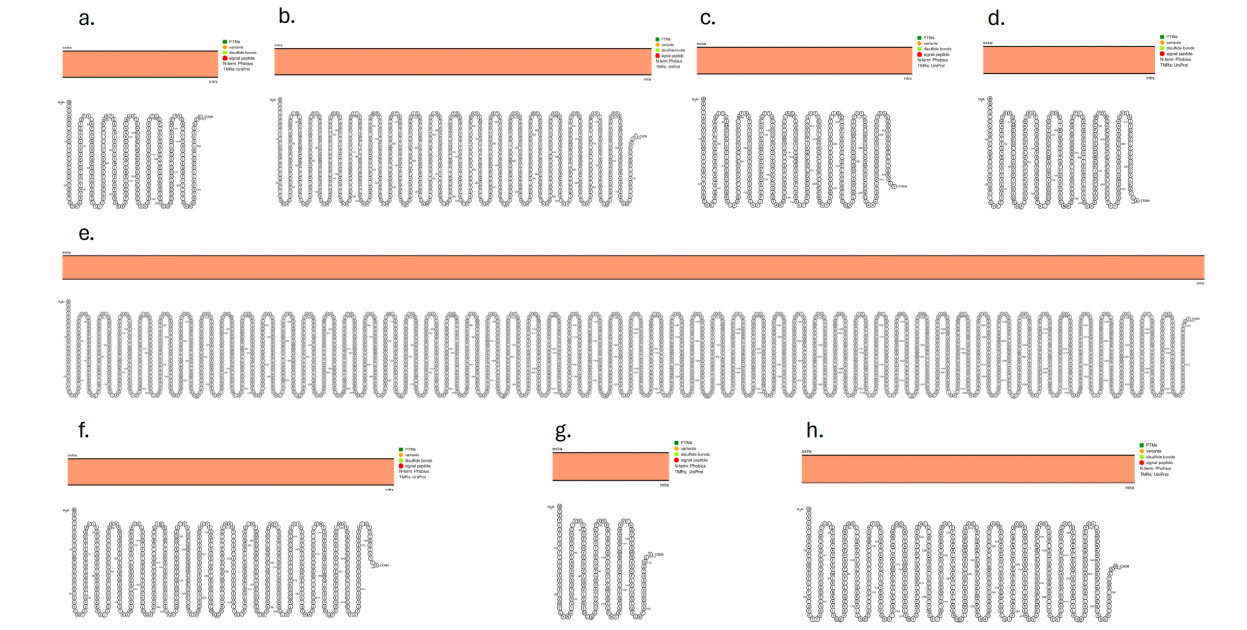


Figure 3. Visualization of proteoforms and interactive integration of annotated and predicted sequence features of main proteins. Nuclear proteins associated with replication and transcription, such as (a) Ell-associated factor Eaf (UniProt ID F1LBV5) and (b) Protein SDA1 (UniProt ID F1KVQ9) in L3-egg ES product. Glycolytic enzymes like (c) fructose-bisphosphate aldolase (UniProt ID F1L5H2) and (d) malate dehydrogenase (UniProt ID F1L8Z9) in L3-lung ESP. Cytoskeletal proteins such as (e) spectrin alpha chain (UniProt ID F1KPR0) and (f) intermediate filament protein ifa-1 (UniProt ID F1KTM8) in both L3-lung and L3-trachea ES product. (g) Peptidyl-prolyl cis-trans isomerase (UniProt ID F1LGZ3), an immunophilin found in L3-Lung ES product. Identified (h) Ezrin-radixin-moesin (ERM) proteins (UniProt ID F1KY69) in the secreted proteomes of larval development stages L3-lung and L3-trachea ES product.

Discussion

This study employed LC-MS/MS to characterize the protein profiles of excretory-secretory (ES) products from different *Ascaris* larval stages (L3-egg, L3-lung, L3-trachea). This is the most comprehensive evaluation of stage-specific ES proteomes during *Ascaris* larval migration to date. Findings reveal intriguing insights into the stage-specific composition of these secretions, potentially reflecting distinct biological

functions during development. The migration of *Ascaris* larvae through the host is a crucial developmental process for the parasite. This study elucidates the similarities and differences in the secreted proteome among L3 developmental stages, offering valuable insights that could spur research focused on identifying novel diagnostic and vaccine targets essential for controlling and eradicating ascariasis [22, 39–41].

Prior research has investigated ES proteins derived from adult worms, uncovering a diverse array of proteins, enzymes, and biomolecules with roles in immunomodulation, immunoevasion, and tissue degradation. Among these are Glycosyl hydrolases, crucial for breaking down complex carbohydrates and integral to the parasite's energy metabolism. Additionally, C-type lectins exhibit a wide range of functions including cell-cell adhesion, immune responses to pathogens, and involvement in apoptosis. Furthermore, Heat shock proteins (HSPs) have been identified, which may modulate host responses to facilitate and sustain infection, among other functions. [22, 40–43]. Furthermore, a study evaluating ES protein of *Ascaris* L3-egg, L3-lung and L4-intestines was completed in two pigs. The majority of the proteins identified were stage-specific but 14-3-3-like protein and a serpin-like protein were conserved amongst the three different larval stages. While pigs are a natural host of ascariasis, use of a pig model is limiting due to size and cost [26]. Here, we used a highly replicated mouse model of ascariasis in order to repeat the experiment in triplicate and identify variation between each larval development stage as well as within each larval development stage.

The identification of stage-specific proteins in our study aligns with previous observations in other parasitic nematodes, where ES products exhibit dynamic changes throughout the life cycle [27, 43–48]. For instance, in *Trichinella spiralis*, infective larvae secrete proteins like excretory-secretory antigen-like protein (TES) and cystatin inhibitor during muscle invasion, potentially aiding tissue penetration and immune modulation [49, 50]. Similarly, *Angiostrongylus cantonensis* ES products change significantly between different developmental stages, with glutathione S-transferase and superoxide dismutase enriched in adult stages, suggesting roles in detoxification and stress management [51, 52].

In the current analysis, a total of 58 unique proteins were identified in the *Ascaris* ES products. Notably, 5 proteins were exclusively identified in L3-egg ES product, 20 proteins unique to proteins were unique to L3-lung ES product, and 3 proteins were distinct to L3-trachea ES product. Additionally, 14 detected proteins were conserved across all three larval developmental stages (Figure 1a, 2a). Protein diversity and protein abundance may be secondary to the active migration. In comparison to the intraluminal L3 egg stage, the L3-trachea stage and L3 lung stage, which require migration through tissue and molting, may necessitate upregulation and secretion of proteins to facilitate these metabolically taxing processes [21, 27, 53, 54].

Our research into L3-egg ES product supports the idea that the GO enrichment of nuclear proteins associated with replication and transcription, such as Ell-associated factor Eaf (UniProt ID F1LBV5) and Protein SDA1 (UniProt ID F1KVQ9), correlates with active cell division and differentiation during this early developmental stage (Figure 2, 3). This finding aligns with previous studies on *Ascaris suum* embryogenesis, where similar proteins were identified [27, 41, 53]. Thus, it appears that ES products in L3-egg may play a crucial role in establishing the foundation for further larval development. Conversely, in L3-lung and L3-trachea ES products, we observed a dominance of cytoplasmic proteins related to energy metabolism and cytoskeletal functions, which aligns well with the increased demands of these later stages [27, 41, 53]. For example, the presence of glycolytic enzymes like fructose-bisphosphate aldolase (UniProt ID F1L5H2) and malate dehydrogenase (UniProt ID F1L8Z9) in L3-lung ES product suggests an adaptation to anaerobic environments during lung migration (Figure 2, 3) [55, 56]. Moreover, the detection of cytoskeletal proteins such as spectrin alpha chain (UniProt ID F1KPR0) and intermediate filament protein ifa-1 (UniProt ID F1KTM8) in both L3-lung and L3-trachea ES product suggests their role in preserving cell shape and aiding in active movement within the host, as has been observed in various nematode models, including *Trichinella britovi* and *Caenorhabditis elegans* (Figure 2, 3) [57–59]. These findings underscore the potential significance of ES products in facilitating the heightened metabolic activity and motility

crucial for the successful migration and establishment of larvae within the host. Further exploration of these specific proteins could yield therapeutic and diagnostic targets for *Ascaris* larval migration, offering valuable insights into proteins essential for helminthic metabolic and motility processes vital to *Ascaris* development.

Furthermore, our analysis revealed several interesting proteins within the ES products specific to particular larval stages. For instance, peptidyl-prolyl cis-trans isomerase (UniProt ID F1LGZ3), an immunophilin found in L3-lung ES product, exhibits activities that isomerize peptidyl-proline bonds, thereby facilitating enhanced protein folding (Figure 2, 3) [60, 61]. Immunophilins, major binding proteins for certain immunosuppressive drugs, have been found in other helminth ES products, including *Trichinella spiralis* early larval development stages [62]. While detailed functional analysis is available for many protozoal immunophilins, relatively little is known about the functionality of helminth immunophilins outside of *Caenorhabditis elegans*, although they are widely expressed within helminth species [61]. Parasite-derived immunophilins could serve as potential targets for diagnostic assays or drug development, especially when considering non-immunosuppressive analogs. Cyclosporin A, along with its non-immunosuppressive counterparts, has demonstrated inhibitory effects on parasites, suggesting promising avenues for therapeutic intervention [60, 61].

Additionally, we identified Ezrin-radixin-moesin (ERM) proteins (UniProt ID F1KY69) in the secreted proteomes of larval development stages L3-lung and L3-trachea ES proteins (Figure 2, 3). ERMs are proteins at the intersection of the cytoskeleton and the extracellular membrane, traditionally considered structural proteins allowing cross-linking of actin to the apical cellular membrane [63]. However, recent studies have revealed significant roles for ERMs in metabolic processes, cell signaling, and activation of the immune response [63, 64]. While several studies have emphasized the role of ERM in protozoa infectivity, particularly in *Trypanosoma cruzi*, there is currently no indication of the involvement of ERM in helminth larval migration in the context of ascariasis [65]. It has been identified that ERM proteins could

be associated with various parasite processes, including invasion, potentially regulating F-actin dynamics and plasma membrane interplay. Additionally, their involvement in the development of several structures has been observed in models such as *C. elegans* [66]. Furthermore, changes in the phosphorylation state of these proteins may be linked to interactions with specific tissues, offering valuable insights into larval changes associated with specific anatomical and parasite-host-tissue interactions during larval migration [63–67].

Although statistically significant differences in protein abundance between stages were not detected, several lines of evidence suggest qualitative differences in the ES proteomes. First, principal component analysis (PCA) revealed distinct clusters for each stage, indicating unique protein profiles (Figure 1b). For instance, L3-egg ES product clustered separately due to the presence of embryonic development proteins like Eaf and SDA1, absent in later stages [39, 41]. Conversely, L3-lung and L3-trachea ES product formed a separate cluster enriched in metabolic enzymes like aldolase and malate dehydrogenase, suggesting a shared focus on energy production. Second, the identification of unique proteins in each stage further supports qualitative differences. For example, L3-egg ES product exclusively contained chromatin remodeling proteins, essential for early development, while L3-lung ES product exhibited specific cytoskeletal proteins like spectrin and intermediate filament proteins, potentially involved in motility during lung migration [55, 56]. Additionally, L3-trachea ES product uniquely possessed stress response proteins like peroxiredoxin, suggesting adaptation to the host environment [55, 56]. Third, the heterogeneity of ES products and inherent variability could mask subtle abundance differences between stages, making statistical detection challenging. For instance, the presence of immunomodulatory proteins in all stages might mask stage-specific variations in their abundance, requiring targeted analyses for accurate comparisons. It is important to highlight the relatively small number of identified proteins might not capture the full complexity of the proteomes [68–72]. Focusing on a broader range of proteins or employing targeted approaches might reveal significant differences between stages. Therefore, while

statistically significant differences in protein abundance weren't observed, the distinct clustering, stage-specific protein groups, and potential limitations suggest qualitative differences in the proteomes of *Ascaris* ES products across developmental stages. Further studies with larger sample sizes and targeted protein analyses are crucial to fully understand these qualitative variations and their functional implications.

These findings pave the way for further investigations into the specific functions of individual proteins and their contributions to the overall biological processes of each larval stage. Studying the downstream effects of Ell-associated factor Eaf through gene expression analysis could elucidate its role in regulating cell cycle genes in L3-egg [39, 41]. While nzyme activity assays could measure the specific activity of fructose-bisphosphate aldolase and malate dehydrogenase in different stages, providing direct evidence for their contribution to energy production [56]. Furthermore, for instance, immunofluorescence microscopy could visualize the localization of spectrin alpha chain and intermediate filament protein ifa-1 in migrating larvae, revealing their role in maintaining specific cellular structures and facilitating movement [58, 59]. Beyond individual proteins, exploring the interactions between ES products and host cells could provide valuable insights into the mechanisms underlying *Ascaris* pathogenesis. For example, investigating how cytokines secreted by host immune cells in response to specific ES proteins could reveal their immunomodulatory potential. Additionally, studying the binding of ES proteins to host cell receptors could identify potential targets for therapeutic intervention [27, 51, 52, 73, 74]. While further investigations are necessary to fully elucidate the functional significance of these proteins and their interactions with host cells, these findings contribute to a deeper understanding of the complex biology of *Ascaris* and its adaptations to the parasitic life cycle.

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Author contributions

SC, GAI, YFW, JDR, JW conceived and designed experiments. GAI, CSR, YFW performed laboratory experiments or clinical sampling. SC, AJ, JDR provided technical support and assisted with data collection. SC, AJ, performed data analysis. SC, GAI, YW, JDR, JW wrote the manuscript. All authors contributed to reviewing the paper and agreed on the present version for submission.

Data availability.

Preprocessing and processing results data are available at https://github.com/scastanedag/Proteome_Larval_Development-Stages_Ascaris_suumv

Supplementary material

Figure S1. ES product proteins and their corresponding subcellular locations at each larval stage.

Figure S2. GO enrichment analysis by Cellular Component. It shows significant results obtained in a. L3-egg and L3-lung.

Figure S3. GO enrichment analysis by Biological Process. It shows significant results obtained in a. L3-egg and L3-lung.

385 **Table S1.** Secreted proteins identified. The Predicted localizations and Predicted signals display the
386 subcellular localizations and sorting signals predicted for the query protein, respectively. The Probability
387 table displays the probability assigned by the model to each of the subcellular localizations.

388

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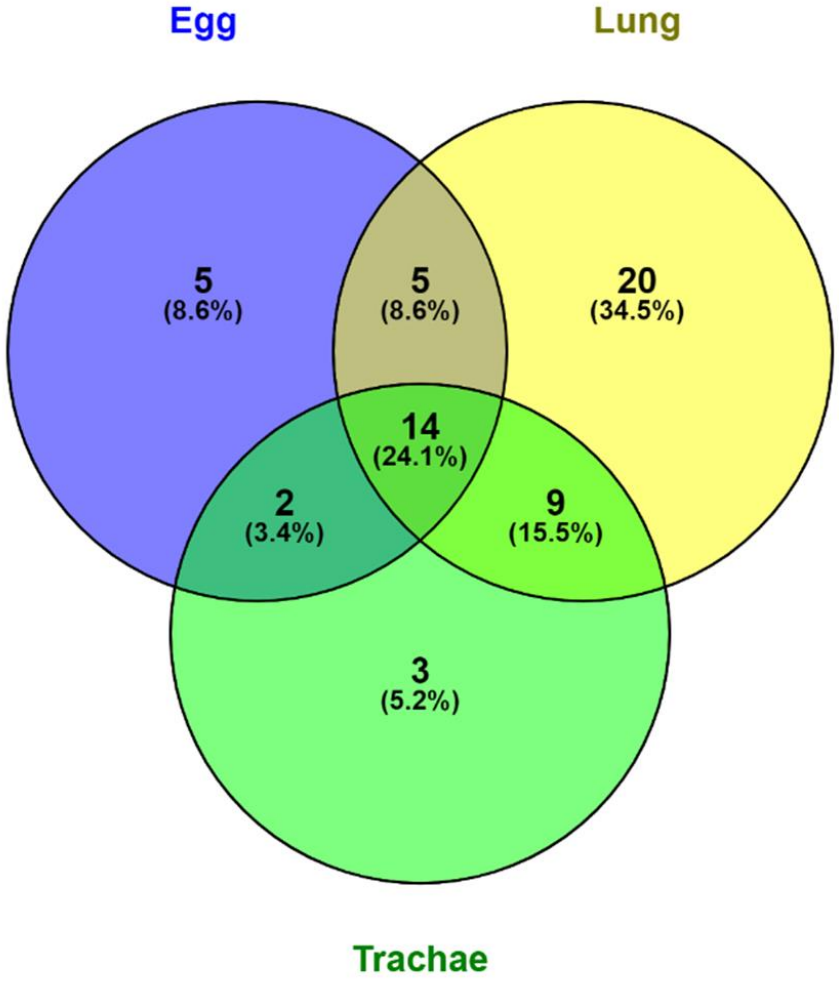
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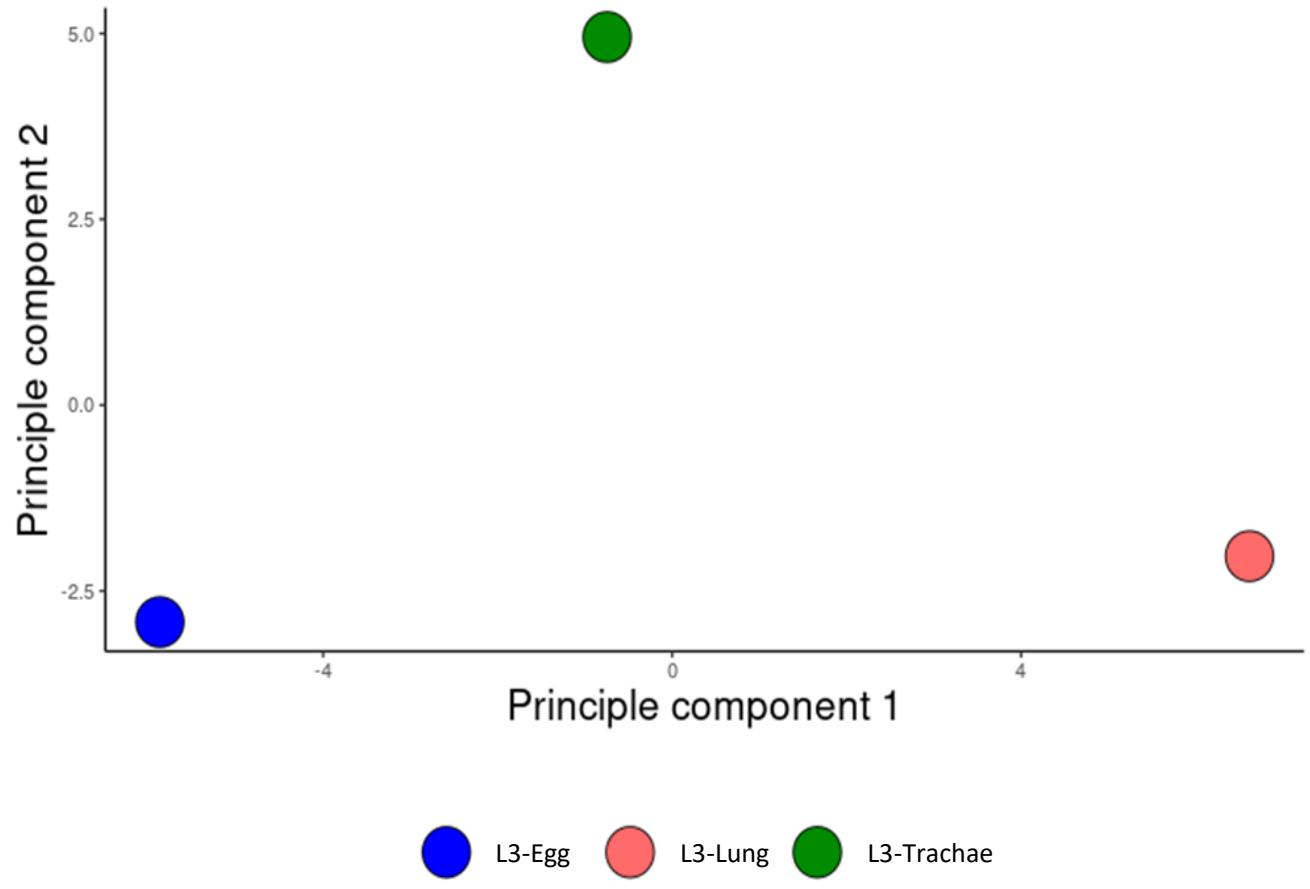
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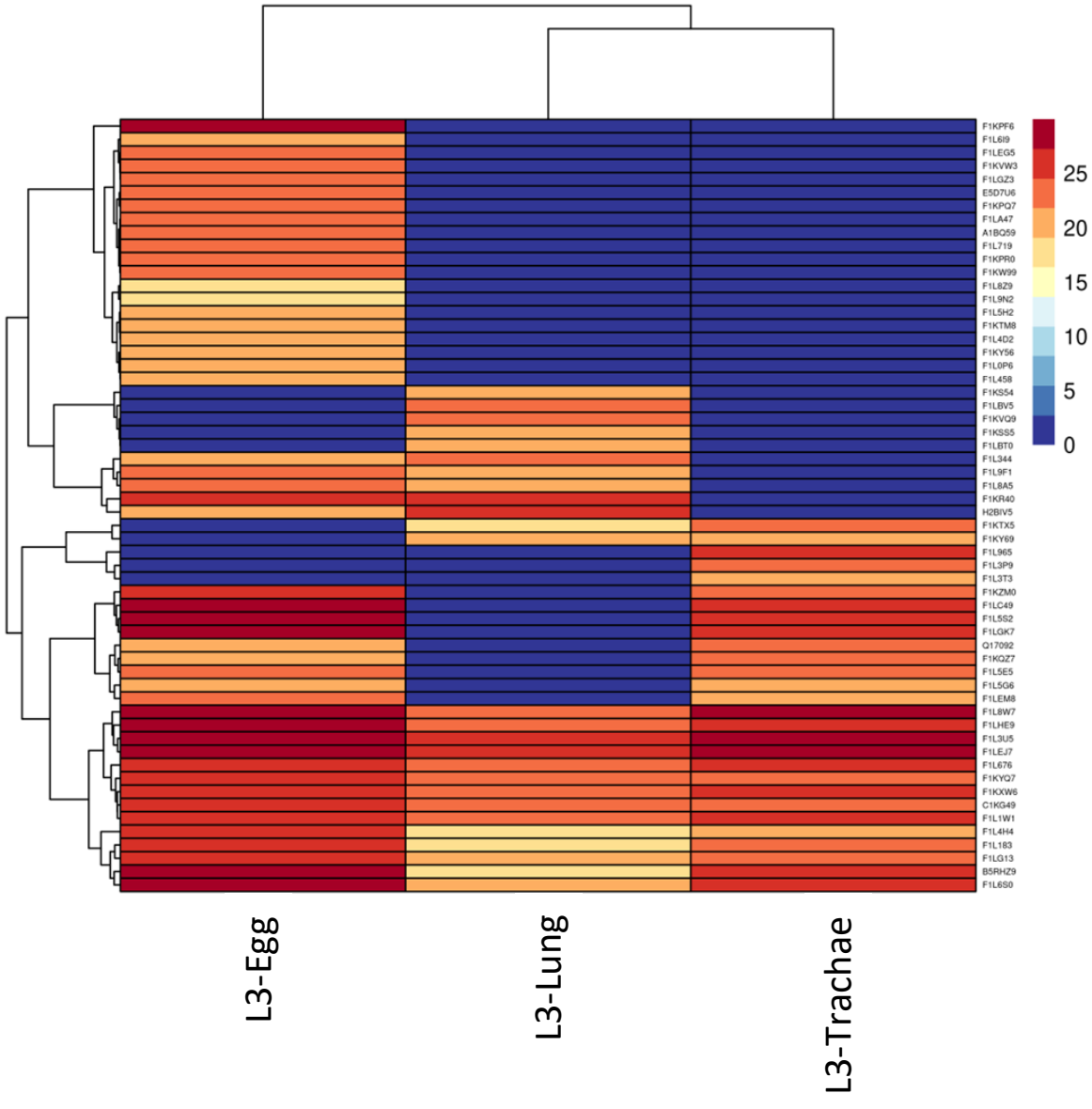
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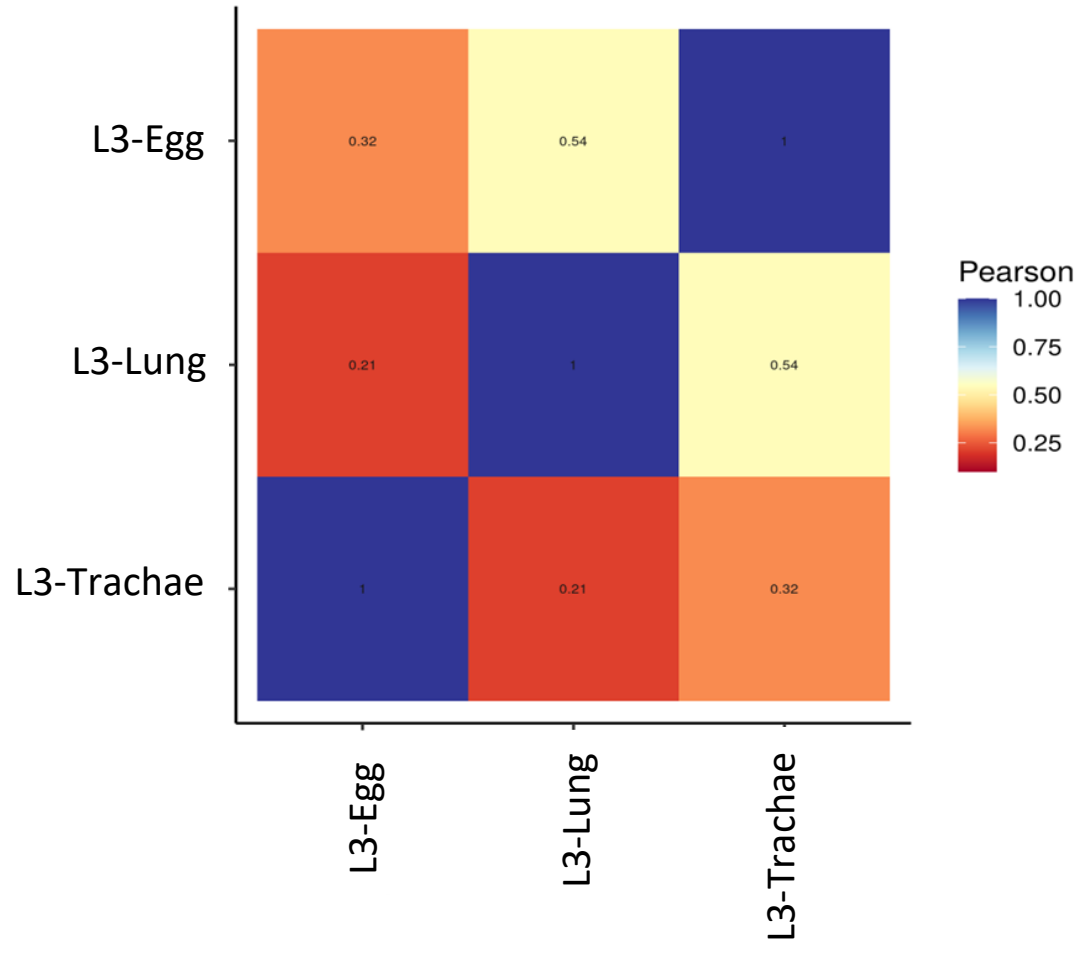
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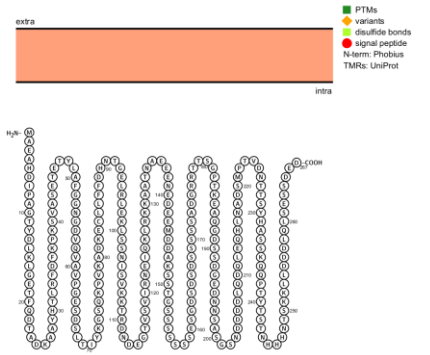
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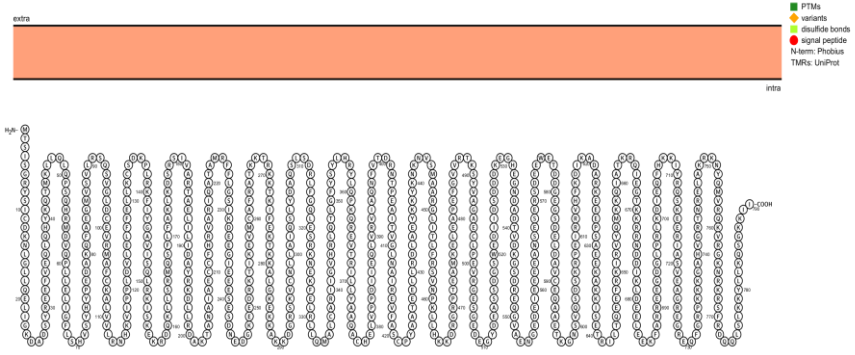
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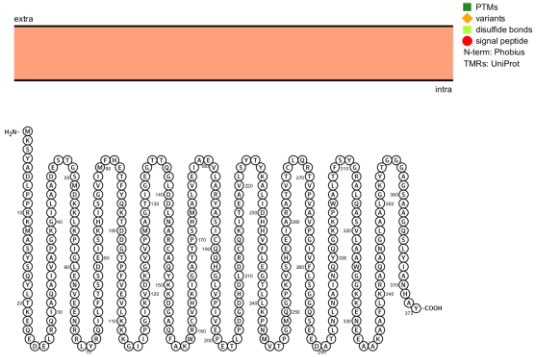
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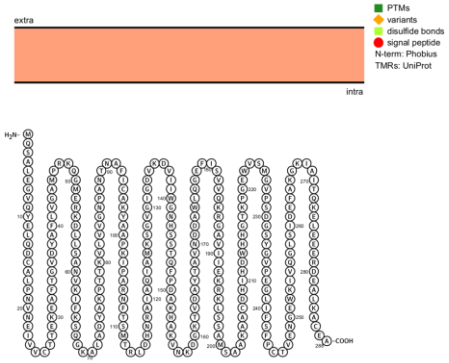
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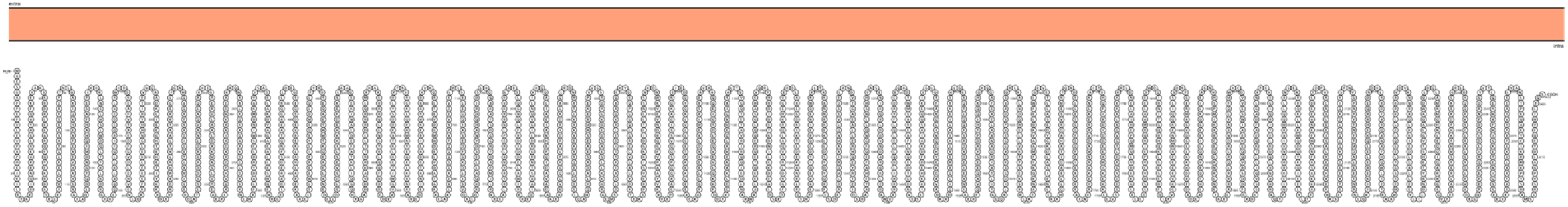
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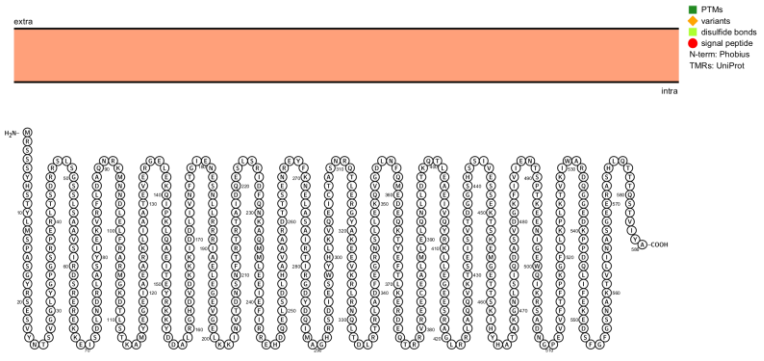
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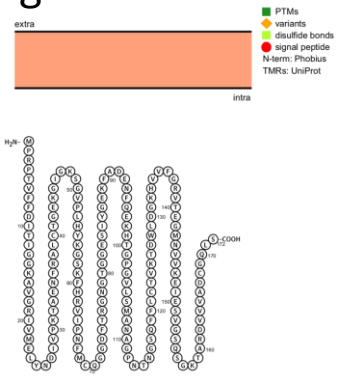
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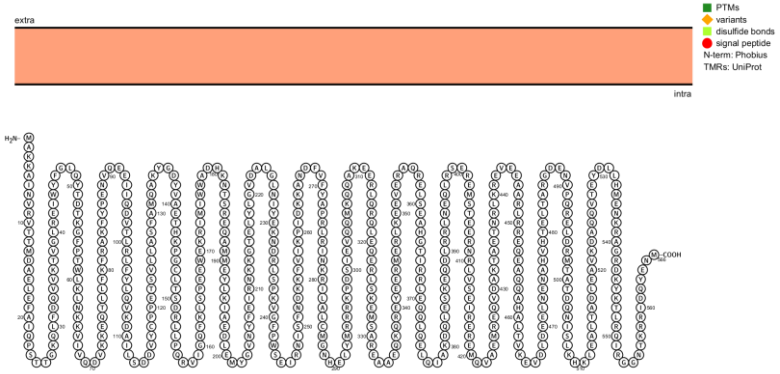
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REVIEW
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Detangling the Crosstalk Between *Ascaris, Trichuris* and Gut Microbiota: What´s Next?

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Helminth infections remain a global public health issue, particularly in low- and middle-income countries, where roundworms from the*Trichuris* and *Ascaris* genera are most prevalent. These geohelminths not only impact human health but most importantly also affect animal well-being, in particular the swine industry. Host-helminth parasite interactions are complex and at the same time essential to understand the biology, dynamics and pathophysiology of these infections. Within these interactions, the immunomodulatory capacity of these helminths in the host has been extensively studied. Moreover, in recent years a growing interest on how helminths interact with the intestinal microbiota of the host has sparked, highlighting how this relationship plays an essential role in the establishment of initial infection, survival and persistence of the parasite, as well as in the development of chronic infections. Identifying the changes generated by these helminths on the composition and structure of the host intestinal microbiota constitutes a field of great scientific interest, since this can provide essential and actionable information for designing effective control and therapeutic strategies. Helminths like *Trichuris* and *Ascaris* are a focus of special importance due to their high prevalence, higher reinfection rates, resistance to anthelmintic therapy and unavailability of vaccines. Therefore, characterizing interactions between these helminths and the host intestinal microbiota represents an important approach to better understand the nature of this dynamic interface and explore novel therapeutic alternatives based on management of host microbiota. Given the extraordinary impact this may have from a biological, clinical, and epidemiological public health standpoint, this review aims to provide a comprehensive overview of current knowledge and future perspectives examining the parasite-microbiota interplay and its impact on host immunity.

Keywords: microbiota, helminths, *Trichuris*, *Ascaris*, host-parasite interactions

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INTRODUCTION

Helminth infections represent a major global public health burden, primarily in developing countries, where lack of basic water, hygiene and sanitation conditions are widespread (Harhay et al., 2010; Hotez et al., 2014; Ojha et al., 2014; Silver et al., 2018) due to stressed socioeconomic conditions. Although many helminth infections are asymptomatic, they can display a variety of

clinical presentations, particularly in children, where they are usually associated with growth retardation, malnutrition, and diarrheal disease, among other conditions. Helminth infections have also been linked with a decreased occurrence of autoimmune diseases presumably based on their immunomodulatory capacity (Cooper and Figueiredo, 2013; Girgis et al., 2013; Giacomin et al., 2015a; Maizels et al., 2018; White et al., 2020). It has been largely hypothesized that individuals with increased exposure to helminths during their first years of life are prone to immunomodulatory effects and immunological tolerance induced by parasites, thus decreasing their chances to develop allergic and autoimmune diseases, a phenomenon also known as the "hygiene hypothesis", later called the "old friends hypothesis" (Altmann, 2009; Chammartin et al., 2013; Stiemsma et al., 2015; Gazzinelli-Guimaraes and Nutman, 2018). It has also been proposed that for certain specific infections such as tuberculosis, helminths may play a relevant role in susceptibility to infection and disease progression, which is correlated with the immunomodulatory properties attributed to helminths (Karo-Atar et al., 2021). Among the most prevalent soil-transmitted helminths worldwide are the genera *Trichuris* and *Ascaris*, which not only affect human health but also have a significant impact on animals and livestock production (Hotez et al., 2008; Harhay et al., 2010; Pullan et al., 2014; Schüle et al., 2014; Else et al., 2020; Gordon et al., 2020).

Studies on the interaction between *Ascaris* and *Trichuris* helminths and host has allowed to determine that in addition to their recognized immunomodulatory capacity, these helminths reveal complex interfaces with the hosts gastrointestinal microbiota, a key factor that may play a fundamental role in parasite survival and persistence (Lee et al., 2014; Zaiss et al., 2015; Zaiss and Harris, 2016; Jenkins et al., 2017; Brosschot and Reynolds, 2018). This host-parasite relationship represents an emerging field of great scientific interest in helminthology and has therefore been subject of intensive scrutiny in recent years. Further evidence showing how the presence of these helminths can relate with changes in the composition and structure of host intestinal microbiota suggests that such interaction may play a determining part in the biology and pathophysiology of the parasite (Lee et al., 2014; Zaiss and Harris, 2016; Ajibola et al., 2019; Cortés et al., 2020; Schachter et al., 2020; Rosa et al., 2021; von Huth et al., 2021). However, contrasting evidence obtained mainly from descriptive studies examining human populations reveals that helminth infections do not exert a significant impact on their interaction with the host microbiota, highlighting that the use of different methodological approaches may derive in heterogeneous results (Cooper et al., 2013; Mejia et al., 2020). Nevertheless, to control potential confusion variables, both for *Trichuris* and *Ascaris*, murine and porcine animal models have been developed and implemented to model the infection process in humans and thus study parasite-host-microbiota interactions, evaluating the changes caused by these helminths in the microbiota in relation to its composition and structure and in order to examine helminth infections and their dynamic interaction with the intestinal microbiota. However, it is also important to highlight the limitations of animal studies, since the similarities and differences in terms of genetics, physiology, diet, etc. must be considered, which is why the use of humanized murine models has been chosen to replicate infection scenarios in humans more efficiently (Lewis et al., 2006; Klementowicz et al., 2012; Nguyen et al., 2015; Williams et al., 2017; Wang et al., 2019; Park and Im, 2020; Yousefi et al., 2021). Likewise, the development and advancement of new sequencing platforms and techniques has promoted a great increase in the availability of data from different approaches. This, together with the continuous innovation of bioinformatics tools, has established novel frameworks that offer interesting strategies in the field of parasite-host-microbiota interactions (Wooley et al., 2010; Simon and Daniel, 2011; Vollmers et al., 2017; Wajid et al., 2022).

Considering the relevance of this field of research, this review will examine in detail the interaction between the intestinal microbiota and helminth parasites, *Trichuris* and *Ascaris*, as well as how these interactions can be potentially influence infection, dissemination, persistence, and survival in the human host.

RELEVANCE OF *TRICHURIS* AND *ASCARIS* HELMINTHS

Trichuriasis is caused by the human whipworm *Trichuris trichiura*, which typically exhibits high parasite burdens that usually translate clinically into a severe constellation of symptoms including diarrhea, abdominal pain, malnutrition, stunted growth and developmental arrest of infected children (Chammartin et al., 2013; World Health Organization Soil-transmitted helminth infections, 2021). The *Trichuris* (Nematoda: Trichuridae) parasite is one of the most highly prevalent intestinal helminths worldwide, mainly in Africa and South America, and is estimated to infect about 500 million people globally (Hotez et al., 2014; Pullan et al., 2014; World Health Organization Soil-transmitted helminth infections, 2021). *Trichuris* was predicted to be responsible for the loss of 0.64 million disability-adjusted life years between 1990 and 2010, ranking tenth amongst all neglected tropical diseases (Hotez et al., 2014).

Similarly, infections to *Ascaris* (Nematoda: Ascarididae) species are also amongst the most cosmopolitan helminth infections worldwide and also included within the Neglected Tropical Diseases roster (Saboyá et al., 2013; Pullan et al., 2014; World Health Organization Soil-transmitted helminth infections, 2021). The overall prevalence of helminth infections exceeds 10% in most developing countries with a large percentage of these attributable to *Ascaris lumbricoides* infections, mostly in endemic regions of Africa and South America (Hotez et al., 2014; Pullan et al., 2014; World Health Organization Soil-transmitted helminth infections, 2021). Ascaridiasis has also been associated with malnutrition and developmental delay due to persistent infections, and the

disease burden is estimated at around 60,000 deaths per year, mostly in children (Harhay et al., 2010; Hotez et al., 2014; Pullan et al., 2014; Jourdan et al., 2018; Corvino et al., 2021) .

A challenging aspect in the management of this parasitic infections is its well-known resistance to routine anti-helminth therapy and thus, their proneness for reinfections, which has stirred the search for new therapeutic alternatives and the development of potential vaccines. This acquires more relevance in endemic regions, where recurrent infections with low parasite loads may lead to chronic and persistent infections (Harhay et al., 2010; Ramanan et al., 2016; Midha et al., 2017; Papaiakovou et al., 2021; Whitman et al., 2021). Factors such as the highly resistant nature of the eggs in moist soil, long standing patency, high rate of reinfections despite effective anti-helminth therapy and the unavailability of vaccines, have all been associated with a high dispersal of *Ascaris* and *Trichuris*, not only in human hosts but also in other vertebrate hosts (Schüle et al., 2014; Easton et al., 2019; Cools et al., 2021). In addition to the human parasite *A. lumbricoides*, ascariasis infection of swine by the highly prevalent species *Ascaris suum* is also known for its pathogenic effects in domestic pigs and its impact in livestock productivity (Dold and Holland, 2011; Velasco et al., 2011; Cooper and Figueiredo, 2013; Miller et al., 2015; Katakam et al., 2016; Williams et al., 2017; Midha et al., 2018; Hansen et al., 2019; Arora et al., 2020; Easton et al., 2020).

HOST-HELMINTH-GUT MICROBIOTA INTERACTIONS

In an attempt to better understand the host-helminth-gut microbiota relationships, most studies have focused on deciphering interactions between helminths and the host immune system given the important immunomodulatory and immunoregulatory effects exerted by these parasites and how this dynamic regulation influences infectiveness, dissemination and persistence in the infected host (McSorley and Maizels, 2012; Giacomin et al., 2015a; Briggs et al., 2016; Maizels et al., 2018; Yasuda and Nakanishi, 2018; White et al., 2020). Also, recent studies have explored the functional consequences of helminth-induced changes in microbiota and its impact in parasite biology and infection dynamics. Interestingly, changes in the relative abundance of certain bacterial taxa as well as the diversity of the intestinal microbiota in presence of helminths have shown to display profound effects on the parasite life cycle by inducing metabolic changes that could promote infectiveness and dissemination, while modulating the host immune response leading to persistence (Berrilli et al., 2012; Li et al., 2012; Cooper et al., 2013; Cantacessi et al., 2014; Glendinning et al., 2014; Lee et al., 2014; Kay et al., 2015; Zaiss et al., 2015; Giacomin et al., 2015b; Midha et al., 2017; Midha et al., 2018; Rosa et al., 2018; Su et al., 2018; Ajibola et al., 2019; Easton et al., 2019; Wang et al., 2019; Mejia et al., 2020; Rosa et al., 2021). Understanding the mechanisms by which helminth parasites influence the complex microbiota ecosystem of the host and how these changes functionally impact the host intestinal microbiota is critically important, as it will pave the way in search of new control strategies and therapeutic approaches.

Studies addressing the relationship of parasites with host microbiota have shown variable results, particularly in human-based descriptive studies (Cooper et al., 2013; Cantacessi et al., 2014; Jenkins et al., 2017; Cortés et al., 2020). This in part, because many methodological and inherent aspects within study groups are prone to introducing biases, thus generating diverse results and conclusions, such as those related to the study population, impact of diet, environmental factors as well as genetic background (Cooper et al., 2013; Rosa et al., 2018; Rinninella et al., 2019). A critical evaluation of current methodologies deserves a separate and dedicated review. However, in light of the above, the use of animal models, essentially pigs and mice, despite certain limitations that must be carefully considered, has allowed to evaluate changes caused on the intestinal microbiota in presence of helminths, which will potentially provide relevant and applicable information to understand infection in humans (Nguyen et al., 2015; Park and Im, 2020). Such approach lends itself to better monitor and control the infectious process and the many variables that influence the composition of the intestinal microbiota of the host (Lewis et al., 2006; Klementowicz et al., 2012; Houlden et al., 2015; Kreisinger et al., 2015; Martín et al., 2016; Douglas, 2019; Wang et al., 2019; Rosa et al., 2021).

One of the first approaches attempting to identify potential relationships between helminth infections and the host derived factors dates back to 1967 by Wescott *et al*, who using a comparative murine model including both germ-free versus conventional mice, showed that infection by the roundworm *Heligmosomoides polygyrus*, (previously *Nematospiroides dubius*) evolved and persisted in conventional mice, thus suggesting, that factors related to the microbiota were directly related to the development and pathophysiology of this parasite (Wescott, 1968). Since then, numerous studies have been carried out with a variety of other helminths to elucidate how this parasite-mammalian microbiota-host relationship influences host immunity and disease course (Glendinning et al., 2014; Reynolds et al., 2015; Gause and Maizels, 2016; Leung et al., 2018; Midha et al., 2021).

HOST-HELMINTH-GUT MICROBIOTA INTERACTIONS: *TRICHURIS*

Several descriptive animal model studies have been implemented in an effort to characterize helminth and host microbiota interactions and to determine the effects of infection on host microbiota composition. One of these studies, which implemented a murine model aiming to evaluate helminth-microbiota interactions in *Trichuris muris* infection, offered the view that infection, persistence, and chronicity with this parasite were associated with changes in the composition of the host microbiota. Moreover, further evidence from *Escherichia coli* cultures revealed that the presence of this bacterium allowed a much quicker and more efficient hatching of *T. muris* eggs, hence

promoting the establishment of infection (Hayes et al., 2010). Subsequently, analyses carried out in a swine animal model, reinforced the idea that the presence of *Trichuris*, in this specific case of *Trichuris suis*, was also associated with changes related to the intestinal host microbiota. Here the researchers identified that changes occurred mainly in the proximal colon exhibiting an increased presence of the *Mucispirillum*, *Succinivibrio* and *Ruminococcus* genera. Likewise, alterations in fatty acid and carbohydrate metabolism were also evident suggesting a possible link to changes at the microbiota level and providing evidence on the potential effects of parasite influence on the enteric microbiota (Li et al., 2012; Wu et al., 2012).

In 2015, Houlden et al. examined how chronic *Trichuris muris* infection could potentially induce changes in microbiota of C57BL/6 mice (Houlden et al., 2015). Here, the authors identified significant microbiota changes occurring between 14- and 28-days post-infection and which persisted up to 91 days of follow up. Such changes were reflected in the relative abundance of certain taxa, alpha and beta diversity of infected mice and predominantly in *Prevotella* and *Parabacteroides* populations. Most importantly, these changes in the microbiota correlated with changes at the metabolome level, which in agreement with previous studies, revealed a reduction in carbohydrate metabolism and a concomitant increase in certain amino acids, a scenario known to promote a permissive environment for parasite development. However, one of the most interesting findings of this study was the fact that, upon elimination of *Trichuris* infection, a complete recovery of the host intestinal microbiota followed with a simultaneous regaining of the Th1 cell population, which was initially skewed to a Th2 profile during the course of infection. This finding is of utmost importance from the helminth-host interface perspective, since it suggests that *Trichuris* infection is capable of evoking immune regulatory mechanisms and adaptations to the host enteric niche as well as modifying the intestinal microbiota composition favoring parasite persistence, with subsequent restoration of homeostasis after infection (Houlden et al., 2015).

Unquestionably, results obtained from animal models offer very interesting observations that fuel knowledge on parasite-microbiota-host interactions while providing fertile ground for further investigations in the field. However, and despite that animal models may accurately replicate *Trichuris* infection as seen in humans, to date, no experimental comparisons have been trialed in order to validate consistent microbiota changes between animal and human models. In a more recent study, Rosa et al. were able to identify changes in the relative abundances of certain bacterial taxa in the context of *Trichuris* infection, which were consistent in both mouse and human microbiota (Rosa et al., 2021). This study is of particular significance as it allowed to experimentally validate the use of *Trichuris muris* infection in mice as a model to assess *Trichuris*-induced modifications of the human gut microbiome (Rosa et al., 2021). Eleven specific bacterial genera of interest were identified, mainly *Escherichia* and *Blautia*; hence, reinforcing the hypotheses of synergy among *Trichuris* and certain bacterial genera to promote infection by inducing changes in the composition and structure of the intestinal host microbiota (Rosa et al., 2021). At the same time, this study served to validate how animal models offer an important methodological advantage in picturing the many aspects related to host-helminth-gut Microbiota interactions, and its application specifically for the case of *Trichuris* sp. Likewise, a study conducted in Tanzania that sought to determine interactions between parasitic helminths and intestinal microbiota in wild tropical primates from Tanzanian habitats, showed that *Trichuris* sp. was associated with a higher bacterial richness and diversity. These findings could have a relevant ecological impact on different primate populations, highlighting again the importance of interactions between helminths and host microbiota (Barelli et al., 2021).

One of the most conclusive studies examining the *Trichuris*-host microbiota regulation hypothesis was recently published by Schachter et al., in 2020 (Schachter et al., 2020). In their study, using a Swiss Webster mouse model, the authors confirmed the occurrence of changes in the intestinal microbiota and the important immunomodulatory effects induced by *Trichuris muris*, which are essential for the establishment of infection, progression of the parasite's life cycle and persistence, as well as to promote chronic infections (Schachter et al., 2020). Furthermore, a subset of dexamethasone-induced tolerant mice mirroring a stage of chronic infection, was followed in order to record changes in the intestinal microenvironment. Here, *T. muris* infected mice developed weight loss, anemia, imbalance of the intestinal microbiota with a significant increase of *Escherichia coli* and *Bacteroides* bacteria, enhanced tissue damage at the intestinal level and changes in the Th1/Th2 immune profile (Schachter et al., 2020). These findings reinforce the fact that certain bacterial taxa are essential for an adequate development of the helminth, while concurrently promoting a permissive intestinal environment for it to thrive. It also emphasizes the need to better understand these interactions in order to elucidate those mechanisms linked to clinical progression of the infection and ultimately to evaluate and implement novel control and treatment strategies (Gazzinelli-Guimaraes et al., 2020; Schachter et al., 2020).

Many of the results found in previously described animal models have been consistent with those evidenced in studies with human populations. Lee et al., 2014, conducted an analysis in an indigenous human population in Malaysia, where they observed an increase in the diversity and abundance of the intestinal microbiota in *Trichuris*-infected subjects, with a preponderance of members of the Paraprevotellaceae family, raising evidence that helminths may have an impact in modulating diversity, bacterial community structure and function of the intestinal microbiota of human hosts, being consistent with findings in mice and primates (Lee et al., 2014; Houlden et al., 2015; Barelli et al., 2021). Interestingly, a previous study carried by Lee et al. hypothesized that the presence of *Trichuris trichiura* was not associated with alterations in the host microbiota, as opposed to *Ascaris lumbricoides* (Cooper et al., 2013). This study conducted in Ecuadorian children failed to demonstrate any changes in the composition and structure of the intestinal microbiota,

highlighting the variation across human studies and demonstrating that discrepancies may be related to other aspects inherent to the populations evaluated such as age, diet, lifestyle, etc.

A recent systematic review evaluated the impact on the microbiota caused by helminths. This study found that in the case of *Trichuris* infection there was a positive association with *Treponema* sp., *Anaerovibrio* sp., *Rikenellaceae* and multiple species of Prevotellaceae. The authors propose that changes in the Firmicutes/Bacteroidetes (Bacillota/Bacteroidota) ratio, which has been shown to be a biologically relevant ratio, are strongly related to the presence of helminths, causing a shift from Bacteroidetes (Bacteroidota) to Firmicutes (Bacillota) and Clostridia, suggesting that helminth-induced enterotyping may have implications for host health (Whitman et al., 2018; Kupritz et al., 2021). Likewise, in general, helminth infections are associated with greater richness and abundance and this difference is even more tailored when co-infections between different helminths occur, thus highlighting that the interaction of helminths with the host has a great relevance in the changes that can be potentially associated with health and disease (Kupritz et al., 2021).

Despite the number of studies carried out so far, the specific mechanisms involved in the *Trichuris*- enteric microbiota interaction remain largely unexplored. Evidence from different studies suggests that *Trichuris* infection is associated with changes in the host microbiota that can potentially lead to increased susceptibility to infection and persistence (Houlden et al., 2015; Vejzagić et al., 2015; Gazzinelli-Guimaraes et al., 2020; Rubel et al., 2020; Rosa et al., 2021), however, further studies are needed to confirm this hypothesis and to understand the mechanisms involved (**Figure 1**). Remarkably, the predominance of certain bacterial taxa has been associated with a better and more efficient hatching of *Trichuris* eggs, as demonstrated in the case of *Escherichia coli* (Wescott, 1968; Rosa et al., 2021). Survival and persistence of the parasite in the intestinal niche is in part driven by modulation of the immune response as a consequence of changes induced at the microbiota level. Likewise, changes related with bacterial composition of the microbiota, particularly the Bacteroidetes (Bacteroidota) (Whitman et al., 2018), including the genera *Prevotella*, *Parabacteroides*, *Paraprevotella*, among others, press for directing further studies in this area since the mechanisms involved in the relationship with specific taxa are yet unclear.

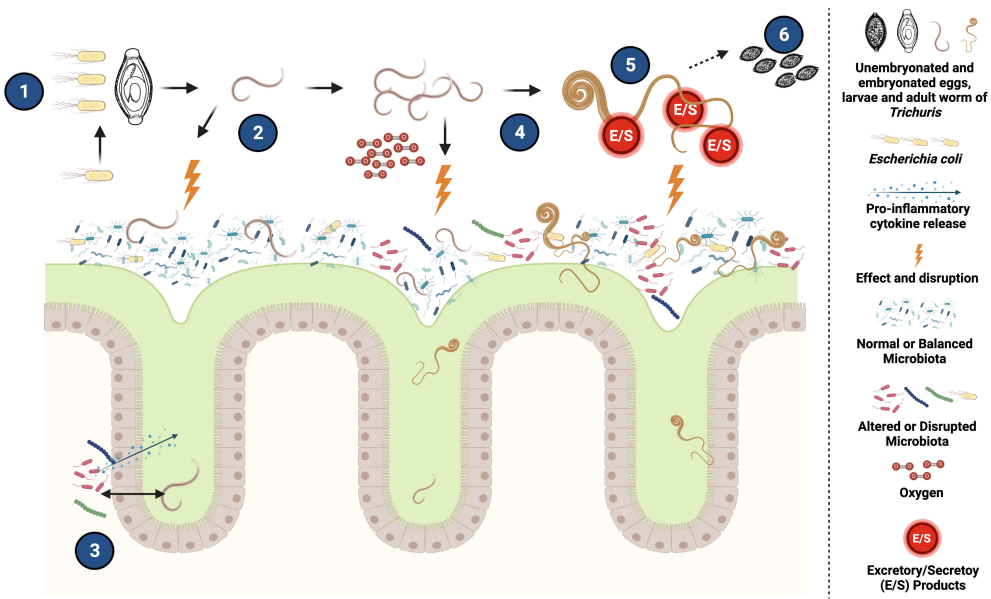

FIGURE 1 | Potential mechanisms of interaction between *Trichuris* and the host intestinal microbiota. (1) After ingestion of the infective stage of *Trichuris*, it has been demonstrated that certain bacteria, such as *Escherichia coli*, can improve the hatching of the eggs, contributing to the establishment of the infection and the development of the parasite. (2) The presence of eggs and released larval stages could cause changes by direct interaction with the host microbiota and by the inflammatory response generated by the parasite (3) Likewise, damage to the intestinal epithelium caused by *Trichuris* can cause translocation of bacteria into the intestinal lumen that may trigger localized inflammatory responses that could potentially affect certain commensal bacterial community of the microbiota influencing the composition and structures of the host bacterial communities. (4) The presence of the parasite in the intestine, together with the changes previously caused in the microbiota, generate an increase in oxygen availability and cause a decrease in strict anaerobic bacteria essential for intestinal health (such as *Faecalibacterium*) and facilitate the increase of facultative aerobes such as some Enterobacteriaceae that in turn contribute to the development of the infection. (5) Adult forms of *Trichuris* can generate Excretory/Secretory (E/S) products that, in addition to having an immunomodulatory effect, can have an impact on certain bacterial groups of the intestinal microbiota, modifying its structure and composition. (6) The adult forms produce eggs that that will be eliminated in the feces. Created with BioRender.com.

In addition to that concerning to the composition and structure of the microbiota, defining the changes related to the metabolome is perhaps another prominent field of study. Available evidence suggests that in presence of chronic *Trichuris* infection, there is an increased concentration in fresh stool samples of amino acids such as phenylalanine, serine, threonine, leucine, ornithine and glycine, with a decrease in the abundance of vitamin D2/D3 derivatives, fatty acids such as butyrate and glycerophospholipid metabolites related with the mice control group (Houlden et al., 2015). These changes appear to be associated with distinct metabolic profiles in *Trichuris* infection while reflecting how this parasite in context of an altered microbiota may utilize different host resources to enable infection and survival. In this sense, *Trichuris* infection seems to trigger a number of helminth-induced changes in the intestine that lead to a less favorable environment for strict anaerobic bacteria, which are responsible for butyrate production and maintenance of intestinal homeostasis, thus, altering bacterial composition and structure to promote its survival. An example of this is the increase in abundance of facultative anaerobes such as *Escherichia coli*, which has shown to be a determinant factor in the hatching of *Trichuris* eggs.

It is also worth mentioning that beyond direct parasite-bacteria interactions, indirect interactions mediated by parasite derived molecules, can also play a role in the immunomodulatory effects leading to modifications of the structure and composition of the host microbiota. For example, it has been described that *Trichuris* exhibits the ability to generate excretion/secretion (E/S) products containing different molecules to facilitate survival and persistence of the helminth. A recent study demonstrated that amongst these E/S products, the p43 protein shows the ability to inhibit IL-13, which is an essential effector cytokine necessary for parasite clearance (Bancroft et al., 2019). Thus p43 exerts its immunomodulatory effects by allowing not only the survival of *Trichuris*, but also progression to chronic stages of infection (Bancroft et al., 2019). These types' of helminth-derived immunomodulatory molecules may also interact indirectly with the host microbiota leading to possible changes in diversity and abundance and at the same time favoring parasite invasion with its consequent pathophysiological chain of events (Bancroft et al., 2019; Schachter et al., 2020; Lawson et al., 2021; Midha et al., 2021).

It is evident from the different studies carried out on *Trichuris*-microbiota-host interactions that this helminth inflicts significant effects on the intestinal microbiota by means of different mechanisms (**Figure 1**). These changes affecting the composition and structure of the host microbiota may lead to a complex regulatory cascade in the intestinal niche which favor helminth infection, survival and persistence, ultimately impacting the course of infection. More studies focusing on how this parasite affects the composition of the enteric microbiota and its impact on host biology are required.

HOST-HELMINTH-GUT MICROBIOTA INTERACTIONS: *ASCARIS*

Studies on *Ascaris*-Microbiota-Host interactions have also been carried out from descriptive designs, both in human populations and animal-based models. A distinct feature for *Ascaris* is that, during this nematode's life cycle, short after ingestion of eggs, the larvae (L3) have the ability to invade mucosal tissue of the gastrointestinal tract and subsequently migrate towards hepatic and pulmonary tissues (Sultan Khuroo, 1996; Nogueira et al., 2016; Midha et al., 2021; Wu et al., 2021). In the lung, infection triggers and inflammatory response characterized by eosinophil infiltration (Wu et al., 2021). The migratory nature of certain parasite stages has also been linked to possible modifications on the composition and structure of the intestinal microbiota. An evidence of this is the fact that translocation of helminth associated bacteria through the intestine barriers can be traced short after, to the lung mucosa. In fact, bacterial translocation can trigger localized inflammatory responses that can potentially affect certain commensal bacterial community of the microbiota (Adedeji et al., 1989; Hübner et al., 2013; Midha et al., 2021). In a study by Cooper et al. in 2013, conducted in Ecuadorian populations the authors present evidence that *Ascaris lumbricoides* in contrast to *Trichuris* does impact intestinal microbiota of the host (Cooper et al., 2013). The authors also suggest that a single infection with *Ascaris lumbricoide*s or co-infection with other helminths can lead to alteration of the microbiota profile by exhibiting lower relative abundances of certain taxa, predominantly Clostridia class and higher relative abundances of bacteria belonging to the genus *Streptococcus*. Similarly, they propose that the presence of this helminth is linked to a lower diversity in the host microbiota (Cooper et al., 2013). These differences between *Ascaris* and *Trichuris*, may be influenced by several factors such as studying samples from a rural population or evaluating the presence of *Ascaris* in context of mixed infections with *Trichuris* and not as a single infection. The results described above were subsequently addressed in a swine model infected with *Ascaris suum*, which sought to find potential effects that could be extrapolated to studies in human infection (Wang et al., 2019). Here, the authors demonstrated that infection with this helminth significantly reduced the alpha diversity of the intestinal microbiota of pigs and that this effect was independent of the parasite load. Also, they were able to determine a differential relative abundance associated with a profile of at least 49 bacterial genera, including *Prevotella* and *Faecalibacterium* (Wang et al., 2019). Additionally, changes in the metabolic profile of stool samples from helminth-infected pigs was observed, noticing a decrease in carbohydrate and amino acid metabolism, in accordance with those studies conducted in *Trichuris* infections (Houlden et al., 2015). As for the case *Trichuris*, discordant results stand out amongst different studies. For example, while the study by (Wang et al., 2019) found a reduced alpha diversity in the intestinal microbiota of pigs infected with *Ascaris suum*, a previous study by Williams et al. in 2017 found that *Ascaris* modified the host's intestinal microbiota with an increased alpha diversity but a concomitant reduction in the relative abundance of the genera *Lactobacillus*, *Ruminococcus* and *Catenibacterium* (Williams et al., 2017). These observations underscore the importance of using appropriate epidemiological designs and animal models inclusive of a greater number of infection-associated variables

that can be controlled and impact on host intestinal microbiota changes, as well as the need to establish experimental protocols and reproducible bioinformatic analyses to allow validation of the results obtained in these research studies.

A recent study carried out in a Thai population, which assessed the presence of helminths, including roundworms (*Ascaris lumbricoides*), whipworms (*Trichuris trichiura*) and hookworms (*Ancylostoma* spp. and *Necator americanus*), allowed to identify changes in the microbiota of infected individuals (Stracke et al., 2021). Although no differences were apparent in reference to alpha diversity at the bacterial level, marked changes impacting beta diversity were noticeable. Likewise, modifications were found for certain bacterial taxa, where a greater abundance of *Akkermansia*, *Prevotella*, *Bacteroides coprophilus* and *Ruminococcus* and a decrease in *Bifidobacterium* was observed. The foregoing facts signal the influence that the presence of helminths such as *Ascaris* and *Trichuris* have on the composition and structure of the host's intestinal microbiota and how this may translate into the pathophysiology of the disease (Stracke et al., 2021). As previously discussed, a key aspect of this study is that it emphasizes that the uncertainty associated with the evaluation on the impact of helminth infection on the intestinal microbiome is probably not only related to the complexity of the interaction but also linked to the technical aspects of the experimental design and analytical approach, weighing the complex limitations involved in the process such as size sampling, collection, preservation and transport of samples, among others (Stracke et al., 2021).

One main aspect that needs to be considered in *Ascaris*-microbiota-host interactions is the fact that infection by this parasite can increase susceptibility to infection by other pathogens, mainly bacterial. In swine models, it has been shown that infection by *Ascaris suum* (Wang et al., 2019) and *Trichuris suis* (Li et al., 2012) are related to a higher relative abundance of *Campylobacter jejuni*, suggesting that these helminths could potentially increase the risk for secondary infections to this bacteria, an aspect of great relevance from a clinical and public health perspective.

As for the case of *Trichuris*, *Ascaris* derived excretory-secretory products may also promote an immunomodulatory response linked to indirect interactions with the intestinal microbiota of the host. E/S products, extracellular vesicles and other metabolites have been identified which may interact in different ways with the host microbiota and may play a key role in parasite biology and pathophysiology of infection (Hansen et al., 2019). In these lines, studies have confirmed that *Ascaris suum* has the ability to induce production of ASABF (*Ascaris suum* antibacterial factor) type antimicrobial peptides (Pillai et al., 2003; Midha et al., 2018) and microbial peptides of the cecropin family (P1 to P4) in the host. These substances have shown to exhibit bactericidal effect against a wide range of microbes, such as gram-positive (*Staphylococcus aureus, Bacillus subtilis* and *Micrococcus luteus*) and gram-negative (*Pseudomonas aeruginosa*, *Salmonella typhimurium*, *Serratia marcescens* and *Escherichia coli*) bacteria (Pillai et al., 2005). This highlights the ability of *Ascaris* to produce a number of nematode-derived metabolites which can exert important modulating effects on the host intestinal microbiota, thus constituting one of the most trending aspects in the field of helminth biology.

The biological characteristics of *Ascaris* infection, which includes multifaceted aspects such as extra-intestinal migration, modulation of the host immune response, and in context of this review, the changes it can inflict on the host's microbiota, altogether blends into a complex network of mechanisms that subvert the helminth-mammalian host relationship allowing the parasite to establish infection and promote its survival in the host (**Figure 2**). Studies in both humans and animal models have defined how *Ascaris* displays its ability to generate changes in both the structure and composition of the host microbiota *via* direct interaction or through production of metabolites and different E/S products, such as antimicrobial peptides, that can further restructure predominant bacterial community. This has a particularly detrimental effect on strict anaerobic bacteria which are essential for proper intestinal functioning such as the case of *Faecalibacterium* (Wang et al., 2019) and *Ruminococcus* (Williams et al., 2017). Alongside, a concomitant increase of facultative anaerobes such as *Streptococcus* (Cooper et al., 2013), which coupled with the immunomodulatory effects of this helminth, allows it to provide a suitable metabolic environment to ensure its survival and reproduction. Thus, it is evident that alterations and subsequent effects on the host microbiota have a fundamental role in the biology and pathophysiology of the parasite paving the way in search for therapeutic alternatives based on the reestablishment of normal host microbiota. Likewise, it is important to highlight that the immunomodulatory capacity of this helminth and its impact on the host microbiota can also promote a greater susceptibility to suffer reinfections or co-infections by other bacterial pathogens. Therefore, enhancing our knowledge on the mechanisms in play during early phases of infection are crucial to succeed in preventing adult worm establishment and the deleterious effects of helminth and host-derived factors during their interaction in the enteric milieu.

CONCLUSIONS AND FUTURE PERSPECTIVES

The study of parasite-host-microbiota interactions is part of a field of great interest as it can provide new insights and provide key aspects in the biology, dynamics and pathophysiology of infection. The high prevalence of helminths in developing countries, high rates of reinfection and resistance to anthelmintics and current unavailability of vaccines against these parasites, stress the need to focus research into elucidating the mechanisms involved in these interactions and thus identify essential aspects pertaining the design of new strategies to control the spread of these helminths as well as novel therapeutic alternatives. In this review, we have included the nematodes *Trichuris* and *Ascaris* since they exhibit the highest prevalence and level of endemicity in low and middle-income countries, as well as to their notorious impact on animal

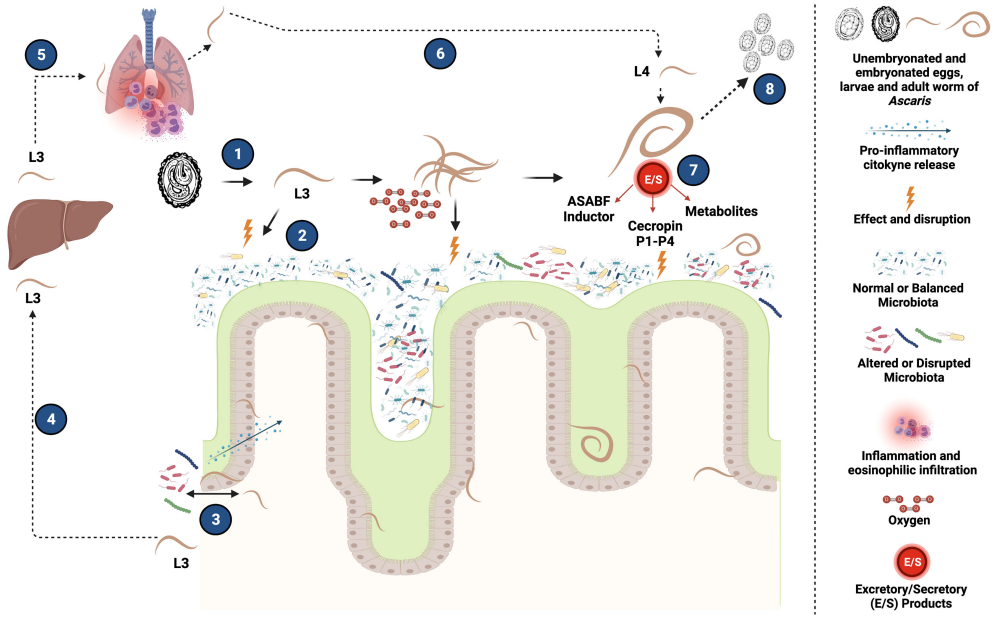

FIGURE 2 | Potential mechanisms of interaction between *Ascaris* and the host intestinal microbiota. (1) After ingestion of the infecting stage of *Ascaris*, L3 larvae are released from embryonated eggs into the host intestine. (2) These larvae can have an effect by direct interactions on the intestinal microbiota and thus propitiate an increase in oxygen availability that promote the increase of facultative aerobic bacteria and the decrease of strict anaerobic bacteria necessary for an adequate intestinal health, generating a disruption of the host microbiota. (3) Additionally, within the life cycle of *Ascaris*, damage to the intestinal epithelium caused to continue its migration produces the translocation of bacteria into the intestinal lumen, that may trigger localized inflammatory responses that could potentially affect certain commensal bacterial community of the microbiota influencing the composition and structures of the host bacterial communities (4) Migration of *Ascaris* L3 larvae to the liver and subsequently (5) from the liver to the lungs, allowing *Ascaris* to complete later phases of its life cycle, generating, among other things, inflammatory processes at the level of the pulmonary tissue characterized by a high infiltration of eosinophils and clinical symptoms such as those evidenced in Loeffler's Syndrome. (6) Larvae released from the lung can migrate back to the intestine and transform into L4, for later completing their development into adult forms. (7) These adult forms have the property of generating Excretory/Secretory (E/S) products and other metabolites with great immunomodulatory capacity, and also with antimicrobial function, such as ASABF inducers and Cecropin P1 to P4 proteins, that can modify the structure and composition of the host intestinal microbiota. (8) Finally, in an environment with now beneficial conditions for *Ascaris*, the adult forms have the ability to produce eggs that will be eliminated in the feces to continue its life cycle and infection process. Created with BioRender.com.

health and the swine industry (Sayasone et al., 2011; Walker et al., 2013; Ojha et al., 2014; Pullan et al., 2014; Dunn et al., 2016; Cortés et al., 2019; Hernández et al., 2019; Sobotková et al., 2019; Else et al., 2020; Gordon et al., 2020).

Different studies have shown that both *Trichuris* and *Ascaris* interact in important ways over the host microbiota through several direct or indirect mechanisms. This interaction provokes profound changes in the composition and structure of the microbiota allowing the parasite to establish, thrive and progress to chronic stages of infection. These changes promote a favorable metabolic, microbial and immunological environment for these parasites, which may lead to a greater susceptibility to acquire superinfections by other pathogenic bacteria such as *Campylobacter*, as has been demonstrated in swine models (Glendinning et al., 2014; Kreisinger et al., 2015; Loke and Lim, 2015; Giacomin et al., 2016; Midha et al., 2017, 2021; Williams et al., 2017; Leung et al., 2018; Lawson et al., 2021; Rosa et al., 2021). In light of these observations, it is essential to continue to conduct studies that allow us assess the potential benefits of probiotics as a novel and effective therapeutic alternative against helminth infections.

In addition, it is necessary to carry out studies, which examine not only bacterial community of the intestinal microbiota, but also eukaryotic community that are also an important component of this microenvironment. Despite advances in sequencing technologies and bioinformatics tools, most studies involving the intestinal microbiota are still limited to bacterial communities and very little is known about how eukaryotes and virus influence and determine the composition, structure and functioning of the host gut microbiota and its interaction with parasites. To date, different protozoa and fungi have been linked with potential changes in the host gut microbiota (Mason et al., 2012; Iebba et al., 2016; Nagel et al., 2016; Stensvold and van der Giezen, 2018; Partida-Rodriguez et al., 2019; Castañeda et al., 2020). Also, it is important to consider that helminth infections can coincide with other protozoa defining intestinal polyparasitism events (Sayasone et al., 2011; Salcedo-Cifuentes et al., 2012; Cimino et al., 2015; Donohue et al., 2019; Geus et al., 2019; Gordon et al., 2020). For example, studies have demonstrated how the presence of the protozoan parasite *Blastocystis* can induce changes in the diversity and abundance of the bacterial microbiota, favoring an increase in taxa such as

Faecalibacterium while decreasing others such as *Bacteroides*. This is similar to the well-known beneficial relationship of this protozoan with the host microbiota by generating an anti-inflammatory environment (Audebert et al., 2016; Iebba et al., 2016; Castañeda et al., 2020). Another case of mutualistic interaction has been described for the protozoan *Tritrichomonas musculis*. This eukaryote shows the ability to activate the host epithelial inflammasome inducing the production of IL 8, which in turn promotes activation of the Th1/Th17 axis, eliciting protection against bacterial infections of the mucous membranes, and ultimately preventing the potential increased risk of suffering inflammatory diseases (Chudnovskiy et al., 2016). Both animal and *in vitro* studies have equally demonstrated that protozoa such as *Giardia* exhibit the capacity to promote alterations in host microbiota, leading to an increased predisposition to develop inflammatory and metabolic diseases (Iebba et al., 2016; Barash et al., 2017; Toro-Londono et al., 2019). As well as eukaryotes interact with the intestinal microbiota, viruses can also influence changes in its composition. The main proportion of the human virome is constituted by bacteriophages which play a key role in the structuring and flow of information amongst bacterial and microbiota components, thus, regulating in a critical way co-evolution events shared between both of these components (Moreno-Gallego et al., 2019; Mukhopadhya et al., 2019; Hufsky et al., 2020). Similarly, it has been hypothesized that the enteric virome may be intimately linked to the etiopathogenesis of gastrointestinal diseases. Both bacteriophages and eukaryote derived viruses may infect host cells and undermine the host's own bacterial microbiota under certain conditions, triggering immunological responses which induce changes in the structure and composition of the microbiota. Therefore, it is essential to recognize not only the role of bacteria, but also eukaryotes and viruses and their interaction across the host-helminth-gut Microbiota interface, as this may reveal key information about the mechanisms associated with these interactions.

Furthermore, it is imperative to look into the helminth's own microbiota, since it not only defines the transient microbiota specific to each stage of the life cycle and its relationship with the host, but also because it can reveal mutualistic associations between certain bacterial groups and the parasite which may prove essential to its biology and ability to infect and disseminate (Jenkins et al., 2019; Formenti et al., 2020). For example, *Wolbachia* bacteria have been found in nematodes of the family Onchocercidae, a family of filarial nematodes, which includes clinically relevant species known to cause lymphatic filariasis and onchocerciasis and which are of vital importance to the parasite since they provide essential metabolites to the filariae as part of a symbiotic relationship (Bouchery et al., 2013). Bacteria of the genus *Neorickettsia* have also been identified in *Fasciola* trematodes through genomic analysis, thus, suggesting a potential mutualistic relationship between these organisms (McNulty et al., 2017). Very little is known about the establishment, structure and function of the microbiota residing in these nematode parasites and therefore, there is a critical need to evaluate these characteristics in an attempt to better decipher the interactions between the parasite and the host microbiota.

There are still many gaps in knowledge on how interactions between the parasite and host microbiota may confer biological advantages that facilitate infection and dissemination, and how these relate to the pathophysiology and clinical manifestations of infection and disease. In order to better understand the complex mechanisms involved in these parasite-microbiota-host interfaces, both in humans and animal models, it becomes critical to design multifaceted investigative approaches (**Figure 3**). Metagenomic analyses using second- and third-generation sequencing technologies can yield key information related to the structure and composition of the microbiota, including prokaryotic, eukaryotic and viral profiles. Such approach can also facilitate the prediction of genes and metabolic pathways that may be differentially expressed in helminth-infected individuals. Transcriptomic and spatial transcriptomic (that enables high-resolution assessment of spatial gene expression across tissue sections) studies will allow evaluation on the differential behavior of gene expression in the enteric niche (or respiratory tract as for the case of *Ascaris*), provide information related to tissue tropism, location and migration patterns of the parasite. Finally, metabolomics mass spectrometry-based studies, for example, from feces and blood samples, could potentially contribute to our knowledge on chemical and metabolic changes related to helminth infections, and how these correlate with alterations in the composition and structure of microbial communities. It is important to emphasize at this point that it is indispensable to carry out these studies under these methodological approaches in such a way that their reproducibility can be assured. It is essential to control, mainly in descriptive studies with humans, the multiple variables that can influence the composition of the microbiota and that can produce biases in the results if they are not appropriately controlled (age, gender, rural or urban population, diet, breastfeeding, route of birth, among others) (Sadowsky et al., 2017; Rinninella et al., 2019). The use of animal models has favored standardization of processes allowing adequate control of most of the variables involved in microbiota analysis. However, it is important to emphasize that in order to translate results derived from mouse studies into a human context, it is necessary to take into account similarities and differences between the gut microbiota of both mice and humans. The comparative physiology of the intestinal tract, the effect of dietary patterns, and differences in genetic backgrounds must also be considered. It is therefore essential to continue to develop and implement humanized gnotobiotic mouse models that allow more concrete and efficient extrapolation of knowledge to the human context (Nguyen et al., 2015; Hugenholtz and de Vos, 2018; Park and Im, 2020). Additionally, the ever-increasing availability of bioinformatics tools should be carefully examined to define their relevance according to the protocols and types of methodologies and analyses, allowing for greater comparability of studies and results. The information obtained from approaches such as those mentioned above are essential for the generation, evaluation and implementation of possible therapeutic alternatives and potential improvements in control mechanisms particularly in areas with

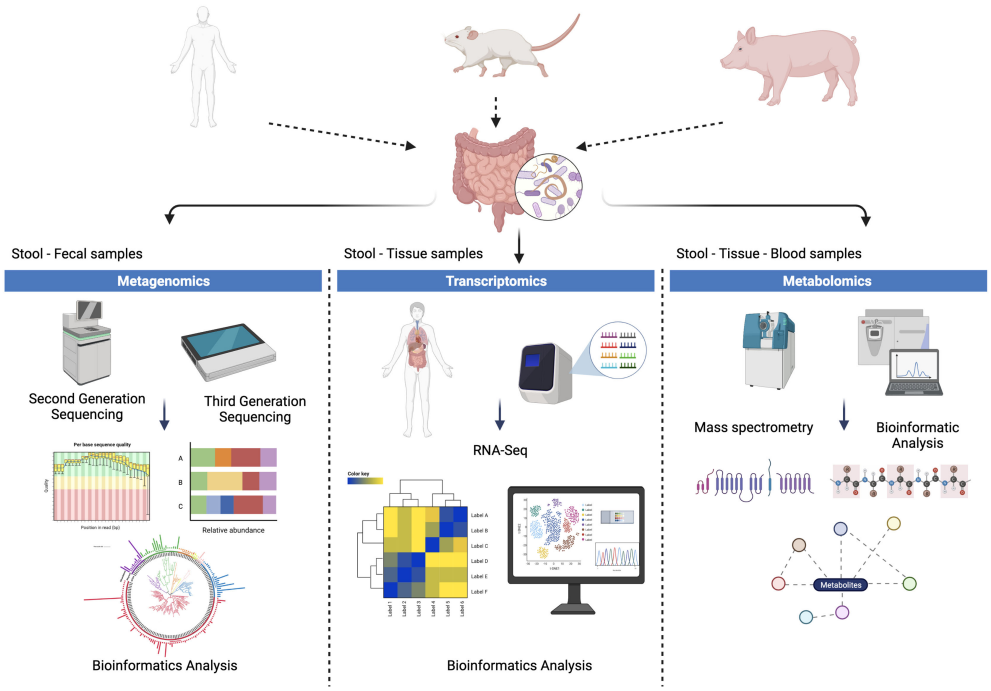

FIGURE 3 | Methodological approaches for the study of parasite-microbiota-host interactions. Studies in humans and in animal models, such as murine and porcine models, facilitate the evaluation of helminth-host microbiota interactions from different approaches such as: (i) metagenomic approaches by means of second and third generation sequencing, (ii) approaches based on transcriptomics and spatial transcriptomics and (iii) metabolomic approaches based on techniques such as mass spectrometry.

high prevalence of these parasites and where reinfections are frequent despite anthelmintic treatment

It has been identified that the microbiome can offer important advantages in the prediction and diagnosis based on the characterization of certain markers, both associated with health and with different types of diseases such as gastric cancer, and it has also been sought to extrapolate this advantage to the prediction of aspects related to parasitic infections in order to establish relationships and even to predict factors such as the parasitic load and the prognosis of the infection (Gupta et al., 2020; Zhang et al., 2021). In this context, Rubel et al. in 2021, in Cameroonian populations, from metataxonomic and metagenomic analyses and through the implementation of machine learning strategies such as Random Forest, managed to identify those members of the order Bacteroidales, which are common members of the intestinal mucosal surfaces, and are known as an important predictive taxon of infections caused by soil-transmitted helminths and conversely, that the presence of *Ruminococcus bromii*, may be a marker of protection against helminth infection (Rubel et al., 2020). This highlights the importance of focusing studies to identify markers that can facilitate the prediction, prognosis and diagnosis of parasitic infections caused by helminths.

Further studies are needed to validate this hypothesis and to establish an appropriate management framework for the potentially applicability and safe use of these alternatives. Thanks to advances in sequencing methodologies and bioinformatics tools and analyses, the development of these studies in humans and animal models will facilitate the integration of essential knowledge for the understanding of parasite-host interactions.

AUTHOR CONTRIBUTIONS

JR and SC conceived the objective of the review. SC, AP-M, and JR wrote and revised the final version of the manuscript. All authors contributed to the article and approved the submitted version.

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Parasite-Microbiota Interactions: A Pathway to Innovative Interventions for Chagas Disease, Leishmaniasis, and Ascariasis

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Abstract

Parasitic infections remain a significant global health challenge, largely due to their complex interactions with host microbiota and immune responses. Advances in microbiome research have revealed that the microbiota significantly influences disease outcomes and treatment efficacy. This perspective delves into how microbiota dynamics affect parasite transmission, pathogenesis, and treatment responses, with a focus on diseases like Chagas disease, leishmaniasis, and ascariasis. The microbiota can either exacerbate or mitigate disease severity, depending on its composition, providing critical insights for novel therapeutic strategies. Emerging approaches discussed include the use of targeted probiotics, prebiotics, and microbiota-modulating drugs to influence parasite dynamics and enhance conventional therapies. The perspective also explores the potential of integrating microbiota knowledge into vaccine design and immunotherapy, aiming to develop vaccines that elicit stronger immune responses and identify new therapeutic targets. A multidisciplinary approach is essential for translating these findings into effective clinical solutions, with future research focusing on validating microbiota-based interventions in clinical settings. In conclusion, the interaction between microbiota and parasitic infections presents a promising avenue for innovative therapies, with the potential to significantly improve global health outcomes.

Key words: Microbiota, Chagas disease, leishmaniasis, *Ascaris*, probiotics, vaccines

Article Highlights

- Parasite-microbiota interactions are crucial in understanding disease outcomes and developing therapeutic strategies.
- *Trypanosoma cruzi* (Chagas disease), prevalent in Latin America, leading to chronic complications like cardiomyopathy and gastrointestinal disorders.

- *Leishmania* spp. (leishmaniasis), affecting over 12 million people globally, causing significant morbidity and mortality.
- *Ascaris lumbricoides* (ascariasis), affecting nearly a quarter of the global population, particularly in regions with poor sanitation, causing malnutrition and stunted growth in children.
- *Trypanosoma cruzi* infection alters the gut microbiota, causing immune dysregulation that exacerbates disease symptoms, particularly in chronic stages like cardiomyopathy.
- Gut and skin microbiota can modulate immune responses, where certain bacterial communities may suppress or enhance inflammation, impacting disease outcomes.
- A more diverse gut microbiota is linked to improved immune regulation, potentially reducing infection severity in ascariasis.
- These parasitic infections share similarities in their interactions with the host microbiota, despite their distinct biological characteristics.
- The potential of microbiota-targeted therapies to improve disease outcomes is being recognized:
- Manipulating the gut microbiota could enhance immune responses and improve treatment efficacy for these parasitic diseases.
- Modulating the microbiota may help mitigate disease progression, providing a complementary therapeutic approach to conventional treatments.
- Comparative research across these three diseases highlights the possibility of broader microbiota-modulating strategies that could be effective across different parasitic infections, particularly in vulnerable tropical and subtropical populations.
- Understanding the interactions between the gut and skin microbiota and parasites like *T. cruzi*, *Leishmania* spp., and *A. lumbricoides* can inform novel therapeutic approaches.
- This review aims to shed light on the mechanisms through which microbial communities influence immune responses and disease outcomes, offering potential microbiota-targeted therapies that could benefit multiple parasitic diseases.
- By comparing the effects of microbiota across different parasitic infections, the review seeks to explore cross-cutting strategies for treating diseases that disproportionately affect at-risk populations.

1. Introduction

The study of parasite-microbiota interactions is gaining significant attention due to its profound implications for disease outcomes and therapeutic strategies. Parasites such as *Trypanosoma cruzi* (Chagas disease), *Leishmania* spp. (leishmaniasis), and *Ascaris lumbricoides* (ascariasis) are major global health threats [1–3] . Chagas disease (CD), endemic to Latin America, is notorious for its chronic complications, including cardiomyopathy and gastrointestinal disorders [2,4,5]. Leishmaniasis, which affects over 12 million people globally, presents in visceral and cutaneous forms, leading to significant morbidity and mortality [3,6]. Ascariasis, caused by the intestinal roundworm *A. lumbricoides*, affects nearly a quarter of the world's population, particularly in areas with poor sanitation, leading to malnutrition and impaired growth in children [1,7].

Recent studies have highlighted the crucial role of the microbiota in these infections. The gut microbiota significantly influences host immunity and parasite survival, impacting the severity

and progression of these diseases [8,9]. For instance, *T. cruzi* infection has been shown to alter the gut microbiota, leading to immune dysregulation that exacerbates disease symptoms [10,11]. Similarly, the microbiota influences the immune responses in leishmaniasis, where certain bacterial communities can either suppress or enhance inflammation, thus affecting disease outcomes [12,13]. In ascariasis, a diverse gut microbiota has been linked to better immune regulation, potentially reducing the severity of infection [14,15]. The potential of microbiota-targeted therapies to modulate these interactions presents a promising avenue for improving treatment efficacy and patient outcomes in parasitic diseases [16,17].

In examining the interactions among *Ascaris lumbricoides*, *Trypanosoma cruzi*, and *Leishmania* species, it is crucial to highlight both their commonalities and differences, particularly regarding their relationships with the host microbiota. While these pathogens differ significantly in their biology—*Ascaris* being a helminth and the other protozoans—they all interact with the host microbiota in ways that can influence disease severity and progression. Research indicates that alterations in the gut and skin microbiota can affect immune responses, potentially exacerbating infection outcomes. For example, changes in microbial composition may modulate inflammatory processes, affecting how the host responds to each pathogen. Understanding these complex microbiota interactions not only sheds light on the mechanisms underlying disease pathogenesis but also opens avenues for innovative therapeutic strategies that leverage microbiota modulation to enhance treatment efficacy across these diverse parasitic infections.

This review seeks to elucidate how these parasitic infections alter the gut microbiota and skin microbiota in the case of leishmaniasis, in turn, how these changes affect the disease's progression and outcomes [18–22]. Although *T. cruzi* (Chagas disease), *Leishmania* species (leishmaniasis), and *Ascaris lumbricoides* (ascariasis) are distinct pathogens, they share significant commonalities: they affect vulnerable populations in similar tropical and subtropical regions and interact with the host microbiota in ways that impact disease severity, immunity, and treatment outcomes. Focusing on these seemingly dissimilar pathogens allows us to explore broader microbiota-modulating strategies that could be effective across multiple parasitic diseases. This comparative approach enriches our understanding of parasite-microbiota interactions, potentially leading to innovative, cross-cutting therapeutic interventions for diseases affecting overlapping at-risk populations.

By examining the multifaceted relationships between the gut microbiota and these parasites, the review aims to provide insights into the mechanisms through which microbial communities influence immune responses and disease severity. Such understanding could inform new therapeutic strategies that leverage microbiota-targeted interventions to improve patient management and treatment efficacy for these prevalent parasitic diseases.

2. Chagas Disease and the Microbiota

CD can manifest with various complications, including cardiomyopathy, gastrointestinal issues, and neurological problems [2]. The diversity in clinical manifestations and outcomes makes CD challenging to manage and study. In addition, the clinical course of CD is highly variable, and not all individuals infected with *T.cruzi* will develop the same severe symptoms or complications. Some differences in the severity of the disease are due to the genetic variability of the *T.cruzi* strain, the geographical distribution, and the genetic variability of the host; however, other

considerations, including the altitude, ethnicity, drug use, and diet, have been associated with the severity of CD. Experimental models showed that mice acutely infected with high parasitemia developed a most severe digestive form of CD, which was associated with a *T. cruzi*-induced intestinal inflammatory response [23–26]. Recently, chronically infected mice showed significant gut tropism with the GI tract as the primary site of *T. cruzi* persistence, possibly contributing to chronic inflammation. In addition, heart disease severity was higher when the infection was disseminated and not restricted to the GI [24,27,28]. However, the mechanisms by which *T.cruzi* infection leads to GI pathology remain unclear, as well as the immune responses, tissue damage, and specific alterations to the GI-specific immunity.

The potential impact of CD on the gut microbiome is still poorly understood. Recent studies have started to uncover the role of gut microbiota in CD, underscoring the need for further research to fully understand how the composition and function of the gut microbiome may influence disease progression and patient outcomes [18,25,26,29,30]. Molina *et al.,* demonstrated that individuals with CD exhibit distinct alterations in their gut microbiota compared to healthy controls [31]. Notably, *Parabacteroides* species are consistently enriched among patients with CD. Other bacterial species, such as *Enterococcus hirae* and *Lactobacillus buchneri*, have also been implicated, although their specific roles remain less clear [31]. These microbiota changes could be linked to the intestinal inflammation and denervation caused by *T. cruzi*, potentially contributing to the gastrointestinal symptoms observed in chronic CD [32]. The perturbations in the gut microbiome during *T. cruzi* infection can result from various factors: (1) Direct parasite-mediated modulation (2) Indirect modulation due to the host's systemic anti-parasitic immune responses [18,25,27,29].

The pathogenesis of CD is driven by *T. cruzi* infection and the host immune response, which is initiated when *T. cruzi* triggers pattern recognition receptors (PRRs) such as toll-like receptors (TLRs) and NOD-like receptors. While these receptors detect microbial signals to initiate protective immune responses during infection, they also recognize ligands produced by commensal microbiota, helping to maintain microbial balance and tissue integrity [33]. TLRs play a dual role in both host defense against pathogens like *T. cruzi* and the regulation of gut microbiota, with TLR5 being particularly important for shaping the microbiota during early life [34]. Commensal-derived molecules, such as polysaccharide A (PSA), engage the TLR2/TLR1 heterodimer and Dectin-1, promoting anti-inflammatory gene expression and regulatory T cell (Treg) differentiation and maintaining immune homeostasis during infection [35]. Early defense against *T. cruzi* involves natural killer (NK) cells and neutrophils, whose activity can be enhanced by gut microbiota via PRR activation. *T. cruzi* evades immune detection by antigenic variation, inducing immune cell apoptosis and modulating cytokine responses, leading to chronic infection [36].

The balance between M1 and M2 macrophages is crucial for determining whether *T.cruzi* infection is controlled or leads to chronic disease. M1 macrophages, which produce proinflammatory cytokines and nitric oxide (NO), are essential for fighting infection, while M2 macrophages, characterized by the expression of arginase 1, act as permissive host cells that allow the parasite to persist [37]. This M1/M2 balance is integral to maintaining gut homeostasis, as macrophages and dendritic cells (DCs), both important antigen-presenting cells, help regulate microbial balance following episodes of inflammation or infection [38]. Microbiota-derived antigens prime dendritic

cells to activate adaptive immunity, though dysbiosis impairs antigen presentation and weakens immune responses [39]. The gut microbiome further regulates the balance between pro-inflammatory Th1 and anti-inflammatory Th2 responses. Beneficial bacteria, such as *Bacteroides* and *Lactobacillus*, produce short-chain fatty acids (SCFAs) like butyrate, which promote regulatory T cell (Treg) differentiation. Tregs and SCFA-producing bacteria induce interleukin-10 (IL-10), which helps control inflammation and prevent tissue damage during *T. cruzi* infection [18].

Castaneda *et al.* have revealed notable changes in the gut microbiome of mice acutely infected with *T. cruzi* compared to healthy controls (Naïve). These alterations include shifts in the abundance of specific bacterial taxa, microbial diversity changes, and microbial community structure disruptions [18]. The perturbations in the gut microbiome during *T. cruzi* infection can result from various factors, the parasite-mediated modulations, as a consequence of the parasite itself directly altering the local GI microenvironment and the host's anti-parasitic immune responses, where the host's immune system responds to combat the *T. cruzi* infection, it can release reactive oxygen and nitrogen species which can have a profound impact on the gut microbiome composition [25] (Figure 1). Understanding the modifications induced by *T. cruzi* in the gut microbiome's composition might hold an alternative strategy for controlling the parasite by manipulating the gut microbiome [10].

Several key proteins in the excretory/secretory (E/S) products of *Trypanosoma cruzi* are involved in host interactions but their effects on the microbiota in Chagas disease remain unexplored. Cruzipain, a cysteine protease, modulates immune responses and aids parasite entry into non-phagocytic cells, promoting survival and replication [40] P21, present in all *T. cruzi* stages, affects phagocytosis signaling [40]. Mucins and trans-sialidases (TS) are crucial for immune evasion and parasite propagation [41]. TSSA is highly antigenic and linked to *T. cruzi* lineages [42]. Additionally, phospholipase A influences host cell signaling, while proteins like superoxide dismutase (SOD) help defend against oxidative stress [43]. Despite these molecules' roles in infection, their interactions with the microbiota in Chagas disease are yet to be investigated.

3. Leishmania modulates gut and skin microbiota

The microbiota plays a pivotal role in influencing the pathogenesis of cutaneous leishmaniasis (CL) and visceral leishmaniasis (VL), caused by *Leishmania* parasites. The effect of *Leishmania* infection on the skin microbiota, particularly in relation to dysbiosis and its implications, is increasingly recognized as a critical factor in the pathology of CL. Research has shown that CL significantly alters the skin microbiota, leading to a dysbiotic state that influences disease progression and inflammation. Infection with *Leishmania braziliensis* and *L. major* results in a skin microbiota characterized by increased *Staphylococcus* and *Streptococcus* species, with this dysbiosis spreading beyond the lesion site to normal skin. In murine models, cutaneous microbiota changes found in mice with CL have been found to be transferred to co-housed naïve mice, indicating a transmissible component. This dysbiosis is associated with enhanced inflammatory responses. In *Leishmania braziliensis* infections, a microbiome dominated by *Staphylococcus spp.* is associated with delayed healing and heightened IL-1 cytokine responses, suggesting that altered skin microbiota modulates immune responses and worsens clinical outcomes. Specifically, *Leishmania* infection increases the abundance of bacterial families like *Staphylococcaceae* and

Streptococcaceae in cutaneous lesions, contributing to local inflammation [44][45]. Additionally, the generation of dysbiotic microbiota in CL is shown to enhance skin inflammation, underscoring the role of microbial imbalance in worsening disease pathology [46]. These studies demonstrate that Leishmania infection induces significant modifications in the skin microbiota, contributing to inflammation and potentially influencing the progression and severity of cutaneous leishmaniasis.

Research has increasingly highlighted the relationship between the microbiome and disease severity in leishmaniasis. Studies show that *Leishmania* infection alters the gut microbiota, particularly in the ileum, while the colon microbiota remains more stable [47]. In cutaneous leishmaniasis, lesions exhibit a less diverse microbiome compared to unaffected skin, with larger wounds and those harboring high parasite loads showing greater abundances of potentially pathogenic organisms, such as *Staphylococcus aureus*, which delays healing by promoting inflammatory responses, including elevated IL-1β markers [22,48]. In visceral leishmaniasis, microbiome disturbances have been linked to histoarchitectural changes in the colon and alterations in the enteric nervous system [49]. Additionally, symptomatic leishmaniasis cases, particularly in dogs, show a shift in microbiome composition, with an overrepresentation of Proteobacteria and reduced Firmicutes, correlating with weakened immune responses [50]. These findings suggest that microbiome alterations may influence the immune response, contributing to disease progression and severity in leishmaniasis [46,51].

Our recent study further underscores the importance of microbial balance in CL pathogenesis [44]. We explored the spatial-temporal distribution of *Leishmania* species and the influence of skin microbiota on disease progression. We observed significant alterations in both prokaryotic and eukaryotic communities within the lesions. Notably, there was an increase in bacterial families such as *Staphylococcaceae* and *Streptococcaceae*, suggesting their role in local inflammation. Additionally, *L. naiffi* emerged as a significant discovery in regions with high CL incidence. These findings highlight the potential contribution of microbial dysbiosis, including fungal components, to the pathogenesis of CL and the need for understanding the *Leishmania* species in skin dysbiosis (Table 1) [44].

Research into the impact of VL on gut microbiota highlights several significant findings [49,52,53]. VL, caused by *Leishmania infantum/donovani* parasites, affects the gut microbiota composition, which is crucial for systemic immune responses necessary to control the infection [13,22]. Studies have shown that *L. infantum* infection in hamsters leads to changes in the gut microbiota, particularly affecting the ileum while the colon remains unchanged. Infected hamsters displayed histoarchitectural changes in the colon, with increased intraepithelial lymphocytes and alterations in the enteric nervous system, suggesting a link between gut microbiota and immune modulation [49,52]. Additionally, canine studies have shown significant differences in gut microbiota composition among infected dogs, particularly in the Firmicutes and Proteobacteria phyla, suggesting that microbiota composition could influence immune responses and disease outcomes [47,50]. These findings underscore the complex interactions between gut microbiota and VL, highlighting the potential for microbiota-targeted interventions to modulate immune responses and improve disease outcomes.

The identification of microbiota-derived biomarkers presents a promising approach for early diagnosis and monitoring of leishmaniasis, with specific shifts in the host microbiota composition

in response to *Leishmania* infection serving as potential early indicators of the disease, even before clinical symptoms appear. For VL, changes in gut microbial populations, such as a decrease in Firmicutes and an increase in Bacteroidetes, have been associated with disease onset, while microbial metabolites like SCFAs or specific microbial DNA signatures in blood or stool could act as non-invasive biomarkers for monitoring progression or therapeutic response. In the context of CL, alterations in skin microbiota, such as increases in bacterial families like *Staphylococcaceae* and *Streptococcaceae* within lesions, highlight the potential role of skin microbes in disease progression and their utility as biomarkers. Given the diverse species of *Leishmania* infecting humans, future studies should systematically investigate the effects of different *Leishmania* species on skin microbiota in CL and gut microbiota in VL to better understand the interplay between microbial communities and disease pathogenesis. Moreover, microbiota-targeted therapies, including probiotics, prebiotics, and fecal microbiota transplants (FMT), offer innovative strategies to enhance the treatment of both CL and VL by improving drug efficacy, reducing side effects, and potentially overcoming drug resistance.

There is limited information regarding how *Leishmania* excretory/secretory (E/S) products specifically interact with the microbiota, but potential mechanisms can be inferred. *Leishmania* E/S products likely modulate host immune responses, indirectly influencing microbiota composition by altering the immune environment [54]. These products may interact with antimicrobial peptides (AMPs), which are produced in response to infections and could affect microbial balance [55]. Additionally, *Leishmania* E/S products may compete with bacteria for nutrients or binding sites in the gut, shaping the microbiota indirectly [56]. Although not well-characterized, *Leishmania* E/S products could also alter the gut environment or metabolic pathways, further influencing microbiota dynamics [57]. However, detailed investigations into these interactions are still lacking.

4. Current Knowledge on the Interaction between *Ascaris* and the Intestinal Microbiota

The interaction between *Ascaris* and the intestinal microbiota is a burgeoning area of research with potential for novel therapeutic interventions. Understanding these interactions can elucidate key aspects of the biology and pathophysiology of these parasites that could be potentially useful in therapeutic and prophylactic control strategies [58–60]. Most studies to date have focused on the interactions between *Ascaris* and the host immune system due to the parasite's significant immunomodulatory and immunoregulatory capacities, which enable it to infect, disseminate, and persist within the host [61–65].

It has been observed that *Ascaris* infection can alter the composition and diversity of the gut microbiota. Adult worms in the intestines cause a decrease in essential anaerobic bacteria such as *Faecalibacterium* and *Ruminococcus*, while increasing facultative anaerobes like *Streptococcus*. This, combined with the immunomodulation generated by the helminth, creates a favorable metabolic environment for the parasite's long-term survival in the intestines [14,20,66–73]. Additionally, a relevant aspect of *Ascaris* infection is that its larvae (L3), after hatching, can invade the mucosal tissue of the gastrointestinal tract and subsequently migrate to hepatic and pulmonary tissues [74]. The migration of these larval stages has been linked to mechanisms that alter the composition and structure of the intestinal microbiota, promoting the translocation of bacteria across intestinal barriers and affecting the liver and lung mucosa [75,76].

The mechanisms by which *Ascaris* influences the gut microbiota are not yet fully understood (Figure 2). However, it is hypothesized that the parasite's metabolic activities, secretions, and the physical presence of its larvae and adults create an environment conducive to the growth of specific microbial populations [64,77]. Additionally, the host's immune response to *Ascaris*, including inflammation and cytokine production, may also contribute to modulating the microbiota composition [75,76,78–80]. Research has shown that *Ascaris* excretory-secretory (E/S) products, such as extracellular vesicles and other metabolites, may elicit an immunomodulatory response, indirectly affecting the host's intestinal microbiota [65]. Various E/S products have been identified, interacting in different ways with the host microbiota and potentially playing critical roles in parasite biology and the infection's pathophysiology [65]. Studies have confirmed that *Ascaris suum* can induce the production of antimicrobial peptides, such as the *Ascaris suum* antibacterial factor (ASABF) and cecropin family peptides (P1 to P4), in the host [75,81–83]. These peptides exhibit bactericidal effects against a broad range of microbes, including gram-positive bacteria (*Staphylococcus aureus*, *Bacillus subtilis*, and *Micrococcus luteus*) and gram-negative bacteria (*Pseudomonas aeruginosa*, *Salmonella typhimurium*, *Serratia marcescens*, and *Escherichia coli*) [75,81–83]. This highlights the roundworm's ability to produce nematode-derived metabolites that significantly modulate the host's intestinal microbiota. This area of research is increasingly gaining prominence and is becoming a key focus in the study of helminth biology.

To fully comprehend the interplay between *Ascaris* and the intestinal microbiota, it is essential to investigate how each influences the other. Very little is known about the establishment, structure, and function of the microbiota residing within the parasite. Therefore, it is a priority to evaluate these characteristics to better decipher the interactions between the parasite and the host microbiota [76,84,85]. Investigating the microbiota associated with *Ascaris* is crucial, as it helps define stage-specific transient microbiota and their interactions with the host. This research uncovers mutualistic relationships between specific bacterial groups and the parasite that are essential for its biology, infectivity, and dissemination [76,84,85]. Studies employing advanced metagenomic and metabolomic techniques in animal models can provide insights into the microbial changes induced by *Ascaris* and the functional consequences of these alterations.

One promising avenue for controlling *Ascaris* infections is the development of probiotics or prebiotics designed to modulate the gut microbiota in favor of the host. Probiotics, which are live beneficial bacteria, or prebiotics, which are non-digestible fibers that promote the growth of beneficial microbes, could be tailored to restore microbial balance disrupted by Ascaris infection [86–88]. For instance, identifying and testing probiotic strains that enhance gut barrier function or stimulate anti-helminthic immune responses could be beneficial. Clinical trials assessing the efficacy of specific probiotic or prebiotic formulations in reducing *Ascaris* burden and improving host health outcomes would be essential for translating these findings into practical interventions. Another innovative strategy involves manipulating the gut microbiota to create an environment hostile to *Ascaris* colonization and survival, potentially through dietary interventions or microbiota-modulating drugs. Exploring dietary interventions rich in fibers and polyphenols, which support beneficial gut bacteria, could offer protective effects against *Ascaris*. Additionally, identifying and targeting microbial taxa and pathways essential for *Ascaris* survival with specific drugs could provide a novel therapeutic approach [86,89]. Understanding the complex and bidirectional interactions between *Ascaris* and the intestinal microbiota has significant implications for infection outcomes and host health. Advancing our understanding of these

interactions, developing microbiota-based interventions, and exploring innovative strategies for microbiota manipulation could pave the way for novel and effective approaches to controlling *Ascaris* infections and enhance our broader understanding of host-parasite-microbiota interactions.

Understanding how changes in the gut microbiota impact *Ascaris* infection outcomes and host immune responses is crucial for developing targeted interventions [83]. Research should aim to identify key microbial taxa or metabolites that modulate the host's immune response to *Ascaris*. For instance, certain bacterial species may produce metabolites that enhance anti-helminthic immunity or, conversely, promote immunosuppression, aiding parasite survival [75,76,79,83,90]. Experimental models, such as gnotobiotic animals with controlled microbiota compositions, can be employed to dissect these complex interactions and identify potential microbial biomarkers of infection susceptibility or resistance.

5. Common Themes and Future Directions

The alterations caused by these parasites may significantly influence the host's immune response, disease progression, and treatment outcomes [18,44,75]. Despite these shared characteristics, the mechanisms by which each parasite interacts with the microbiota can vary considerably. For instance, *T. cruzi*, the causative agent of CD, may indirectly alter the gut microbiota through systemic immune modulation. In contrast, *Leishmania* species, responsible for leishmaniasis, may directly interact with gut microbes due to their close association with macrophages and dendritic cells. *Ascaris*, being a gastrointestinal helminth, directly impacts the gut microbiota through physical interactions and metabolic products [18,44,75].

Common mechanisms across these infections include the disruption of gut barrier integrity, immune modulation, and metabolic alterations. Unique features involve specific immune pathways activated, such as the Th1/Th2 response balance in helminth infections versus inflammatory responses in protozoan infections. Additionally, the route of entry and localization of the parasites influence their interactions with the gut microbiota—intestinal for *Ascaris*, cutaneous and visceral for *Leishmania*, and systemic for *T. cruzi* [18,44,75].

Future research should leverage multi-omics approaches, including genomics, transcriptomics, proteomics, and metabolomics, to comprehensively map the interaction networks between parasites and the microbiota. This integrative approach can uncover crucial molecular pathways and biomarkers essential for understanding parasite-host-microbiota dynamics [18,75,84,91]. Longitudinal studies both in humans and in animal models which favored standardization of processes allowing adequate control of most of the variables involved in microbiota analysis, are vital for capturing the temporal dynamics of parasite-microbiota interactions, revealing how microbiota changes throughout infection, during treatment, and post-clearance, thus providing insights into optimal stages for microbiota modulation [75,92,93]. Additionally, research should focus on the microbiota's role in modulating host resistance to infections and vaccine efficacy, as understanding how different microbiota compositions influence immune responses can lead to improved vaccine formulations and strategies [18,94]. Personalized medicine approaches, which tailor microbiota-based therapies to individual microbiome profiles, offer promising potential for more effective treatments. Customizing probiotics, prebiotics, and microbiota transplants to enhance host resilience against specific parasites could improve infection outcomes and reduce disease burden [86,95,96]. Comparative analyses of parasite-microbiota interactions across

diseases such as Chagas disease, leishmaniasis, and *Ascaris* infections highlight the complexity and specificity of these relationships. Future research should focus on multi-omics, longitudinal studies, vaccine interactions, and personalized therapies to develop innovative interventions and enhance control and treatment of parasitic diseases (Figure 3).

6. Therapeutic Implications and Innovation

Current treatments for *T. cruzi*, *Leishmania*, and *Ascaris* infections vary significantly but have common challenges, including side effects and limited efficacy in chronic or severe cases. For *T. cruzi* (Chagas disease), benznidazole and nifurtimox are the primary drugs used. These are most effective in the acute phase but less so in chronic infections [97]. Common side effects include skin reactions, gastrointestinal disturbances, and potential neurological toxicity, which often limits their use in some patients. *Leishmania* infections (leishmaniasis) are treated with pentavalent antimonials (e.g., sodium stibogluconate), amphotericin B, and miltefosine [98]. Side effects vary by treatment but can include pancreatitis, nephrotoxicity, hepatotoxicity, and gastrointestinal issues, making long-term or high-dose treatments difficult for patients. For *Ascaris* infections (ascariasis), treatment typically involves albendazole or mebendazole. These drugs are generally well-tolerated, but side effects can include mild gastrointestinal discomfort, dizziness, and, in rare cases, liver function alterations [99]. Each of these treatments, while essential, presents challenges in terms of efficacy and tolerability, highlighting the need for safer and more effective therapeutic options.

Understanding the components of the microbiota and their mechanisms of action in inducing pathological changes or exerting beneficial effects, along with disease-protective activities, is crucial for influencing microbiota composition [100]. The microbiota plays a critical role in training and modulating the immune system [8]. Higher microbial diversity can prevent the colonization of pathogenic organisms, limiting their ability to establish and proliferate through competitive exclusion and the production of antimicrobial substances [101] . Additionally, a diverse microbiota helps maintain immune homeostasis, reducing inappropriate inflammatory responses and improving the body's ability to respond to infections [8], enhancing and promoting the development of regulatory T cells and the production of anti-inflammatory cytokines. Microbiota-target interventions such as prebiotics, probiotics, symbiotics, antibiotic gut depletion, and fecal transplants can delay the onset and progression of parasitic diseases.

The development of probiotics, prebiotics, and synbiotics specifically targeted at parasitic infections holds significant promise [102,103]. By understanding the intricate interactions between gut microbiota and parasites, tailored probiotic strains can be engineered to enhance the microbiota's ability to outcompete or inhibit parasites. Prebiotics can be designed to selectively stimulate beneficial microbial populations that could suppress parasitic growth or enhance host immune responses [104]. Synbiotics, combining both probiotics and prebiotics, could synergistically modulate the microbiota to achieve therapeutic outcomes. These interventions have the potential to offer adjunctive treatments that complement existing antiparasitic therapies, potentially improving efficacy and reducing side effects [86].

Integrating microbiota-modulating drugs with conventional antiparasitic treatments is a promising avenue for enhancing therapeutic efficacy [103,105]. Drugs that specifically target and modify the

microbiota could improve drug absorption, metabolism, and activity of antiparasitic agents. For example, microbiota-modulating drugs could enhance the bioavailability of antiparasitics or reduce drug resistance by altering microbial composition. The idea that drugs targeting and modifying the microbiota could improve drug absorption, metabolism, and activity of antiparasitic agents is supported by several studies, which highlight the role of the gut microbiota in drug metabolism and its potential to influence drug efficacy and resistance [106]. The gut microbiota can significantly impact the pharmacokinetics of drugs, including their absorption, distribution, metabolism, and excretion (ADME), potentially altering therapeutic effects and toxicity. This suggests that modulating the microbiota could enhance drug bioavailability and reduce resistance. The microbiome's enzymatic transformation of drugs affects their bioavailability, bioactivity, and toxicity, and understanding these interactions could lead to strategies for modulating the microbiome to improve drug efficacy [107]. Thus, the gut microbiota is considered a key metabolic organ that can affect drug efficacy by altering drug bioavailability and bioactivity, opening up possibilities for regulating gut microbes to enhance therapeutic effects and clinical outcomes [108]. Understanding the microbiota's role in drug metabolism and parasite-host interactions could lead to innovative therapies that optimize treatment regimens and outcomes.

The interplay between microbiota and immune responses is crucial for vaccine development and immunotherapy. By leveraging insights into how microbiota influence immune system priming and pathogen interactions, researchers can design vaccines that harness these microbial effects to enhance immunity against parasitic infections. The gut microbiota plays a significant role in modulating immune responses to vaccines, acting as a natural adjuvant and potentially enhancing vaccine efficacy by influencing both mucosal and systemic immune responses [109,110]. For instance, oral vaccines using genetically modified bacteria have demonstrated improved immune responses, indicating that microbiota-targeted interventions could boost vaccine effectiveness against parasitic infections [111,112]. Additionally, the microbiota influences vaccine-induced immune responses by shaping B cell and T cell repertoires essential for effective antigen-specific responses, which can be leveraged to design vaccines that harness microbial effects to enhance immunity against parasitic diseases [112]. Strategies like prebiotics, probiotics, and synbiotics are being explored to modulate the microbiota and improve vaccine immunogenicity, providing a novel approach to enhance disease prevention and treatment when used alongside immunotherapy [110,111]. Integrating microbiota knowledge into vaccine and immunotherapy development could thus significantly enhance the effectiveness of treatments for parasitic diseases.

The concept of fecal microbiota transplantation (FMT) as a potential treatment for parasitic infections is an emerging area of research. Although FMT is primarily utilized for treating recurrent *Clostridioides difficile* infections, there is growing interest in its application for parasitic diseases [113]. FMT has been proposed as a potential treatment for intestinal parasite infections, such as giardiasis and worm infections, with the idea that restoring a healthy microbial community could disrupt parasite life cycles or enhance the host's natural defenses against these infections [114]. While research into FMT's efficacy for other conditions, including parasitic diseases, is ongoing, the potential for FMT to address chronic or drug-resistant parasitic infections is being explored, though more scientific evidence is needed to establish its safety and effectiveness [115]. This evidence highlights the potential of FMT as an innovative approach to treating parasitic infections by modulating the gut microbiota, although further research is necessary to fully understand its therapeutic capabilities and limitations in this context.

The therapeutic options discussed here are largely speculative. While exploring novel treatments is essential, a clear connection to these particular pathogens is necessary for advancing therapeutic development. Current therapies, although effective in some cases, face significant challenges related to toxicity, limited efficacy in chronic infections, and the emergence of drug resistance, underscoring the need for more targeted and evidence-based interventions tailored to each disease. Continued efforts are needed to study and develop new therapeutic interventions for these diseases.

7. Conclusions

The study of parasite-microbiota interactions holds significant promise for advancing therapeutic strategies against parasitic diseases. Our findings underscore the crucial role that microbiota play in modulating parasite infections and influencing treatment efficacy. Future research should prioritize the development of tailored probiotics, prebiotics, and synbiotics specifically designed to target parasitic infections, as well as explore microbiota-modulating drugs that can enhance conventional therapies. Additionally, fecal microbiota transplantation presents a novel therapeutic option worth investigating.

To fully realize these potential advancements, a concerted effort is needed from the scientific community and funding agencies. By adopting a multidisciplinary approach that integrates microbiology, immunology, and therapeutic development, we can uncover new therapeutic avenues and significantly improve disease management. Investing in this research will not only advance our understanding but also lead to more effective and sustainable strategies for combating parasitic diseases.

Future perspective

The future of parasitic disease management may well hinge on a deeper understanding of the intricate relationships between parasites and the microbiota. The exploration of these interactions could lead to the development of innovative therapies, such as targeted probiotics, prebiotics, and synbiotics, designed specifically to combat parasitic infections. Additionally, the potential of microbiota-modulating drugs and fecal microbiota transplantation as therapeutic tools should not be underestimated. Moving forward, a collaborative, multidisciplinary approach that brings together microbiology, immunology, and therapeutic development will be crucial. Such an approach will not only deepen our comprehension of these complex biological systems but also pave the way for more effective and sustainable interventions, ultimately transforming the landscape of parasitic disease treatment.

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Author contributions

JDR and CP conceived the idea, JDR, CP, SC, JW wrote the manuscript. All authors approved the final version of the manuscript.

Disclosures

None

Figures/Tables

Figure 1. A schematic overview of the proposed interplay mechanism between gut microbiome, host immunity, and *T. cruzi*. The microbiota resides in the lumen and outer mucus layer of the intestine. A. Naïve B. *T.cruzi* Infection Acute phase: Parasite infection disrupts gut microbiota. Dysregulated microbiota signals are transmitted via DCs and macrophages, promoting inflammatory T-cell subsets (Th1, Th2, Th17) and B-cell activation, yielding protective IgA to restore microbial balance. C. Early chronic phase: *T. cruzi* infects the intestinal epithelium, controlled by a balanced Th1/Th2/Th17 response. Parasite-driven mechanisms affect B cell-mediated responses. D. Late chronic phase: Parasites disseminate, causing dysbiosis with Th1 and Th17 reduction and Th2 increase. Elevated inflammatory cytokines (IFN-γ, TNFα) result from *T. cruzi* actions on DC functionality, impacting adaptive immune responses.

Figure 2. Interaction between *Ascaris* and the host intestinal microbiota. After ingestion of the infective stage of *Ascaris*, L3 larvae are released from embryonated eggs into the host intestine and begin their life cycle. During migration, the interplay between *Ascaris* and the intestinal microbiota is essential to understand not only changes in the host but also the microbiota residing within the parasite. These interactions involve mutualistic relationships between specific bacterial groups and the parasite that are crucial for its biology, infectivity, and dissemination.

Figure 3. Future Directions. Future research from longitudinal studies in animal models and natural hosts must implement multi-omics approaches, including genomics, transcriptomics, proteomics, and metabolomics. To better understand parasite-host-microbiome interactions, it is essential to use metabarcoding and metagenomics analyses. These approaches can identify metabolic pathways and differentially abundant taxa that may be relevant to parasite infection and persistence. Understanding these functional features and parasite-host-microbiome interactions will facilitate the design of studies aimed at elucidating the role of the microbiota in modulating host resistance to infections and the efficacy of vaccines. Personalized medicine approaches using microbiota-based therapies tailored to individual microbiome profiles hold promise for more effective treatments.

Table 1. Cutaneous Leishmaniasis and microbiota correlation consensus. Showing different studies and their different findings relating CL disease with prokaryote microbiota associations.

Reference annotations

***Hossain E, Khanam S, Dean DA, et al. Mapping of host-parasite-microbiome interactions reveals metabolic determinants of tropism and tolerance in Chagas disease. Sci Adv [Internet]. 2020 [cited 2021 Jun 15];6(30):eaaz2015.**

This study highlights the changes in parasite burden and microbiota across different sections of the gastrointestinal (GI) tract in mice, providing important insights into the interactions between Trypanosoma cruzi *and the gut microbiota. By examining these dynamics, the research sheds light on how* T. cruzi *influences and is influenced by the gut microbial communities, potentially affecting disease progression and immune responses. This information could be valuable for developing microbiota-targeted therapies to improve the management and treatment of Chagas disease.*

***Castañeda S, Muñoz M, Hotez PJ, et al. Microbiome Alterations Driven by Trypanosoma cruzi Infection in Two Disjunctive Murine Models . Microbiol Spectr [Internet]. 2023 [cited 2024 Aug 9];11(3).**

This study is the first to employ metagenomics to analyze the acute phase of Chagas disease, revealing how specific metabolic pathways contribute to microbiota dysbiosis and exacerbate disease severity.

***Jaimes J, Patiño LH, Herrera G, et al. Prokaryotic and eukaryotic skin microbiota modifications triggered by Leishmania infection in localized Cutaneous Leishmaniasis. PLoS Negl Trop Dis [Internet]. 2024 [cited 2024 Aug 9];18(3):1–25.**

This study utilizes next-generation sequencing to evaluate the impact of Leishmania *parasite diversity on the skin microbiota, providing insights into how variations in the parasite may influence microbial communities and affect disease outcomes.*

***Amorim CF, Lovins VM, Singh TP, et al. Multiomic profiling of cutaneous leishmaniasis infections reveals microbiota-driven mechanisms underlying disease severity. Sci Transl Med [Internet]. 2023 [cited 2024 Aug 9];15(718).**

This study employs a state-of-the-art multiomics approach to unravel the complex interactions between Leishmania *and the host microbiota in cutaneous leishmaniasis. By integrating metagenomics, transcriptomics, and metabolomics, the research provides a comprehensive view of how microbial communities influence disease progression, immune responses, and tissue damage. This advanced methodology not only deepens our understanding of the host-microbiota-parasite interplay but also paves the way for novel microbiota-targeted therapeutic strategies to mitigate disease severity and improve treatment outcomes.*

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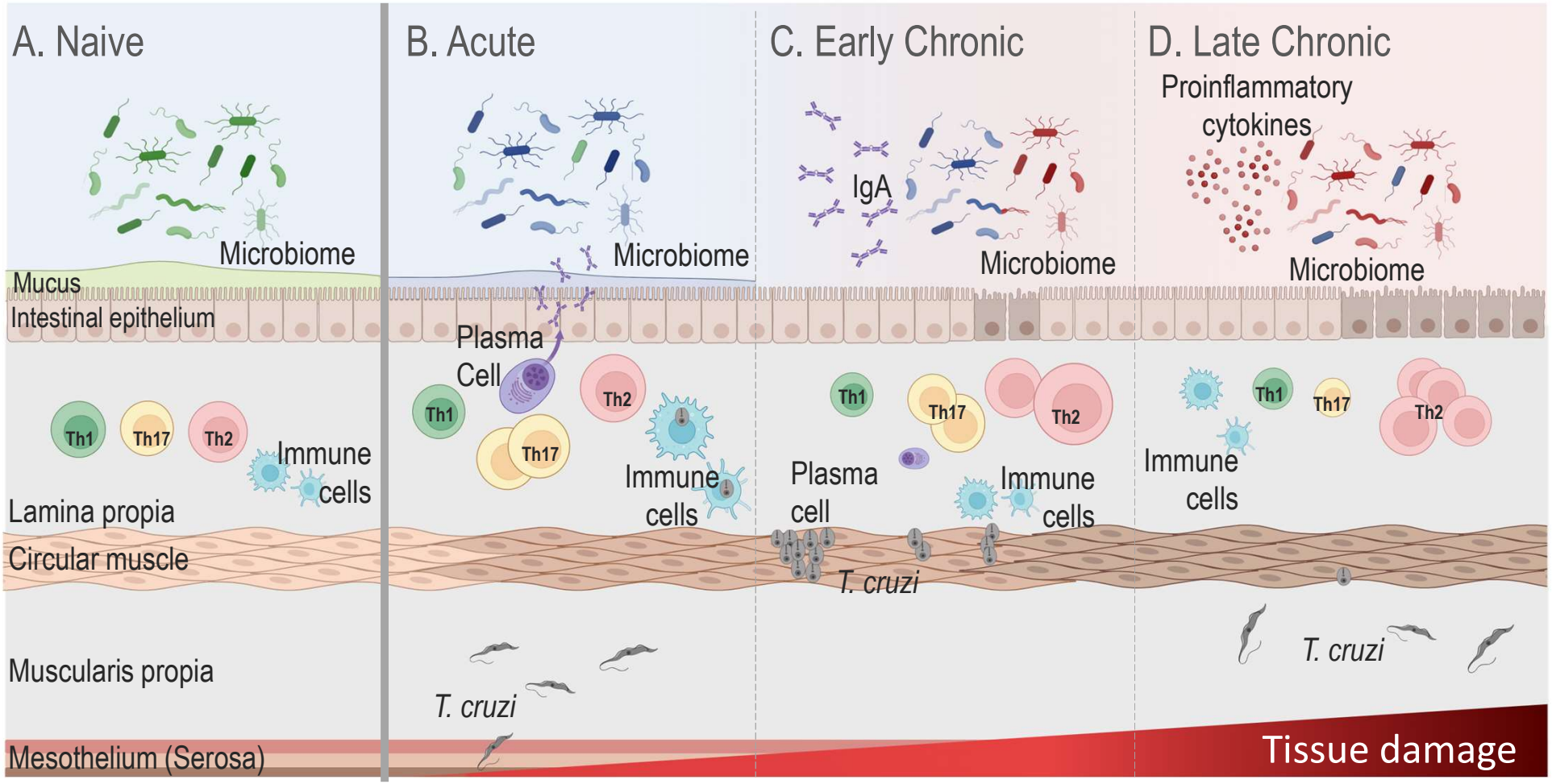
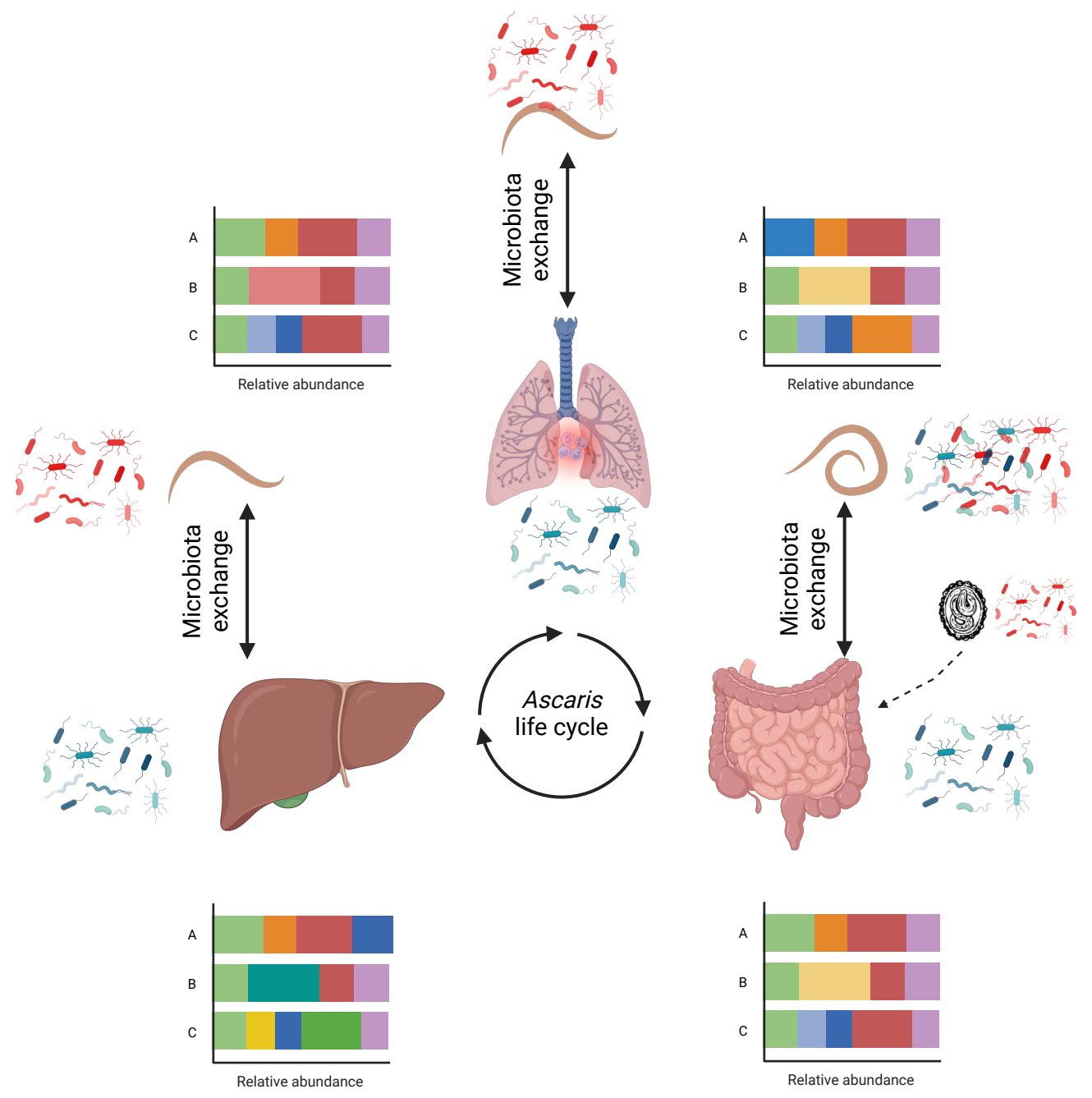

A. Naive
B. Acute
C. Early Chronic
D. Late Chronic
Proinflammatory cytokines
IgA
Microbiome
Mucus
Intestinal epithelium
Plasma Cell
Th1
Th17
Th2
Immune cells
Lamina propia
Circular muscle
Plasma cell
T. cruzi
Muscularis propia
Mesothelium (Serosa)
Tissue damage


Microbiota exchange
A
B
C
Relative abundance
Microbiota exchange
A
B
C
Relative abundance
Microbiota exchange
Microbiota exchange
Ascaris life cycle
A
B
C
Relative abundance
A
B
C
Relative abundance

Animal models

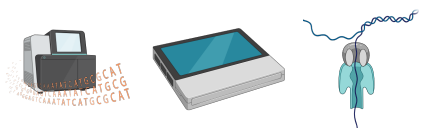
Natural Hosts

Longitudinal Designs

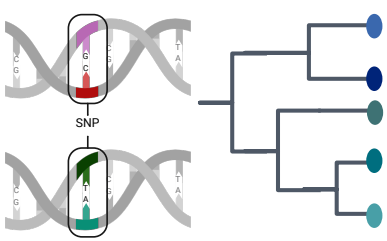
Animal models

Natural Hosts

Genomics

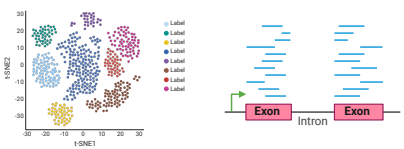


Sequencing platforms

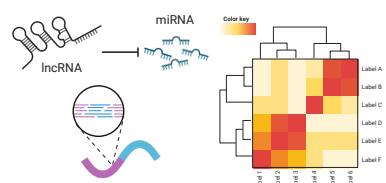


Assembly, annotation, comparative genomics

Transcriptomics

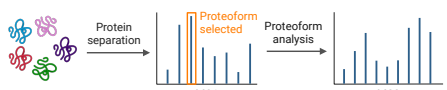


RNA-seq technologies and pipelines

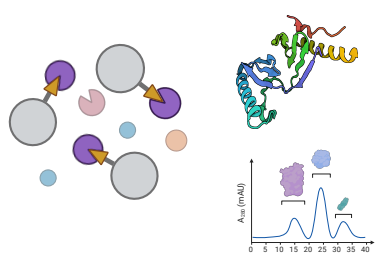


Differential expression analysis, non-coding RNAs

Proteomics and Metabolomics

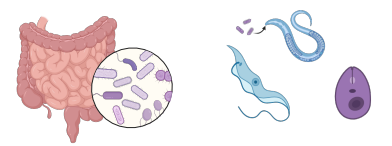


Techniques and pipelines

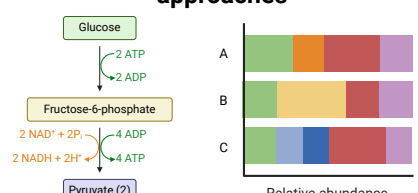


Identifying, protein-protein interactions, functional proteomics

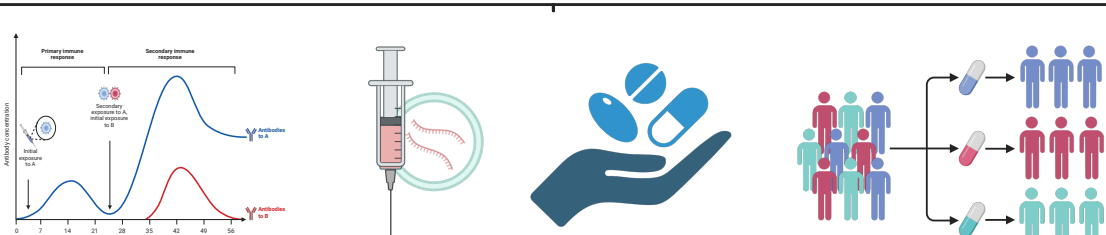
Helminths-Host-Microbiome Interactions



Metabarcoding and metagenomics approaches



Identification of differentially abundant taxa and metabolic genes and pathways



Vaccine, probiotic and prebiotic development. Personalized medicine approaches using microbiota-based therapies

7. CONCLUSIONES

Capítulo 1.

- La presencia generalizada de parásitos intestinales en la población rural de Las Guacas, especialmente la alta frecuencia de *Blastocystis*, subraya la necesidad de implementar estrategias direccionadas al mejoramiento en el suministro y calidad del agua y en las prácticas de saneamiento. Las condiciones socioeconómicas desfavorables, junto con la ausencia de tratamiento adecuado del agua y la eliminación inadecuada de excretas, aumentan el riesgo de infecciones transmitidas por el agua y facilitan la transmisión zoonótica, ya que las mascotas en la comunidad también albergan parásitos que afectan tanto a humanos como a animales.

- La coinfección y el poliparasitismo observados en la comunidad sugieren interacciones complejas entre los parásitos y el huésped, que pueden influir en la presentación clínica y en la dinámica de transmisión. Los resultados de este estudio refuerzan la importancia de una aproximación integrada de "Una Sola Salud" para abordar la prevención y el control de estas infecciones, considerando no solo a los humanos, sino también a los animales y al ambiente como actores clave en los ciclos de transmisión de los parásitos.

- La colonización por *Blastocystis* está asociada con un aumento en la diversidad bacteriana del intestino, un factor que generalmente se relaciona con mejores resultados de salud. Un microbioma intestinal diverso mejora la resiliencia frente a alteraciones como infecciones y cambios en la dieta.

- En individuos positivos para *Blastocystis*, se observan ciertos taxones bacterianos, como *Bacteroides uniformis*, *Oscillibacter sp.* y *Prevotella copri*, en mayor abundancia. Estos taxones son conocidos por su papel en la absorción de nutrientes, el metabolismo energético y los efectos antiinflamatorios.

- El impacto de *Blastocystis* en el microbioma intestinal varía según la región geográfica y el estilo de vida, con efectos más marcados en poblaciones rurales no occidentalizadas donde el consumo de dietas ricas en vegetales y alimentos fermentados es característica.

- Estos hallazgos resaltan la importancia de la dieta en la composición del microbioma, sugiriendo que *Blastocystis* podría contribuir a los beneficios para la salud, siendo más evidente en individuos con dietas ricas en fibra y vegetales, comunes en entornos rurales.

- Diferentes niveles de carga de *Blastocystis* (alta, media, baja) se asocian con variaciones en la abundancia de ciertos taxones microbianos. Las cargas más altas están relacionadas con taxones como *Alistipes* y *Lachnospira*, conocidos por sus propiedades antiinflamatorias. Una relación dependiente de la dosis sugiere que la abundancia de *Blastocystis* puede influir en el grado en que modula el microbiota intestinal.

- Los individuos positivos para *Blastocystis* presentan perfiles distintos de microbiota eucariota, con mayor abundancia de *Entamoeba coli*, mientras que los grupos negativos muestran más *Saccharomyces cerevisiae* y *Candida albicans*.

- Las posibles interacciones entre *Blastocystis* y otros eucariotas, incluidos los hongos, requieren más investigación para comprender sus efectos en el ecosistema intestinal.

- Un clasificador de *machine learning*, específicamente un *random forest*, distingue efectivamente entre individuos positivos y negativos para *Blastocystis* basándose en la composición de la microbiota, identificando taxones clave como *Faecalibacterium prausnitzii* y *Bacteroides ovatus*. Estos taxones están asociados con propiedades antiinflamatorias y el metabolismo de carbohidratos, lo que sugiere que *Blastocystis* puede fomentar un entorno intestinal favorable para funciones microbianas beneficiosas.

- El estudio contribuye a la hipótesis de que *Blastocystis* podría actuar como un organismo comensal que apoya un microbioma diverso y funcionalmente rico.

- Las investigaciones futuras deberían centrarse en dilucidar los mecanismos por los cuales *Blastocystis* influye en la salud intestinal, con énfasis en estudios longitudinales y métodos avanzados como la metagenómica y la metabolómica.

- Entre las limitaciones se incluyen un tamaño de muestra relativamente pequeño y la falta de datos dietéticos detallados. Es necesario realizar estudios longitudinales con conjuntos de datos complementarios para llegar a conclusiones que permitan determinar direccionalidad y causalidad.

- En resumen, los hallazgos sugieren que la colonización por *Blastocystis* podría desempeñar un papel beneficioso en la configuración de la microbiota intestinal, especialmente en poblaciones rurales no occidentalizadas. Su presencia se asocia con una mayor diversidad microbiana y con taxones vinculados a funciones metabólicas e inmunológicas mejoradas, lo que apoya la noción de *Blastocystis* como un comensal más que como un patógeno.

Capítulo 2.

- En este estudio se propuso evaluar el impacto de la infección por *T. cruzi* en la composición y función de la microbioma intestinal en un modelo murino. Se observó que ambos modelos de ratón presentaron parasitemia detectable a partir del segundo día post-infección (dpi), siendo más elevados los niveles en ratones BALB/c en comparación con C57BL/6.
- Los ratones infectados desarrollaron esplenomegalia, un hallazgo consistente con estudios previos que utilizan cepas quimioluminiscentes.
- Análisis de citoquinas en sueros mostraron una respuesta inmune proinflamatoria predominante en C57BL/6, con incremento de IFN-γ, TNF-α e IL-6. En contraste, los ratones BALB/c mostraron niveles más altos de citoquinas antiinflamatorias como IL-10 e IL-4.
- A través de análisis metagenómicos, se identificaron los mismos taxones con mayores abundancias relativas en ambos modelos: *Lactobacillus johnsonii*, *Akkermansia muciniphila*, *Bacteroides thetaiotaomicron*, *Bacteroides pseudolongum*, *Anaerostipes finegoldii*, *Fusobacterium rodentium* y *Staphylococcus xylosus*.
- Se identificó una correlación negativa entre la reducción de *L. johnsonii* e IL-10 en BALB/c y entre *A. muciniphila* con IL-22 e IFN-γ en C57BL/6.
- Se evidenció que, con el transcurso de la infección por *T. cruzi*, se presentó una reducción de bacterias como *L. johnsonii* y *B. thetaiotaomicron*, las cuales son esenciales para mantener la barrera intestinal y regular la respuesta inmune, así como para procesos metabólicos cruciales como la biosíntesis de ácidos grasos de cadena corta (AGCC).
- La predicción funcional a partir de datos metagenómicos reveló que las alteraciones en las abundancias relativas de los taxones bacterianos descritos se asociaron con una disminución en varios procesos metabólicos, incluyendo la biosíntesis de AGCC en ratones BALB/c. La reducción de AGCC podría afectar la regeneración de tejidos en un entorno proinflamatorio, facilitando así la colonización por patógenos.
- *B. thetaiotaomicron* desempeña un papel crucial en procesos metabólicos esenciales para la fisiología del huésped. La disminución en la abundancia relativa de este taxón podría estar directamente implicada en la disminución de las capacidades metabólicas del microbioma durante la infección por *T. cruzi*.
- Se sugiere que la interacción de *T. cruzi* con el microbioma intestinal podría estar relacionada con la generación de perfiles inmunológicos que favorecen un ambiente proinflamatorio.
- Los cambios en el microbioma intestinal identificados en este estudio sugieren alteraciones en los perfiles inmunológicos y en varias vías metabólicas esenciales que favorecen los

procesos asociados con la infección. Los hallazgos destacan la necesidad de investigar nuevas alternativas terapéuticas para la enfermedad de Chagas, incluyendo el uso de probióticos que podrían ayudar a resolver infecciones y reducir cargas parasitarias.

- Es fundamental realizar estudios transcriptómicos y metabolómicos para corroborar los resultados funcionales predichos.
- Se necesita investigar los efectos en la fase crónica de la infección y establecer la causalidad y direccionalidad de los cambios en el microbioma y la respuesta inmune.
- Este estudio es el primero en reportar MAGs en huéspedes durante un proceso infeccioso por *T. cruzi*, abriendo nuevas oportunidades de investigación sobre las interacciones parásito-microbioma.

Capítulo 3.

- Este estudio ha caracterizado de manera detallada la microbiota asociada a las larvas de *Ascaris* en diversas etapas de desarrollo, evidenciando la complejidad de las interacciones entre el parásito y el hospedero durante el ciclo migratorio larval.

- Se encontraron diferencias significativas en la composición y diversidad de la microbiota entre los grupos analizados, destacando la predominancia de *Proteobacteria* en la microbiota de *Ascaris*, lo que sugiere su papel fundamental en la biología del parásito.

- Los perfiles de microbiota observados en los tejidos del hospedero resaltan la intrincada interacción entre las comunidades microbianas del hospedero y del parásito, sugiriendo un impacto potencial en la salud del hospedero y en el éxito del parásito.

- La evaluación de la microbiota de las larvas de *Ascaris* y sus cambios a lo largo del ciclo larval proporciona información valiosa sobre cómo el parásito se adapta y se beneficia de su entorno, específicamente, de la microbiota del hospedero.

- Se observaron cambios significativos en la abundancia de géneros específicos durante el ciclo migratorio larval. Por ejemplo, los géneros *Lactobacillus*, y *Lachnospiraceae NK4A136* mostraron un aumento en su abundancia en las larvas aisladas del hígado y pulmón en comparación con las larvas en etapas iniciales. Esto sugiere que la adquisición horizontal de estas bacterias del hospedero durante la migración es probable.

- Los hallazgos de este estudio abren nuevas posibilidades para investigar estrategias de control de helmintos, al resaltar la importancia de las interacciones entre microbiota y parásitos en la persistencia y patogenicidad de *Ascaris*.

- Es esencial que futuras investigaciones se centren en comprender los roles funcionales de las comunidades bacterianas dentro de *Ascaris* y los mecanismos de adquisición de estas bacterias, tanto vertical como horizontalmente, para obtener una comprensión más completa de estas interacciones.

- A pesar de los avances logrados, es importante reconocer las limitaciones del estudio, incluyendo el uso de un modelo animal no nativo para *Ascaris*. Se recomienda considerar modelos porcinos en futuros estudios para validar los hallazgos y su aplicabilidad a la infección en humanos.

- En relación con el análisis proteómico, este estudio llevó a cabo una evaluación completa de los proteomas de productos de ES en las diferentes etapas larvales de *Ascaris*, utilizando LC-MS/MS para identificar 58 proteínas únicas en los productos ES de las larvas L3-egg, L3-lung y L3-trachea. Las diferencias en la composición proteica entre estas etapas sugieren funciones biológicas específicas durante el desarrollo, reflejando la complejidad del proceso migratorio del parásito a través del hospedero. Se identificaron 5 proteínas exclusivas de L3-egg, 20 de L3-lung y 3 de L3-trachea, mientras que 14 proteínas fueron conservadas en todas las etapas.

- La identificación de proteínas específicas por etapa proporciona hallazgos valiosos. Por ejemplo, la presencia de factores nucleares asociados a la replicación y transcripción, como Ell-associated factor Eaf (UniProt ID F1LBV5) y Protein SDA1 (UniProt ID F1KVQ9) en L3-egg, sugiere un papel crítico en la división celular y diferenciación, fundamentales para el desarrollo inicial de larvas. En contraste, las proteínas en L3-lung y L3-trachea reflejan una mayor necesidad de proteínas relacionadas con el metabolismo energético, como fructosa-bisfosfato aldolasa (UniProt ID F1L5H2) y malato deshidrogenasa (UniProt ID F1L8Z9), evidenciando la adaptación a entornos anaeróbicos durante la migración a través del pulmón.

- La identificación de proteínas como peptidil-prolilo isomerasa (UniProt ID F1LGZ3) y las proteínas ERM (UniProt ID F1KY69) en las etapas L3-lung y L3-trachea sugiere que estas podrían jugar roles en la modulación inmune y la migración larval. Dada la función de las proteínas de choque térmico (HSPs) en la adaptación del parásito al hospedero, estas características podrían ser objetivos potenciales para intervenciones terapéuticas. Las variaciones en la expresión proteica podrían contribuir a la capacidad del parásito para evadir respuestas inmunitarias y establecer infecciones persistentes.

- Aunque no se detectaron diferencias estadísticamente significativas en la abundancia de proteínas entre las etapas, se observaron diferencias cualitativas evidentes en los perfiles

proteómicos, como lo indica el análisis de componentes principales (PCA). La heterogeneidad de los productos ES y su variabilidad inherente podrían haber enmascarado sutiles diferencias en la abundancia, lo que sugiere la necesidad de enfoques más amplios en futuras investigaciones. El uso de modelos porcinos, que son hospederoes naturales de *Ascaris*, también se recomienda para validar estos hallazgos.

- Las diferencias en la composición proteica y los roles funcionales identificados en las distintas etapas larvales sugieren que estos productos ES podrían ser valiosos para el desarrollo de nuevos diagnósticos y objetivos de vacunas. La caracterización de las interacciones entre las proteínas ES, las células del hospedero y la microbiota del hospedero, podría abrir vías para nuevas estrategias terapéuticas, al identificar dianas moleculares y novedosas estrategias relevantes para la intervención en la ascariasis.

- Este estudio proporciona una visión integral sobre la dinámica proteómica de las larvas de *Ascaris*, destacando la importancia de las proteínas ES en su ciclo de vida y su interacción con el hospedero. Los hallazgos pueden guiar investigaciones futuras dirigidas a desentrañar los mecanismos de patogenicidad de *Ascaris*, así como a desarrollar herramientas efectivas para el control de ascariasis.

- Las interacciones entre helmintos y la microbiota del hospedero son complejas y multidimensionales, influidas por una variedad de factores, incluidos los componentes eucariotas y virales. Estas interacciones no solo afectan la patogénesis de las infecciones por helmintos, sino que también tienen implicaciones significativas para la salud del hospedero.

- La microbiota intestinal desempeña un papel crucial en la susceptibilidad y la respuesta a las infecciones por helmintos. Los cambios en la composición y la diversidad de la microbiota pueden facilitar la colonización y persistencia de estos parásitos, lo que subraya la necesidad de investigar estrategias que modulen la microbiota como posibles enfoques terapéuticos.

- Es esencial adoptar enfoques de investigación integrados que combinen diferentes estrategias ómicas para comprender los mecanismos involucrados, la causalidad y direccionalidad de estas interacciones.

8. PERSPECTIVAS

La comprensión de las interacciones entre los parásitos intestinales, sus hospederoes y el microbioma es un campo de creciente interés, con múltiples áreas en las que se requieren avances para mejorar el conocimiento sobre las dinámicas y consecuencias de estas interacciones. Los estudios futuros deben enfocarse en explorar tanto las bases mecanísticas de la relación parásito-microbioma como en el desarrollo de aplicaciones prácticas para el control de infecciones y el manejo de la salud pública.

- **Estudios longitudinales en parásitos intestinales**

La necesidad de estudios longitudinales es fundamental para caracterizar la evolución temporal de la interacción entre los parásitos, el microbioma y el hospedero. Estos estudios permitirían evaluar cómo las infecciones intestinales crónicas, las intervenciones terapéuticas o los cambios ambientales afectan la composición y función del microbioma a lo largo del tiempo. Además, permitirían elucidar la direccionalidad y causalidad de los cambios observados, lo cual es crucial para determinar si las alteraciones en el microbioma contribuyen a la patogénesis de las infecciones parasitarias o si, en cambio, son una consecuencia de estas. La implementación de cohortes con seguimiento prolongado en poblaciones vulnerables y el uso de técnicas multiómicas podría arrojar luz sobre los factores subyacentes que modulan la resistencia o susceptibilidad a las infecciones.

- **Investigaciones transcriptómicas y metabolómicas**

El empleo de la transcriptómica y la metabolómica ofrece un enfoque integral para el estudio de las interacciones entre parásitos y microbioma. Los análisis transcriptómicos permiten identificar cambios en la expresión génica en respuesta a la infección, proporcionando información sobre las vías moleculares implicadas en la adaptación del parásito y del hospedero. De manera complementaria, la metabolómica ofrece una visión directa de los metabolitos producidos y consumidos en estas interacciones, lo que es fundamental para identificar rutas metabólicas alteradas. En modelos de infección crónica con parásitos como *Trypanosoma cruzi*, estas tecnologías pueden ayudar a caracterizar las adaptaciones funcionales del microbioma y la respuesta inmune del hospedero, así como a identificar biomarcadores potenciales para la detección y el monitoreo de la enfermedad.

- **Desarrollo de modelos crónicos de infección**

La creación de modelos experimentales que simulen infecciones crónicas es esencial para entender la relación a largo plazo entre parásitos y microbiota. Los modelos de infección por *Trypanosoma cruzi*, entre otros parásitos, ofrecen la posibilidad de estudiar cómo las infecciones persistentes modifican la ecología microbiana y cómo los cambios en el microbioma influyen en la progresión de la enfermedad y la respuesta inmune. Además, estos modelos pueden proporcionar información

valiosa sobre la plasticidad del microbioma en respuesta a diferentes etapas de la infección y ayudar a evaluar el impacto de intervenciones terapéuticas dirigidas a restaurar el equilibrio microbiano.

- **Exploración de comunidades bacterianas en *Ascaris* y otros helmintos**

Los parásitos intestinales como *Ascaris* albergan diversas comunidades bacterianas asociadas, cuya composición y función son poco comprendidas. Futuras investigaciones deben centrarse en el análisis de estos microbiomas para elucidar cómo se establecen las interacciones simbióticas entre el parásito y las bacterias, incluyendo los mecanismos de transmisión vertical y horizontal. Un conocimiento detallado de estas asociaciones podría revelar nuevas dianas para el desarrollo de estrategias de control basadas en la manipulación del microbioma asociado a los parásitos, favoreciendo así un enfoque terapéutico complementario.

- **Interacciones eucariotas y virales**

Es esencial ampliar el foco de la investigación para considerar las interacciones de eucariotas y virus con helmintos y el microbioma. La microbiota intestinal no se limita solo a bacterias, sino que también incluye hongos, protistas y virus, todos los cuales juegan un papel en la dinámica de las infecciones parasitarias. La integración de estudios de viromas y micobiomas junto con la caracterización de la microbiota bacteriana podría proporcionar una comprensión más completa de cómo los diferentes componentes del microbioma interactúan entre sí y con el parásito para influir en la salud del hospedero.

- **Avances en bioinformática y análisis de datos**

El desarrollo continuo de herramientas bioinformáticas es esencial para manejar la complejidad de los datos generados en los estudios multiómicos. La integración de datos de secuenciación metagenómica, transcriptómica y metabolómica mediante enfoques de análisis avanzados, como la inteligencia artificial y el aprendizaje automático, facilitará la identificación de patrones emergentes y la inferencia de redes funcionales de interacciones. Además, es fundamental estandarizar los métodos de análisis para mejorar la reproducibilidad entre estudios, lo que permitirá la comparación de datos obtenidos en diferentes contextos y regiones geográficas.

- **Implicaciones clínicas y de salud pública**

Los hallazgos sobre las interacciones parásito-microbioma tienen implicaciones directas en la práctica clínica y en las políticas de salud pública. La identificación de marcadores microbianos específicos asociados a la susceptibilidad a infecciones puede facilitar el desarrollo de pruebas de diagnóstico más precisas. Además, el enfoque de modulación del microbioma como intervención terapéutica podría ser una estrategia prometedora para prevenir o tratar infecciones helmínticas,

especialmente en poblaciones con acceso limitado a medicamentos. La implementación de programas de control basados en la microbiota podría reducir la prevalencia de infecciones intestinales y mejorar la salud intestinal en comunidades endémicas.

9. PRODUCTOS DE LA TESIS

Todos los productos derivados de la formación doctoral y la tesis correspondiente se indican a continuación. Los soportes respectivos se adjuntan con el documento.

9.1. Artículos científicos:

- **Artículo 1: Castañeda, S**, Acosta, Claudia Patricia, Vasquez-A, Luis Reinel, Patiño, Luz H, Mejía, Rojelio, Ramírez JD. Molecular detection of intestinal parasites in a rural community of Colombia: A one health approach to explore potential environmental–zoonotic transmission. Zoonoses Public Health [Internet]. 2024 [cited 2024 Oct 23];71(6):723–735. https://doi.org/10.1111/zph. 13138.

- **Artículo 2: Castañeda S**, Stensvold CR, Andersen LOBB, Acosta, Claudia Patricia, Vasquez-A, Luis Reinel, Ramírez JD. Impact of *Blastocystis* Colonization and Burden on Gut Microbiota Composition in a Non-Westernized Rural Population from Colombia. *Sometido a PLOS Neglected Tropical Diseases (Q1).*

- **Artículo 3: Castañeda S,** Muñoz M, Hotez PJ, Bottazzi ME, Paniz-Mondolfi AE, Jones KM, Mejia R, Poveda C, Ramírez JD. Microbiome Alterations Driven by *Trypanosoma cruzi* Infection in Two Disjunctive Murine Models. Microbiology Spectrum [Internet]. 2023 [cited 2023 Jun 16];11(3). https://doi.org/10.1128/spectrum.00199-23.

- **Artículo 4: Castañeda S,** R,Poveda Charlie Suarez-Reyes, Yifan Wu, Noah Haugen, Patiño, Luz H, Weatherhead JE, Ramírez JD. Microbiota Dynamics During *Ascaris* Larval Migration: Implications for Host Microbial Communities. *Sometido a Microbial Pathogenesis (Q1).*

- **Artículo 5: Castañeda S**, Adeniyi-Ipadeola G, Wu Y, Suarez-Reyes C, Jain A, Ramírez JD, Weatherhead JE. Characterizing Excretory-Secretory Products Proteome Across Larval Development Stages in *Ascaris suum*. *Sometido a Microbes and Infectio*n *(Q1)* https://doi.org/10.1101/2024.07.03.601870.

- **Artículo 6: Castañeda S,** Paniz-Mondolfi A, Ramírez JD. 2024. Detangling the Crosstalk Between *Ascaris, Trichuris,* and Gut Microbiota: What´s Next? Frontiers in Cellular and Infection Microbiology [Internet]. 2022. https://doi.org/10.3389/fcimb.2022.852900.

- **Artículo 7:** Ramírez JD, **Castañeda S,** Weatherhead JE, R,Poveda. 2024. Parasite-Microbiota Interactions: A Pathway to Innovative Interventions for Chagas Disease, Leishmaniasis, and Ascariasis**.** *Sometido a Future Microbiology (Q1).*

9.2. Capítulo de libro:

- **Castañeda S**, Ramírez JD. 2024 Libro: Recent Advances in Parasitomics. Capítulo: From Genes to Worms: A Deep Dive into Helminth Omics. Springer; 2024th edition (2024).

9.3. Presentaciones En Eventos Científicos:

- Poster: ***In silico*** **prediction of gut microbial genes as potential targets for** ***Fasciola hepatica*** **EV-associated miRNAs.** 7th GEIVEX symposium, Palma, Mallorca, España, Octubre 23-25, 2024.

- Poster: **Nanovesicles from frozen homogenized** ***Fasciola hepatica*** **adults exhibit similar properties to extracellular vesicles from parasitic cultures.** 2nd MOVE Symposium. Serbia, October 2024.

- Ponente Mesa Redonda de **Análisis bioinformáticos en Parasitología** con la presentación: ***"Estudio de las interacciones parásito-hospedero-microbioma en infecciones causadas por protozoos y helmintos"***. XXIII Congreso de la Sociedad Española de Parasitología, Sevilla, España, 2 al 5 de septiembre de 2024.

- Presentación Oral: **Molecular Detection of Intestinal Parasites in a Rural Community of Colombia: A One Health Approach to Explore Potential Environmental-Zoonotic Transmission**. XXIII Congreso de la Sociedad Española de Parasitología, Sevilla, España, 2 al 5 de septiembre de 2024.

- Presentación Oral: **Microbiome Alterations Driven By** ***Trypanosoma cruzi*** **Infection In Two Disjunctive Mouse Models.** XVIII Congreso Colombiano de Parasitología y Medicina Tropical. Diciembre 2022.

- Presentación Oral: **Microbiome Alterations Driven By** ***Trypanosoma cruzi*** **Infection In Two Disjunctive Mouse Models.** 15th International Congress of Parasitology. Copenhagen, Dinamarca. Agosto 21 - 26, 2022.

9.4. Cursos:

- COST Action. *Blastocystis* Under One Health. ***Blastocystis*** **and the Gut Microbiome - a bioinformatics training school.** Barcelona. Abril 2024

- Universidad EAN. **Curso Python**. Mar 2023.
- Universidad Javeriana – Universidad Peruana Cayetano Heredia. **International Course on Outbreak Analysis, Modeling and Response in Public Health**. Jul 2021.
- Universidad del Rosario. **Structuring of public investment projects in science, technology and innovation and innovation.** Jun 2021.
- Universidad del Valle. **Fundamentals of Genomics for Health Research.** Mayo 2021
- Winter Genomics. **Metagenome**. Mayo 2021
- RSG/iGEM Ecuador. **UNIX Boot Camp: Linux and Bash Scripting (Basic - Advanced modules)**. Mayo 2021
- Biofreelancer. **Bioinformatics Course**: **Linux**. Enero 2021.
- Pontificia Universidad Javeriana. **Course: R as a Geographical Information System (GIS) Tool**. Diciembre 2020
- Biofreelancer. **R for beginners**. Diciembre 2020
- The School of Biomedical Informatics UTHealth. **Population Health**. Diciembre 2020.
- The School of Biomedical Informatics UTHealth. **Care Coordination & Interoperable Health IT Systems**. Diciembre 2020.
- Welcome Genome Campus Advanced Course. Sanger Institute. **Bacterial genomes II: Accessing and analyzing microbial genome data using ARTEMIS.** Noviembre 2020. Certificado: https://www.futurelearn.com/certificates/2i4dj6n
- Welcome Genome Campus Advanced Course. Sanger Institute. **Bacterial genomes I: from DNA to proteinfunction using bioinformatics.** Octubre 2020. Certificado: https://www.futurelearn.com/certificates/34jw3ei
- Welcome Genome Campus Advanced Course. Sanger Institute. **Working with Protozoan Parasite Database Resources** (Virtual) 5-9 Octubre 2020.

9.5. Pasantía Internacional

2024. Laboratory of Parasitology, Department of Bacteria, Parasites & Fungi of Statens Serum Institut, Copenhagen, Denmark

Inicialmente se desarrolló una colaboración en Laboratory of Parasitology, Department of Bacteria, Parasites & Fungi of Statens Serum Institut (SSI), Copenhagen, Dinamarca, bajo la tutoría del Prof. Rune Stensvold. Durante esta estancia, el trabajo se centró en la identificación

molecular y tipificación de parásitos intestinales en humanos y animales, principalmente *Blastocystis*, así como en la evaluación de los cambios en los perfiles del microbioma asociados con estos parásitos, mediante la secuenciación del ADN ribosomal 16S y 18S en muestras de humanos y mascotas de una población rural del departamento del Cauca (suroeste de Colombia), y sus posibles implicaciones en las interacciones parásito-hospedero-microbioma del hospedero. Durante la estancia en el SSI, se participó activamente en actividades tales como:

- Análisis de laboratorio seco de las secuencias de ADN ya obtenidas.

- Perfilado del microbioma en laboratorio húmedo (secuenciación de genes 16S + 18S) a partir del ADN genómico de heces, utilizando el protocolo y la línea de análisis interna (BION).

- Análisis de laboratorio seco de los datos de salida de la secuenciación del microbioma, incluyendo la evaluación de la calidad de las secuencias y el análisis de la diversidad del microbioma mediante R.

Como producto de este trabajo se ha generado el artículo:

- **Castañeda S,** Stensvold CR, Andersen LOBB, Acosta, Claudia Patricia, Vasquez-A, Luis Reinel, Ramírez JD. Impact of *Blastocystis* Colonization and Burden on Gut Microbiota Composition in a Non-Westernized Rural Population from Colombia. *Submitted*.

Así mismo, gracias al trabajo realizado durante esta pasantía, tuve la oportunidad de ser elegido como **Early Career Researcher Lead.** *Blastocystis* under One Health. COST (European Cooperation in Science and Technology), específicamente como líder de los grupos de trabajo WG2: *Blastocystis* collection and databases (https://blastocystis-cost.com/working-groups/wg2-blastocystis-collection-and-databases/) y del grupo WG3: Omics' generation and analysis (https://blastocystis-cost.com/working-groups/wg3-omics-generation-and-analysis/).

2024. Laboratorio de Bioquímica y Biología Molecular de Parásitos del Grupo Parásitos y Salud, Universitat de València, España.

Así mismo, tuve la oportunidad de realizar una pasantía en el Laboratorio de Bioquímica y Biología Molecular de Parásitos del Grupo Parásitos y Salud, Universitat de València, España, bajo la tutoría del Prof. Antonio Marcilla. Durante esta estancia, el trabajo se centró en el estudio de la acción de las vesículas extracelulares (VEs) en las interacciones parásito-hospedero-microbioma en las infecciones por helmintos, usando como modelo biológico *Fasciola hepatica*. Usando enfoques bioinformáticos y experimentales el estudio se centró en evaluar cómo ciertos

componentes de las VEs, particularmente miRNAs generados por el parásito, pueden tener un efecto en la microbiota del hospedero.

Durante la estancia se participó activamente en actividades tales como:

- Análisis experimental: aislamiento de VEs de *Fasciola hepatica*, microscopía electrónica y confocal, cultivos celulares, entre otros.

- Desarrollo de pipelines bioinformáticos para la predicción de targets de miRNA en bacterias componentes de la microbiota del hospedero.

- Análisis proteómicos de VEs aisladas de *Fasciola hepatica.*

Como resultado de este trabajo se han generado los siguiente productos:

Poster: ***In silico* prediction of gut microbial genes as potential targets for *Fasciola hepatica* EV-associated miRNAs.** 7th GEIVEX symposium, Palma, Mallorca, España, Octubre 23-25, 2024.

Poster: **Nanovesicles from frozen homogenized *Fasciola hepatica* adults exhibit similar properties to extracellular vesicles from parasitic cultures.** 2nd MOVE Symposium. Serbia, October 2024.

9.6. Beca

Becas de Excelencia Doctoral del Bicentenario. Sistema General de Regalías (Departamento del Cauca)

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ANEXOS

Se adjunta carpeta con artículos, certificados y demás documentos.