UNIVERSIDAD SAN FRANCISCO DE QUITO USFQ

Colegio de Posgrados

Factors modulating the human gut resistome

Sara Gabriela Cifuentes Rodríguez

Paúl A. Cárdenas, Ph.D.
Thesis Committee Chairperson

Manuscript style thesis submitted in partial fulfillment of the requirements for the Degree of Doctor of Philosophy (Ph.D.) in Microbiology

Tesis estilo manuscrito presentada en cumplimiento parcial de los requisitos para el Grado de Doctor en Filosofía (Ph.D.) en Microbiología

Quito, 21 de diciembre de 2022

UNIVERSIDAD SAN FRANCISCO DE QUITO USFQ

COLEGIO DE POSGRADOS

We recommended acceptance of this thesis in partial fulfillment of the candidate´s requirements for the Degree of Doctor of Philosophy in Microbiology
Recomendamos la aceptación de esta tesis en cumplimiento parcial de los requisitos de la candidata al Grado de Doctor en Filosofía en Microbiología

Sara Gabriela Cifuentes Rodríguez

Firmas

Paúl A. Cárdenas Ph.D. ______________________________

Director del Trabajo de Titulación

Gabriel Trueba Ph.D. ______________________________

Director del Programa de Doctorado en Microbiología

Carlos Valle Ph.D. ______________________________

Decano del Colegio de Ciencias Biológicas y Ambientales COCIBA

Hugo Burgos Ph.D. ______________________________

Decano del Colegio de Posgrados

Miembros del comité de tesis

Manuel E. Baldeón Ph.D. ______________________________

Paúl A. Cárdenas Ph.D. ______________________________

Patricio Rojas Ph.D. ______________________________

Gabriel Trueba Ph.D ______________________________

Quito, 21 de diciembre de 22

© DERECHOS DE AUTOR

Por medio del presente documento certifico que he leído todas las Políticas y Manuales de la Universidad San Francisco de Quito USFQ, incluyendo la Política de Propiedad Intelectual USFQ, y estoy de acuerdo con su contenido, por lo que los derechos de propiedad intelectual del presente trabajo quedan sujetos a lo dispuesto en esas Políticas.

Asimismo, autorizo a la USFQ para que realice la digitalización y publicación de este trabajo en el repositorio virtual, de conformidad a lo dispuesto en la Ley Orgánica de Educación Superior del Ecuador.

Nombre del estudiante: Sara Gabriela Cifuentes Rodríguez

Código de estudiante: 208766

C.I.: 1002942751

Lugar y fecha: Quito, 21 de diciembre de 2022

ACLARACIÓN PARA PUBLICACIÓN

Nota: El presente trabajo, en su totalidad o cualquiera de sus partes, no debe ser considerado como una publicación, incluso a pesar de estar disponible sin restricciones a través de un repositorio institucional. Esta declaración se alinea con las prácticas y recomendaciones presentadas por el Committee on Publication Ethics COPE descritas por Barbour et al. (2017) Discussion document on best practice for issues around theses publishing, disponible en http://bit.ly/COPETheses.

UNPUBLISHED DOCUMENT

Note: The following graduation project is available through Universidad San Francisco de Quito USFQ institutional repository. Nonetheless, this project – in whole or in part – should not be considered a publication. This statement follows the recommendations presented by the Committee on Publication Ethics COPE described by Barbour et al. (2017) Discussion document on best practice for issues around theses publishing available on http://bit.ly/COPETheses.

DEDICATORIA

Dedico este trabajo a mis hermanas Amanda, Laura y Elsa, y a mis padres Elsa y Mauro quienes son una inspiración constante.

AGRADECIMIENTOS

Agradezco a Paúl Cárdenas por su liderazgo, por la confianza depositada en mí, por su guía y retroalimentación constante en el desarrollo de este trabajo.

A los otros miembros de mi Comité de Tesis, Gabriel Trueba, Patricio Rojas y Manuel Baldeón por contribuir enormemente con este trabajo de investigación, y en mi desarrollo académico y profesional.

A los coautores que no han sido mencionados previamente, Jay Graham, Liseth Salinas, Fernanda Loayza, Carlos Saraiva, María Belén Prado, Henry Cohen, Marco Fornasini cuya colaboración ha sido muy valiosa en la colección de muestras y datos demográficos, análisis e interpretación de datos y revisión de manuscritos.

A la Universidad San Francisco de Quito, al Colegio de Ciencias Biológicas y Ambientales, al Colegio de Posgrados y al Instituto de Microbiología (IM) por acogerme nuevamente como parte de su comunidad y financiar mis estudios.

A cada uno de los profesores, asistentes, y a todo el personal que mantiene en pie el IM porque han hecho posible que este instituto funcione de la mejor manera y que sus estudiantes reciban la mejor instrucción humana, teórica y práctica.

A todas mis compañeras del Programa de Doctorado por su ejemplo, amistad y reciprocidad.

A todos mis compañeros de varios ciclos del Programa de Maestría que también ha sido modelos para seguir.

A todas las personas que fueron reclutadas para participar en los estudios descritos en este trabajo de titulación.

RESUMEN

La resistencia a los antimicrobianos (RAM) amenaza gravemente la salud pública global. Sin estrategias urgentes para frenar y combatir la diseminación de la RAM se espera que para el 2050, 10 millones de personan mueran cada año por esta amenaza. Los países de ingresos económicos bajos y medios son más vulnerables al impacto por la RAM. Es importante abordar la RAM desde el punto de vista de una sola salud y desarrollar estudios para entender la evolución, el surgimiento y los factores que favorecen la diseminación de la RAM. La metagenómica "shotgun" ha surgido como una herramienta para explorar no solo la composición taxonómica microbiana y el potencial funcional de las comunidades microbianas en una muestra, sino también para llevar a cabo estudios de vigilancia en diferentes tipos de muestras complejas como la microbiota intestinal. Siendo la microbiota intestinal un ecosistema complejo, puede favorecer el intercambio de genes de resistencia a los antimicrobianos (GRAs) entre bacterias comensales y patogénicas. Por lo tanto, nosotros buscábamos estudiar los factores que modulan el resistoma intestinal humano usando metagenómica shotgun. En nuestro primer estudio encontramos que la cohabitación con animales domésticos y la cercanía a animales de granja no afecta la estructura general del resistoma intestinal en niños. Sin embargo, genes que confieren resistencia a tetraciclinas fueron significativamente más abundantes en niños expuestos a animales que en aquellos no expuestos. Nuestro segundo estudio demostró además que el resistoma intestinal en niños es altamente variable en el tiempo y es más parecido entre niños de 4, 5 y 7 años de edad que entre niños de corta edad. Además, pudimos observar que genes de importancia clínica eran transitorios en la microbiota intestinal de los niños estudiados. Finalmente, nuestro interés por encontrar estrategias para modificar la microbiota intestinal y su resistoma nos llevó a desarrollar estudios usando probióticos durante una

terapia con antibióticos. Si bien la suplementación con probióticos durante la terapia de erradicación de *Helicobacter pylori* no tiene fuerte evidencia de proteger la composición de la microbiota intestinal, si pudimos demostrar que puede reducir significativamente la abundancia de GRAs.

ABSTRACT

Antimicrobial resistance (AMR) severely threatens public health globally. Without urgent strategies to block and combat the spread of antimicrobial resistance, it is expected that by 2050, 10 million people will die each year from AMR. Low- and middle-income countries (LMICs) are most vulnerable to the impact of AMR. It is crucial to approach AMR from the point of view of a one-health and to develop studies to understand the evolution, emergence, and factors that favor the dissemination of AMR. Shotgun metagenomics has emerged as a tool to explore the microbial taxonomic composition and functional potential of microbial communities in a sample and to conduct surveillance studies on different types of complex samples, such as the gut microbiota. Being the gut microbiota a complex ecosystem, it can favor the exchange of resistance genes between commensal and pathogenic bacteria. Therefore, we sought to study the factors that modulate the human gut resistome using shotgun metagenomics. In our first study, we found that cohabitation with domestic animals and proximity to food animals do not affect children's general structure of the gut resistome. However, genes conferring resistance to tetracyclines were significantly more abundant in children exposed to animals than in those not exposed. Our second study further demonstrated that gut resistome in children is highly variable over time and is more similar among older children than younger children. In addition, we could observe that genes of clinical importance were transient in the gut microbiota of the children studied. Finally, our interest in finding strategies to modify the gut microbiota and its resistome led us to develop studies using probiotics during antibiotic therapy. Although probiotic supplementation during *Helicobacter pylori* eradication therapy does not have strong

evidence of protecting the composition of the gut microbiota, we were able to show that it can significantly reduce the abundance of certain ARGs.

TABLA DE CONTENIDO

DEDICATORIA 5

AGRADECIMIENTOS 6

RESUMEN 7

ABSTRACT 9

CHAPTER 1. GENERAL INTRODUCTION 16

Antimicrobial resistance: a global challenge 16

Gut microbiota: a reservoir of resistance determinants and a horizontal gene transfer contributor 17

Strategies to combat antimicrobial resistance: manipulation of the intestinal resistome 19

References 22

CHAPTER 2. EVALUATION OF CHANGES IN THE FECAL RESISTOME ASSOCIATED WITH CHILDREN'S EXPOSURE TO DOMESTIC ANIMALS AND FOOD ANIMAL PRODUCTION 31

Highlights 32

Abstract 33

Graphical abstract 34

Introduction 35

Materials and Methods 36

Results 38

Discussion 41

References 44

CHAPTER 3. TEMPORAL VARIABILITY OF THE FECAL RESISTOME IN HEALTHY CHILDREN LIVING IN PERI-URBAN COMMUNITIES OF QUITO, ECUADOR 48

Abstract 49

Introduction 49

Materials and Methods 50

Results 56

Discussion 70

References 75

CHAPTER 4. SYSTEMATIC REVIEW: THE EFFECT OF SUPPLEMENTARY PROBIOTICS ON GUT MICROBIOTA STRUCTURE DURING ERADICATION THERAPY AGAINST *HELICOBACTER PYLORI* 89

Abstract 90

Introduction 91

Materials and Methods 92

Results 94

Discussion 102

References 104

CHAPTER 5. *SACCHAROMYCES BOULARDII* CNCM I-745 SUPPLEMENTATION MODIFIES THE FECAL RESISTOME DURING *HELICOBACTER PYLORI* ERADICATION THERAPY 111

Abstract 112

Introduction 113

Materials and Methods 114

Results 118

Discussion 128

References 136

CHAPTER 6. GENERAL CONCLUSIONS 147

CHAPTER 7. PROSPECTS 149

References 150

ANEXOS 153

Anexo A. Chapter 2. Supporting information 153

Anexo B. Chapter 4. Supporting information 186

ÍNDICE DE TABLAS

Table 1. Summary of findings of the studies included in this review. 99

Table 2. Differential abundance of args between interventions immediately after treatments (week 3) *. 125

ÍNDICE DE FIGURAS

Chapter 1

Figure 1. Fecal resistome of children in different exposure groups to domestic and farmed animals……………………………………………………………………39

Figure 2. Structure of the fecal resistome among the three risk groups based on exposure levels to animals…………………………………………………………..40

Chapter 2

Figure 1. Study design and sampling periods……………………..………………..51

Figure 2. Composition of the children's fecal resistome based on sampling periods…58

Figure 3. Composition of the children's fecal resistome based on age…………..……….59

Figure 4. Beta diversity analysis of the children's fecal resistome……………………60

Figure 5. Temporal dynamics of the children's resistome in individual 79 (household 79)…………..……………………………………………………………………….62

Figure 6. Correlation between resistome and children's fecal microbiome…..……....65

Figure 7. Children's fecal microbiota structure is compared by sampling periods……67

Figure 8. Children's fecal microbiota structure is compared by age...…………..……69

Chapter 3

Figure 1. PRISMA flowchart of study selection………………………………………95

Figure 2. Risk of bias analysis using the Rob2b tool …………………………………97

Chapter 4

Figure 1. Study design and antibiotic resistant genes identified in fecal samples from patients who received eradication therapy against *H. pylori*…………………………119

Figure 2. Normalized composition profiling of the fecal resistome..……………..…..121

Figure 3. Differential abundance of ARGs comparison among the three-time points in (A) Sb group and (B) in the control group…..…………………………………..……124

Figure 4. Procrustes analysis of the fecal microbiota and its resistome…………...….126

Figure 5. Regularized Canonical Correlation Analysis (rCCA) between fecal resistome and microbiome……………………………………….………...…………….128

CHAPTER 1. GENERAL INTRODUCTION

Antimicrobial resistance: a global challenge

Antimicrobial resistance (AMR) is one of today's most significant global health challenges (Centers for Disease Control and Prevention, 2019). Antimicrobial resistance seriously threatens advances in modern medicine, infectious disease response capabilities, and advances in health care, life expectancy, and food safety (Centers for Disease Control and Prevention. It is estimated that without urgent action to combat this problem, by 2050, approximately 10 million deaths will be attributed to AMR each year (O'Neill, 2016). Recently, a comprehensive review of the global burden of AMR during 2019, applying predictive statistical models, reported that 1.27 million deaths were attributed to bacterial AMR, while 4.95 million deaths were associated with bacterial AMR (Antimicrobial Resistance Collaborators, 2022).

Low- and middle-income countries (LMICs) are more vulnerable to this threat (Laxminarayan et al., 2020; Nadimpalli et al., 2020; Sulis et al., 2022). Antimicrobial resistance deaths are more frequent in LMICs than in other counties (Antimicrobial Resistance Collaborators, 2022). In 2021, approximately 84% of the world's population lived in LMICs (See: https://data.worldbank.org/). Notably, in informal urban settlements (Nadimpalli et al., 2020), several factors can favor the emergence and spread of AMR, such as densely populated areas, cohabiting or living in proximity with domestic animals and food animals, deficiencies in access to drinking water, hygiene, sanitation, quality health services and lack of control in the use of antimicrobials in humans, animals, environment, and crops (Nadimpalli et al., 2020; Sulis et al., 2022).

Antimicrobial resistance is a One-health issue (Kim & Cha, 2021); all three humans, animal, and environmental domains have clear and close connections to AMR (Robinson et al., 2016). In humans, globally attributed deaths from antimicrobial-

resistant bacterial pathogens include infections due to *Escherichia coli*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Streptococcus pneumoniae*, *Acinetobacter baumannii*, and *Pseudomonas aeruginosa* among the most common (Antimicrobial Resistance Collaborators, 2022). In animals, although the burden of disease by AMR has not been appropriately estimated, it is suggested that in China and the United States of America, the proportion of the total use of antibiotics in a country in animal and veterinary agriculture is 84.3% (Q.-Q. Zhang et al., 2015) and 72% (Food and Drug Administration, 2014), respectively. These data show that the global consumption of antimicrobials in agriculture exceeds that of humans (Robinson et al., 2016). Another contributor to the AMR crisis is the environment, which includes soil, water, air, and built environments (Surette & Wright, 2017). The environment is the largest source of some antimicrobial resistance genes (ARGs) with the potential to be mobilized to pathogenic organisms (Surette & Wright, 2017). The environmental dimension may be more critical in LMICs because, in these locations, there is little management of both animal and human waste and antibiotic residues from industry (Larsson & Flach, 2022). Therefore, the environment plays a crucial role in AMR's evolution, transmission, and expansion when environmental contaminants converge (Larsson & Flach, 2022). Humans are one of the most numerous mammalians on earth (Bar-On et al., 2018) and probably the largest source of waste (Tortell, 2020). Additionally, humans carry some of the most dangerous antimicrobial resistances such as carbapenemases which are used exclusively in humans (Gupta et al., 2011).

Gut microbiota: a reservoir of resistance determinants and a horizontal gene transfer contributor

The human gut is one of the richest and most diverse microbial habitats in the human body (Leviatan et al., 2022; Luckey, 1972). This ecosystem is known as the gut

microbiota and is made up of prokaryotes, viruses, and eukaryotes (Clemente et al., 2012). The microbiota plays a paramount role in the health and disease of its host (Clemente et al., 2012). The microbiota is also a reservoir of opportunistic pathogenic bacteria and a diverse collection of ARGs (Schaik, 2015). Mechanisms of action of antimicrobial resistance include reduced permeability, antibiotic efflux, changes in expression, antibiotic degradation or modification, target protection, and target modification (Boolchandani et al., 2019). When commensal and pathogenic bacteria share the same ecological niche, the opportunity for horizontal gene transfer (HGT) increases (Smillie et al., 2011). The capture, storage, and dissemination of ARGs are commanded by mobile genetic elements (MGEs), such as phages, plasmids, genomic islands, integrons, insertion sequences, and transposons (Partridge et al., 2018). The support of the bacterial adaptive capacity rests on the HGT, and the knowledge reached so far about MGEs is focused on isolates of clinical strains (Partridge et al., 2018). Therefore, understanding more broadly the ecology of antimicrobial-resistant bacteria and their commensal neighbors within complex microbial communities requires culture-independent approaches such as shotgun metagenomics (Boolchandani et al., 2019; S. Singh et al., 2019).

Shotgun metagenomics is based on high-throughput sequencing technologies that, combined with computational pipelines, allow profiling of the microbial taxonomic composition and the functional potential (in this case, the antibiotic resistance pattern) of all microbial members of a community or sample (Pehrsson et al., 2016; Waskito et al., 2022). In addition, shotgun metagenomics overcomes the limitations attributed to amplifying 16S rRNA gene regions. For example, shotgun metagenomics bypasses the biases introduced by having to choose a region to amplify (Rintala et al., 2017), has a better taxonomic resolution (Laudadio et al., 2018), and has a better capability to

describe the complexity of microbial communities (Durazzi et al., 2021; Laudadio et al., 2018). When combined with deconvolution techniques such as Hi-C, shotgun metagenomics can determine the genetic context of ARGs, their mobile capacity (if found within MGEs), and reconstruct metagenomes (Press et al., 2017; Stalder et al., 2019). Metagenomics has also emerged as a tool for AMR surveillance (Duarte et al., 2021; Ko et al., 2022) and for finding new solutions in health care (De, 2019).

Strategies to combat antimicrobial resistance: manipulation of the intestinal resistome

The Global Action Plan launched by the WHO in 2015 aims for measures to combat AMR framed around five strategic objectives. Objective two refers to strengthening knowledge and the scientific base through surveillance and research (World_Health_Organization, 2015). An essential aspect of this objective is understanding the conditions related to the appearance, propagation, and circulation of antimicrobial resistance in the human population and developing basic studies whose results translate into new interventions to address antimicrobial resistance. Regarding new interventions to combat antibiotic resistance, the potential benefits of functional foods (food with a positive impact on an individual's health) to modulate the microbiota and the intestinal resistome emerge (Tsigalou et al., 2020).

Several microbiota-based interventions that modulate gut microbiota ecology have been described, such as fecal microbiota transplantation, fecal filtrate transplantation, diet, fermented foods, prebiotics, probiotics, postbiotics, synbiotics, and phage therapy (Hitch et al., 2022; Tsigalou et al., 2020). There are reports of attempts to modify the gut microbiota to prevent or eradicate the colonization of multidrug-resistant (MDR) bacteria (Wieërs et al., 2020). Some works have successfully used fecal microbiota transplantation (FMT) for decolonizing extended-spectrum beta-lactamases

producing *Escherichia coli* (R. Singh et al., 2014), carbapenem-resistant *Enterobacteriaceae,* carbapenem-resistant *Pseudomonas* spp., methicillin-resistant *Staphylococcus aureus*, and vancomycin-resistant *Enterococcus faecalis* (Crum-Cianflone et al., 2015). Furthermore, the study by Millan et al. not only demonstrated the effectiveness of universal donor FMT in reducing resistant pathogenic bacteria (for example, members of the Proteobacteria phylum: *Klebsiella*, *Escherichia*) but also reduced the abundance of ARGs of different types such as beta-lactam, multidrug efflux pumps, fluoroquinolone, and antibiotic inactivation genes in patients with recurrent *Clostridium difficile* infection compared to healthy donors or healthy controls (Millan et al., 2016). Additionally, probiotics such as *Lactobacillus plantarum* and *Lactobacillus fermentum* have been shown *in vitro* to have the ability to inhibit the growth of naturally resistant nosocomial pathogens such as *Acinetobacter baumannii*, and *Pseudomonas aeruginosa* (Dallal et al., 2017). In critically ill preterm infants, oral administration of *Lactobacillus casei* subspecies *rhamnosus*, *Lactobacillus reuteri*, and *Lactobacillus rhamnosus* effectively prevented gastrointestinal *Candida* colonization and consequently reduced the risk of developing candidemia (Singhi & Kumar, 2016).

However, there is little evidence of functional foods positive and significant effects on the modulation of the human gut resistome. The work of Oliver et al. with a North American population suggests that diets rich in fiber and low protein intake correlate with a lower abundance of ARGs and, therefore, can be considered a potential intervention to reduce the impact of AMR (Oliver et al., 2022). However, two other studies with Brazilian and Dutch populations have not demonstrated significant associations of any signature between gut resistome and long-term dietary habits (Silva et al., 2021; Stege et al., 2022). In a study evaluating a dietary intervention targeting the

gut microbiota in children with obesity or Prader-Willi syndrome (3-16 years of age), the authors demonstrated that this intervention significantly reduced gut resistome (Wu et al., 2016). The previously described dietary intervention study (C. Zhang et al., 2015) included whole grains, traditional Chinese medicinal foods, and prebiotics that significantly reduced the abundance of 27% of ARGs (Wu et al., 2016).

In conclusion, since the intestinal microbiota is a very diverse microbial ecosystem that houses a vast collection of ARGs, it is essential to study the factors that modulate the structure and composition of the resistome to develop tools or new interventions that allow us to prevent the spread of AMR.

References

Antimicrobial Resistance Collaborators. (2022). Global burden of bacterial antimicrobial resistance in 2019: a systematic analysis. *Lancet*, *399*(10325), 629–655. https://doi.org/10.1016/s0140-6736(21)02724-0

Bar-On, Y. M., Phillips, R. & Milo, R. (2018). The biomass distribution on Earth. *Proceedings of the National Academy of Sciences*, *115*(25), 6506–6511. https://doi.org/10.1073/pnas.1711842115

Boolchandani, M., D'Souza, A. W. & Dantas, G. (2019). Sequencing-based methods and resources to study antimicrobial resistance. *Nature Reviews Genetics*, *20*(6), 356–370. https://doi.org/10.1038/s41576-019-0108-4

Centersfor Disease Control and Prevention. (2019). *Antibiotic Resistance Threats in the United States, 2019* (p. 150). https://www.cdc.gov/drugresistance/pdf/threats-report/2019-ar-threats-report-508.pdf

Clemente, J. C., Ursell, L. K., Parfrey, L. W. & Knight, R. (2012). The Impact of the Gut Microbiota on Human Health: An Integrative View. *Cell*, *148*(6), 1258–1270. https://doi.org/10.1016/j.cell.2012.01.035

Crum-Cianflone, N. F., Sullivan, E. & Ballon-Landa, G. (2015). Fecal Microbiota Transplantation and Successful Resolution of Multidrug-Resistant-Organism Colonization. *Journal of Clinical Microbiology*, *53*(6), 1986–1989. https://doi.org/10.1128/jcm.00820-15

Dallal, M. M. S., Davoodabadi, A., Abdi, M., Hajiabdolbaghi, M., Yazdi, M. K. S., Douraghi, M. & Bafghi, S. M. T. (2017). Inhibitory effect of Lactobacillus plantarum and Lb. fermentum isolated from the faeces of healthy infants against nonfermentative bacteria causing nosocomial infections. *New Microbes and New Infections*, *15*, 9–13. https://doi.org/10.1016/j.nmni.2016.09.003

De, R. (2019). Metagenomics: aid to combat antimicrobial resistance in diarrhea. *Gut Pathogens*, *11*(1), 47. https://doi.org/10.1186/s13099-019-0331-8

Duarte, A. S. R., Röder, T., Gompel, L. V., Petersen, T. N., Hansen, R. B., Hansen, I. M., Bossers, A., Aarestrup, F. M., Wagenaar, J. A. & Hald, T. (2021). Metagenomics-Based Approach to Source-Attribution of Antimicrobial Resistance Determinants – Identification of Reservoir Resistome Signatures. *Frontiers in Microbiology*, *11*, 601407. https://doi.org/10.3389/fmicb.2020.601407

Durazzi, F., Sala, C., Castellani, G., Manfreda, G., Remondini, D. & Cesare, A. D. (2021). Comparison between 16S rRNA and shotgun sequencing data for the taxonomic characterization of the gut microbiota. *Scientific Reports*, *11*(1), 3030. https://doi.org/10.1038/s41598-021-82726-y

Food and Drug Administration. (2014). *Antimicrobials Sold or Distributed for Use in Food-ProducingAnimals. Summary Report* (p. 58). https://www.fda.gov/media/94906/download

Gupta, N., Limbago, B. M., Patel, J. B. & Kallen, A. J. (2011). Carbapenem-Resistant Enterobacteriaceae: Epidemiology and Prevention. *Clinical Infectious Diseases*, *53*(1), 60–67. https://doi.org/10.1093/cid/cir202

Hitch, T. C. A., Hall, L. J., Walsh, S. K., Leventhal, G. E., Slack, E., Wouters, T. de, Walter, J. & Clavel, T. (2022). Microbiome-based interventions to modulate gut ecology and the immune system. *Mucosal Immunology*, 1–19. https://doi.org/10.1038/s41385-022-00564-1

Kim, D.-W. & Cha, C.-J. (2021). Antibiotic resistome from the One-Health perspective: understanding and controlling antimicrobial resistance transmission. *Experimental & Molecular Medicine*, *53*(3), 301–309. https://doi.org/10.1038/s12276-021-00569-z

Ko, K. K. K., Chng, K. R. & Nagarajan, N. (2022). Metagenomics-enabled microbial surveillance. *Nature Microbiology*, *7*(4), 486–496. https://doi.org/10.1038/s41564-022-01089-w

Larsson, D. G. J. & Flach, C.-F. (2022). Antibiotic resistance in the environment. *Nature Reviews. Microbiology*, *20*(5), 257–269. https://doi.org/10.1038/s41579-021-00649-x

Laudadio, I., Fulci, V., Palone, F., Stronati, L., Cucchiara, S. & Carissimi, C. (2018). Quantitative Assessment of Shotgun Metagenomics and 16S rDNA Amplicon Sequencing in the Study of Human Gut Microbiome. *OMICS: A Journal of Integrative Biology*, *22*(4), 248–254. https://doi.org/10.1089/omi.2018.0013

Laxminarayan, R., Boeckel, T. V., Frost, I., Kariuki, S., Khan, E. A., Limmathurotsakul, D., Larsson, D. G. J., Levy-Hara, G., Mendelson, M., Outterson, K., Peacock, S. J. & Zhu, Y.-G. (2020). The Lancet Infectious Diseases Commission on antimicrobial resistance: 6 years later. *The Lancet Infectious Diseases*, *20*(Lancet Infect Dis 18 2018), e51–e60. https://doi.org/10.1016/s1473-3099(20)30003-7

Leviatan, S., Shoer, S., Rothschild, D., Gorodetski, M. & Segal, E. (2022). An expanded reference map of the human gut microbiome reveals hundreds of previously unknown species. *Nature Communications*, *13*(1), 3863. https://doi.org/10.1038/s41467-022-31502-1

Luckey, T. D. (1972). Introduction to intestinal microecology. *American Journal of Clinical Nutrition*, *25*(12), 1292–1294. https://doi.org/10.1093/ajcn/25.12.1292

Millan, B., Park, H., Hotte, N., Mathieu, O., Burguiere, P., Tompkins, T. A., Kao, D. & Madsen, K. L. (2016). Fecal Microbial Transplants Reduce Antibiotic-resistant Genes in Patients With Recurrent Clostridium difficile Infection. *Clinical Infectious Diseases*, *62*(12), 1479–1486. https://doi.org/10.1093/cid/ciw185

Nadimpalli, M. L., Marks, S. J., Montealegre, M. C., Gilman, R. H., Pajuelo, M. J., Saito, M., Tsukayama, P., Njenga, S. M., Kiiru, J., Swarthout, J., Islam, M. A., Julian, T. R. & Pickering, A. J. (2020). Urban informal settlements as hotspots of antimicrobial resistance and the need to curb environmental transmission. *Nature Microbiology*, *5*(6), 787–795. https://doi.org/10.1038/s41564-020-0722-0

Oliver, A., Xue, Z., Villanueva, Y. T., Durbin-Johnson, B., Alkan, Z., Taft, D. H., Liu, J., Korf, I., Laugero, K. D., Stephensen, C. B., Mills, D. A., Kable, M. E. & Lemay, D. G. (2022). Association of Diet and Antimicrobial Resistance in Healthy U.S. Adults. *MBio*, *13*(3), e00101-22. https://doi.org/10.1128/mbio.00101-22

O'Neill, J. (2016). *Tackling drug-resistant infections globally: final report and recommendations. The Review on Antimicrobial Resistance*. Review on Antimicrobial Resistance. https://wellcomecollection.org/works/thvwsuba

Partridge, S. R., Kwong, S. M., Firth, N. & Jensen, S. O. (2018). Mobile Genetic Elements Associated with Antimicrobial Resistance. *Clinical Microbiology Reviews*, *31*(4). https://doi.org/10.1128/cmr.00088-17

Pehrsson, E. C., Tsukayama, P., Patel, S., Mejía-Bautista, M., Sosa-Soto, G., Navarrete, K. M., Calderon, M., Cabrera, L., Hoyos-Arango, W., Bertoli, M. T., Berg, D. E., Gilman, R. H. & Dantas, G. (2016). Interconnected microbiomes and resistomes in low-income human habitats. *Nature*, *533*(7602), 212–216. https://doi.org/10.1038/nature17672

Press, M. O., Wiser, A. H., Kronenberg, Z. N., Langford, K. W., Shakya, M., Lo, C.-C., Mueller, K. A., Sullivan, S. T., Chain, P. S. G. & Liachko, I. (2017). Hi-C deconvolution of a human gut microbiome yields high-quality draft genomes and reveals plasmid-genome interactions. *BioRxiv*, 198713. https://doi.org/10.1101/198713

Rintala, A., Pietilä, S., Munukka, E., Eerola, E., Pursiheimo, J.-P., Laiho, A., Pekkala, S. & Huovinen, P. (2017). Gut Microbiota Analysis Results Are Highly Dependent on the 16S rRNA Gene Target Region, Whereas the Impact of DNA Extraction Is Minor. *Journal of Biomolecular Techniques : JBT*, *28*(1), 19–30. https://doi.org/10.7171/jbt.17-2801-003

Robinson, T. P., Bu, D. P., Carrique-Mas, J., Fèvre, E. M., Gilbert, M., Grace, D., Hay, S. I., Jiwakanon, J., Kakkar, M., Kariuki, S., Laxminarayan, R., Lubroth, J., Magnusson, U., Ngoc, P. T., Boeckel, T. P. V. & Woolhouse, M. E. J. (2016). Antibiotic resistance is the quintessential One Health issue. *Transactions of The*

Royal Society of Tropical Medicine and Hygiene, *110*(7), 377–380. https://doi.org/10.1093/trstmh/trw048

Schaik, W. van. (2015). The human gut resistome. *Philosophical Transactions of the Royal Society B: Biological Sciences*, *370*(1670), 20140087. https://doi.org/10.1098/rstb.2014.0087

Silva, S. F. da, Reis, I. B., Monteiro, M. G., Dias, V. C., Machado, A. B. F., Silva, V. L. da & Diniz, C. G. (2021). Influence of Human Eating Habits on Antimicrobial Resistance Phenomenon: Aspects of Clinical Resistome of Gut Microbiota in Omnivores, Ovolactovegetarians, and Strict Vegetarians. *Antibiotics*, *10*(3), 276. https://doi.org/10.3390/antibiotics10030276

Singh, R., Nood, E., Nieuwdorp, M., Dam, B., Berge, I. J. M., Geerlings, S. E. & Bemelman, F. J. (2014). Donor feces infusion for eradication of Extended Spectrum beta-Lactamase producing Escherichia coli in a patient with end stage renal disease. *Clinical Microbiology and Infection*, *20*(11), O977–O978. https://doi.org/10.1111/1469-0691.12683

Singh, S., Verma, N. & Taneja, N. (2019). The human gut resistome: Current concepts & future prospects. *The Indian Journal of Medical Research*, *150*(4), 345–358. https://doi.org/10.4103/ijmr.ijmr_1979_17

Singhi, S. C. & Kumar, S. (2016). Probiotics in critically ill children. *F1000Research*, *5*, F1000 Faculty Rev-407. https://doi.org/10.12688/f1000research.7630.1

Smillie, C. S., Smith, M. B., Friedman, J., Cordero, O. X., David, L. A. & Alm, E. J. (2011). Ecology drives a global network of gene exchange connecting the human microbiome. *Nature*, *480*(7376), 241–244. https://doi.org/10.1038/nature10571

Stalder, T., Press, M. O., Sullivan, S., Liachko, I. & Top, E. M. (2019). Linking the resistome and plasmidome to the microbiome. *The ISME Journal*, *13*(10), 2437–2446. https://doi.org/10.1038/s41396-019-0446-4

Stege, P. B., Hordijk, J., Shetty, S. A., Visser, M., Viveen, M. C., Rogers, M. R. C., Gijsbers, E., Dierikx, C. M., Plaats, R. Q. J. van der, Duijkeren, E. van, Franz, E., Willems, R. J. L., Fuentes, S. & Paganelli, F. L. (2022). Impact of long-term dietary habits on the human gut resistome in the Dutch population. *Scientific Reports*, *12*(1), 1892. https://doi.org/10.1038/s41598-022-05817-4

Sulis, G., Sayood, S. & Gandra, S. (2022). Antimicrobial resistance in low- and middle-income countries: current status and future directions. *Expert Review of Anti-Infective Therapy*, *20*(2), 147–160. https://doi.org/10.1080/14787210.2021.1951705

Surette, M. D. & Wright, G. D. (2017). Lessons from the Environmental Antibiotic Resistome. *Annual Review of Microbiology*, *71*, 309-329. https://doi.org/10.1146/annurev-micro-090816-093420

Tortell, P. D. (2020). Earth 2020: Science, society, and sustainability in the Anthropocene. *Proceedings of the National Academy of Sciences*, *117*(16), 8683–8691. https://doi.org/10.1073/pnas.2001919117

Tsigalou, C., Konstantinidis, T., Stavropoulou, E., Bezirtzoglou, E. E. & Tsakris, A. (2020). Potential Elimination of Human Gut Resistome by Exploiting the Benefits of

Functional Foods. *Frontiers in Microbiology*, *11*, 50. https://doi.org/10.3389/fmicb.2020.00050

Waskito, L. A., Rezkitha, Y. A. A., Vilaichone, R., Wibawa, I. D. N., Mustika, S., Sugihartono, T. & Miftahussurur, M. (2022). Antimicrobial Resistance Profile by Metagenomic and Metatranscriptomic Approach in Clinical Practice: Opportunity and Challenge. *Antibiotics*, *11*(5), 654. https://doi.org/10.3390/antibiotics11050654

Wieërs, G., Belkhir, L., Enaud, R., Leclercq, S., Foy, J.-M. P. de, Dequenne, I., Timary, P. de & Cani, P. D. (2020). How Probiotics Affect the Microbiota. *Frontiers in Cellular and Infection Microbiology*, *9*, 454. https://doi.org/10.3389/fcimb.2019.00454

World_Health_Organization. (2015). *Global Action Plan on Antimicrobial Resistance* (p. 45). World Health Organization. https://www.who.int/publications/i/item/9789241509763

Wu, G., Zhang, C., Wang, J., Zhang, F., Wang, R., Shen, J., Wang, L., Pang, X., Zhang, X., Zhao, L. & Zhang, M. (2016). Diminution of the gut resistome after a gut microbiota-targeted dietary intervention in obese children. *Scientific Reports*, *6*(1), 24030. https://doi.org/10.1038/srep24030

Zhang, C., Yin, A., Li, H., Wang, R., Wu, G., Shen, J., Zhang, M., Wang, L., Hou, Y., Ouyang, H., Zhang, Y., Zheng, Y., Wang, J., Lv, X., Wang, Y., Zhang, F., Zeng, B., Li, W., Yan, F., … Zhao, L. (2015). Dietary Modulation of Gut Microbiota Contributes to Alleviation of Both Genetic and Simple Obesity in Children. *EBioMedicine*, *2*(8), 968–984. https://doi.org/10.1016/j.ebiom.2015.07.007

Zhang, Q.-Q., Ying, G.-G., Pan, C.-G., Liu, Y.-S. & Zhao, J.-L. (2015). Comprehensive Evaluation of Antibiotics Emission and Fate in the River Basins of China: Source Analysis, Multimedia Modeling, and Linkage to Bacterial Resistance. *Environmental Science & Technology*, *49*(11), 6772–6782. https://doi.org/10.1021/acs.est.5b00729

J Glob Antimicrob Resist. **2022 Oct 3;31:212-215. doi: 10.1016/j.jgar.2022.09.009.**

CHAPTER 2. EVALUATION OF CHANGES IN THE FECAL RESISTOME ASSOCIATED WITH CHILDREN'S EXPOSURE TO DOMESTIC ANIMALS AND FOOD ANIMAL PRODUCTION

Sara G. Cifuentes[a], Jay Graham[b], Fernanda Loayza[a], Carlos Saraiva[a], Liseth Salinas[a], Gabriel Trueba[a] , Paúl A. Cárdenas[a,*]

[a] Universidad San Francisco de Quito USFQ, Colegio de Ciencias Biológicas y Ambientales, Instituto de Microbiología, Quito, Pichincha, Ecuador

[b] University of California, Environmental Health Sciences Division, Berkeley, California, USA

*Corresponding author: Paúl A. Cárdenas. Mailing address: Universidad San Francisco de Quito USFQ, Instituto de Microbiología, Vía Interoceánica y Diego de Robles, Cumbayá, Ecuador.

Email: pacardenas@usfq.edu.ec

Highlights

- The pediatric gut microbiota is a reservoir of antimicrobial resistance genes (ARG).
- The abundance of specific ARGs is significantly different between risk groups.
- Animal exposure did not affect the overall pediatric gut resistome structure.

Abstract

Background

The pediatric gut microbiota is a reservoir of antimicrobial resistance genes. Environmental factors such as a child's exposure to fecal contamination and antimicrobial resistance genes of animal origin likely shape the resistome of infants and children.

Objective

This study measured how different levels of exposure to domestic or food animals affect the structure of the intestinal resistome in children between 1 to 7 years of age.

Methods

One hundred and nineteen fecal samples from 39 children were analyzed according to the level of exposure to domestic or food animals and categorized into three risk groups. Using high-throughput sequencing with an Illumina NovaSeq 6000 SP platform, we performed fecal resistome analyses using the Resfinder database. Additionally, ResistoXplorer was used to characterize the resistomes of children differentially exposed to domestic animals.

Results

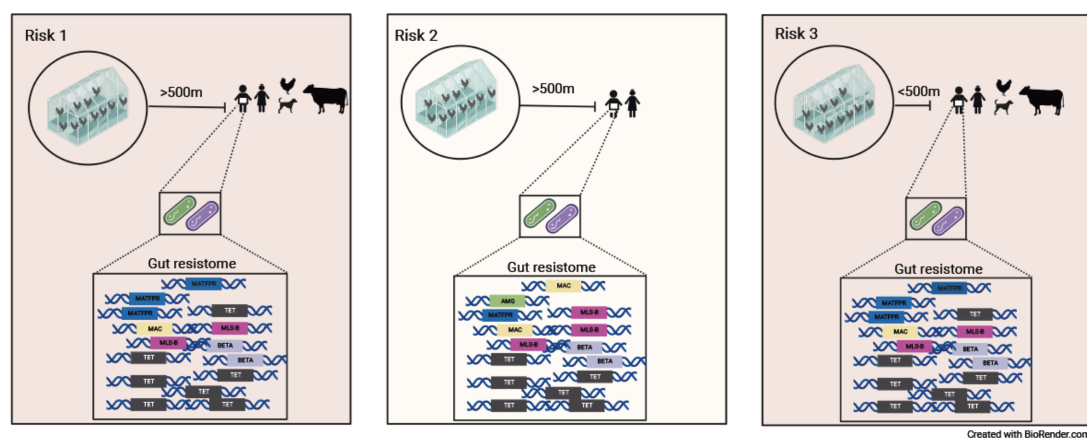
Our data indicated that specific ARGs such as those that confer resistance to MATFPR (macrolide, aminoglycoside, tetracycline, fluoroquinolone, phenicol, and rifamycin) and tetracyclines were statistically less abundant in the group of children without exposure to animals (group 2), compared with the groups exposed to domestic and food animals

(group 1 and 3). However, the overall resistome structure among the children was not affected by the different levels of exposure to animals.

Conclusion

This study suggests that animal exposure is a risk factor for young children acquiring specific antimicrobial resistance genes from domestic animals or animal production areas. However, the overall resistome structure was not affected.

Graphical abstract



Keywords:

Gut resistome, children, domestic animals, food animal production, Ecuador

Introduction

The gut microbiota is a densely populated ecosystem and an essential reservoir of antimicrobial resistance genes (ARGs) (Penders et al., 2013). Even children who have not recently been exposed to the selective pressure of antimicrobials are found to have a large and diverse collection of ARGs (Moore et al., 2013). The gut resistome (collection of resistance genes in the intestinal microbiota) is enriched and shaped by environmental factors, including diet, certain drugs (Roy et al., 2020), and the exposure to antibiotics used clinically and during animal production according to the geographical context in which they live (Forslund et al., 2014). It has been reported that cohabitation of humans with domestic animals in the agricultural context, and the improper handling of excreta, favor the exchange of ARGs between humans and animals in rural areas (Pehrsson et al., 2016). Thus, it has been shown that phylogenetic composition and resistance determinants of the human fecal microbiota are more similar to the microbiota of soil close to chicken coops than to the soil of any other place in rural areas (Pehrsson et al., 2016).

In particular, infants and young children in rural areas in low- and middle-income countries (LMIC) where small-scale animal production is commonly practiced are exposed to zoonotic enteric pathogens due to coprophagia or through various exposures such as hands, toys, cutlery, flies, and food (Ngure et al., 2013). In semi-rural communities near Quito, Ecuador, a study found that several third-generation cephalosporin-resistant *Escherichia coli* strains isolated from the feces of young children, dogs, and chickens had acquired different resistance determinants and replicons. However, the authors found clonal transmission of this bacterial species between humans and domestic animals (Salinas et al., 2021). Therefore, in this analysis, we hypothesized that children who live near animal production areas or own domestic

animals have a richer and more diverse resistome than those who live in households that are not exposed to animals.

Materials and Methods

Study population and fecal samples

The present study was conducted from August 2018 to September 2021 in peri-urban and rural areas east of Quito. The parishes of Tumbaco, Puembo, Tababela, Pifo, Yaruquí, Checa, and El Quinche are characterized by agricultural activities and small to large-scale animal production, particularly chickens. Children from 3 months to 7 years old were included, and three risk groups for exposure of children to domestic and food animals were established. Risk group 1: children who lived more than 500 meters away from chicken operations and who had domestic animals at home (n = 18, 44 fecal samples); risk group 2: children who lived at a distance greater than 500 meters from chicken operations and did not own domestic animals (n = 15, 38 fecal samples), and risk group 3: children who lived less 500 meters away from chicken operations and had domestic animals at home (n = 13, 37 fecal samples) (Figure 1a). Thirty-nine children were included in the present study (several children moved to other places and changed their risk groups; that is why the number of children in each group does not coincide with the total number of children included in the study). The caregivers were instructed to collect and store child fecal samples (Salinas et al., 2021) until field staff could pick them up in a few hours and transport them at approximately 4°C to the laboratory. The caregivers delivered between 1 to 5 serial samples from 5 months to 1 year apart between collections. One hundred nineteen fecal samples were obtained and stored at -80°C until further analysis.

DNA extraction and sequencing

Five hundred mg of fecal material from each sample was used for the FastDNA ™ SPIN Kit for Soil (MP Biomedicals, USA) to obtain 60ul of DNA stored at -20C^{o} until further analysis. The quality and quantity of the DNA were measured with a Nanodrop (NanoDrop ND-2000, Thermo Scientific) using the absorption ratios of 260/280 and 260/230. The DNA was lyophilized and sent to the High Throughput Sequencing Facility at the University of North Carolina in Chapel Hill, NC, USA, where the library preparation (KAPA libraries using the Mantis system) and the control quality were carried out. The Illumina NovaSeq 6000 SP platform was used for sequencing and generated 2X150bp paired-end sequences.

Bioinformatic analysis

The child fecal resistome analysis protocol followed the AMrPlusPlus pipeline (https://megares.meglab.org/amrplusplus/latest/html/). Briefly, the raw sequences were processed to improve their quality and remove contamination using the FastQC and Trimmomatic tools, respectively (Andrews, 2010; Bolger et al., 2014). We removed the host genome to conduct the resistome analysis using the Resistome Analyzer tool (https://github.com/cdeanj/resistomeanalyzer) together with the ResFinder database (Zankari et al., 2012). This last step generated tables of short-read counts assigned to a specific resistance gene. Only reads with an 80% or more identity threshold in the gene fraction (Noyes et al., 2016) were included in the subsequent analyses.

Statistical analysis

The graphical representation and statistical analyses were carried out using the web tool ResistoXplorer (Dhariwal et al., 2021). Before applying any statistical tests, the data were filtered to remove low-quality data. Subsequently, the Cumulative Sum Scaling (CSS) method (Paulson et al., 2013), popularly used in analyzing metagenomic data, was used. We applied the diversity measures with the Chao1, Pielous's evenness, and Shannon indices applying the Kruskal-Wallis statistical method for the alpha diversity analysis. We used the NMDS ordination method with the Bray-Curtis index and the PERMANOVA statistical method for the beta diversity analysis. For the differential abundance analysis of ARGs, we used edgeR (M. D. Robinson et al., 2010) with the Trimmed Mean of M-values (TMM) method(Pereira et al., 2018) to normalize the data. Significant differences were declared with a P-value <0.05, and in the case of differential abundance analysis, adjusted P-values (false discovery rate (FDR)) <0.05 were used by applying the Benjamini–Hochberg method (Benjamini & Hochberg, 1995).

Results

In the 119 fecal samples analyzed, 339 different ARGs were found that confer resistance to 8 classes of antibiotics (Figure 1b). The most abundant ARGs conferred resistance to tetracyclines (75%), MLS_B (macrolide, lincosamides, and streptogramin B) (9%), and beta-lactams (8%). The relative abundance profile showed that ARGs conferring resistance to tetracyclines, lincosamides, and MATFPRs (macrolide, aminoglycoside, tetracycline, fluoroquinolone, phenicol, and rifamycin) were less abundant in risk group 2 compared to the other two risk groups. In risk group 2,

however, resistance to macrolides, MLS_B, and MS_B (macrolide and streptogramin B) were more abundant compared to risk groups 1 and 3. A differential abundance analysis confirmed that ARG alleles conferring resistance to MATFPR (*mdf*(A)*_1_Y08743*, FDR <0.001) and to tetracyclines (*tet*(O/W)*_1_AM889118*, FDR <0.05, and *tet*(32)*_2_EF626943*, FDR <0.05) were statistically less abundant in group 2, compared with group 1 and 3. On the other hand, the ARG allele against MLS_B (*erm*(G)*_1_M15332*, FDR <0.001) was significantly more abundant in the group of risk 2 compared to the other two risk groups.

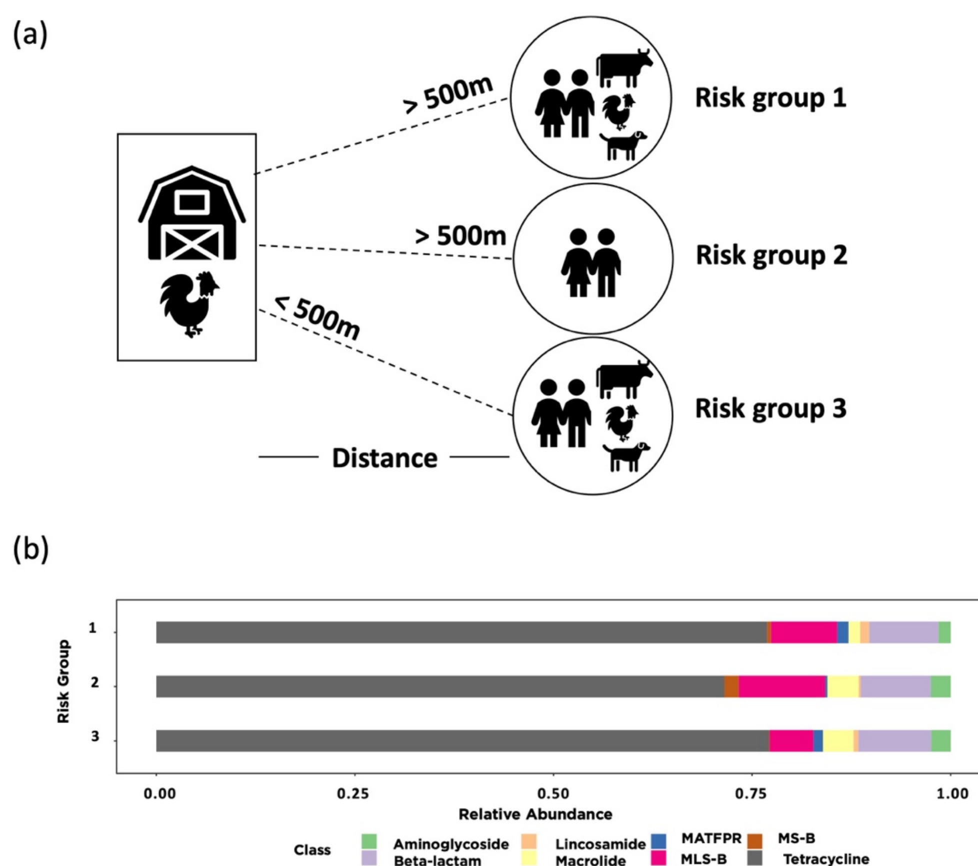


Figure 1. Fecal resistome of children in different exposure groups to domestic and farmed animals. (a) Study design and risk groups. (b) Relative abundance in the proportion of fecal ARGs (classified by class of antimicrobials) grouped in different risk groups.

The alpha diversity analysis showed no significant differences in ARGs' richness, evenness, and diversity between the three risk groups (Figure 2a). Also, when we carried out an ordination analysis to compare the dissimilarities between fecal samples, it was observed that there were no significant differences between fecal samples based on the children's fecal resistome (Figure 2b).

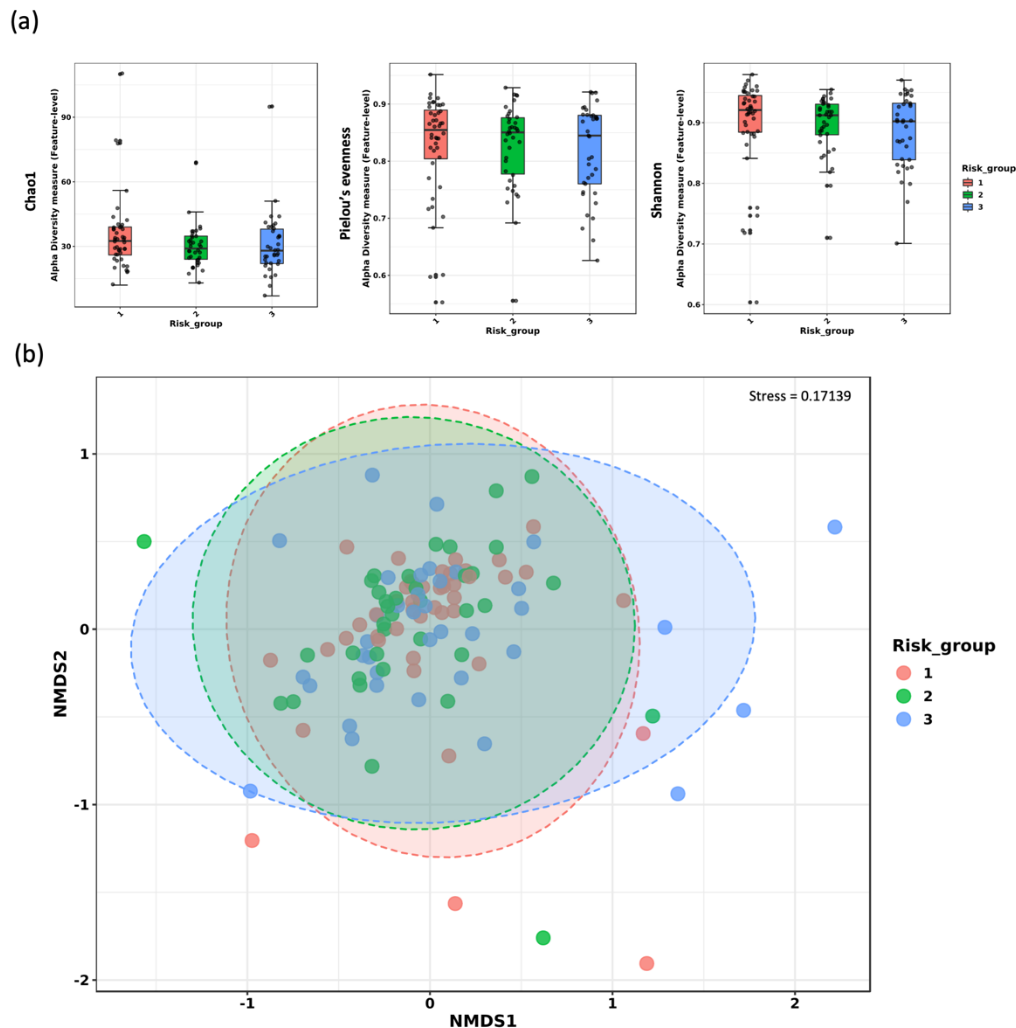


Figure 2. Structure of the fecal resistome among the three risk groups based on exposure levels to animals. (a) Alpha diversity was measured with the Chao1 (richness), Pielou's evenness (evenness), and Shannon (diversity) indices. Central black horizontal lines indicate median values; the 25th and 75th percentiles are indicated (boxes), and the whiskers extend from each end of the box to the most extreme values. P-values were obtained using the Kruskal-Wallis test, with P <0.05 as the significance threshold. (b)

NMDS plot based on a Bray-Curtis dissimilarity matrix; significance was obtained using the PERMANOVA test.

Discussion

In this study, we explored whether cohabitation with domestic animals and closeness to animal production areas were related to changes in the intestinal resistome of children between 1 and 7 years of age. Our findings indicated that whole children's fecal resistome structure did not change due to different levels of exposure to domestic and food animal production. However, there were specific resistance genes (*mdf*(A), *tet*(O/W), *tet*(32), *erm*(G)) whose abundance were significantly different between risk groups.

Similarly, a previous report that evaluated the fecal resistome of one-year-old children shows that the ARGs that confer resistance to tetracyclines and beta-lactams are the most frequent and abundant (Li et al., 2021). However, the authors also frequently found ARGs that confer resistance to fluoroquinolones, unlike our finding where we found ARGs against MLS_B. Genes that confer resistance to tetracyclines (*tet*(O/W) and *tet*(32)) were significantly less abundant in the group of children who do not own domestic animals and live far from chicken operations. It can be explained by the fact that tetracyclines are one of the most used antibiotics worldwide in veterinary medicine for the prevention and treatment of diseases and as growth promoters (Chowdhury et al., 2014; Kyselková et al., 2015). Furthermore, the ARGs *tet*(O), *tet*(W), and *tet*(32) are genes frequently found in the gut of chickens and cattle (Juricova et al., 2021; Kyselková et al., 2015). The relative abundance of *Escherichia coli* was reduced in the risk group 2 compared to groups 1 and 3 (data not shown). Due to *mdf*(A) being a membrane transporter frequently found in *E. coli,* it could explain

that *mdf*(A) gene was significantly less abundant in risk group 2 compared with the other two groups.

We have shown that the whole structure of the fecal resistome was not modified by animal-human cohabitation or its proximity to animal husbandry areas. Nevertheless, the proximity of young children to domestic animals poses a risk to the acquisition of certain ARGs. The fecal contamination of the environment where young children live is possibly the most likely source of ARGs of animal origin (Salinas et al., 2021).

Further research is needed to address antimicrobial resistance transmission from a One Health perspective. This preliminary study is the starting point for using metagenomics to study the evolution and transmission dynamics of antimicrobial-resistant bacteria in the human-animal-environment triad in this geographic area.

Acknowledgments

We are grateful for the work of Soledad Sarsoza and all the personnel involved in collecting of epidemiological data and fecal samples in the field, such as Valeria Garzón, Paúl Barahona, Gabriela Heredia, Cristian Sisalema, and Josue Barahona. Additionally, we acknowledge the great work and support in the methodology and bioinformatics tools of Belén Prado; of course, we thank the parents and children involved in the present study.

Data availability

The sequences were deposited in the European Nucleotide Archive under accession project number **PRJEB50568**.

Funding

This work was supported in part by the National Institutes of Health (NIH) (award number R01AI135118). PC was funded by NIH Fogarty International Center and the Global Health Equity Scholars Program (award number D43TW010540). The funders had no role in study design, data collection, interpretation, or the decision to submit the work for publication.

Competing Interest

None declared

Ethical approval

The Ethics Committee for Research in Human Beings at the Universidad San Francisco de Quito USFQ (code 2017-178M) and the Committee for Protection of Human Subjects (CPHS) at the University of California–Berkeley (Federal wide Assurance #6252) approved the study. The parents or guardians signed the informed consent form, and the children, who were old enough, consented once the research protocol was fully explained.

References

Andrews, S. (2010). *FastQC: a quality control tool for high throughput sequence data*. https://www.bioinformatics.babraham.ac.uk/projects/fastqc/

Benjamini, Y. & Hochberg, Y. (1995). Controlling the False Discovery Rate: A Practical and Powerful Approach to Multiple Testing. *Journal of the Royal Statistical Society*, *57*(1), 289–300. https://doi.org/10.1111/j.2517-6161.1995.tb02031.x

Bolger, A. M., Lohse, M. & Usadel, B. (2014). Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformatics*, *30*(15), 2114–2120. https://doi.org/10.1093/bioinformatics/btu170

Chowdhury, P. R., McKinnon, J., Wyrsch, E., Hammond, J. M., Charles, I. G. & Djordjevic, S. P. (2014). Genomic interplay in bacterial communities: implications for growth promoting practices in animal husbandry. *Frontiers in Microbiology*, *5*, 394. https://doi.org/10.3389/fmicb.2014.00394

Dhariwal, A., Junges, R., Chen, T. & Petersen, F. C. (2021). ResistoXplorer: a web-based tool for visual, statistical and exploratory data analysis of resistome data. *NAR Genomics and Bioinformatics*, *3*(1), lqab018. https://doi.org/10.1093/nargab/lqab018

Forslund, K., Sunagawa, S., Coelho, L. P. & Bork, P. (2014). Metagenomic insights into the human gut resistome and the forces that shape it. *BioEssays*, *36*(3), 316–329. https://doi.org/10.1002/bies.201300143

Juricova, H., Matiasovicova, J., Kubasova, T., Cejkova, D. & Rychlik, I. (2021). The distribution of antibiotic resistance genes in chicken gut microbiota commensals. *Scientific Reports*, *11*(1), 3290. https://doi.org/10.1038/s41598-021-82640-3

Kyselková, M., Jirout, J., Vrchotová, N., Schmitt, H. & Elhottová, D. (2015). Spread of tetracycline resistance genes at a conventional dairy farm. *Frontiers in Microbiology*, *6*, 536. https://doi.org/10.3389/fmicb.2015.00536

Li, X., Stokholm, J., Brejnrod, A., Vestergaard, G. A., Russel, J., Trivedi, U., Thorsen, J., Gupta, S., Hjelmsø, M. H., Shah, S. A., Rasmussen, M. A., Bisgaard, H. & Sørensen, S. J. (2021). The infant gut resistome associates with E. coli, environmental exposures, gut microbiome maturity, and asthma-associated bacterial composition. *Cell Host & Microbe*, *29*(6), 975-987.e4. https://doi.org/10.1016/j.chom.2021.03.017

Moore, A. M., Patel, S., Forsberg, K. J., Wang, B., Bentley, G., Razia, Y., Qin, X., Tarr, P. I. & Dantas, G. (2013). Pediatric Fecal Microbiota Harbor Diverse and Novel Antibiotic Resistance Genes. *PLoS ONE*, *8*(11), e78822. https://doi.org/10.1371/journal.pone.0078822

Ngure, F. M., Humphrey, J. H., Mbuya, M. N. N., Majo, F., Mutasa, K., Govha, M., Mazarura, E., Chasekwa, B., Prendergast, A. J., Curtis, V., Boor, K. J. & Stoltzfus, R. J. (2013). Formative Research on Hygiene Behaviors and Geophagy among Infants and Young Children and Implications of Exposure to Fecal Bacteria. *The American Journal of Tropical Medicine and Hygiene*, *89*(4), 709–716. https://doi.org/10.4269/ajtmh.12-0568

Noyes, N. R., Yang, X., Linke, L. M., Magnuson, R. J., Cook, S. R., Zaheer, R., Yang, H., Woerner, D. R., Geornaras, I., McArt, J. A., Gow, S. P., Ruiz, J., Jones, K. L., Boucher, C. A., McAllister, T. A., Belk, K. E. & Morley, P. S. (2016). Characterization of the resistome in manure, soil and wastewater from dairy and beef production systems. *Scientific Reports*, *6*(1), 24645. https://doi.org/10.1038/srep24645

Paulson, J. N., Stine, O. C., Bravo, H. C. & Pop, M. (2013). Differential abundance analysis for microbial marker-gene surveys. *Nature Methods*, *10*(12), 1200–1202. https://doi.org/10.1038/nmeth.2658

Pehrsson, E. C., Tsukayama, P., Patel, S., Mejía-Bautista, M., Sosa-Soto, G., Navarrete, K. M., Calderon, M., Cabrera, L., Hoyos-Arango, W., Bertoli, M. T., Berg, D. E., Gilman, R. H. & Dantas, G. (2016). Interconnected microbiomes and resistomes in low-income human habitats. *Nature*, *533*(7602), 212–216. https://doi.org/10.1038/nature17672

Penders, J., Stobberingh, E. E., Savelkoul, P. H. M. & Wolffs, P. F. G. (2013). The human microbiome as a reservoir of antimicrobial resistance. *Frontiers in Microbiology*, *4*, 87. https://doi.org/10.3389/fmicb.2013.00087

Pereira, M. B., Wallroth, M., Jonsson, V. & Kristiansson, E. (2018). Comparison of normalization methods for the analysis of metagenomic gene abundance data. *BMC Genomics*, *19*(1), 274. https://doi.org/10.1186/s12864-018-4637-6

Robinson, M. D., McCarthy, D. J. & Smyth, G. K. (2010). edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. *Bioinformatics*, *26*(1), 139–140. https://doi.org/10.1093/bioinformatics/btp616

Roy, C. I. L., Bowyer, R. C. E., Carr, V. R., Costeira, R., Castillo-Fernandez, J. E., Martin, T. C., Spector, T. D., Steves, C. J., Moyes, D., Forslund, S. K. & Bell, J. T. (2020). Host genetic and environmental factors shape the human gut resistome. *BioRxiv*, 2020.05.18.092973. https://doi.org/10.1101/2020.05.18.092973

Salinas, L., Loayza, F., Cárdenas, P., Saraiva, C., Johnson, T. J., Amato, H., Graham, J. P. & Trueba, G. (2021). Environmental Spread of Extended Spectrum Beta-Lactamase (ESBL) Producing Escherichia coli and ESBL Genes among Children and Domestic Animals in Ecuador. *Environmental Health Perspectives*, *129*(2), 027007. https://doi.org/10.1289/ehp7729

Zankari, E., Hasman, H., Cosentino, S., Vestergaard, M., Rasmussen, S., Lund, O., Aarestrup, F. M. & Larsen, M. V. (2012). Identification of acquired antimicrobial resistance genes. *Journal of Antimicrobial Chemotherapy*, *67*(11), 2640–2644. https://doi.org/10.1093/jac/dks261

CHAPTER 3. TEMPORAL VARIABILITY OF THE FECAL RESISTOME IN HEALTHY CHILDREN LIVING IN PERI-URBAN COMMUNITIES OF QUITO, ECUADOR

Sara G. Cifuentes[1], Jay Graham[2], Fernanda Loayza[1], Carlos Saraiva[1], Liseth Salinas[1], Gabriel Trueba[1], Paúl A. Cárdenas[1*]

1 Universidad San Francisco de Quito USFQ, Colegio de Ciencias Biológicas y Ambientales, Instituto de Microbiología, Quito, Pichincha, Ecuador

2 University of California, Environmental Health Sciences Division, Berkeley, California, USA

***Corresponding author:** Paúl A. Cárdenas.

E-mail: pacardenas@usfq.edu.ec

Abstract

The gut microbiome is a source of antimicrobial resistance genes (ARGs), and little is known about the temporal dynamics of the gut resistome over relatively short periods of time. In the present study, we measured the fecal resistome longitudinally in children ages three months to 7 years old and evaluated changes in the diversity and relative abundance of antimicrobial resistance genes over time. From a cohort of 29 children, we obtained 104 fecal samples (range: 3 to 5 fecal samples per child for three years). Next-generation sequencing with an Illumina NovaSeq 6000 platform was used to characterize children's resistome and fecal microbiota according to sampling periods and age. The relative abundance of ARGs within each child's fecal sample varied significantly over time based on hierarchical clustering analysis. We observed transitory elevations for some clinically important ARGs (e.g., extended-spectrum beta-lactamases-ESBLs, colistin, and fluoroquinolone resistance) among 29 samples. We found that the fecal samples from children aged three months to 7 years old that shared similar bacterial profiles also had similar resistomes. This finding highlights how the taxonomic composition of child feces influences the specific ARGs making up the resistome.

Introduction

The rise in antimicrobial resistance (AMR) threatens global public health by attenuating the ability of antimicrobials to treat infections (Martens & Demain, 2017; Medina & Pieper, 2016). This is further complicated by the fact that there is limited availability of new treatment options for infections.

The children's gut microbiota harbors a diverse collection of antimicrobial resistance genes (ARGs) (Lebeaux et al., 2021; X. Li et al., 2021; Moore et al., 2013) known collectively as the resistome (Perry et al., 2014; Wright, 2007). The gut bacteria comprise a complex ecosystem in which ARGs are shared among commensal or pathogenic counterparts (Karami et al., 2007; Salyers et al., 2004).

Some environmental factors are thought to shape the gut resistome, such as the presence of antimicrobial-resistant bacteria in the food chain (Heuer et al., 2009; Verraes et al., 2013), consumption of antimicrobials (Jernberg et al., 2007, 2010) socioeconomic status, consumption of alcohol, vegetables, fruits, legumes, and even statin-type medications (Roy et al., 2020). Children under two years of age receive more antibiotic prescriptions compared to children over 2 and teenagers (Hicks et al., 2015).

Until now, studies of children's resistome have focused on the impact of factors such as type of birth or postpartum environment (Gibson et al., 2016; Lebeaux et al., 2021; X. Li et al., 2021; Pärnänen et al., 2018; Rahman et al., 2018); however, little attention has been given to the resistome evolution from infants to school-age children. We used metagenomic analysis (Hendriksen et al., 2019; Pehrsson et al., 2016) to characterize the temporal dynamics of the fecal resistome from children aged three months to 7 years old in a peri-urban community.

Materials and Methods

Patients and samples

We included 104 fecal samples belonging to 29 children (3 months -7 years old) in this study (this cohort is part of a more extensive longitudinal study encompassing 374 households). Surveys collected demographic information, antimicrobial consumption,

and breastfeeding. Time-series fecal samples were collected in 5 different sampling periods (SP), where SP 1 began in 2018 (beginning of recruitment) and SP 5 ended in 2021. The children lived in Quito's peri-urban, including the parishes of Tumbaco, Puembo, Tababela, Pifo, Yaruquí, Checa, and El Quinche. Twenty-four, 25, 27, 7, and 21 fecal samples were obtained from participants at SP 1, 2, 3, 4, and 5, respectively. Only those children who had provided at least three fecal samples were eligible for further analysis. Figure 1 outlines the study design. The Ethics Committee for Research in Human Beings at the Universidad San Francisco de Quito USFQ (code 2017-178M) and the Committee for Protection of Human Subjects (CPHS) at the University of California–Berkeley (Federal wide Assurance #6252) approved this study. The parents or guardians signed the written informed consent form.

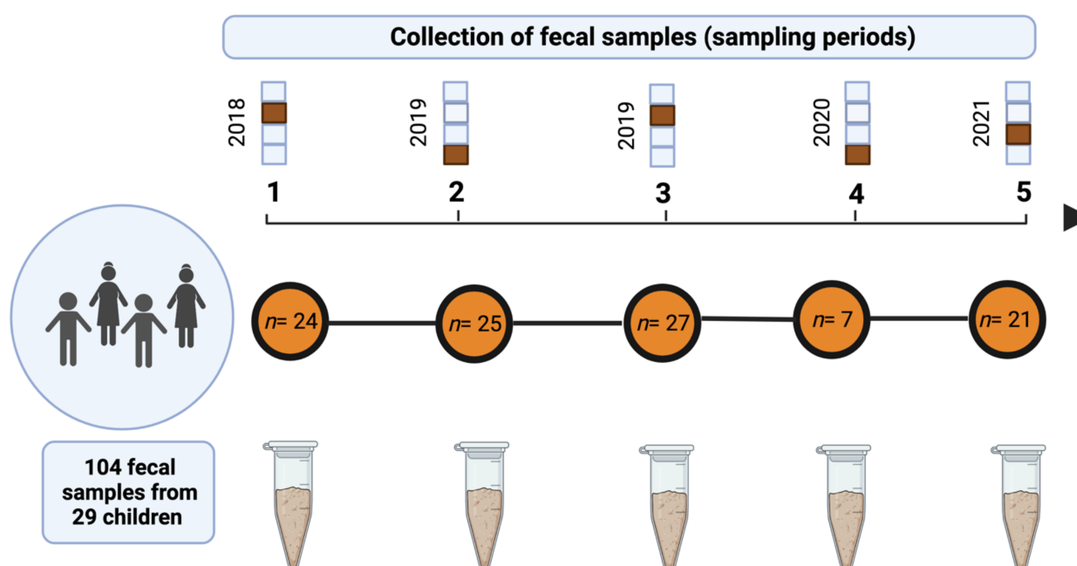


Figure 1. Study design and sampling periods. The brown boxes in the years indicate the trimester when the samples were collected, and it must read from bottom to top. The

numbers inside the orange circles indicate the number of fecal samples collected in each cycle. This illustration was created using Biorender (https://biorender.com/).

Escherichia coli isolation

Ten fecal samples from SP 1 were plated on MacConkey agar (Difco, Sparks, Maryland) supplemented with ceftriaxone (2 mg/L) and incubated for 18 hours at 37ºC. After incubation, if present, up to 5 lactose-positive colonies were selected and grown on Chromocult agar (Merk KGaA, Darmstadt, Germany) to identify putative *E. coli*. Further confirmation of *E. coli* isolates was performed with API RapiD-20E (bioMérieux, Marcy l'Etoile, France) as described previously (Salinas et al., 2021). All confirmed third-generation cephalosporin-resistant *E. coli* (3GCR-EC) were preserved at −80°C in Trypticase Soy Broth (Difco, Sparks, MD) with 20% glycerol.

DNA extraction and sequencing

DNA from fecal samples was extracted using the FastDNA™ SPIN Kit for Soil (MP Biomedicals, USA) at the Instituto de Microbiología at Universidad San Francisco de Quito. During the DNA extraction process, samples were randomly chosen from each group, approximately 20 samples each time. 60ul of DNA was obtained and stored at -20 °C until further analysis. The quality and quantity of DNA were quantified with a nanodrop (NanoDrop ND-2000, Thermo Scientific) using absorption ratios of 260/280 and 260/230. Lyophilized samples were sent to the High Throughput Sequencing Facility at the University of North Carolina in Chapel Hill, NC, USA, to prepare the genetic library (KAPA libraries using the Mantis system), quality control, and sequencing.

Sequencing was performed on an Illumina NovaSeq 6000 platform that generated 2x150bp paired-end (PE) sequences at a sequencing depth of 10 million reads. The sequences were deposited in the European Nucleotide Archive under accession project PRJEB50568.

The DNA from 22 3GCR-EC isolates was obtained using Wizard®Genomic DNA Purification (Promega, Madison, USA), and whole genome sequencing using an Illumina MiSeq platform with Nextera XT to generate 2x250bp PE sequences was performed at The University of Minnesota. The sequences were deposited in the European Nucleotide Archive under accession project PRJEB37285.

Bioinformatic analysis of the resistome

The identification of antimicrobial resistance genes was based on the AmrPlusPlus pipeline (for more information, you can review the documentation http://megares.meglab.org/amrplusplus/latest/html/). Raw sequences were quality controlled using FastQC (Andrews, 2010) and multiQC (Ewels et al., 2016) before and after removing low-quality nucleotides, low-quality reads, and adapters (TreSeq3-PE) with Trimmomatic (Bolger et al., 2014). The steps used during trimming were: ILLUMINACLIP: TruSeq3-PE.fa:2:30:10, SLIDINGWINDOW:4:15, MINLEN:36. Next, reads belonging to the host (*Homo sapiens* GRCh38 reference genome) were mapped and removed using the BWA aligner (H. Li, 2013) and Samtools (H. Li et al., 2009), respectively. We carried out the alignment to the Resfinder (Bortolaia et al., 2020; Zankari et al., 2012) database (database of acquired ARGs). The generation of the reading count table was built using the ResistomeAnalyzer tool. Only those ARG reads with at least 80% (Noyes et al., 2016) of the gene fraction threshold were used for graphical generation and statistical analysis.

Escherichia coli genomes

Acquired ARGs in the whole genomes of 3GCR-EC were identified using the Resfinder database (Bortolaia et al., 2020; Zankari et al., 2012) with at least 80% of the gene length and identity of 100% by the ABRicate tool (version 0.8.13) (https://github.com/tseemann/ABRicate).

Taxonomic assignment

Metaphlan2 (Version 2.6.0.0) (Segata et al., 2012; Truong et al., 2015) on the Galaxy platform (Afgan et al., 2018) was used to obtain the count of reads assigned to different taxonomic levels. We used all the default parameters, except that the analysis type was changed to "profiling metagenomes in relative abundances and estimate the number of reads coming from each clade." The estimated number of reads from each clade was used for the integrative analysis between the resistome and the microbiome.

Escherichia coli strain-level metagenomic profiling

Proteobacteria, particularly *E. coli*, have previously been shown to contribute to more abundant and diverse ARGs in human intestinal resistome (X. Li et al., 2021); therefore, we selected to study *E. coli*. For the metagenomic profiling at the *E. coli* strain level, we used the PanPhlan 3.1 tool (Beghini et al., 2021). The pangenome-based phylogenomic analysis was based on three tasks, downloading pangenomes, mapping samples against pangenomes, and profiling *E. coli* strains (https://github.com/SegataLab/panphlan). PanPhlan 3.1, without assembly, aligns reads to a database of pangenomes, identifies

which genes are present or absent, and functionally characterizes strains within this species (Beghini et al., 2021; Scholz et al., 2016). The heatmap visualization and hierarchical clustering analysis (using R studio version 2022.2.1.461, ComplexHeatmap (Gu et al., 2016) package) were built from the matrix that PanPhlan 3.1 provides and includes the presence/absence of each gene family within each dominant strain identified in each sample.

Statistical Analysis

The normalization process, statistical analysis, and visualization of the resistome were carried out with the web tool ResistoXplorer (Dhariwal et al., 2021). While MicrobiomeAnalyst was used to conduct the process of normalization, statistical analysis, and visualization of the microbiome (Chong et al., 2020; Dhariwal et al., 2017). Before the normalization process, data filtering was performed to reduce low-quality and non-informative features. The Cumulative Sum Scaling (CSS) (Paulson et al., 2013) method was used for the normalization process before diversity analyses (in the resistome and microbiome analyses).

The Chao1 and Shannon diversity indices were applied to the study of alpha diversity. If three or more independent samples were compared, the Kruskal-Wallis method was used. The NMDS ordination method was used for the beta diversity analysis using the PERMANOVA statistical method and the Bray-Curtis index distance method. Procrustes analysis was applied to evaluate the association between taxonomic composition and ARGs. The parameters used included 9999 permutations, species at the taxonomic level, genes at the profile level, and the methods of ordering and distancing were the same as described in the beta diversity analysis.

Hierarchical clustering analysis and heatmap visualization used gene alleles as profile level and the Euclidean clustering distance. The samples were grouped by SP or household as experimental factors, and relative abundances were scaled by genes (row). Correlation analysis was performed at the level of mechanisms of action using Pearson's correlation coefficient. Clustered Image Map (CIM) was used to visualize the correlation between the resistome and microbiome. The differences were declared significant when P-values were <0.05.

Results

High-throughput sequencing produced approximately 1.19 billion reads from the 104 fecal samples. After trimming, all sequences had a Phred score greater than Q30 (mean = Q36). The trimming process extracted approximately 22% of the readings. Among all the samples analyzed, 451,021 were the total reads aligned to 5 mechanisms of action that confirmed resistance to 9 classes of antimicrobials and 317 different alleles. The mechanisms of action identified were target protection, enzymatic inactivation, increased efflux, target modification, and enzymatic modification. ARGs, grouped by class of antimicrobials, conferred resistance to tetracyclines (75%), beta-lactams (10%), MLS-B (macrolide, lincosamide, and streptogramin B) (7%), macrolides (3%), aminoglycosides (2%), MS-B (macrolide, streptogramin B) (1%), MATFPR (macrolide, aminoglycoside, tetracycline, fluoroquinolone, phenicol, and rifampicin) (1%), lincosamides (1%), and phenicols (< 1%).

The structure of the children’s fecal resistome varies over time

We assessed whether the structure of the children's fecal resistome could change over time. Only 6.7% (7/104) of the children in this study received antimicrobials in the last three months before collecting fecal samples (Supplementary Figure 1). Ninety six out of 104 children's caregivers provided information about breastfeeding, and only 18.7% (18 out of 96) of the children were breastfed. Figures 2 and 3 show the children's fecal resistome as a function of cumulative relative abundance, comparing the 5 SP and the age. ARGs are represented according to the class of antibiotics that confer resistance (Figures 2A and 3A) or the mechanism of action (Figures 2B and 3B, respectively). The composition of the fecal resistome showed changes in the cumulative relative abundance of tetracyclines, beta-lactams, MS-B, and lincosamides without a pattern related to the SP or age. On the other hand, ARGs that confer resistance to MATFPR (macrolide, aminoglycoside, tetracycline, fluoroquinolone, phenicol, and rifampicin) and macrolides showed a progressive reduction from SP 1 to SP 5 and as age advances. Additionally, ARGs' proportions confer resistance to MLS-B, and aminoglycosides remained stable over time. Related to the mechanisms of action, it can be observed that enzyme inactivation increased when target protection was reduced or vice versa. However, no negative correlation was found between these two mechanisms of action (Supplementary Table 1). The proportion of the other mechanisms of action remained stable over time.

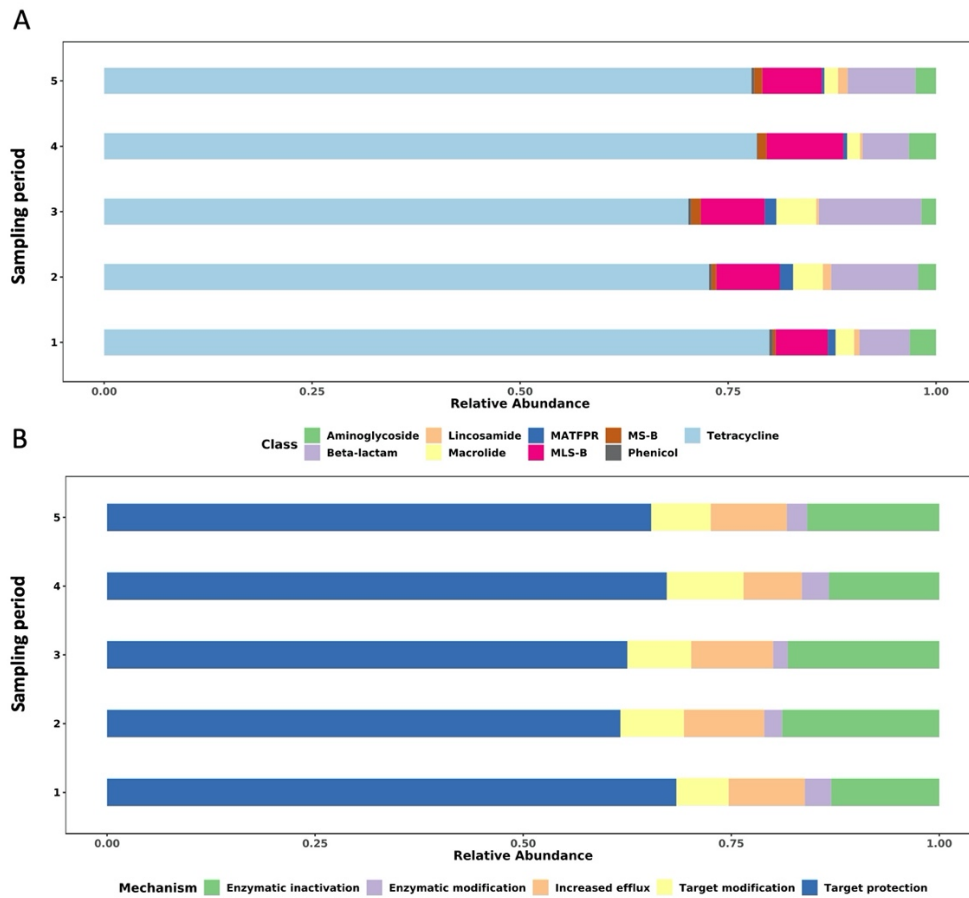


Figure 2. Composition of the children's fecal resistome based on sampling periods. Cumulative relative abundance compared among longitudinal sampling periods measured in proportions. Different colors refer to different ARGs grouped by A) antimicrobial classes and B) mechanisms of action.

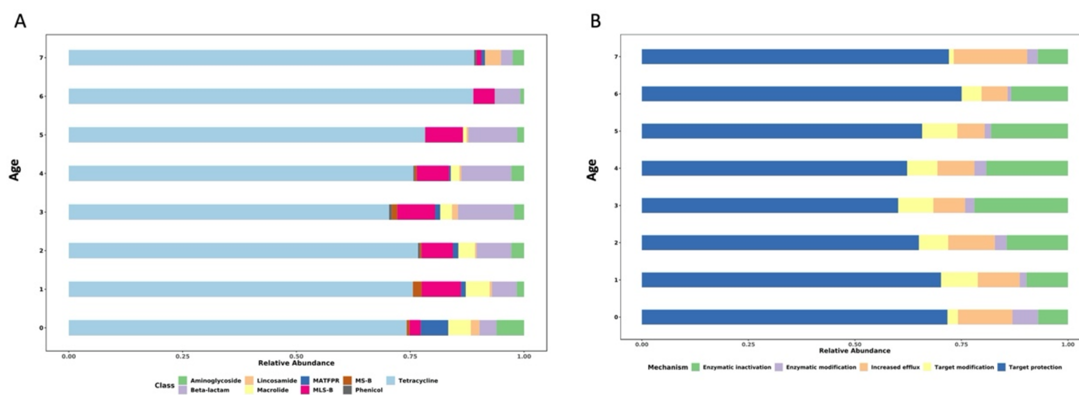


Figure 3. Composition of the children's fecal resistome based on age. Cumulative relative abundance compared among ages measured in proportions. Different colors refer to different ARGs grouped by A) antimicrobial classes and B) mechanisms of action.

Because we observed changes in the children's fecal resistome composition, we wanted to assess alpha and beta diversity over time by SP and age. Supplementary Figure 2 shows a slight and sustained increase in richness (Chao1, Kruskal-Wallis: P= 0.39001) and diversity (Shannon, Kruskal-Wallis: P= 0.25615) among the five SP analyzed. Nonetheless, such differences were not statistically significant (Supplementary Figures 2 A and B). When analyzing richness (Chao1, Kruskal-Wallis: P= 0.016711) and the diversity (Shannon, Kruskal-Wallis: P= 0.017) by age, different behavior of the resistome was observed, with alternating increases and decreases from one year to another. Both analyses showed statistically significant differences in fecal resistome changes (Supplementary Figures 2 C and D). The ordination analysis using NMDS plots in which we compared the fecal resistome between the 5 SP showed no statistically significant differences in the dissimilarities (PERMANOVA: $P < 0.193$) (Figure 4A). In addition, it was shown that children's fecal resistomes (from the same individual) were not similar at different time points (see straight lines connecting different samples in the same individual in Figure 4). Interestingly, we found statistically significant differences when samples were grouped by age (PERMANOVA: $P < 0.044$). We noticed that children's fecal resistome resembles each other more at older ages, especially at four, five, and seven years old (Figure 4B).

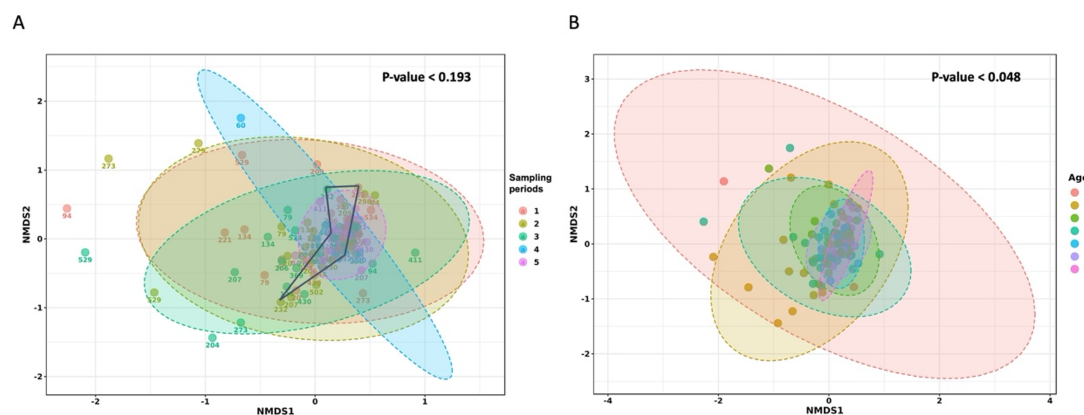


Figure 4. Beta diversity analysis of the children's fecal resistome. Comparisons were made by A) sampling periods and by B) age. Solid dark gray lines connect each sample from child 232, showing that resistomes do not cluster per individual. Beta diversity is visualized on NMDS plots using the PERMANOVA test and the Bray-Curtis distance method (Stress = 0.14825 and 0.14861, respectively).

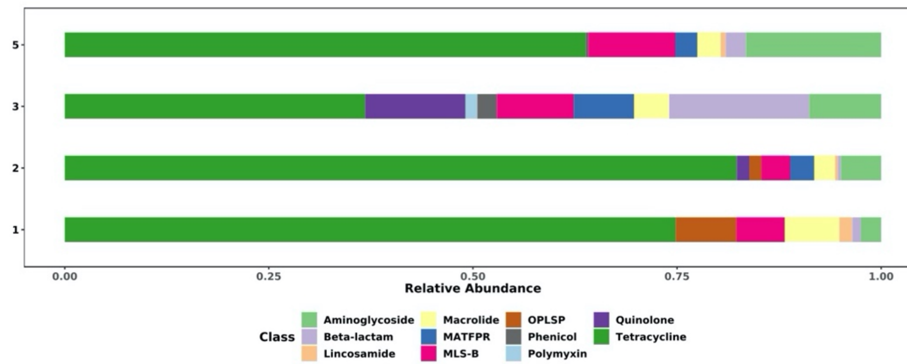
The children's gut resistome is highly dynamic within the same individual

Figure 5 shows that ARGs abundance (at the antimicrobial class and allele levels) also changed over time. Although Figure 5 shows individual 79 (household 79), the same dynamics could be evidenced in each of the other 28 individuals (Supplementary Figures 3- 30). In this individual, the relative abundance of ARGs grouped by antimicrobial class varies considerably in the four SP analyzed (Figure 5A). In SP 1, the presence of ARGs that confer resistance to OPLSP (oxazolidinone, phenicol, lincosamide, streptogramin A, pleuromutilin) was observed, which was reduced in SP 2 and disappeared in the following SP. In the same way, quinolone resistance appeared in SP 2, and the relative abundance of this group of ARGs increased in SP 3 and disappeared in SP 5. It is noteworthy that in SP 3, individual 79 suffers from the most substantial alteration in the structure of the fecal resistome, and it is not related to antimicrobial consumption. An increase in the relative

abundance of several ARGs (beta-lactams, quinolones, MLS-B, MATFPR, polymyxins, and phenicol) was observed. When SP 5 was compared with SP 3, a slight recovery of the ARG abundance was observed (Figure 5A).

The heatmap and the hierarchical clustering analysis showed that each allele's abundance varied between samples in the four SPs (Figure 5B). In SP 3, we observed the most considerable alteration of the resistome. The relative abundance of resistance gene alleles *bla*MIR-3_1_AY743435, *bla*OXA-1_1_HQ170510 (beta-lactams), *oqx*A_1_EU370913, *oqx*B_1_EU370913, *qnr*B5_1_DQ303919 (quinolones), and *mcr*-10_1_MN179494 (polymyxins) increased only in the SP 3 compared with the other SPs, evidencing a transitory introduction and elevation of ARGs of medical importance in the fecal resistome of this individual. The same behavior occurred in other individuals' fecal resistome; ARGs identified in one SP were absent in the other SP (Supplementary Figures 3-30).

A



B

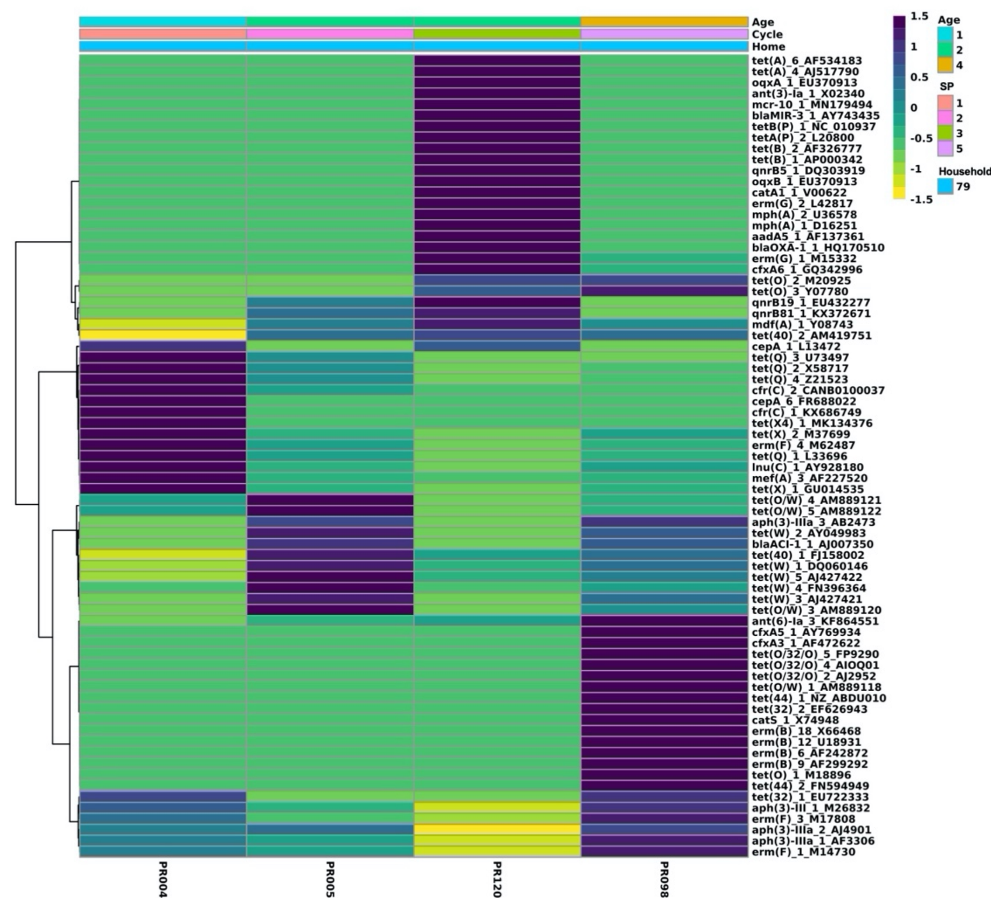


Figure 5. Temporal dynamics of the children's resistome in individual 79 (household 79). A) The relative abundance of fecal resistome was compared by sampling periods (numbers indicate SP). The relative abundance was evaluated in proportions. B) Heatmap and hierarchical clustering analysis of fecal resistome. Rows are gene alleles, and

columns are samples. Relative abundances were scaled by ranks (genes). Euclidean distances were used, and the grouping was by cycle.

We also found a variable pattern of dominant *E. coli* strains within the same individual (Supplementary Figure 31). We used the PanPhlan 3.1 tool (Beghini et al., 2021; Scholz et al., 2016) to characterize *E. coli* strains from our metagenomic data using 200 reference pangenomes. One dominant *E. coli* strain was detected for each sample, and only in 45 samples out of 104 samples, we recovered *E. coli* strains. Forty-four dominant *E. coli* strains did not cluster with the reference pangenomes (using hierarchical clustering analysis with Euclidean distances), so the reference pangenomes do not appear in the figure and only the pangenomes identified in the samples are shown in the heatmap (Supplementary Figure 31). No dominant *E. coli* strain in each sample remained in the same individual over time.

During the development of the present study, we isolated 22 3GCR-EC strains from 10 fecal samples belonging to SP 1, which were included in this metagenomic study. The most prevalent 3GCR-encoding gene was *bla*$_{CTX-M}$ in 14 isolates (*bla*$_{CTX-M-15}$, n= 6; *bla*$_{CTX-M-55}$, n= 5; *bla*$_{CTX-M-27}$, n= 3) followed by *bla*$_{TEM-1B}$ in 5 isolates. The metagenomic analysis failed to detect the 3GCR genes in these samples. We identified beta-lactamase encoding genes belonging to two ESBL families in 16 (72.73%) of the 22 whole genomes of 3GCR-EC from 7 fecal samples. Three isolates carried *bla*$_{CTX-M-55}$ and *bla*$_{TEM-1B}$ genes, and one isolate also harbored a *bla*$_{CMY-2}$ gene. All the ARGs identified in the whole genomes of 3GCR-EC are shown in the S2 Table.

The dynamics of the children's resistome correlate with the dynamics of the microbiome

The Procrustes analysis confirmed a significant positive correlation (correlation coefficient = 0.6383; $P < 0001$) between the composition of the bacterial communities and the resistome (Figure 6A), suggesting that fecal samples with similar bacterial profiles have similar resistomes. Figure 6B shows the ARGs grouped by class and their correlation with bacterial families. The abundance of bacteria belonging to the families *Enterobacteriaceae* and *Prevotellaceae* was positively and strongly correlated with ARGs that confer resistance to MATFPR and beta-lactams, respectively. In contrast, the abundance of bacteria belonging to families *Clostridiaceae*, *Ruminococcaceae*, *Rikenellaceae*, and *Porphyromonadaceae* correlated strongly and negatively with ARGs that confer resistance to beta-lactams, MATFPR, MATFPR, and MS-B, respectively.

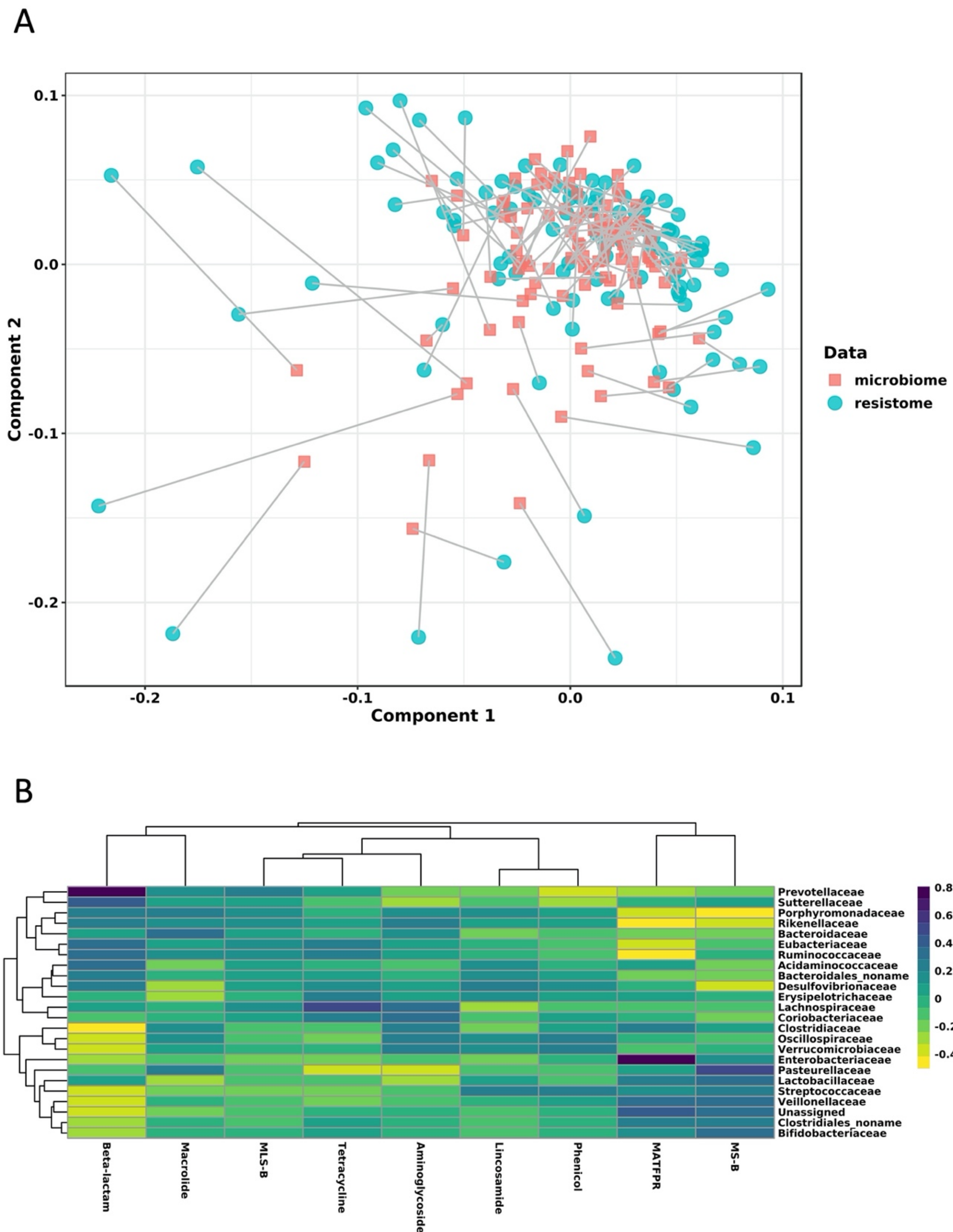


Figure 6. Correlation between resistome and children's fecal microbiome. A) Procrustes analysis using the NMDS ordination method, the distance method was Bray-Curtis Index, and we used 9999 permutations. B) Clustered Image Map (CIM); the taxonomic profile level used was family, and the profile level for ARGs was by antimicrobial class. The yellow color indicates a negative correlation, and the dark blue color indicates a positive

correlation. We used Euclidean distance for the analysis, and the clustering algorithm used was complete.

To explore the fecal resistome and microbiome composition temporally, we measured the correlation between the two. The taxonomic analysis showed that the microbial composition became more similar among individuals in the latter SPs (PERMANOVA, $P < 0.05$) (Figures 7A, 7C, 8A, 8C). Additionally, taxonomic richness (Figures 7B and 8B) and diversity (Figures 7B and 8B) also increased significantly over time based on the SP (Chao1: Kruskal-Wallis, P = 0.0060563; Shannon: Kruskal-Wallis, P = 0.0034064, respectively) or the age (Chao1: Kruskal-Wallis, P = 0.0060563; Shannon: Kruskal-Wallis, P = 0.0040378, respectively). Beta diversity analysis (NMDS) of the microbiota and resistome diversity showed that children's fecal samples were most similar to each other (using the Bray-Bray distance method) over time. Taken together, these data suggest that there is a correlation between changes in the gut microbiota and resistome. Although microbial richness and diversity increased significantly as children aged (sampling period 1 to 5 or 6 months to 7 years old), we did not show that the same occurs with the fecal resistome.

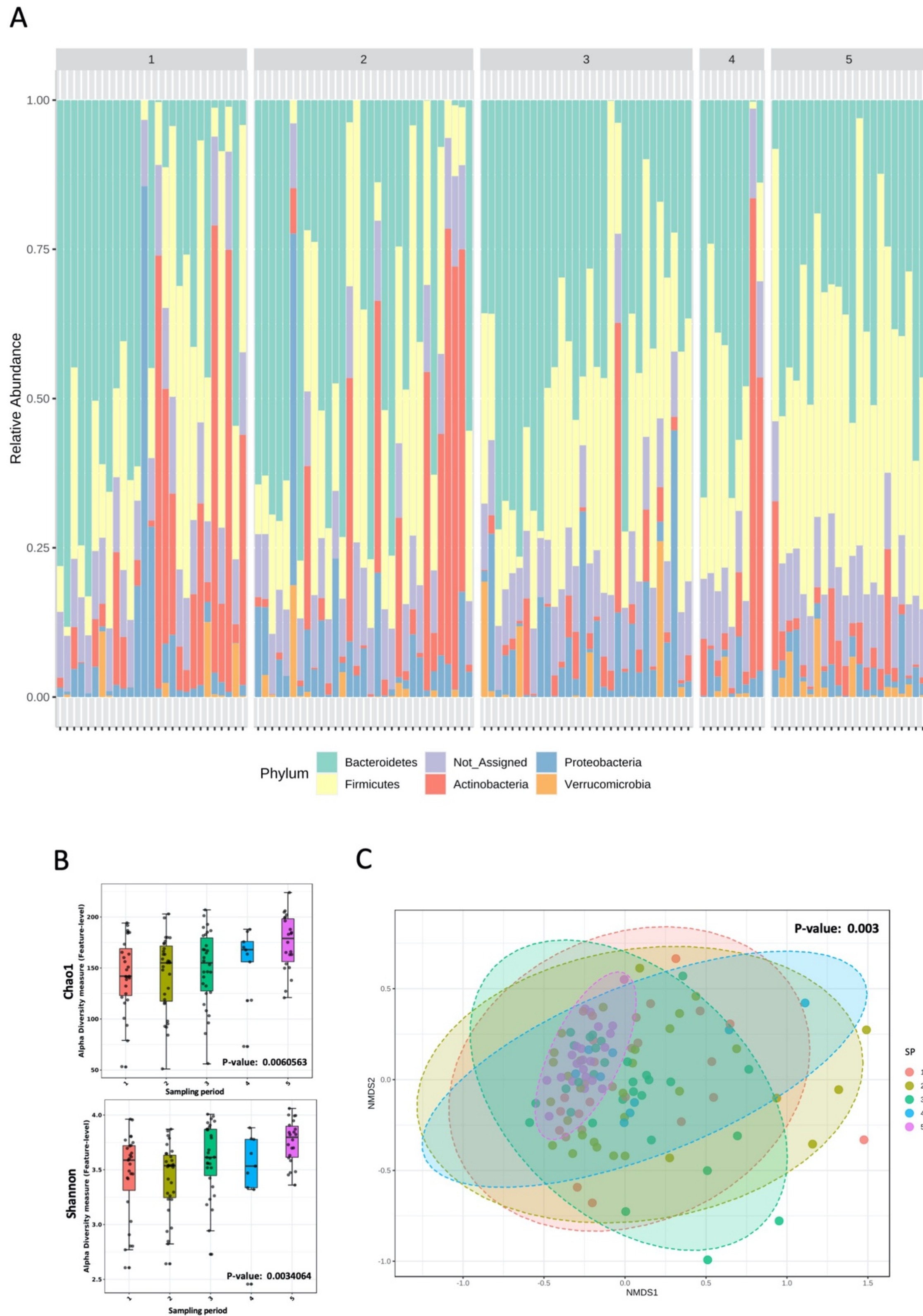


Figure 7. Children's fecal microbiota structure is compared by sampling periods. A) Composition of the fecal microbiota is represented in relative abundance and measured in proportions, and the different colors refer to different taxonomic phyla. B) Alpha

diversity is represented by richness (Chao1 index) and diversity (Shannon index). The taxonomic level was species and strains, and the statistical method used was Kruskal-Wallis. C) Beta diversity is visualized in the NMDS plot using the PERMANOVA test and the Bray-Curtis distance method.

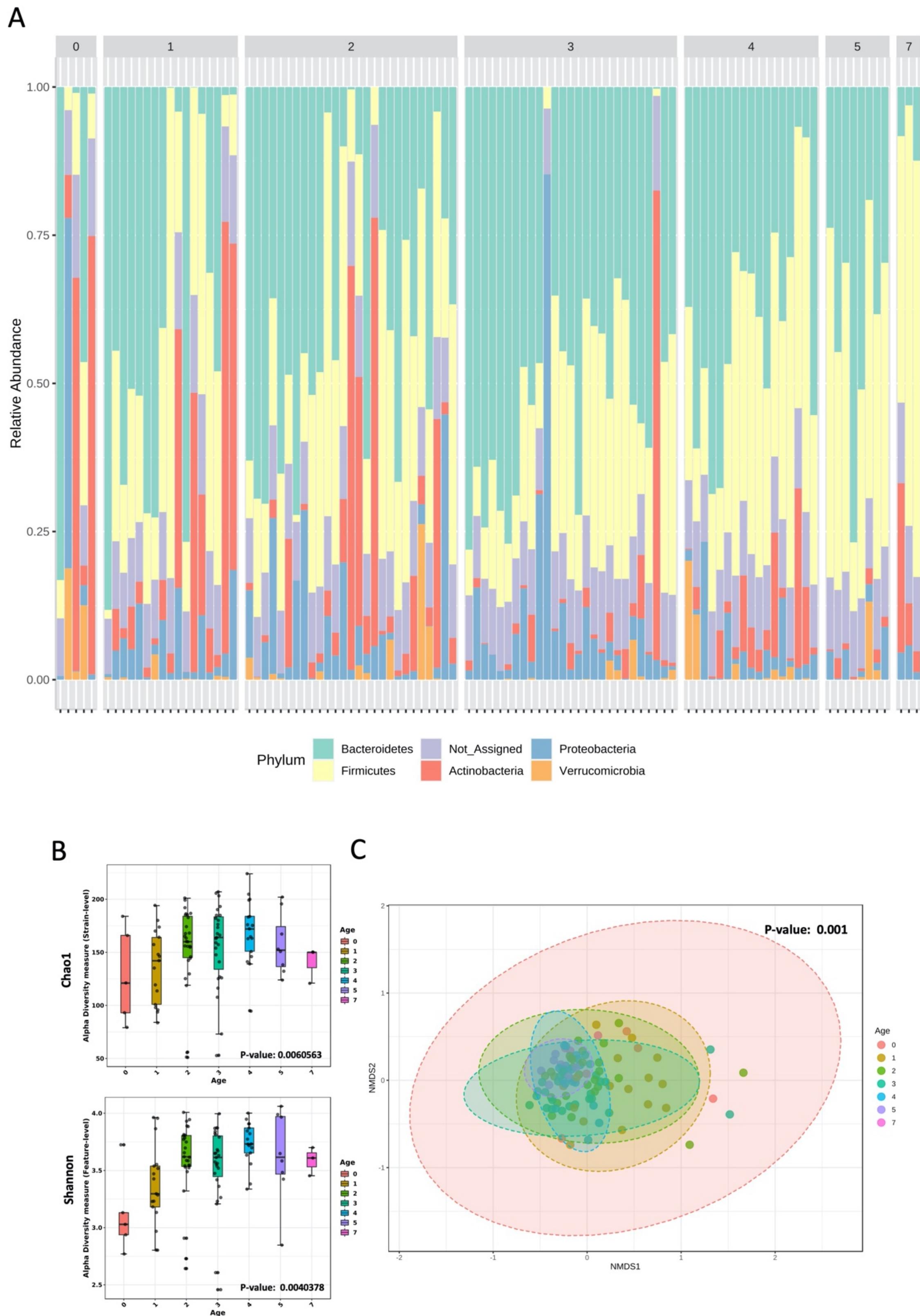


Figure 8. Children's fecal microbiota structure is compared by age. A) Composition of the fecal microbiota is represented in relative abundance and measured in proportions, and the different colors refer to different taxonomic phyla. B) Alpha diversity is

represented by richness (Chao1 index) and diversity (Shannon index). The taxonomic level was species and strains, and the statistical method used was Kruskal-Wallis. C) Beta diversity is visualized in the NMDS plot using the PERMANOVA test and the Bray-Curtis distance method.

Discussion

This study showed that the children's fecal resistome is highly dynamic over time and strongly correlated with intestinal microbiota changes. In addition, the resistome tends to be more similar within older children than among very young children (Figure 4B), perhaps influenced by a more stable and mature microbiota (Kurokawa et al., 2007; Palmer et al., 2007). We found that sample PR102 from SP3 had a significantly higher abundance of ARGs without any apparent cause (Figure 5 A and B). Further, seven children who received antimicrobials before any sampling period showed no increased abundance of ARGs (Supplementary Figure 1). These results corroborate a recent study of the same children population, in which significant overtime changes in antimicrobial resistance in (cultured) commensal *E. coli* were observed without any apparent environmental factor (Calderón et al., 2022). Other studies have observed the persistence of resistance genes over several years in adults under antibiotic therapy (Jernberg et al., 2007; Löfmark et al., 2006) and antimicrobial therapy selects multi-resistant bacteria that remain for several months or years (Lester et al., 2006; Shoemaker et al., 2001). Plasmids that carry resistance determinants can persist for several months in an infant not subjected to antibiotic therapy (Gumpert et al., 2017). These unexplained ARGs elevations may be due to random events, some antimicrobial genes have been disseminated in bacterial populations for 80 years since the industrial production of antimicrobials (D'Costa et al., 2011) and may not reduce bacterial fitness anymore.

The five types of ARGs found in this study coincide with previous studies in adults under antimicrobial therapy in Quito (Cifuentes et al., 2022). Several studies in adults (carried out in Ecuador (Cifuentes et al., 2022), China, Denmark, Spain (Hu et al., 2013), and Pakistan (Afridi et al., 2021)) demonstrate that the most prevalent ARGs in the human fecal resistome are against tetracyclines, beta-lactams, and MLS-B. On the other hand, the study by Li et al. on fecal resistome in Danish infants showed that ARGs that confer resistance to tetracyclines, quinolones, and beta-lactams are the most frequent genes in this group of children analyzed (X. Li et al., 2021). We found quinolone resistance sporadically in our cohort compared with the abovementioned study.

The ARGs frequency in our study may be explained by antimicrobial use among children under five years old globally (Browne et al., 2021). Also, tetracyclines are the most frequently used antibiotics in domestic animals (Granados-Chinchilla & Rodríguez, 2017; Roberts & Roberts, 2001) and ARGs can spread from animal bacteria to human bacteria through the food chain (Verraes et al., 2013) or the surrounding environment (Blaak et al., 2015; Zhang et al., 2022). Overtime changes in fecal resistome (and microbiome) could also result from the introduction of non-digestible carbohydrates in infants (Wu et al., 2016), gastrointestinal infections (Hansen et al., 2021), or the environment in which children develop (Szekeres et al., 2018).

We isolated *E. coli* with *bla*$_{\text{CTX-M-15, 55, 27}}$ (resistant to 3rth generation cephalosporins) from 3 fecal samples whose microbiomes were negative for this gene. The gene *bla*$_{\text{CTX-M}}$ is associated mainly with *E. coli* which was detected in only 45 (42%) microbiomes. Additionally, most *bla*$_{\text{CTX-M}}$-carrying *E. coli* lineages are not numerically dominant in the human microbiota (Calderón et al., 2022); this gene may cause a reduction of fitness in most commensal *E. coli* (McNally et al., 2016).

Given that the fecal resistome changes correlate positively and significantly with the fecal microbiota (demonstrated by Procrustes analysis), we argue that the fecal resistome also takes on a generic adult-like shape over time in the same way. The fecal microbial community in children becomes progressively more similar to the adult ones (Gibson et al., 2015; Palmer et al., 2007; Putignani et al., 2014). Interpersonal variation is more significant among children than adults (Yatsunenko et al., 2012); during the neonatal period and infancy, dynamic changes in microbial diversity occur (Vatanen et al., 2019; Yassour et al., 2018). Interestingly, clinically relevant ARGs encoding ESBLs, colistin, or fluoroquinolone resistance were transient in the children's fecal resistome (Figure 5 A and B). Additionally, as in previous studies (Calderón et al., 2022; Martinson et al., 2019; Richter et al., 2018), we demonstrated the dynamic turnover of *E. coli* strains in the same child (Supplementary Figure 31). Kurokawa et al. suggested that the functional uniformity of the adult-type microbiota (from non-infant children and adults) is more complex than infant microbiota which is unstable due to its immature nature (Kurokawa et al., 2007).

We identified that fecal samples with similar bacterial taxonomic profiles have similar resistomes, and, therefore, the taxonomic composition may influence the resistome (Pehrsson et al., 2016). Previous metagenomic studies with infant fecal samples showed that Proteobacteria, particularly *Escherichia coli*, are positively correlated with increased resistome diversity and abundance (Lebeaux et al., 2021; X. Li et al., 2021). In the present study, we evidenced a positive correlation between microbiota composition and the presence of some ARGs: *Prevotellaceae* family with beta-lactams and *Enterobacteriaceae* with MATFPR, and at the same time, negative correlations between *Clostridiaceae* with beta-lactams, *Ruminococcaceae* with MATFPR, *Rikenellaceae* with

MATFPR, and *Porphyromonadaceae* with MS-B. We demonstrated that factors that modify the gut microbiota also affect the resistome.

The current study has some limitations. Due to its ability to transmit among hosts and high conjugation rate (Martinson & Walk, 2020), the Centers for Disease Control (USA) considers *E. coli* (and other Enterobacterales) as one of the most important bacteria associated with the antimicrobial crisis (Centers for Disease Control and Prevention, 2019). However, *E. coli* is also a minor component of the human microbiota (Rooney et al., 2022). In this study, we were able to detect *E. coli* in only 42% of the samples, which may indicate that 10 million reads are insufficient to have a more comprehensive view of the strains of *E. coli* dynamics and the composition of the fecal resistome. It has been shown that increasing sequencing depth (≥ 50 million reads) can improve the ability to detect low-abundance genes (Zaheer et al., 2018) and taxa such as *E. coli* (Rooney et al., 2022).

To our knowledge, this is the first longitudinal study that evaluated the children's fecal resistome beyond infancy. This work focuses on understanding the extent of dissemination of resistance determinants in the child population of rural and peri-urban areas, the intestinal dynamics, and the interconnection with the microbiota. These results will allow for establishing measures and new hypotheses to mitigate antimicrobial resistance and manipulate the gut microbiota to modify the resistome.

Acknowledgments

We thank Soledad Sarsoza and all the personnel involved in the collection of epidemiological data and fecal samples in the field, such as Valeria Garzón, Paúl Barahona, Gabriela Heredia, Cristian Sisalema, and Josue Barahona. Additionally, we appreciate the great work and support in the methodology and bioinformatics tools of

Belén Prado. Finally, we thank the parents and children involved in the present study. The Galaxy server that was used for some calculations is in part funded by Collaborative Research Centre 992 Medical Epigenetics (DFG grant SFB 992/1 2012) and German Federal Ministry of Education and Research (BMBF grants 031 A538A/A538C RBC, 031L0101B/031L0101C de.NBI-epi, 031L0106 de.STAIR (de.NBI)).

References

Afgan, E., Baker, D., Batut, B., van den Beek, M., Bouvier, D., Čech, M., Chilton, J., Clements, D., Coraor, N., Grüning, B. A., Guerler, A., Hillman-Jackson, J., Hiltemann, S., Jalili, V., Rasche, H., Soranzo, N., Goecks, J., Taylor, J., Nekrutenko, A. & Blankenberg, D. (2018). The Galaxy platform for accessible, reproducible and collaborative biomedical analyses: 2018 update. *Nucleic Acids Research*, *46*, 537–544. https://doi.org/10.1093/nar/gky379

Afridi, O. K., Ali, J. & Chang, J. H. (2021). Fecal Microbiome and Resistome Profiling of Healthy and Diseased Pakistani Individuals Using Next-Generation Sequencing. *Microorganisms*, *9*(3), 616. https://doi.org/10.3390/microorganisms9030616

Andrews, S. (2010). *FastQC: a quality control tool for high throughput sequence data*. https://www.bioinformatics.babraham.ac.uk/projects/fastqc/

Beghini, F., McIver, L. J., Blanco-Míguez, A., Dubois, L., Asnicar, F., Maharjan, S., Mailyan, A., Manghi, P., Scholz, M., Thomas, A. M., Valles-Colomer, M., Weingart, G., Zhang, Y., Zolfo, M., Huttenhower, C., Franzosa, E. A. & Segata, N. (2021). Integrating taxonomic, functional, and strain-level profiling of diverse microbial communities with bioBakery 3. *ELife*, *10*, e65088. https://doi.org/10.7554/elife.65088

Blaak, H., Hoek, A. H. A. M. van, Hamidjaja, R. A., Plaats, R. Q. J. van der, Heer, L. K., Husman, A. M. de R. & Schets, F. M. (2015). Distribution, Numbers, and

Diversity of ESBL-Producing E. coli in the Poultry Farm Environment. *PLoS ONE*, *10*(8), e0135402. https://doi.org/10.1371/journal.pone.0135402

Bolger, A. M., Lohse, M. & Usadel, B. (2014). Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformatics*, *30*(15), 2114–2120. https://doi.org/10.1093/bioinformatics/btu170

Bortolaia, V., Kaas, R. S., Ruppe, E., Roberts, M. C., Schwarz, S., Cattoir, V., Philippon, A., Allesoe, R. L., Rebelo, A. R., Florensa, A. F., Fagelhauer, L., Chakraborty, T., Neumann, B., Werner, G., Bender, J. K., Stingl, K., Nguyen, M., Coppens, J., Xavier, B. B., … Aarestrup, F. M. (2020). ResFinder 4.0 for predictions of phenotypes from genotypes. *Journal of Antimicrobial Chemotherapy*, *75*(12), dkaa345-. https://doi.org/10.1093/jac/dkaa345

Browne, A. J., Chipeta, M. G., Haines-Woodhouse, G., Kumaran, E. P. A., Hamadani, B. H. K., Zaraa, S., Henry, N. J., Deshpande, A., Reiner, R. C., Day, N. P. J., Lopez, A. D., Dunachie, S., Moore, C. E., Stergachis, A., Hay, S. I. & Dolecek, C. (2021). Global antibiotic consumption and usage in humans, 2000–18: a spatial modelling study. *The Lancet. Planetary Health*, *5*(12), e893–e904. https://doi.org/10.1016/s2542-5196(21)00280-1

Calderón, D., Cárdenas, P. A., Prado-Vivar, B., Graham, J. P. & Trueba, G. (2022). A longitudinal study of dominant E. coli lineages and antimicrobial resistance in the gut of children living in an upper middle-income country. *Journal of Global Antimicrobial Resistance*, *29*, 136–140. https://doi.org/10.1016/j.jgar.2022.03.002

Centers for Disease Control and Prevention. (2019). *Antibiotic Resistance Threats in the United States, 2019* (p. 150). https://www.cdc.gov/drugresistance/pdf/threats-report/2019-ar-threats-report-508.pdf

Chong, J., Liu, P., Zhou, G. & Xia, J. (2020). Using MicrobiomeAnalyst for comprehensive statistical, functional, and meta-analysis of microbiome data. *Nature Protocols*, *15*(3), 799–821. https://doi.org/10.1038/s41596-019-0264-1

Cifuentes, S. G., Prado, M. B., Fornasini, M., Cohen, H., Baldeón, M. E. & Cárdenas, P. A. (2022). Saccharomyces boulardii CNCM I-745 supplementation modifies the fecal resistome during Helicobacter pylori eradication therapy. *Helicobacter*, *27*(2), e12870. https://doi.org/10.1111/hel.12870

D'Costa, V. M., King, C. E., Kalan, L., Morar, M., Sung, W. W. L., Schwarz, C., Froese, D., Zazula, G., Calmels, F., Debruyne, R., Golding, G. B., Poinar, H. N. & Wright, G. D. (2011). Antibiotic resistance is ancient. *Nature*, *477*(7365), 457–461. https://doi.org/10.1038/nature10388

Dhariwal, A., Chong, J., Habib, S., King, I. L., Agellon, L. B. & Xia, J. (2017). MicrobiomeAnalyst: a web-based tool for comprehensive statistical, visual and meta-analysis of microbiome data. *Nucleic Acids Research*, *45*(W1), W180–W188. https://doi.org/10.1093/nar/gkx295

Dhariwal, A., Junges, R., Chen, T. & Petersen, F. C. (2021). ResistoXplorer: a web-based tool for visual, statistical and exploratory data analysis of resistome data. *NAR Genomics and Bioinformatics*, *3*(1), lqab018. https://doi.org/10.1093/nargab/lqab018

Ewels, P., Magnusson, M., Lundin, S. & Käller, M. (2016). MultiQC: summarize analysis results for multiple tools and samples in a single report. *Bioinformatics*, *32*(19), 3047–3048. https://doi.org/10.1093/bioinformatics/btw354

Gibson, M. K., Crofts, T. S. & Dantas, G. (2015). Antibiotics and the developing infant gut microbiota and resistome. *Current Opinion in Microbiology*, *27*, 51–56. https://doi.org/10.1016/j.mib.2015.07.007

Gibson, M. K., Wang, B., Ahmadi, S., Burnham, C.-A. D., Tarr, P. I., Warner, B. B. & Dantas, G. (2016). Developmental dynamics of the preterm infant gut microbiota and antibiotic resistome. *Nature Microbiology*, *1*(4), 16024. https://doi.org/10.1038/nmicrobiol.2016.24

Granados-Chinchilla, F. & Rodríguez, C. (2017). Tetracyclines in Food and Feedingstuffs: From Regulation to Analytical Methods, Bacterial Resistance, and Environmental and Health Implications. *Journal of Analytical Methods in Chemistry*, *2017*, 1315497. https://doi.org/10.1155/2017/1315497

Gu, Z., Eils, R. & Schlesner, M. (2016). Complex heatmaps reveal patterns and correlations in multidimensional genomic data. *Bioinformatics*, *32*(18), 2847–2849. https://doi.org/10.1093/bioinformatics/btw313

Gumpert, H., Kubicek-Sutherland, J. Z., Porse, A., Karami, N., Munck, C., Linkevicius, M., Adlerberth, I., Wold, A. E., Andersson, D. I. & Sommer, M. O. A. (2017). Transfer and Persistence of a Multi-Drug Resistance Plasmid in situ of the Infant Gut Microbiota in the Absence of Antibiotic Treatment. *Frontiers in Microbiology*, *8*, 1852. https://doi.org/10.3389/fmicb.2017.01852

Hansen, Z. A., Cha, W., Nohomovich, B., Newton, D. W., Lephart, P., Salimnia, H., Khalife, W., Shade, A., Rudrik, J. T. & Manning, S. D. (2021). Comparing gut resistome composition among patients with acute Campylobacter infections and healthy family members. *Scientific Reports*, *11*(1), 22368. https://doi.org/10.1038/s41598-021-01927-7

Hendriksen, R. S., Munk, P., Njage, P., Bunnik, B. van, McNally, L., Lukjancenko, O., Röder, T., Nieuwenhuijse, D., Pedersen, S. K., Kjeldgaard, J., Kaas, R. S., Clausen, P. T. L. C., Vogt, J. K., Leekitcharoenphon, P., Schans, M. G. M. van de, Zuidema, T., Husman, A. M. de R., Rasmussen, S., Petersen, B., … Aarestrup, F. M. (2019). Global monitoring of antimicrobial resistance based on metagenomics analyses of urban sewage. *Nature Communications*, *10*(1), 1124. https://doi.org/10.1038/s41467-019-08853-3

Heuer, O. E., Kruse, H., Grave, K., Collignon, P., Karunasagar, I. & Angulo, F. J. (2009). Human Health Consequences of Use of Antimicrobial Agents in Aquaculture. *Clinical Infectious Diseases*, *49*(8), 1248–1253. https://doi.org/10.1086/605667

Hicks, L. A., Bartoces, M. G., Roberts, R. M., Suda, K. J., Hunkler, R. J., Taylor, T. H. & Schrag, S. J. (2015). US Outpatient Antibiotic Prescribing Variation According to Geography, Patient Population, and Provider Specialty in 2011. *Clinical Infectious Diseases*, *60*(9), 1308–1316. https://doi.org/10.1093/cid/civ076

Hu, Y., Yang, X., Qin, J., Lu, N., Cheng, G., Wu, N., Pan, Y., Li, J., Zhu, L., Wang, X., Meng, Z., Zhao, F., Liu, D., Ma, J., Qin, N., Xiang, C., Xiao, Y., Li, L., Yang, H., … Zhu, B. (2013). Metagenome-wide analysis of antibiotic resistance genes in a large

cohort of human gut microbiota. *Nature Communications*, *4*(1), 2151. https://doi.org/10.1038/ncomms3151

Jernberg, C., Löfmark, S., Edlund, C. & Jansson, J. K. (2007). Long-term ecological impacts of antibiotic administration on the human intestinal microbiota. *The ISME Journal*, *1*(1), 56–66. https://doi.org/10.1038/ismej.2007.3

Jernberg, C., Löfmark, S., Edlund, C. & Jansson, J. K. (2010). Long-term impacts of antibiotic exposure on the human intestinal microbiota. *Microbiology*, *156*(11), 3216–3223. https://doi.org/10.1099/mic.0.040618-0

Karami, N., Martner, A., Enne, V. I., Swerkersson, S., Adlerberth, I. & Wold, A. E. (2007). Transfer of an ampicillin resistance gene between two Escherichia coli strains in the bowel microbiota of an infant treated with antibiotics. *Journal of Antimicrobial Chemotherapy*, *60*(5), 1142–1145. https://doi.org/10.1093/jac/dkm327

Kurokawa, K., Itoh, T., Kuwahara, T., Oshima, K., Toh, H., Toyoda, A., Takami, H., Morita, H., Sharma, V. K., Srivastava, T. P., Taylor, T. D., Noguchi, H., Mori, H., Ogura, Y., Ehrlich, D. S., Itoh, K., Takagi, T., Sakaki, Y., Hayashi, T. & Hattori, M. (2007). Comparative Metagenomics Revealed Commonly Enriched Gene Sets in Human Gut Microbiomes. *DNA Research: An International Journal for Rapid Publication of Reports on Genes and Genomes*, *14*(4), 169–181. https://doi.org/10.1093/dnares/dsm018

Lebeaux, R. M., Coker, M. O., Dade, E. F., Palys, T. J., Morrison, H. G., Ross, B. D., Baker, E. R., Karagas, M. R., Madan, J. C. & Hoen, A. G. (2021). The infant gut resistome is associated with E. coli and early-life exposures. *BMC Microbiology*, *21*(1), 201. https://doi.org/10.1186/s12866-021-02129-x

Lester, C. H., Frimodt-Møller, N., Sørensen, T. L., Monnet, D. L. & Hammerum, A. M. (2006). In vivo transfer of the vanA resistance gene from an Enterococcus faecium isolate of animal origin to an E. faecium isolate of human origin in the intestines of human volunteers. *Antimicrobial Agents and Chemotherapy*, *50*(2), 596–599. https://doi.org/10.1128/aac.50.2.596-599.2006

Li, H. (2013). Aligning sequence reads, clone sequences and assembly contigs with BWA-MEM. *ArXiv*.

Li, H., Handsaker, B., Wysoker, A., Fennell, T., Ruan, J., Homer, N., Marth, G., Abecasis, G., Durbin, R. & Subgroup, 1000 Genome Project Data Processing. (2009). The Sequence Alignment/Map format and SAMtools. *Bioinformatics*, *25*(16), 2078–2079. https://doi.org/10.1093/bioinformatics/btp352

Li, X., Stokholm, J., Brejnrod, A., Vestergaard, G. A., Russel, J., Trivedi, U., Thorsen, J., Gupta, S., Hjelmsø, M. H., Shah, S. A., Rasmussen, M. A., Bisgaard, H. & Sørensen, S. J. (2021). The infant gut resistome associates with E. coli, environmental exposures, gut microbiome maturity, and asthma-associated bacterial composition. *Cell Host & Microbe*, *29*(6), 975-987.e4. https://doi.org/10.1016/j.chom.2021.03.017

Löfmark, S., Jernberg, C., Jansson, J. K. & Edlund, C. (2006). Clindamycin-induced enrichment and long-term persistence of resistant Bacteroides spp. and resistance genes. *Journal of Antimicrobial Chemotherapy*, *58*(6), 1160–1167. https://doi.org/10.1093/jac/dkl420

Martens, E. & Demain, A. L. (2017). The antibiotic resistance crisis, with a focus on the United States. *The Journal of Antibiotics*, *70*(5), 520–526. https://doi.org/10.1038/ja.2017.30

Martinson, J. N. V., Pinkham, N. V., Peters, G. W., Cho, H., Heng, J., Rauch, M., Broadaway, S. C. & Walk, S. T. (2019). Rethinking gut microbiome residency and the Enterobacteriaceae in healthy human adults. *The ISME Journal*, *13*(9), 2306–2318. https://doi.org/10.1038/s41396-019-0435-7

Martinson, J. N. V. & Walk, S. T. (2020). Escherichia coli Residency in the Gut of Healthy Human Adults. *EcoSal Plus*, *9*(1). https://doi.org/10.1128/ecosalplus.esp-0003-2020

McNally, A., Oren, Y., Kelly, D., Pascoe, B., Dunn, S., Sreecharan, T., Vehkala, M., Välimäki, N., Prentice, M. B., Ashour, A., Avram, O., Pupko, T., Dobrindt, U., Literak, I., Guenther, S., Schaufler, K., Wieler, L. H., Zhiyong, Z., Sheppard, S. K., … Corander, J. (2016). Combined Analysis of Variation in Core, Accessory and Regulatory Genome Regions Provides a Super-Resolution View into the Evolution of Bacterial Populations. *PLoS Genetics*, *12*(9), e1006280. https://doi.org/10.1371/journal.pgen.1006280

Medina, E. & Pieper, D. H. (2016). How to Overcome the Antibiotic Crisis, Facts, Challenges, Technologies and Future Perspectives. *Current Topics in Microbiology and Immunology*, 3–33. https://doi.org/10.1007/82_2016_492

Moore, A. M., Patel, S., Forsberg, K. J., Wang, B., Bentley, G., Razia, Y., Qin, X., Tarr, P. I. & Dantas, G. (2013). Pediatric Fecal Microbiota Harbor Diverse and Novel

Antibiotic Resistance Genes. *PLoS ONE*, *8*(11), e78822. https://doi.org/10.1371/journal.pone.0078822

Noyes, N. R., Yang, X., Linke, L. M., Magnuson, R. J., Cook, S. R., Zaheer, R., Yang, H., Woerner, D. R., Geornaras, I., McArt, J. A., Gow, S. P., Ruiz, J., Jones, K. L., Boucher, C. A., McAllister, T. A., Belk, K. E. & Morley, P. S. (2016). Characterization of the resistome in manure, soil and wastewater from dairy and beef production systems. *Scientific Reports*, *6*(1), 24645. https://doi.org/10.1038/srep24645

Palmer, C., Bik, E. M., DiGiulio, D. B., Relman, D. A. & Brown, P. O. (2007). Development of the Human Infant Intestinal Microbiota. *PLoS Biology*, *5*(7), e177. https://doi.org/10.1371/journal.pbio.0050177

Pärnänen, K., Karkman, A., Hultman, J., Lyra, C., Bengtsson-Palme, J., Larsson, D. G. J., Rautava, S., Isolauri, E., Salminen, S., Kumar, H., Satokari, R. & Virta, M. (2018). Maternal gut and breast milk microbiota affect infant gut antibiotic resistome and mobile genetic elements. *Nature Communications*, *9*(1), 3891. https://doi.org/10.1038/s41467-018-06393-w

Paulson, J. N., Stine, O. C., Bravo, H. C. & Pop, M. (2013). Differential abundance analysis for microbial marker-gene surveys. *Nature Methods*, *10*(12), 1200–1202. https://doi.org/10.1038/nmeth.2658

Pehrsson, E. C., Tsukayama, P., Patel, S., Mejía-Bautista, M., Sosa-Soto, G., Navarrete, K. M., Calderon, M., Cabrera, L., Hoyos-Arango, W., Bertoli, M. T., Berg, D. E., Gilman, R. H. & Dantas, G. (2016). Interconnected microbiomes and resistomes in

low-income human habitats. *Nature*, *533*(7602), 212–216. https://doi.org/10.1038/nature17672

Perry, J. A., Westman, E. L. & Wright, G. D. (2014). The antibiotic resistome: what's new? *Current Opinion in Microbiology*, *21*, 45–50. https://doi.org/10.1016/j.mib.2014.09.002

Putignani, L., Chierico, F. D., Petrucca, A., Vernocchi, P. & Dallapiccola, B. (2014). The human gut microbiota: a dynamic interplay with the host from birth to senescence settled during childhood. *Pediatric Research*, *76*(1), 2–10. https://doi.org/10.1038/pr.2014.49

Rahman, S. F., Olm, M. R., Morowitz, M. J. & Banfield, J. F. (2018). Machine Learning Leveraging Genomes from Metagenomes Identifies Influential Antibiotic Resistance Genes in the Infant Gut Microbiome. *MSystems*, *3*(1), e00123-17. https://doi.org/10.1128/msystems.00123-17

Richter, T. K. S., Hazen, T. H., Lam, D., Coles, C. L., Seidman, J. C., You, Y., Silbergeld, E. K., Fraser, C. M. & Rasko, D. A. (2018). Temporal Variability of Escherichia coli Diversity in the Gastrointestinal Tracts of Tanzanian Children with and without Exposure to Antibiotics. *MSphere*, *3*(6), e00558-18. https://doi.org/10.1128/msphere.00558-18

Roberts, I. C., Marilyn & Roberts, M. (2001). Tetracycline Antibiotics: Mode of Action, Applications, Molecular Biology, and Epidemiology of Bacterial Resistance. *Microbiology and Molecular Biology Reviews*, *65*(2), 232–260. https://doi.org/https://doi.org/10.1128/MMBR.65.2.232-260.2001

Rooney, A. M., Raphenya, A. R., Melano, R. G., Seah, C., Yee, N. R., MacFadden, D. R., McArthur, A. G., Schneeberger, P. H. H. & Coburn, B. (2022). Performance Characteristics of Next-Generation Sequencing for the Detection of Antimicrobial Resistance Determinants in Escherichia coli Genomes and Metagenomes. *MSystems*, e00022-22. https://doi.org/10.1128/msystems.00022-22

Roy, C. I. L., Bowyer, R. C. E., Carr, V. R., Costeira, R., Castillo-Fernandez, J. E., Martin, T. C., Spector, T. D., Steves, C. J., Moyes, D., Forslund, S. K. & Bell, J. T. (2020). Host genetic and environmental factors shape the human gut resistome. *BioRxiv*, 2020.05.18.092973. https://doi.org/10.1101/2020.05.18.092973

Salinas, L., Loayza, F., Cárdenas, P., Saraiva, C., Johnson, T. J., Amato, H., Graham, J. P. & Trueba, G. (2021). Environmental Spread of Extended Spectrum Beta-Lactamase (ESBL) Producing Escherichia coli and ESBL Genes among Children and Domestic Animals in Ecuador. *Environmental Health Perspectives*, *129*(2), 027007. https://doi.org/10.1289/ehp7729

Salyers, A. A., Gupta, A. & Wang, Y. (2004). Human intestinal bacteria as reservoirs for antibiotic resistance genes. *Trends in Microbiology*, *12*(9), 412–416. https://doi.org/10.1016/j.tim.2004.07.004

Scholz, M., Ward, D. V., Pasolli, E., Tolio, T., Zolfo, M., Asnicar, F., Truong, D. T., Tett, A., Morrow, A. L. & Segata, N. (2016). Strain-level microbial epidemiology and population genomics from shotgun metagenomics. *Nature Methods*, *13*(5), 435–438. https://doi.org/10.1038/nmeth.3802

Segata, N., Waldron, L., Ballarini, A., Narasimhan, V., Jousson, O. & Huttenhower, C. (2012). Metagenomic microbial community profiling using unique clade-specific marker genes. *Nature Methods*, *9*(8), 811–814. https://doi.org/10.1038/nmeth.2066

Shoemaker, N. B., Vlamakis, H., Hayes, K. & Salyers, A. A. (2001). Evidence for extensive resistance gene transfer among Bacteroides spp. and among Bacteroides and other genera in the human colon. *Applied and Environmental Microbiology*, *67*(2), 561–568. https://doi.org/10.1128/aem.67.2.561-568.2001

Szekeres, E., Chiriac, C. M., Baricz, A., Szőke-Nagy, T., Lung, I., Soran, M.-L., Rudi, K., Dragos, N. & Coman, C. (2018). Investigating antibiotics, antibiotic resistance genes, and microbial contaminants in groundwater in relation to the proximity of urban areas. *Environmental Pollution*, *236*, 734–744. https://doi.org/10.1016/j.envpol.2018.01.107

Truong, D. T., Franzosa, E. A., Tickle, T. L., Scholz, M., Weingart, G., Pasolli, E., Tett, A., Huttenhower, C. & Segata, N. (2015). MetaPhlAn2 for enhanced metagenomic taxonomic profiling. *Nature Methods*, *12*(10), 902–903. https://doi.org/10.1038/nmeth.3589

Vatanen, T., Plichta, D. R., Somani, J., Münch, P. C., Arthur, T. D., Hall, A. B., Rudolf, S., Oakeley, E. J., Ke, X., Young, R. A., Haiser, H. J., Kolde, R., Yassour, M., Luopajärvi, K., Siljander, H., Virtanen, S. M., Ilonen, J., Uibo, R., Tillmann, V., … Xavier, R. J. (2019). Genomic variation and strain-specific functional adaptation in the human gut microbiome during early life. *Nature Microbiology*, *4*(3), 470–479. https://doi.org/10.1038/s41564-018-0321-5

Verraes, C., Boxstael, S. V., Meervenne, E. V., Coillie, E. V., Butaye, P., Catry, B., Schaetzen, M.-A. de, Huffel, X. V., Imberechts, H., Dierick, K., Daube, G., Saegerman, C., Block, J. D., Dewulf, J. & Herman, L. (2013). Antimicrobial Resistance in the Food Chain: A Review. *International Journal of Environmental Research and Public Health*, *10*(7), 2643–2669. https://doi.org/10.3390/ijerph10072643

Wright, G. D. (2007). The antibiotic resistome: the nexus of chemical and genetic diversity. *Nature Reviews Microbiology*, *5*(3), 175–186. https://doi.org/10.1038/nrmicro1614

Wu, G., Zhang, C., Wang, J., Zhang, F., Wang, R., Shen, J., Wang, L., Pang, X., Zhang, X., Zhao, L. & Zhang, M. (2016). Diminution of the gut resistome after a gut microbiota-targeted dietary intervention in obese children. *Scientific Reports*, *6*(1), 24030. https://doi.org/10.1038/srep24030

Yassour, M., Jason, E., Hogstrom, L. J., Arthur, T. D., Tripathi, S., Siljander, H., Selvenius, J., Oikarinen, S., Hyöty, H., Virtanen, S. M., Ilonen, J., Ferretti, P., Pasolli, E., Tett, A., Asnicar, F., Segata, N., Vlamakis, H., Lander, E. S., Huttenhower, C., … Xavier, R. J. (2018). Strain-Level Analysis of Mother-to-Child Bacterial Transmission during the First Few Months of Life. *Cell Host & Microbe*, *24*(1), 146-154.e4. https://doi.org/10.1016/j.chom.2018.06.007

Yatsunenko, T., Rey, F. E., Manary, M. J., Trehan, I., Dominguez-Bello, M. G., Contreras, M., Magris, M., Hidalgo, G., Baldassano, R. N., Anokhin, A. P., Heath, A. C., Warner, B., Reeder, J., Kuczynski, J., Caporaso, J. G., Lozupone, C. A., Lauber, C., Clemente, J. C., Knights, D., … Gordon, J. I. (2012). Human gut

microbiome viewed across age and geography. *Nature*, *486*(7402), 222–227. https://doi.org/10.1038/nature11053

Zaheer, R., Noyes, N., Polo, R. O., Cook, S. R., Marinier, E., Domselaar, G. V., Belk, K. E., Morley, P. S. & McAllister, T. A. (2018). Impact of sequencing depth on the characterization of the microbiome and resistome. *Scientific Reports*, *8*(1), 5890. https://doi.org/10.1038/s41598-018-24280-8

Zankari, E., Hasman, H., Cosentino, S., Vestergaard, M., Rasmussen, S., Lund, O., Aarestrup, F. M. & Larsen, M. V. (2012). Identification of acquired antimicrobial resistance genes. *Journal of Antimicrobial Chemotherapy*, *67*(11), 2640–2644. https://doi.org/10.1093/jac/dks261

Zhang, Z., Zhang, Q., Wang, T., Xu, N., Lu, T., Hong, W., Penuelas, J., Gillings, M., Wang, M., Gao, W. & Qian, H. (2022). Assessment of global health risk of antibiotic resistance genes. *Nature Communications*, *13*(1), 1553. https://doi.org/10.1038/s41467-022-29283-8

CHAPTER 4. SYSTEMATIC REVIEW: THE EFFECT OF SUPPLEMENTARY PROBIOTICS ON GUT MICROBIOTA STRUCTURE DURING ERADICATION THERAPY AGAINST *HELICOBACTER PYLORI*

Sara Gabriela Cifuentes[1], María Fernanda Zurita[1], Paúl Andrés Cárdenas[1]

1 Universidad San Francisco de Quito USFQ

Corresponding author: pacardenas@usfq.edu.ec

Abstract

All patients infected with *Helicobacter pylori* should be treated with eradication therapy to restore gastric function and prevent developing chronic and malignant diseases. However, managing this infection includes a triple or quadruple therapeutic regimen whose drugs such as antibiotics and proton pump inhibitors can alter the gut microbiota with temporary or lasting effects. Probiotics are positioned as an alternative to protect and restore changes in the structure and function of the gut microbiota during therapies with antibiotics. We aimed to evaluate the effects of probiotic supplementation on gut microbiota abundance and diversity during antibiotic treatment against *H. pylori* infections. We systematically searched articles in MEDLINE, Embase, and Web of Science databases and the Cochrane Central Register of Controlled Trials (CENTRAL) clinical trials registry. Of 494 articles, only eight clinical trials were included, which recruited 581 participants between 15 and 70 years of age. The studies were very heterogeneous in eradication therapies against *H.pylori*, in the strains of probiotics used as supplements, in the time of administration and dose. This heterogeneity and other factors, such as the small sample size in most clinical trials, may have also contributed to the highly variable results. Thus, we could not conclude whether or not the use of probiotics is an effective measure to protect the structure of the intestinal microbiota during the administration of an eradication therapy against *H. pylori*.

Introduction

Although antimicrobials revolutionized the way infectious diseases are managed, the use or misuse of antibiotics can select multi-resistant bacteria(English & Gaur, 2009), altering the intestinal microbiota's structure and function(Francino, 2016; Vangay et al., 2015). Gut dysbiosis is the imbalance of a microbial community due to alterations in the abundance and diversity of community members associated with a disease(Messer & Chang, 2018). The use of antibiotics can contribute to this intestinal microbial imbalance temporarily or permanently (Cochetière et al., 2005; Dethlefsen & Relman, 2011; Huse et al., 2008). Gut dysbiosis may also be responsible for gastrointestinal adverse effects suffered by patients treated with antimicrobials, for example, during *H. pylori* eradication therapy (Hsu et al., 2018). Antimicrobial therapies with a broad spectrum of action significantly impact the gut microbiota (Francino, 2016). Additionally, gastrointestinal adverse effects can affect patient adherence to antimicrobial therapy against *H. pylori* (O'Connor et al., 2009).

Probiotics have been frequently used to improve intestinal dysbiosis in digestive diseases such as irritable bowel syndrome and infectious or antibiotic-associated diarrhea (Gareau et al., 2010). The use of probiotics as supplementation during the treatment of *H. pylori* to improve the bacterium's eradication rate and reduce the incidence of adverse effects has shown positive results (Shi et al., 2019; F. Wang et al., 2017). Possible mechanisms of action of probiotics in the intestinal environment are associated with their interaction with host cells (Baarlen et al., 2011), modulation of the function of the intestinal barrier (Wieërs et al., 2020), the immune system(Fata et al., 2018), and host metabolism (Dewulf et al., 2013). However, so far, no scientific literature has been analyzed to evaluate the usefulness of probiotics in restoring or protecting the intestinal

microbiota during the eradication of *H. pylori.* The evaluation of the effect of probiotics on the structure of the intestinal microbiota during the administration of antibiotics could give us information on how to protect the gut microbiota and if its beneficial effects are reproducible with different strains of probiotics. We aimed to evaluate the effects of probiotic supplementation on gut microbiota abundance and diversity during antibiotic treatment against *H. pylori* infections.

Materials and Methods

Data sources, bibliographic search, and selection criteria

The search for bibliographic sources began after the protocol was registered in PROSPERO on November 8th, 2021, under CRD42021282907 (See: https://www.crd.york.ac.uk/prospero/display_record.php?ID =CRD42021282907). The literature source search strategy was developed using the MEDLINE, Embase, and Web of Science databases and the Cochrane Central Register of Controlled Trials (CENTRAL) clinical trials registry. Search terms used included 'gut microbiota' [Title/Abstract] AND 'helicobacter pylori' [Title/Abstract] AND probiotic [Title/Abstract] AND 'clinical trial' [Title/Abstract]. When the searches did not return good bibliographic sources, we also considered using synonyms, related terms, and variant spellings for the word microbiota, such as 'microbiome' OR 'flora' OR 'microflora.' Also, we use the terms' gut' OR 'intestine.'

We included randomized controlled trials or controlled clinical trials because we analyzed the effect of probiotics (intervention) (Higgins et al., 2019) on the gut microbiota. The studies included participants of any age and diagnosed with *H. pylori* infections. The diagnosis of an *H. pylori* infection was based on endoscopy, rapid urease test, or non-invasive tests such as urease breath test, or stool antigen test (Y.-K.

Wang et al., 2015). The studies must evaluate two groups. The intervention group received probiotic supplementation, and the control group (comparator) received no intervention or placebo. The primary outcome focused on changes in gut microbiota composition during *H. pylori* eradication therapy by probiotics. The specific measurements were alpha, beta diversity, or relative abundance. The studies included in this review evaluated the abundance and diversity changes from the baseline and compared the intervention and control groups. We excluded studies that recruited patients diagnosed with malignancies in the stomach or intestines and those studies where only abstracts were available.

Two reviewers (SGC and MFZ) extracted all data from included studies independently using a standardized protocol. Protocol standardization was developed with pre-reviewer training, preliminary extractions, and improvements to the extraction instrument. Extraction forms were used in Excel in which general information such as abstract, accession number (unique identifier), affiliation or address, article identifier or digital object identifier (DOI), source, resource, and year of publication were included. The specific information incorporated characteristics of the study and methods used, baseline characteristics of the patients, diagnostic methods to identify *H. pylori*, dose, time, duration of the intervention and the comparator, outcomes, results on alpha, beta diversity, relative abundance of the gut microbiota, adverse effects, and conclusions. Discrepancies were resolved by discussion or by including a third reviewer (PAC). The reference manager was Papers v4.32.2067 (x64). The adapted PRISMA (Preferred Reporting Items for Systematic Reviews and RMeta -Analyses) (Page et al., 2021) flowchart of study selection is in Figure 1.

Assessment of risk of bias

The risk of bias assessment was carried out using the Cochrane risk of bias tool for randomized trials (RoB 2) (Sterne et al., 2019) in the Excel version. The tool allowed the evaluation of the following criteria: random sequence generation, allocation concealment, blinding, incomplete outcome data, and selective reporting. The reviewers (SGC and MFZ) independently performed a risk-of-bias analysis in each of the included trials. If the reviewers had discrepancies, the third reviewer (PAC) resolved the disagreements or through discussion.

Results

The search based on the PICO (patient/population, intervention, comparison, and outcomes) question yielded 494 articles. After screening by titles and abstracts, 29 articles were evaluated according to the inclusion and exclusion criteria. Eight primary scientific articles (Cárdenas et al., 2020; Chen et al., 2018; Dore et al., 2022; Kakiuchi et al., 2020; Oh et al., 2016; Tang et al., 2021; Wu et al., 2019; C. Yang et al., 2021), and clinical trials, were included in this study (Figure 1). A total of 581 patients were included in this systematic review; ages ranged from 15 to 70 years old. Six studies were open-label and did not use a placebo or another intervention as a comparator. Only one study clearly explained that it is double-blind, and the remaining article, although it used a placebo, does not describe that it is a double-blind study. The countries described in this study belong mainly to China (4 studies), the Republic of Korea, Japan, Italy, and Ecuador. The oldest article was published in 2015, and the most recent in 2022. Therapies to eradicate *H. pylori* varied across the six studies, with only two studies using the same therapy. Only the works by Chen et al. (2018) and Tang et al. (2021) used quadruple therapy, the other studies administered triple therapy.

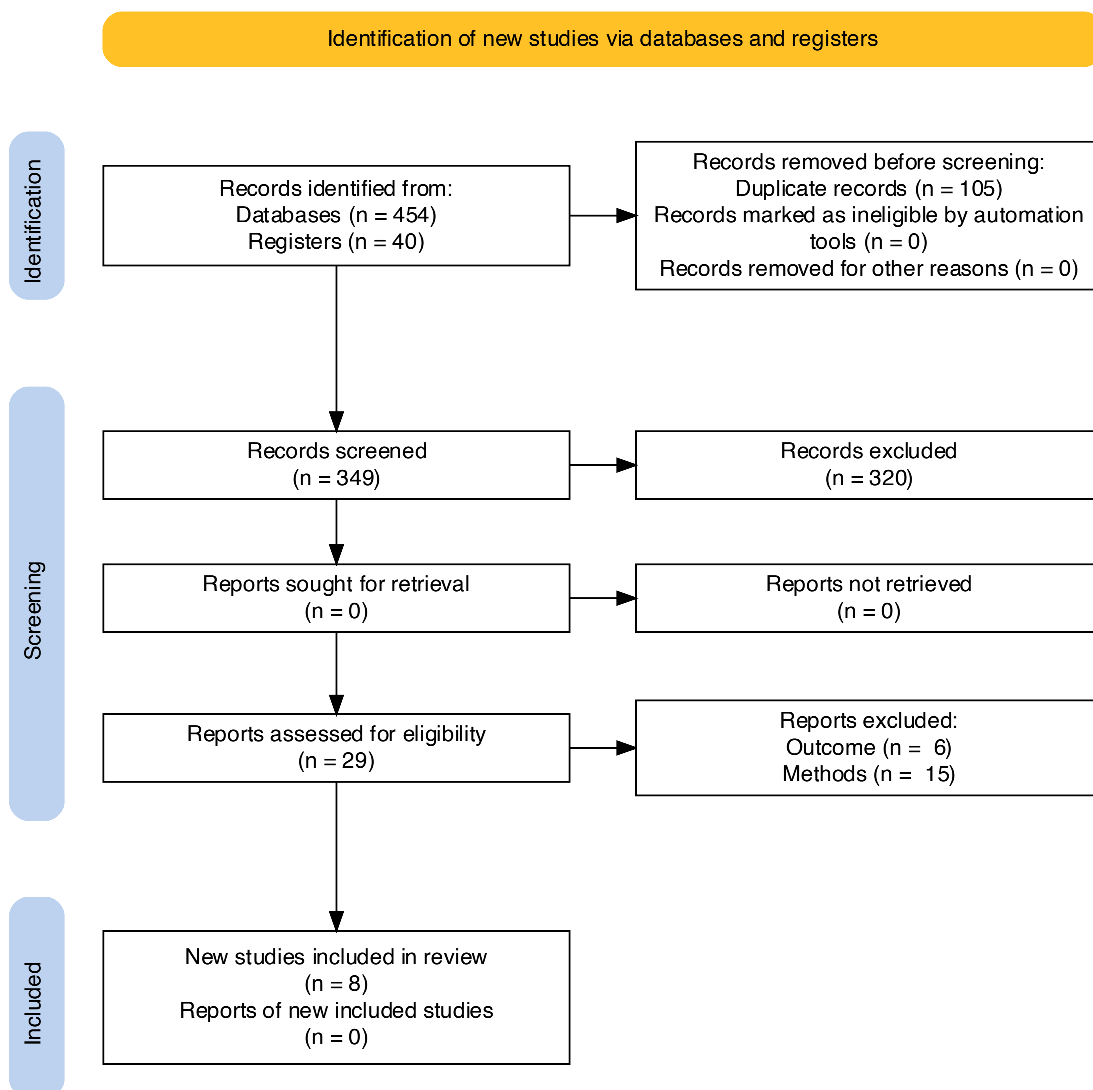


Figure 1. PRISMA flowchart of study selection.

Table 1 shows the characteristics of the participants recruited in the studies included in this review and general findings on the use of probiotics and the analysis of the intestinal microbiota. The use of probiotics was variable across the studies. Three studies used the same combination of *Bacillus subtilis* and *Enterococcus faecium* probiotics but with different doses. The other studies used *Lactobacillus reuteri*, killed *Lactobacillus reuteri* cells, *Enterococcus faecium*, *Saccharomyces boulardii* CNCM I-745, and *Clostridium butiricum* (Table 1). Three studies did not have detailed information on how many CFUs were used in each administered dose.

Probiotic administration time was also widely variable, from 7 days to 6 weeks. While 7 of 8 studies administered probiotics simultaneously as eradication therapy, one study administered probiotics after *H. pylori* eradication therapy. Only two studies used a placebo as a comparator, while six used no intervention.

Gut microbiota analysis

All studies had a time-point assessment of the gut microbiota before starting eradication therapy or probiotic administration. Seven studies evaluated changes in the gut microbiota immediately after antibiotic eradication therapy was completed, but one study evaluated it two weeks after eradication therapy, missing the possibility of capturing the most significant changes that occur in the microbiota during or immediately after the completion of antimicrobial therapy.

The analysis method for studying the intestinal microbiota was the amplification of the 16S rRNA gene in all the studies; however, six studies amplified the V3-V4 variable regions, one study V1-V3 and one study only the V4 variable region. Four studies evaluated alpha, beta diversity, and relative abundance together, and only two studies included differential abundance analysis to explore abundance between intervention groups. The findings for changes in microbiota structure between the two intervention groups are as follows: two studies showed that alpha diversity (using the Shannon or Chao1 or Evenness indices) was significantly higher in the group that received probiotics immediately after the eradication therapies. Only one study demonstrated these same changes eight weeks after eradication therapy, although it did not assess changes in the gut microbiota after antibiotic therapy. Two studies demonstrated no significant difference in alpha diversity between the intervention groups after treatments, and two did not describe pairwise comparisons between the two intervention

groups. One study did not use statistical analyzes for between-group comparisons. Only one study demonstrated that the bacterial composition between the two intervention groups was significantly different using beta diversity analysis. Two studies did not apply beta diversity analysis, and one study, although applied it, did not report pairwise comparisons for the intervention groups. In general, and in most of the studies, the differences between the probiotic and control groups disappeared after the time elapsed (2, 4, 6, or 8 weeks after the eradication treatments). The relative abundances of bacteria at the phylum, class, family, or genus level were completely variable across studies (See Table 1). Two studies obtained contradictory findings regarding the *Escherichia-Shigella* genus between the probiotic and control groups after the completion of eradication treatments.

Risk of bias analysis

Using the Rob2 tool (a revised tool for assessing risk of bias in randomized trials), only one study had no risk of bias in the five fields analyzed: random sequence generation, allocation concealment, blinding, incomplete outcome data, and selective reporting. Three studies were cataloged with some concerns since they did not report allocation concealment methods and because they were open-label studies. Four studies had a severe risk of bias problems in 4 criteria: random sequence generation, allocation concealment, blinding, and incomplete outcome data, except selective reporting (See Figure 2).

Unique ID	Study ID	Experimental	Comparator	Outcome	Weight	D1	D2	D3	D4	D5	Overall
RB02	SR09	Intervention	No Intervention	Microbiota	1	-	-	-	+	+	-
RB03	SR10	Intervention	No intervention	Microbiota	1	+	!	+	+	+	!
RB05	SR08	Intervention	No intervention	Microbiota	1	!	!	+	+	+	!
RB07	SR013	Intervention	Placebo	Microbiota	1	+	+	+	+	+	+
RB09	SR019	Intervention	No intervention	Microbiota	1	-	!	+	-	+	-
RB11	SR20	Intervention	Placebo	Microbiota	1	-	!	+	!	+	!
RB13	SR23	Intervention	No intervention	Microbiota	1	+	!	+	-	+	-
RB15	SR27	Intervention	Bismuth	Microbiota	1	+	!	-	+	+	-

\+ Low risk
! Some concerns
\- High risk

D1 Randomisation process
D2 Deviations from the intended interventions
D3 Missing outcome data
D4 Measurement of the outcome
D5 Selection of the reported result

Figure 2. Risk of bias analysis using the Rob2 tool.

Table 1. Summary of findings of the studies included in this review.

Study	Country	Patients (n)	Age (yr)	Eradication therapy (ET) regimens and dosage	Amplicon/ shutgun sequencing	Probiotic (strains)	Probiotic dosis	Duration of the probiotic	Comparator	Time of assessment (weeks)	Methods of assessment (microbiota changes)	Key findings comparing the two groups*	Limitations
Dore et al., 2022	Italy	22	> 18	Rabeprazole (20 mg twice daily), tetracycline (500 mg twice daily) and metronidazole (500 mg twice daily) for 10 days	rRNA 16S, amplicon sequencing (V4)	*Lactobacillus reuteri* DSM 17938 y *L. reuteri* ATCC PTA 6475	2x10^8/tablet/twice daily	27 days	No intervention	w0, w2, w6-7	Alpha, beta diversity and differential abundance	Alpha diversity: no significant differences Beta diversity: no significant differences Differential abundance: the phylum Verrucomicrobiota and the families Akkermansiaceae and Acidaminococcaceae were more abundant in the control group.	Open-label study and small sample
Tang et al., 2021	China (multi center)	151	18-65	Esomeprazole (20 mg twice daily), amoxicillin (1000 mg twice daily), furazolidone (100 mg twice daily), bismuth potassium citrate (220 mg twice daily) for two weeks	rRNA 16S, amplicon sequencing (V3-V4)	*Enterococcus faecium* and *Bacillus subtilis*	*Ef* 4.5x10^8 and *Bs* 5.0x10^7/cap /three times daily	4 weeks (2 weeks with ET and the next two weeks without ET	Placebo (maltodextrin) three times daily	w0, w2, w4, w6, w8	Alpha, beta diversity and relative abundance	Alpha diversity: no significant differences Beta diversity: microbial composition was significantly different and at week 4 Relative abundance: *Enterococcus, Citrobacter,* and *Oscillospira* were significantly increased in the probiotic group, while *Dialister*, *Anaerotruncus*, and *Megasphaera* were mainly increased in the placebo group.	It did not include differential abundance analyses to compare abundances between microorganisms.
Yang et al., 2021	China	198	18-70	Esomeprazole (20 mg twice daily) before meals, amoxicillin (1 g twice daily) after meals, and clarithromycin (500 mg twice daily) after meals for 2 weeks	rRNA 16S, amplicon sequencing (V3-V4)	Dead cells of *Lactobacillus reuteri* DSM17648	1 × 10^10 dead cells/ packet/ 2 packets twice daily	4 weeks (2 weeks alone and the next two weeks with ET)	Starch identical to non-viable *L. reuteri* DSM17648 in weight, appearance, and taste	w0, w2, w8	Alpha, beta diversity and relative abundance	Alpha diversity: significant more diverse in the probiotic group at week 8 after ET. Beta diversity: no significant differences at week 8 Relative abundance: *Faecalibacterium* and *Suldoligranulum* in the probiotic group were significantly higher than that in the placebo group at week 8.	It did not report changes in the gut microbiota immediately after H. pylori treatment ended. It did not include differential abundance analyses to compare abundances between microorganisms.
Kakiuchi et al., 2020	Japan	49	15	Vonoprazan (20 mg twice daily), amoxicillin (750 mg twice daily), and clarithromycin (400 mg twice daily) for 7 days	rRNA 16S, amplicon sequencing (V3-V4)	*Enterococcus faecium* 129 BIO 3B-R	3 tablets/daily	7 days with the ET	No intervention	w0, w1, w8-12	Alpha, beta diversity and relative abundance	Alpha diversity: significant more diverse in the probiotic group than the control group. Beta diversity: no significant differences Relative abundance: *Blautia* was significantly more abundant in the probiotic group. *Bifidobacterium* and *Collinsella* were significantly less abundant in the control group.	Open-label study and small sample. It did not include differential abundance analyses to compare abundances between microorganisms.

Study	Country	Patients (n)	Age (yr)	Eradication therapy (ET) regimens and dosage	Amplicon/ shutgun sequencing	Probiotic (strains)	Probiotic dosis	Duration of the probiotic	Comparator	Time of assessment (weeks)	Methods of assessment (microbiota changes)	Key findings comparing the two groups*	Limitations
Cárdenas et al., 2020	Ecuador	38	18-55	Amoxicillin (1 g tree times daily, tinidazole 1 g (four times daily), and omeprazole (40 mg twice daily) for 2 weeks	rRNA 16S, amplicon sequencing (V3-V4)	*Saccharomyces boulardii* CNCM I-745, presentation not specified	Approximately 22.5 × 10^9 CFU/750mg /daily	2 weeks with ET	No intervention	w0, w2, w6	Alpha, beta diversity and relative abundance	Alpha diversity: significant more diverse in the probiotic group than in the control group. Beta diversity: no significant differences Relative abundance: Gammaproteobacteria (*Escherichia* spp. and another *Enterobacteriaceae* OTUs) were more abundant in the probiotic group than in the control group. Bacteroides (*Prevotella*) and Clostridia (*Lachnospira* and *Ruminococcus*) showed a lower abundance in the control group.	Open-label study and small sample. It did not include differential abundance analyses to compare abundances between microorganisms.
Wu et al., 2019	China	40	18-65	Esomeprazole (20 mg twice daily) before meals, amoxicillin (1 g twice daily) after meals, and clarithromycin (500 mg twice daily) after meals for 2 weeks	rRNA 16S, amplicon sequencing (V3-V4)	*Bacillus subtilis* and coated capsules BSEF (*Bacillus subtilis* and *Enterococcus faecium*)	Capsules of 500 mg/ 3 times daily	6 weeks after ET	No intervention	w0, w2, w6, w10	Beta diversity and differential abundance	**Findings of probiotics never given at the same time as antibiotics** Alpha diversity: not applied Beta diversity: pairwise comparisons not reported Differential abundance: abundance was significantly higher in the probiotic group compared to the control group at 2, 6, and 10 weeks after ET. At the genus levels, 23, 28, and 11 genera were more abundant in the probiotic group than the control group at 2-, 6-, and 10-weeks post ET.	Open-label study and small sample. Probiotics never given at the same time as antibiotics. It did not report alpha diversity analysis.
Chen et al., 2018	China	63	18-70	Pantoprazole (40mg twice daily), amoxicillin (1000mg twice daily), furazolidone (100mg twice daily), colloidal bismuth pectin (400mg twice daily) for 2 weeks	rRNA 16S, amplicon sequencing (V4) and ITS1 and ITS2 regions	*Clostridium butyricum* CBM588	Tablets of 40 mg/ 3 times daily	2 weeks with ET	No intervention	w0, w2, w8	Alpha diversity and relative abundance	Alpha diversity: pairwise comparisons not reported Beta diversity: pairwise comparisons not reported Relative abundance: only focused on describing *C. butiricum* changes but no other microorganisms in the samples.	Open-label study and small sample. It did not include differential abundance analyses to compare abundances between microorganisms.

Study	Country	Patients (n)	Age (yr)	Eradication therapy (ET) regimens and dosage	Amplicon/ shutgun sequencing	Probiotic (strains)	Probiotic dosis	Duration of the probiotic	Comparator	Time of assessment (weeks)	Methods of assessment (microbiota changes)	Key findings comparing the two groups*	Limitations
Oh el al., 2015	Republic of Korea	20	Adults (range not specified)	Clarithromycin (500 mg twice daily) , amoxicillin (1000 mg twice daily) , and lansoprazole (30 mg) for 2 weeks	rRNA 16S, amplicon sequencing (V1-V3)	*Enterococcus faecium* and *Bacillus subtilis*	*Ef* 9x10^8 and *Bs* 1X10^81/ tablet/ twice daily	2 weeks with ET	No intervention	w0, w2	Alpha diversity and relative abundance	Alpha diversity: diversity higher in the probiotic group compared to the control group (not applied statistical analysis). Relative abundance: the microbiota composition in all subjects changed in the control group than in the probiotic group.	Open-label study and small sample. There was not any statistical analysis for pairwise comparisons. It did not include differential abundance analyses to compare abundances between microorganisms.

*Comparisons at the end of eradication therapy (ET).

Discussion

The present study has not been able to support with solid evidence that the use of probiotics (viable and non-viable) as a supplement protects the gut microbiota during the use of combinations of drugs utilized in triple or quadruple eradication therapy against *H. pylori*. However, neither was able to rule it out.

Evidence has shown that antibiotics (Dong & Gupta, 2018; Duan et al., 2022) and proton pump inhibitors (Lombardo et al., 2010) produce alterations in the gut microbiota related to a reduction in the diversity and changes in the abundance of specific taxa. The work of Duan et al. (2020) describes several strategies to alleviate the damage caused by the administration of antibiotics, including probiotic supplementation. Although probiotics can attenuate the adverse effects of antibiotics (Leclercq et al., 2017), there are some explanations for why we have not been able to demonstrate the positive effect of probiotics in protecting the gut microbiota during eradication therapy against *H. pylori* in this systematic review.

We included eight studies in this work, and the insufficient number of studies in this area may be because of the interest in understanding the role of the gut microbiota in health and disease using next-generation sequencing techniques has emerged a few decades ago (Ananthakrishnan et al., 2019). Additionally, the small sample size in all the clinical trials could have influenced that clear differences are not evident when comparing the probiotic group and the comparator group and that it is impossible to generalize these results to the entire target population (Gupta et al., 2016).

Another essential aspect that has weakened the findings reported by the included studies is having conducted single-blind studies. These studies can fall into biases that can lead to inflated effect size and even increase the risk of type I error (David & Khandhar, 2022).

The heterogeneity throughout the studies has not allowed us to implement a meta-analysis in this work (Charrois, 2015). Regarding probiotics, three studies used the same bacterial strains (different doses), and the other used different strains. Evidence has shown that different probiotic strains can trigger different effects in participants (DeGruttola et al., 2016). In addition, the timing of probiotic administration was highly variable across the eight studies, and not all studies evaluated gut microbiota changes simultaneously. All these variations among the studies included in this systematic review have not allowed us to firmly conclude whether probiotics are effective in reverting the diversity and abundance of the intestinal microbiota to pre-treatment states or in attenuating the changes produced by eradication therapy against *H. pylori*.

It is still too early to rule out that probiotic supplementation during *H.pylori* eradication therapy is effective in protecting the structure and function of the gut microbiota. However, more studies with a larger sample size in each evaluated group, double- or triple-blind, are required to reduce the type I error or inflate the effect size. Furthermore, studies need to report their findings, even if no differences exist between the groups evaluated. Also, it is necessary to develop studies where differential abundance analyses are implemented (L. Yang & Chen, 2022) to evaluate the changes between species or genera of bacteria affected by the interventions or find biomarkers. We suggest combining metagenomics with other omics, such as metabolomics (O'Connell, 2020), to study the mechanisms behind probiotics and incorporate other probiotics, such as yeasts (Alkalbani et al., 2022).

References

Alkalbani, N. S., Osaili, T. M., Al-Nabulsi, A. A., Olaimat, A. N., Liu, S.-Q., Shah, N. P., Apostolopoulos, V. & Ayyash, M. M. (2022). Assessment of Yeasts as Potential Probiotics: A Review of Gastrointestinal Tract Conditions and Investigation Methods. *Journal of Fungi*, *8*(4), 365. https://doi.org/10.3390/jof8040365

Ananthakrishnan, A. N., Singal, A. G. & Chang, L. (2019). The Gut Microbiome and Digestive Health – A New Frontier. *Clinical Gastroenterology and Hepatology*, *17*(2), 215–217. https://doi.org/10.1016/j.cgh.2018.10.040

Baarlen, P. van, Troost, F., Meer, C. van der, Hooiveld, G., Boekschoten, M., Brummer, R. J. M. & Kleerebezem, M. (2011). Human mucosal in vivo transcriptome responses to three lactobacilli indicate how probiotics may modulate human cellular pathways. *Proceedings of the National Academy of Sciences*, *108*(Supplement 1), 4562–4569. https://doi.org/10.1073/pnas.1000079107

Cárdenas, P. A., Garcés, D., Prado-Vivar, B., Flores, N., Fornasini, M., Cohen, H., Salvador, I., Cargua, O. & Baldeón, M. E. (2020). Effect of Saccharomyces boulardii CNCM I-745 as complementary treatment of Helicobacter pylori infection on gut microbiome. *European Journal of Clinical Microbiology & Infectious Diseases*, *39*(7), 1365–1372. https://doi.org/10.1007/s10096-020-03854-3

Charrois, T. L. (2015). Systematic Reviews: What Do You Need to Know to Get Started? *CJHP*, *68*(2), 144–148.

Chen, L., Xu, W., Lee, A., He, J., Huang, B., Zheng, W., Su, T., Lai, S., Long, Y., Chu, H., Chen, Y., Wang, L., Wang, K., Si, J. & Chen, S. (2018). The impact of Helicobacter pylori infection, eradication therapy and probiotic supplementation on gut microenvironment homeostasis: An open-label, randomized clinical trial. *EBioMedicine*, *35*, 87–96. https://doi.org/10.1016/j.ebiom.2018.08.028

Cochetière, M. F. D. L., Durand, T., Lepage, P., Bourreille, A., Galmiche, J. P. & Doré, J. (2005). Resilience of the dominant human fecal microbiota upon short-course antibiotic challenge. *Journal of Clinical Microbiology*, *43*(11), 5588–5592. https://doi.org/10.1128/jcm.43.11.5588-5592.2005

David, S. & Khandhar, P. (2022). *StatPearls [Internet]*. Treasure Island (FL): StatPearls Publishing. https://www.ncbi.nlm.nih.gov/books/NBK546641

DeGruttola, A. K., Low, D., Mizoguchi, A. & Mizoguchi, E. (2016). Current Understanding of Dysbiosis in Disease in Human and Animal Models. *Inflammatory Bowel Diseases*, *22*(5), 1137–1150. https://doi.org/10.1097/mib.0000000000000750

Dethlefsen, L. & Relman, D. A. (2011). Incomplete recovery and individualized responses of the human distal gut microbiota to repeated antibiotic perturbation. *Proceedings of the National Academy of Sciences*, *108*(Supplement 1), 4554–4561. https://doi.org/10.1073/pnas.1000087107

Dewulf, E. M., Cani, P. D., Claus, 1 Sandrine P, Fuentes, S., Puylaert, P. G., Neyrinck, A. M., Bindels, L. B., Vos, W. M. de, Gibson, G. R., Thissen, J.-P. & Delzenne1, N. M. (2013). Insight into the prebiotic concept: lessons from an exploratory, double blind intervention study with inulin-type fructans in obese women. *Gut*, *62*, 1112–1121. https://doi.org/10.1136/gutjnl-2012-303304

Dong, T. S. & Gupta, A. (2018). Influence of Early Life, Diet, and the Environment on the Microbiome. *Clinical Gastroenterology and Hepatology*, *17*(2), 231–242. https://doi.org/10.1016/j.cgh.2018.08.067

Dore, M. P., Sau, R., Niolu, C., Abbondio, M., Tanca, A., Bibbò, S., Loria, M., Pes, G. M. & Uzzau, S. (2022). Metagenomic Changes of Gut Microbiota following Treatment of Helicobacter pylori Infection with a Simplified Low-Dose Quadruple Therapy with Bismuth or Lactobacillus reuteri. *Nutrients*, *14*(14), 2789. https://doi.org/10.3390/nu14142789

Duan, H., Yu, L., Tian, F., Zhai, Q., Fan, L. & Chen, W. (2022). Antibiotic-induced gut dysbiosis and barrier disruption and the potential protective strategies. *Critical Reviews in Food Science and Nutrition*, *62*(6), 1427–1452. https://doi.org/10.1080/10408398.2020.1843396

English, B. K. & Gaur, A. H. (2009). The Use and Abuse of Antibiotics and the Development of Antibiotic Resistance. In A. Finn, N. Curtis & A. Pollard (Eds.), *Hot Topics in Infection and Immunity in Children VI* (Vol. 659, pp. 73–82). https://doi.org/10.1007/978-1-4419-0981-7_6

Fata, G. L., Weber, P. & Mohajeri, M. H. (2018). Probiotics and the Gut Immune System: Indirect Regulation. *Probiotics and Antimicrobial Proteins*, *10*(1), 11–21. https://doi.org/10.1007/s12602-017-9322-6

Francino, M. P. (2016). Antibiotics and the Human Gut Microbiome: Dysbioses and Accumulation of Resistances. *Frontiers in Microbiology*, *6*, 1543. https://doi.org/10.3389/fmicb.2015.01543

Gareau, M. G., Sherman, P. M. & Walker, W. A. (2010). Probiotics and the gut microbiota in intestinal health and disease. *Nature Reviews Gastroenterology & Hepatology*, *7*(9), 503–514. https://doi.org/10.1038/nrgastro.2010.117

Gupta, K., Attri, J., Singh, A., Kaur, H. & Kaur, G. (2016). Basic concepts for sample size calculation: Critical step for any clinical trials! *Saudi Journal of Anaesthesia*, *10*(3), 328–331. https://doi.org/10.4103/1658-354x.174918

Higgins, J. P., Savović, J., Page, M. J., Sterne, J. A. & Group, on behalf of the R. D. (2019). *Revised Cochrane risk-of-bias tool for randomized trials (RoB 2)*.

Hsu, P., Pan, C., Kao, J. Y., Tsay, F., Peng, N., Kao, S., Wang, H., Tsai, T., Wu, D., Chen, C., Tsai, K. & Group, the T. A. D. (TARD) S. (2018). Helicobacter pylori eradication with bismuth quadruple therapy leads to dysbiosis of gut microbiota with an increased relative abundance of Proteobacteria and decreased relative abundances of Bacteroidetes and Actinobacteria. *Helicobacter*, *23*(4), e12498. https://doi.org/10.1111/hel.12498

Huse, S. M., Dethlefsen, L., Huber, J. A., Welch, D. M., Welch, D. M., Relman, D. A. & Sogin, M. L. (2008). The Pervasive Effects of an Antibiotic on the Human Gut Microbiota, as Revealed by Deep 16S rRNA Sequencing. *PLoS Biology*, *6*(11), e280. https://doi.org/10.1371/journal.pbio.0060280

Kakiuchi, T., Mizoe, A., Yamamoto, K., Imamura, I., Hashiguchi, K., Kawakubo, H., Yamaguchi, D., Fujioka, Y., Nakayama, A., Okuda, M. & Matsuo, M. (2020). Effect of probiotics during vonoprazan-containing triple therapy on gut microbiota in Helicobacter pylori infection: A randomized controlled trial. *Helicobacter*, *25*(3), e12690. https://doi.org/10.1111/hel.12690

Leclercq, S., Mian, F. M., Stanisz, A. M., Bindels, L. B., Cambier, E., Ben-Amram, H., Koren, O., Forsythe, P. & Bienenstock, J. (2017). Low-dose penicillin in early life induces long-term changes in murine gut microbiota, brain cytokines and behavior. *Nature Communications*, *8*(1), 15062. https://doi.org/10.1038/ncomms15062

Lombardo, L., Foti, M., Ruggia, O. & Chiecchio, A. (2010). Increased Incidence of Small Intestinal Bacterial Overgrowth During Proton Pump Inhibitor Therapy. *Clinical Gastroenterology and Hepatology*, *8*(6), 504–508. https://doi.org/10.1016/j.cgh.2009.12.022

Messer, J. S. & Chang, E. B. (2018). Physiology of the Gastrointestinal Tract. In H. M. Said (Ed.), *Chapter 36 - Microbial Physiology of the Digestive Tract and Its Role in Inflammatory Bowel Diseases* (pp. 795–810). Academic Press. https://doi.org/10.1016/b978-0-12-809954-4.00036-0

O'Connell, T. M. (2020). The Application of Metabolomics to Probiotic and Prebiotic Interventions in Human Clinical Studies. *Metabolites*, *10*(3), 120. https://doi.org/10.3390/metabo10030120

O'Connor, J. P. A., Taneike, I. & O'Morain, C. (2009). Review: Improving compliance with Helicobacter pylori eradication therapy: when and how? *Therapeutic Advances in Gastroenterology*, *2*(5), 273–279. https://doi.org/10.1177/1756283x09337342

Oh, B., Kim, B., Kim, J. W., Kim, J. S., Koh, S., Kim, B. G., Lee, K. L. & Chun, J. (2016). The Effect of Probiotics on Gut Microbiota during the Helicobacter pylori Eradication: Randomized Controlled Trial. *Helicobacter*, *21*(3), 165–174. https://doi.org/10.1111/hel.12270

Page, M. J., McKenzie, J. E., Bossuyt, P. M., Boutron, I., Hoffmann, T. C., Mulrow, C. D., Shamseer, L., Tetzlaff, J. M., Akl, E. A., Brennan, S. E., Chou, R., Glanville, J., Grimshaw, J. M., Hróbjartsson, A., Lalu, M. M., Li, T., Loder, E. W., Mayo-Wilson, E., McDonald, S., … Moher, D. (2021). The PRISMA 2020 statement: an updated guideline for reporting systematic reviews. *BMJ*, *372*, n71. https://doi.org/10.1136/bmj.n71

Shi, X., Zhang, J., Mo, L., Shi, J., Qin, M. & Huang, X. (2019). Efficacy and safety of probiotics in eradicating Helicobacter pylori: A network meta-analysis. *Medicine*, *98*(15), e15180. https://doi.org/10.1097/md.0000000000015180

Sterne, J. A. C., Savović, J., Page, M. J., Elbers, R. G., Blencowe, N. S., Boutron, I., Cates, C. J., Cheng, H.-Y., Corbett, M. S., Eldridge, S. M., Emberson, J. R., Hernán, M. A., Hopewell, S., Hróbjartsson, A., Junqueira, D. R., Jüni, P., Kirkham, J. J., Lasserson, T., Li, T., … Higgins, J. P. T. (2019). RoB 2: a revised tool for assessing risk of bias in randomised trials. *BMJ*, *366*, l4898. https://doi.org/10.1136/bmj.l4898

Tang, B., Tang, L., Huang, C., Tian, C., Chen, L., He, Z., Yang, G., Zuo, L., Zhao, G., Liu, E., Wang, S., Lin, H., He, J. & Yang, S. (2021). The Effect of Probiotics Supplementation on Gut Microbiota After Helicobacter pylori Eradication: A Multicenter Randomized Controlled Trial. *Infectious Diseases and Therapy*, *10*(1), 317–333. https://doi.org/10.1007/s40121-020-00372-9

Vangay, P., Ward, T., Gerber, J. S. & Knights, D. (2015). Antibiotics, Pediatric Dysbiosis, and Disease. *Cell Host & Microbe*, *17*(5), 553–564. https://doi.org/10.1016/j.chom.2015.04.006

Wang, F., Feng, J., Chen, P., Liu, X., Ma, M., Zhou, R., Chang, Y., Liu, J., Li, J. & Zhao, Q. (2017). Probiotics in Helicobacter pylori eradication therapy: Systematic review and network meta-analysis. *Clinics and Research in Hepatology and Gastroenterology*, *41*(4), 466–475. https://doi.org/10.1016/j.clinre.2017.04.004

Wang, Y.-K., Kuo, F.-C., Liu, C.-J., Wu, M.-C., Shih, H.-Y., Wang, S. S., Wu, J.-Y., Kuo, C.-H., Huang, Y.-K. & Wu, D.-C. (2015). Diagnosis of Helicobacter pylori infection: Current options and developments. *World Journal of Gastroenterology*, *21*(40), 11221–11235. https://doi.org/10.3748/wjg.v21.i40.11221

Wieërs, G., Belkhir, L., Enaud, R., Leclercq, S., Foy, J.-M. P. de, Dequenne, I., Timary, P. de & Cani, P. D. (2020). How Probiotics Affect the Microbiota. *Frontiers in Cellular and Infection Microbiology*, *9*, 454. https://doi.org/10.3389/fcimb.2019.00454

Wu, L., Wang, Z., Sun, G., Peng, L., Lu, Z., Yan, B., Huang, K. & Yang, Y. (2019). Effects of anti-H. pylori triple therapy and a probiotic complex on intestinal microbiota in duodenal ulcer. *Scientific Reports*, *9*(1), 12874. https://doi.org/10.1038/s41598-019-49415-3

Yang, C., Liang, L., Lv, P., Liu, L., Wang, S., Wang, Z. & Chen, Y. (2021). Effects of non-viable Lactobacillus reuteri combining with 14-day standard triple therapy on Helicobacter pylori eradication: A randomized double-blind placebo-controlled trial. *Helicobacter*, *26*(6), e12856. https://doi.org/10.1111/hel.12856

Yang, L. & Chen, J. (2022). A comprehensive evaluation of microbial differential abundance analysis methods: current status and potential solutions. *Microbiome*, *10*(1), 130. https://doi.org/10.1186/s40168-022-01320-0

Helicobacter. **2022 Apr;27(2):e12870. doi: 10.1111/hel.12870.**

CHAPTER 5. *SACCHAROMYCES BOULARDII* CNCM I-745 SUPPLEMENTATION MODIFIES THE FECAL RESISTOME DURING *HELICOBACTER PYLORI* ERADICATION THERAPY

Sara Gabriela Cifuentes[1], María Belén Prado[1], Marco Fornasini[2], Henry Cohen[3], Manuel Eduardo Baldeón[2], Paúl Andrés Cárdenas[1]

[1] Universidad San Francisco de Quito, Institute of Microbiology, Quito, Ecuador; [2] Universidad Internacional del Ecuador, Facultad de Ciencias Médicas de la Salud y la Vida, Quito, Ecuador; [3] Clínica de Gastroenterología Prof. Dra. Carolina Olano, Facultad de Medicina, Montevideo, Uruguay

Abstract

Background

The gut microbiota is a significant reservoir of antimicrobial resistance genes (ARGs). The use and misuse of antimicrobials can select multi-resistant bacteria and modify the repertoire of ARGs in the gut. Developing effective interventions to manipulate the intestinal resistome would allow us to modify the antimicrobial resistance risk.

Materials and Methods

Applying shotgun metagenomics, we compared the composition of fecal resistome from individuals treated with triple therapy for *Helicobacter pylori* plus *Saccharomyces boulardii* CNCM-I 745 (*Sb*) versus triple antibiotherapy without *S. boulardii* (control) before, after, and one month after treatments. DNA samples were sequenced on an Illumina NovaSeq 6000 platform. Reads were trimmed and filtered for quality, and the reads classified as host genome were removed from further analysis. We used the ResFinder database for resistome analysis and the web-based tool ResistoXplorer and RStudio for graphical representation and statistical analysis.

Results

We identified 641 unique ARGs in all fecal samples, conferring resistance to 18 classes of antibiotics. The most prevalent ARGs found in at least 90% of the samples before the treatments were against tetracyclines, MLS-B (macrolide, lincosamide, streptogramin B), beta-lactams, and aminoglycosides. Differential abundance analysis allowed the identification of ARGs significantly different between treatment groups. Thus, immediately after the treatments, the abundance of ARGs that confer resistance to lincosamides, tetracyclines, MLS-B, and two genes in the beta-lactam class (*cfxA*2 and *cfxA*3) was significantly lower in the group that received *Sb* than in the control group (edgeR, FDR <0.05).

Conclusion

Our study demonstrated that the addition of *S. boulardii* CNCM-I 745 to the conventional antibiotic eradication therapy for *H. pylori* reduced the abundance of ARGs, particularly those genes that confer resistance to lincosamides, tetracyclines, MLS-B, and a few genes in the beta-lactams class.

Keywords

Saccharomyces boulardii CNCM I-745, *Helicobacter pylori,* resistome, antibiotic resistance, antibiotic resistance genes

Introduction

Antibiotic resistance is a growing public health problem worldwide.(World Health Organization, 2015) By 2050, an estimated 10 million deaths will occur because of antimicrobial resistance.(O'Neill, 2016) Abuse and misuse of antibiotics can select multi-resistant bacteria(Francino, 2016) and increase antimicrobial resistance genes (ARGs) repertoire.(Modi et al., 2013) Additionally, the administration of antibiotics can enhance the risk of colonization by multi-resistant bacteria.(Tacconelli et al., 2009) The gut microbiota is an essential reservoir of ARGs.(Forslund et al., 2013; Penders et al., 2013) The gut resistome is dynamic due to the exchange of resistance determinants among microbial communities through mobile genetic elements (MGEs), including plasmids, phages, or transposons.(Davies & Davies, 2010) Therefore, finding and developing strategies to manipulate the gut resistome becomes essential to benefit the patients and public health.

Novel strategies, including diet and functional foods, have been used to modify the gut resistome positively.(Tsigalou et al., 2020) Previous work showed that a dietary intervention consisting of whole grains, traditional Chinese medicinal foods, and prebiotics can significantly reduce fecal ARGs and alleviate metabolic syndrome in obese Chinese children.(Wu et al., 2016) Moreover, *Saccharomyces boulardii* CNCM I-745 (*Sb*) can reduce dysbiosis(Moré & Swidsinski, 2015) and has shown antagonistic and antimicrobial properties against various pathogens.(Pais et al., 2020) In a previous study, we showed that in patients treated with a *Helicobacter pylori* eradication therapy, *Sb* reduces the abundance of Clostridia and Bacteroides, bacteria that are traditionally multi-resistant, and the patients have a lower frequency of gastrointestinal symptoms that could be related to favorable changes in gut microbiota composition.(Cárdenas et al., 2020)
The use of high-throughput sequencing technologies makes possible to study a microbial community through metagenomic analysis from a structural and functional point of view. Applying this approach for the fecal resistome study will allow us to understand its relationship with the hosts and its dynamics within the community. Therefore, we sought to study the effect of *Sb* on the fecal resistome in individuals treated with eradication therapy against *H. pylori*.

Materials and Methods

Clinical investigation and samples

The study design and subjects' recruitment of the study have been described previously.(Cárdenas et al., 2020) Sixty-four fecal samples (one biological sample for each participant and no replicates) were used in the present study. We compared the

composition of the fecal resistome from individuals treated with triple therapy for *H. pylori* (amoxicillin 1 g three times a day, tinidazole 1 g once a day, and omeprazole 40 mg two times a day) plus *S. boulardii* CNCM I-745 (approximately 22.5 x 10^9 CFU) (*Sb*) versus triple therapy without *Sb* (control) at three-time points: before treatments (week 0), immediately after treatments (week 3), and one month after treatments (week 7). The number of samples in each intervention group and time-points are described in figure 1A. The Ethics Committee of the Universidad de Las Americas approved the study (code number: 2016-0101). The clinical trial was registered at ClinicalTrials.gov (No. NCT04786938). Written informed consent forms were obtained from participants once patients received a full explanation of the research protocol.

DNA extraction, library preparation, and sequencing

The details of DNA extraction were described previously (Cárdenas et al., 2020). Before sending the DNA samples for sequencing, total DNA concentration and purity (NanoDrop ND-2000, Thermo Scientific 260/280 and 260/230 absorbance ratios) were verified. DNA samples were lyophilized and sent for DNA sequencing at the High Throughput Sequencing Facility at the University of North Carolina at Chapel Hill, NC, USA, for library preparation, next-generation sequencing, and quality control. KAPA libraries using the Mantis system were prepared per the manufacturer's instructions, and samples were run on an Illumina NovaSeq 6000 platform to generate 2×150bp paired-end sequences. The data for this study were deposited in the European Nucleotide Archive (ENA) at the European Bioinformatics Institute (EMBL-EBI) under accession number PRJEB47358 (https://www.ebi.ac.uk/ena/browser/view/PRJEB47358).

Sequence analysis and resistome annotation

The code used for the resistome pipeline was adapted from the AMrPlusPlus pipeline (https://megares.meglab.org/amrplusplus/latest/html/). Briefly, we performed quality control of raw sequence data using FastQC (version 0.11.9)(Andrews, 2010) and multiQC (version 1.9).(Ewels et al., 2016) Trimmomatic (version 0.39)(Bolger et al., 2014) was used to remove adapter contamination and low-quality reads applying these trimming steps and the associated parameters for paired-end reads: ILLUMINACLIP: TruSeq3-SE:2:30:10, LEADING:3, TRAILING:3 SLIDINGWINDOW:4:15, MINLEN:36. The reads were matched to the *Homo sapiens* (GRCh38) reference genome using BWA(H. Li, 2013) and then removed with Samtools.(H. Li et al., 2009) For resistome analysis, we applied Resistome Analyzer (https://github.com/cdeanj/resistomeanalyzer) using the ResFinder database (downloaded: October 23th, 2020)(Zankari et al., 2012) that gave the total count of reads aligned to the target gene. Reads assigned to ARGs with at least 80% gene fraction threshold were used to compare the group treated with *Sb* (n=33) and the control group (n=31).

Taxonomy

The unassembled reads without the host genome were used for taxonomic analyzes. To profile the structure and composition of fecal microbiota, we used Methaplan2 (Version 2.6.0.0)(Truong et al., 2015) available in the Galaxy platform(Afgan et al., 2018) with default parameters. Only the type of analysis was changed to: "profiling metagenomes in terms of relative abundances and estimate the number of reads coming from each clade." The estimated number of reads was used for the integrative analysis.

Statistical and integrative analysis

RStudio (Version V4.0.3) was used for graphical representation using the libraries "*ggplot2,*" "*reader,*" and "*esquisse.*" Additionally, we used the web-based tool ResitoXplorer(Dhariwal et al., 2021) to visualize and generate a statistical analysis of the composition profiling, clustering analysis, differential abundance testing, and integrative analysis. The filtration process removed low-quality or non-informative ARGs to improve the statistical tests using ResitoXplorer's default parameters (low count and low variance filters). We applied two popular scaling methods to normalize metagenomic data(Lin & Peddada, 2020) and potentially remove biases that may have been introduced during the sampling or sequencing process.(Weiss et al., 2017) We used the Cumulative Sum Scaling (CSS) method(Paulson et al., 2013) for data normalization before the relative, actual abundance, and diversity analysis. We used edgeR(Robinson et al., 2010) included in the ResitoXplorer analysis workflow for differential analysis of gene abundance where the normalization process occured with trimmed mean of M-values (TMM).(Pereira et al., 2018)

In the study of alpha diversity, we used the diversity measures Chao1 and Shannon indexes, applying the statistical methods of Mann-Whitney and Kruskal-Wallis when two or three independent samples were compared, respectively. For beta diversity analysis, we used the ordination method PCA and the statistical method PERMANOVA. Procrustes analysis(Peres-Neto & Jackson, 2001) was applied to test the association between ARG composition and taxonomic composition in the fecal microbiota. We used the following parameters: 9999 permutations, species as taxonomic level, and genes as profile level with the ordination and distance methods PCoA and Bray-Curtis Index. Regularized Canonical Correlation Analysis (rCCA)(González et al., 2012) was also carried out: species were used as a taxonomic level, and the class was used as profile level

with the default parameters. Differences were declared significant with a P-value <0.05. The differential abundance analysis used an adjusted P-value (<0.05) for false discovery rate (FDR) using the Benjamini–Hochberg method.(Benjamini & Hochberg, 1995) Correlations were considered significant at a P-value <0.05.

Results

Figure 1A shows the study design and how the samples were distributed between the intervention groups (*Sb* and control) and the three-time points (weeks 0, 3, and 7).

High throughput sequencing of sixty-four DNA samples produced approximately 1.1 billion sequences, ranging from 5 to 40 million reads per sample. The mean sequence quality (Phred score) was 36. All samples had Phred scores greater than Q30. We identified 641 unique ARGs in all fecal samples, conferring resistance to 18 classes of antibiotics with at least five mechanisms of action that included enzymatic inactivation, enzymatic modification, increased antibiotic efflux, target modification, and target protection (Figure 1B). The most prevalent ARGs (core resistome) found in at least 90% of the samples before the treatments (week 0) were against tetracyclines, MLS-B (macrolide, lincosamide, streptogramin B), beta-lactams, and aminoglycosides. *TetW, tetQ, tet32, tetX, tetO, tet40, ermF, and ant6* gene families comprised most alignments based on the same prevalence within these four classes of antimicrobials. These findings indicate that these ARGs were prevalent in the human intestine of individuals infected with *H. pylori*.

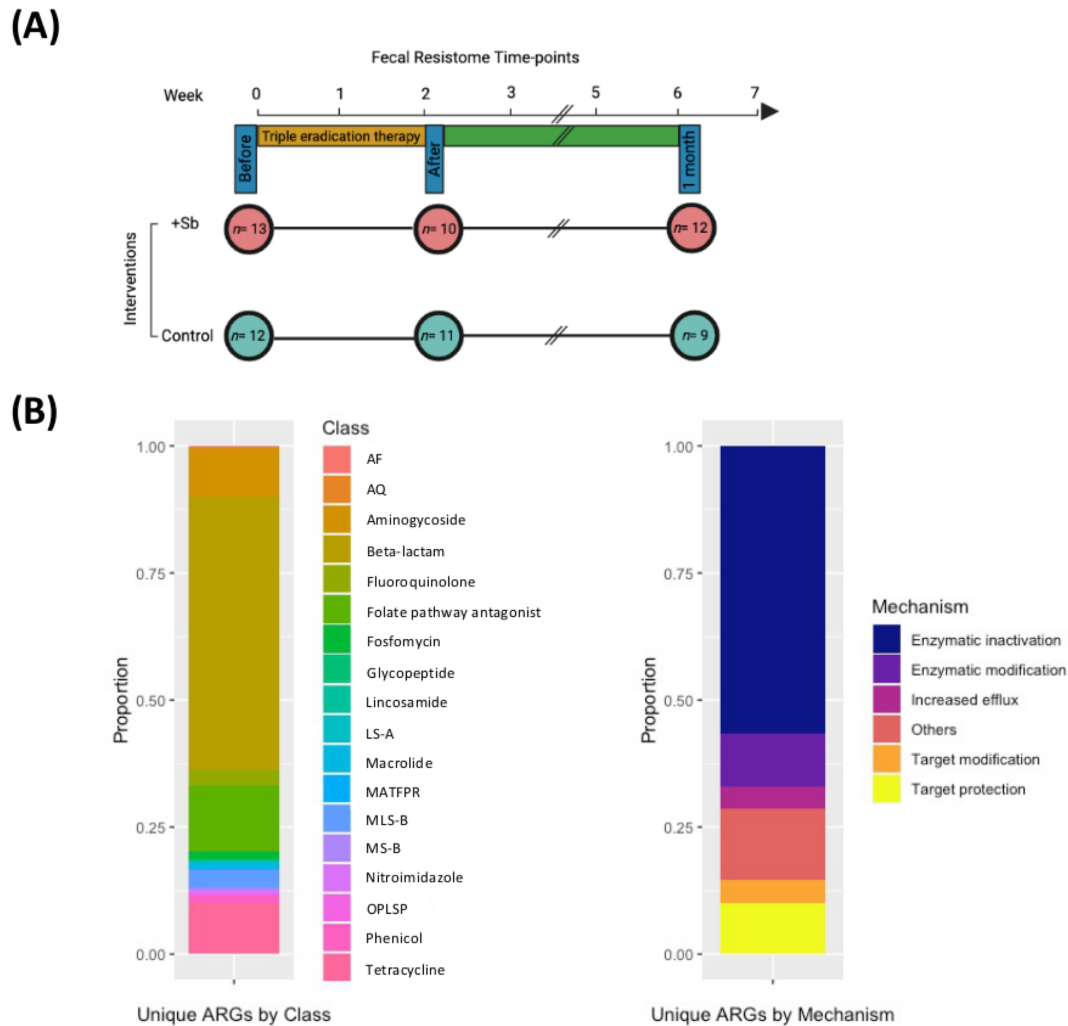


Figure 1. Study design and antibiotic resistant genes identified in fecal samples from patients who received eradication therapy against *H. pylori.* (A) Treatment groups, time points of analysis and number of samples studied. (B) Antibiotic resistant genes grouped in proportions by antimicrobial activity and mechanism of action. Aminoglycoside, fluoroquinolone (AF), aminoglycoside, quinolone (AQ), lincosamide, streptogramin A (LS-A), macrolide, aminoglycoside, tetracycline, fluoroquinolone, phenicol, rifamycin (MATFPR), macrolide, lincosamide, streptogramin B (MLS-B), macrolide, streptogramin B (MS-B), oxazolidinone, phenicol, lincosamide, streptogramin A, pleuromutilin (OPLSP).

Effect of *S. boulardii* CNCM I-745 on the abundance and diversity of the fecal resistome

To explore the changes of the human fecal resistome after an eradication therapy against *H. pylori* supplemented with *Sb*, we investigated the abundance profiling of ARGs between interventions and within treatments at every time point. The relative abundance of ARGs observed in each of the samples showed no substantial change in the individual resistome before and one month after treatments in both the *Sb* and control treatment groups (Figure 2A). However, when the composition profiling of the fecal resistome was evaluated immediately after the treatments (week 3), it was evident that the relative abundance of ARGs changed in some individuals in the groups that received the probiotic and in the control group (Figure 2A). The relative abundance by time points in both *Sb* and control groups (Supplementary Figure 1) showed that ARGs that confer resistance against tetracyclines were reduced immediately after treatments in the group that received *Sb*. On the other hand, at the same time point (week 3) in the group that did not receive *Sb*, an increase in the relative abundance was observed in ARGs against tetracyclines, lincosamides, and MATFPR (macrolide, aminoglycoside, tetracycline, fluoroquinolone, phenicol, rifampicin) (Supplementary Figure 1). When we evaluated the actual abundance of ARGs among each intervention group, we observed that at the end of the antibiotic treatments (week 3), the group that received *Sb* showed a reduction in the abundance of ARGs that confer resistance to tetracyclines, MLS-B, lincosamides, and beta-lactams and an increase in macrolides, aminoglycosides, and quinolones compared with the group that did not receive the probiotic (Figure 2B). Observing such differences, especially after the treatments (week 3), we decided to perform an ordination analysis (beta diversity analysis) to confirm whether the fecal samples were different based on the

intervention or the three-time points. Beta diversity showed that the fecal samples were similar to each other and that there was no distinctive pattern based on their resistome (PERMANOVA, P> 0.05) (Supplementary Figure 2A and 2B).

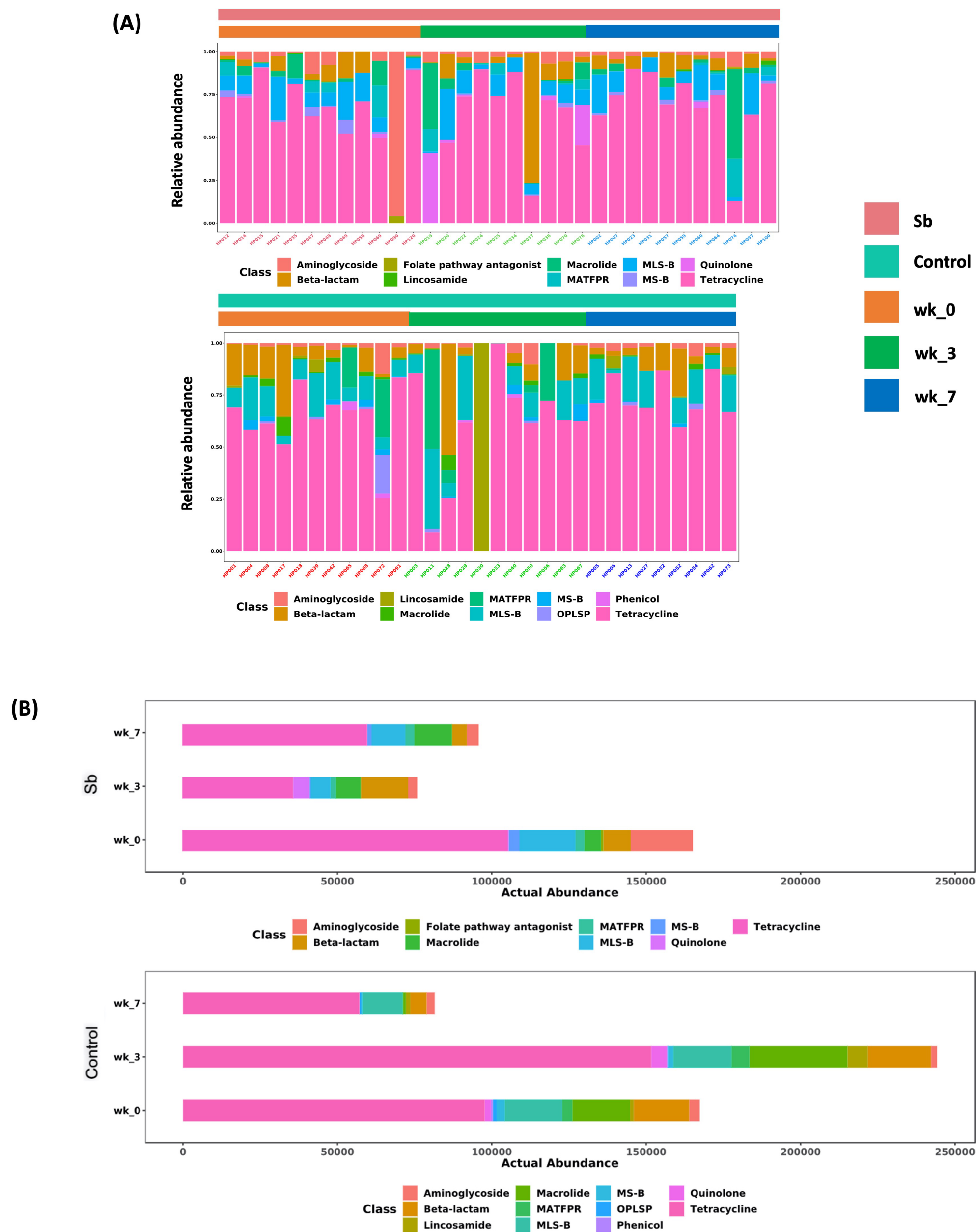


Figure 2. Normalized composition profiling of the fecal resistome. (A) Relative abundance of antibiotic resistant genes of *S. boulardii* and control treatment groups; (B) actual abundance of ARG of *S. boulardii* and control treatment groups. Macrolide,

lincosamide, streptogramin B (MLS-B), macrolide, aminoglycoside, tetracycline, fluoroquinolone, phenicol, rifamycin (MATFPR), macrolide, streptogramin B (MS-B); oxazolidinone, phenicol, lincosamide, streptogramin A, pleuromutilin (OPLSP).

Subsequently, we sought to identify whether the administration of *Sb* would modify the alpha diversity of ARGs in patients treated with the eradication therapy against *H. pylori*. Alpha diversity analysis showed a progressive reduction of ARGs among the three-time points in the two treatment groups (*Sb* and control) when richness (Chao1 index) and diversity (Shannon index) were evaluated. Although these differences were not significant, the pattern of reduction was more evenly staggered in the group that received *Sb* than in the control group (Kruskal-Wallis, $P > 0.05$) (Supplementary Figure 3). When we compared the alpha diversity of the ARGs between the two intervention groups at the three-time points, it could be observed that there were no significant differences between the *Sb* and control groups before, after, and one month after the treatment both in the richness analysis (Chao1), as in the diversity analysis (Shannon) (Mann-Whitney, $P > 0.05$) (Supplementary Figure 4). The comparisons described above were not made with paired samples. Therefore, we were interested in carrying out pairwise comparisons on the diversity of ARGs among the three-time points in the intervention groups (*Sb* and control). However, the limited number of samples (n= 3-7) could have negatively affected the statistical power to find significant differences (Kruskal-Wallis, $P > 0.05$).

Modification of the differential abundance of ARGs in individuals treated with *S. boulardii* CNCM I-745

To identify the abundance of specific ARGs in *Sb* and control groups, we applied a differential abundance analysis using RNAseq count data analysis that has been previously tested for metagenomic studies.(Jonsson et al., 2016) In the *Sb* group, a significant reduction in the abundance of ARGs that confer resistance to aminoglycosides and folate pathway antagonists was observed between week 0 and week 3 (edgeR, FDR = 0.00033559, and FDR = 0.00050066, respectively) and between week 0 and week 7 (edgeR, FDR = 0.00032621, and FDR = 0.0051479, respectively) (Figure 3A). Similarly, ARGs that confer resistance to lincosamides and tetracyclines in the control group showed a significant reduction between week 0 and week 3 (edgeR, FDR = 0.02483 and FDR = 0.026823, respectively) (Figure 3B). However, the abundance of ARGs that confer resistance to quinolones was significantly more abundant after treatments (week 3) than at baseline, and its abundance was almost absent one month after the eradication therapy in both intervention groups *Sb* and control (edgeR, FDR < 0.05) (Figure 3A and 3B).

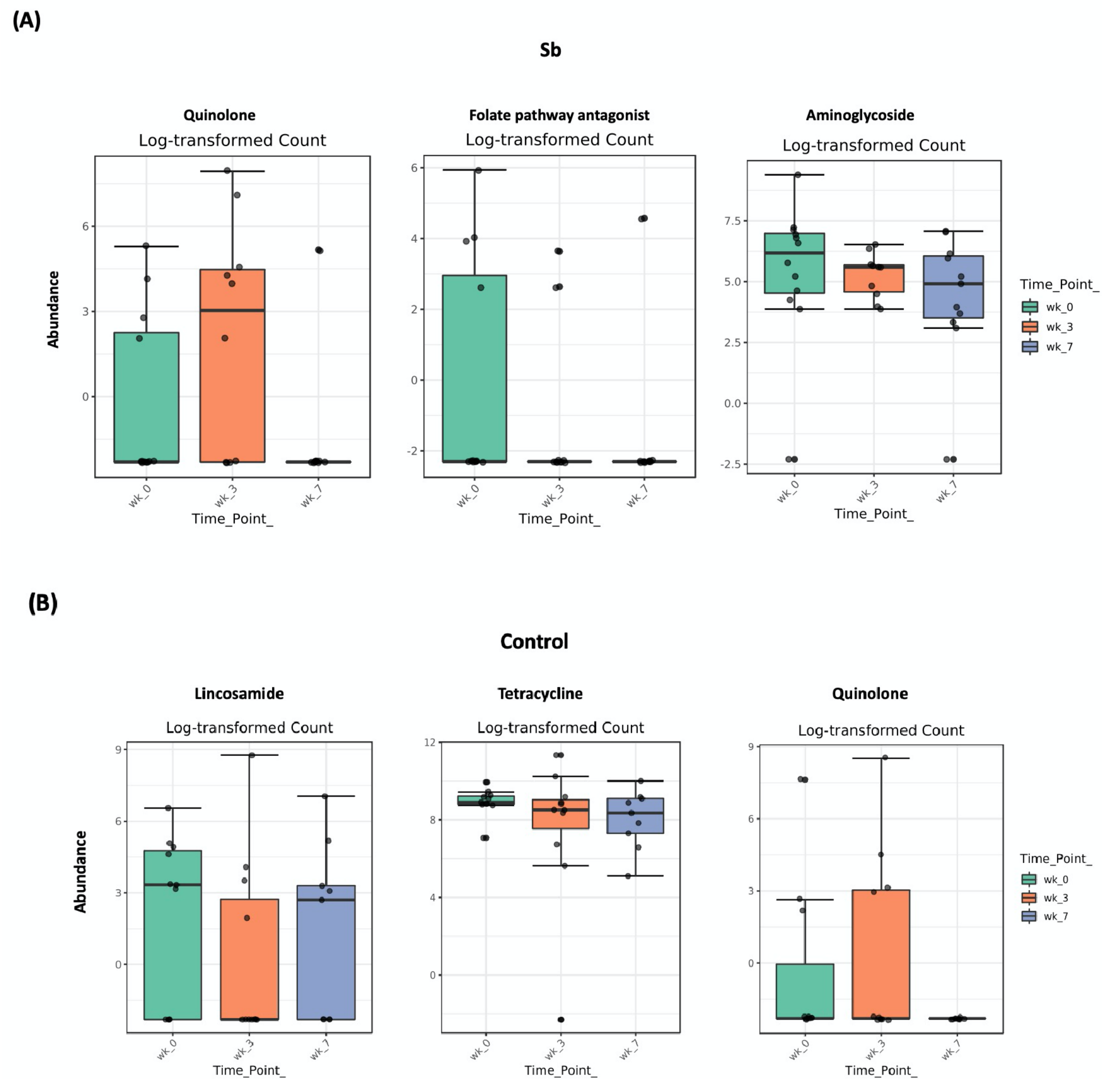


Figure 3. Differential abundance of ARGs comparison among the three-time points in (A) Sb group and (B) in the control group. Median values are indicated by central black horizontal lines; the 25th and 75th percentiles are indicated (boxes), and the whiskers extend from each end of the box to the most extreme values. Only significant differences are shown.

Subsequently, we wanted to evaluate the differential abundance of the ARGs between intervention groups (*Sb* and control) immediately after treatments (week 3). Interestingly,

we found that the abundance of ARGs against lincosamides, tetracyclines, and MLS-B was significantly lower in the group that received *Sb* compared to the group that did not receive the probiotic (edgeR, FDR < 0.05) (Table 1). When we evaluated the differential abundance at the gene level, the genes *lnuC*, *msrD*, *cfxA2*, and *cfxA3* were significantly less abundant in the group that received *Sb* compared to the one that did not receive the probiotic (edgeR, FDR <0.05) (Table 1). These significant differences did not remain one month after the treatments ended. We carried out the same analyses described above only with paired samples (at all three-time points and between two-time points). However, we did not likely find significant differences due to the low number of samples (n = 3-7). Only in the group that received *Sb* was it observed that when comparing the fecal resistomes before and after receiving the treatments, the abundance of ARGs that confer resistance to lincosamides was significantly lower (Supplementary Figure 5).

Table 2. Differential abundance of ARGs between interventions immediately after treatments (week 3) *.

Class			
ARGs	**log2FC †**	**P-values**	**FDR**
Lincosamide	13.119	4.8531E-06	4.3678E-05
Tetracycline	3.9152	0.0010275	0.0046236
MLS-B	3.1129	0.0077152	0.023145
Gene			
ARGs	**log2FC**	**P-values**	**FDR**
lnu(C)_1_AY928180	8.9177	6.9162E-05	0.0041497
msr(D)_2_AF274302	5.4297	0.00017585	0.0052755
*cfx*A2_1_AF504914	3.9669	0.0009865	0.01973
*cfx*A3_1_AF472622	4.0981	0.0020164	0.030246

* The upper part of the table shows the ARGs grouped according to the class of antibiotics to which they confer resistance, while the lower part of the table refers to each gene. Statistical analysis based on RNA-seq at the gene level; only significant findings are shown in the table and the differences favor the group treated with Sb.
† Log2FC: log 2-fold-change, which is the log difference between *Sb* and control groups. log2FC positive numbers indicate less ARGs abundance in the group *Sb.*

Integrative analysis between the resistome and the fecal microbiota

Later, we wanted to show that the changes observed in the resistome correlate with the changes in the fecal microbiota of patients treated with the eradication therapy against *H. pylori.* We carried out an integration analysis in which we included the ARG counts and taxonomic counts of the fecal microbiota of all the participants, regardless of the interventions. We applied Procrustes analysis to test the association between ARGs composition and taxonomic composition (the inter-omic relationship) in the fecal microbiota. This analysis revealed that regardless of treatment, the composition of ARGs was significantly associated (procrustean randomization tests, P = 0.0001) with the taxonomic composition of the fecal microbiota (Figure 4).

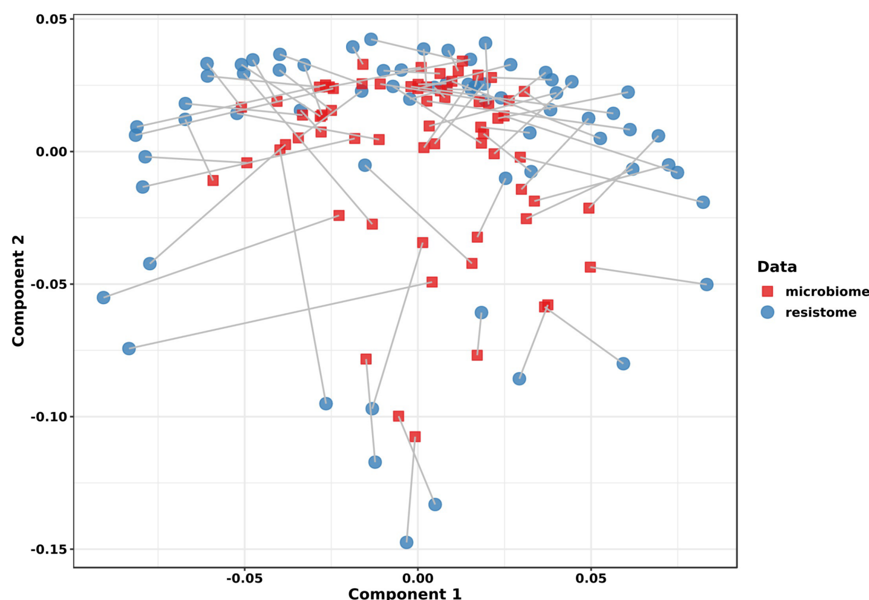


Figure 4. Procrustes analysis of the fecal microbiota and its resistome. The red rectangular dots refer to the microbiota, and the blue round dots refer to the resistome.

Sum of Squares = 0.3616; Correlation coefficient (squared m12) = 0.799; P = 1e-04. Ordination Method: PCoA, distance method: Bray-Curtis Index, 9999 permutations.

Finally, in our previous study, the fecal microbiota in patients treated against *H. pylori* with or without *Sb* was evaluated using the marker gene 16S rRNA, and we demonstrated a lower abundance of Bacteroides (*Prevotella*) and Clostridia (*Lachnospira* and *Ruminococcus*) and a greater abundance of Gammaproteobacteria (*Escherichia* spp.) in the group that received the probiotic.(Cárdenas et al., 2020) Thus, we wanted to evaluate which ARGs were associated with these taxonomic groups described previously and modified with the probiotic therapy within the present study. Using Regularized Canonical Correlation Analysis (rCCA), we found that the presence of the *Enterobacteriaceae* family was negatively associated with ARGs that confer resistance to lincosamides, tetracyclines, MLS-B, and beta-lactams and was positively and strongly associated with quinolones and MATFPR. In the same way, the presence of the *Prevotellaceae* family was positively associated with ARGs that confer resistance to lincosamides and beta-lactams. Thus, a positive relationship was evidenced between the presence of the *Lachnospiraceae* family with MLS-B and the *Ruminococcaceae* family with beta-lactams (Figure 5A and 5B). These data suggest that the reduction in the abundance of ARGs that confer resistance to lincosamides, tetracyclines, MLS-B, and specific genes that confer resistance to beta-lactams were associated with changes in the fecal microbiota (reduction of *Prevotellaceae*, *Lachnospiraceae*, *Ruminococcaceae*). At the same time, the significant increase in ARGs in the group that received the probiotic were positively correlated with the increase in the abundance of members of the *Enterobacteriaceae* family found in our previous study.

Figure 5. Regularized Canonical Correlation Analysis (rCCA) between fecal resistome and microbiome. (A) Correlation Circle Plot and (B) Clustered Image Map (CIM). Correlation threshold for the correlation circle plot: 0.4. Dark colors show positive correlations, while light colors show negative correlations in CIM.

Discussion

Because antimicrobial resistance is a threat to public health and the human gut an essential reservoir of ARGs, it is critical to identify potential interventions able to reduce the abundance of ARGs in the gut before, during, and after the administration of antibiotics. In addition, to limit horizontal gene transfer between bacteria in a densely populated environment such as the intestine. The use of functional foods rich in indigestible carbohydrates(Wu et al., 2016) and probiotics are candidate interventions to decrease ARGs (Tsigalou et al., 2020). In particular, the probiotic *S. boulardii*, being a yeast, is not adversely affected by short or prolonged periods of antibiotic treatment(Czerucka et al., 2007) To our knowledge, the present study is the first to evaluate and demonstrate

beneficial modifications of the human fecal resistome using *S. boulardii* CNCM-I 745 during *H. pylori* eradication therapy.

In the present study, ARGs that confer resistance to tetracyclines, MLS-B, and beta-lactams were the most prevalent genes found in the human intestine, and these data are consistent with other studies on fecal resistome in humans.(Afridi et al., 2021; Hu et al., 2013) A previous work in China shows that the *ermF* gene (MLS-B) is highly prevalent in the Chinese population. Strikingly in the present study, we found that the *ermF* gene was also prevalent in more than 90% of the samples from patients infected with *H. pylori* before receiving antibiotic treatment.(Feng et al., 2018) This finding may be because, in Ecuador, antibiotics are used without any medical prescription,(Quizhpe et al., 2017) and in China, the rate of antibiotic consumption is very high, even in viral infections.(Heddini et al., 2009) The resistance mechanisms found in our study were similar to those reported by Feng et al.,(Feng et al., 2018) which include enzyme inactivation, enzyme modification, and increased efflux. These mechanisms are in all the populations studied.(Feng et al., 2018) Furthermore, a large cohort study on fecal resistomes in healthy individuals from China, Denmark, and Spain, confirmed high abundance of tetracycline resistance genes. Otherwise, they also find genes that confer resistance to vancomycin and bacitracin (Hu et al., 2013), which were not common in our study. The connection between being a fecal carrier of bacteria that carry ARGs, and the consumption of antibiotics has previously been reported (Barbosa & Levy, 2000; Levy et al., 1988). The most prevalent resistance genes found in all participants in our study have been associated with the use of antibiotics or their analogs approved for use in animals (Forslund et al., 2013).

Few studies have evaluated the fecal resistome in patients treated for infection with *H. pylori*. Olekhnovich et al. demonstrate that the administration of quadruple eradication therapy increases the relative abundance of some genes that belonged to the MLS-B (*erm*B), beta-lactam (CFX group), and tetracycline (*tetQ*) classes and reduces the relative abundance of other members of the tetracycline class (*tetW* and *tetO*) (Olekhnovich et al., 2019). We demonstrated that the relative abundance of ARGs that confer resistance to tetracyclines, MLS-B, lincosamides, and beta-lactams were exclusively reduced in the group that received *Sb*. In contrast, the relative abundance of ARGs against quinolones, aminoglycosides, and macrolides increased in this same intervention group. It is expected that by administering an antibiotic, the resistome becomes enriched with resistance genes due to selective pressure to which the bacteria is exposed,(Willmann et al., 2015) and in our study, we evidenced such changes. It should also be noted that ARGs against nitroimidazoles do not appear because the filtration process (see material and methods) removed genes in very low abundance that can give unreliable results. However, the increase in ARGs against quinolones, aminoglycosides, and macrolides is not explained by specific selection but by co-selection. Different genes can coexist in MGEs so that co-selection can stabilize the resistance phenotype even in the absence of specific selection.(Wright, 2007) Furthermore, two previous studies carried out by Willman et al. show that administering an antimicrobial alone can generate positive or negative selective pressure on ARGs that confer resistance to classes of antimicrobials unrelated to the antibiotics administered.(Willmann et al., 2015, 2019) In other words, different antibiotics have specific effects on the intestinal resistome.(Willmann et al., 2019) Something striking in our study is the enrichment of ARGs against quinolones in the group that received *Sb*. The rCCA analysis showed a strong correlation between the *Enterobacteriaceae* family and ARGs against quinolones. This result is consistent with

the finding in our previous study, where we evidenced that *Escherichia spp*. and other members of the *Enterobacteriaceae* family were significantly more abundant in the group that received *Sb* than those that did not receive *Sb.*(Cárdenas et al., 2020) It can be explained by the fact that there are inter-kingdom interactions between fungi and bacteria where members of the *Enterobacteriaceae* family interact with fungi assisting them in their colonization in the intestinal niche.(Sovran et al., 2018)

The fecal resistome structure based on alpha diversity analysis showed the highest diversity before the treatments and the lowest one month after the end of the treatments, regardless of the interventions (Sb and control). These findings are consistent with the data shown by Ghanbari et al. in a study carried out with weaned pigs that received a single antimicrobial (oxytetracycline) when evaluating the effects of antimicrobials on fecal resistome before and after treatments.(Ghanbari et al., 2019) Although they show that the richness and diversity of fecal resistome are more significant in the group that received antimicrobial therapy than in the group that did not receive such therapy, we did not include in our study patients not treated with *H. pylori* eradication therapy.

Moreover, we demonstrated that *Sb* supplementation in patients treated with triple eradication therapy against *H. pylori* could modify the abundance of resistance genes once the treatments were completed. When we evaluated the differential abundance at the class level, *Sb* could significantly reduce the abundance of ARGs that confer resistance to lincosamides, tetracyclines and MLS-B compared to the control group. When evaluated at the gene level, it could significantly reduce the abundance of *lnu*C, *msr*D, *cfxA2*, and *cfxA3*. The genes identified in this study are acquired resistance genes.(Zankari et al., 2012) Acquired ARGs are frequently transferred in MGEs between different parts

of the same genome or between genomes.(Hoek et al., 2011) A critical and worrying aspect during the administration of antibiotics is the potential surge of horizontal gene transfer (HGT).(J. Li et al., 2019) We believe that the beneficial effects of *Sb* in reducing the abundance of ARGs in patients treated with a triple eradication therapy against *H. pylori* are due to its metabolic and structural products. First, *Sb* beneficially modulates the intestinal microbiota towards a healthy microbiota thanks to the fact that it also functions as a postbiotic due to the compounds in its cell walls such as glycans, mannoproteins, and chitin that are substrates for the microbial fermentation of bacteria that produce short-chain fatty acids(Pais et al., 2020) The reduction of ARGs abundance in the group that received *Sb* may explain the modulation of the intestinal microbiota. Our previous work demonstrated a lower abundance in the *Sb* group of Bacteroides (*Prevotella*) and Clostridia (*Lachnospira* and *Ruminococcus*), bacteria that have been shown to carry resistance genes against lincosamides, tetracyclines, and MLS-B (Champion et al., 1988; Sabino et al., 2019; Veloo et al., 2019). Second, *Sb* can modulate and activate the immune system in a general way due to its cell wall components, allowing it to combat pathogens in the early stages (Moré & Swidsinski, 2015). Third, *Sb* produces saturated fatty acids (FAs) such as capric acid (Murzyn et al., 2010). Capric acid and other saturated FAs possess antimicrobial properties (Carballeira, 2008; Huang et al., 2014; Murzyn et al., 2010; Yang et al., 2018) and synergistic effects with other antimicrobials (Suchodolski et al., 2021). Previous studies show that synthetic FAs have HGT inhibitory properties (Casillas-Vargas et al., 2021). Inhibition of conjugation occurs on the donor cell, affecting several pathogenic hosts such as *Escherichia coli*, *Salmonella enterica*, *Pseudomonas putida*, and *Agrobacterium tumefaciens* (Getino et al., 2015). Likewise, FAs can interfere with quorum sensing, inhibiting biofilm formation, virulence, and

motility without affecting the cell growth rate (Casillas-Vargas et al., 2021; Cui et al., 2019).

The mycobiome (fungal microbiota) occupies a small proportion of the microbiota of the human intestine, has low diversity, and is dominated by the yeast genera *Saccharomyces*, *Malassezia*, and *Candida*.(Nash et al., 2017) Nash et al. employing data from the Human Microbiome Project and using sequences from the Internal Transcribed Spacer 2 (ITS2) show that the *Candida* and *Saccharomyces* genera present a strong negative correlation(Nash et al., 2017) In addition, Murzyn et al. demonstrate with *in vitro* experiments that capric acid secreted by *Sb* can inhibit the morphological transition of yeast to the filamentous form and, to a lesser extent, inhibit the adhesion and biofilm formation.(Murzyn et al., 2010) Perhaps because of the little attention is given to fungi that colonize various barrier surfaces of the human body, there is a gap in understanding the effect of the resident fungal microbiota on the human intestinal resistome.

One of the most critical limitations of this study was that not all participants provided a stool sample at the three-time points, which negatively affected the study's statistical power, particularly when analyzing paired samples. In addition, the follow-up duration of one month after treatments did not allow us to show whether *Sb* could promote the recovery of the fecal resistome or maintain it at lower levels compared to the moment before receiving antimicrobial therapy.

In conclusion, we have demonstrated for the first time that *S. boulardii* CNCM-I 745, during a triple eradication therapy against *H. pylori,* can positively modify the gut resistome by reducing the abundance of various ARGs. The findings of the present work

support the use of drugs or functional foods, including probiotics, as measures in the fight against antibiotic resistance. It will be essential to carry out more extensive randomized clinical trials with different probiotics to determine their respective effects on ARGs.

Acknowledgements

The following study received funding from BIOCODEX and the Universidad San Francisco de Quito. None of the funders intervened in study design, participant enrollment, metagenomics or statistical analysis, interpretation results, and construction of conclusions. We are grateful to Francisco Guarner for his suggestions on the methodology and Achal Dhariwal, who kindly solved problems related to the use of ResitoXplorer. The Galaxy server that was used for some calculations is in part funded by Collaborative Research Centre 992 Medical Epigenetics (DFG grant SFB 992/1 2012) and German Federal Ministry of Education and Research (BMBF grants 031 A538A/A538C RBC, 031L0101B/031L0101C de.NBI-epi, 031L0106 de.STAIR (de.NBI)).

Conflict of interest

Dr. Cohen works at advisory boards and lectures for BIOCODEX. The authors report no other conflicts of interest in this work.

Author contributions

All authors contributed significantly to the work of this manuscript. SC, PC, BP analyzed and interpreted data. SC drafted the paper and was involved in manuscript writing. PC, MEB, MF and HC designed the study. BP, MF, MEB, HC, PC critically revised and edited the manuscript. All authors approved the final version of the article.

References

Afgan, E., Baker, D., Batut, B., van den Beek, M., Bouvier, D., Čech, M., Chilton, J., Clements, D., Coraor, N., Grüning, B. A., Guerler, A., Hillman-Jackson, J., Hiltemann, S., Jalili, V., Rasche, H., Soranzo, N., Goecks, J., Taylor, J., Nekrutenko, A. & Blankenberg, D. (2018). The Galaxy platform for accessible, reproducible and collaborative biomedical analyses: 2018 update. *Nucleic Acids Research*, *46*, 537–544. https://doi.org/10.1093/nar/gky379

Afridi, O. K., Ali, J. & Chang, J. H. (2021). Fecal Microbiome and Resistome Profiling of Healthy and Diseased Pakistani Individuals Using Next-Generation Sequencing. *Microorganisms*, *9*(3), 616. https://doi.org/10.3390/microorganisms9030616

Andrews, S. (2010). *FastQC: a quality control tool for high throughput sequence data*. https://www.bioinformatics.babraham.ac.uk/projects/fastqc/

Barbosa, T. M. & Levy, S. B. (2000). The impact of antibiotic use on resistance development and persistence. *Drug Resistance Updates*, *3*(5), 303–311. https://doi.org/10.1054/drup.2000.0167

Benjamini, Y. & Hochberg, Y. (1995). Controlling the False Discovery Rate: A Practical and Powerful Approach to Multiple Testing. *Journal of the Royal Statistical Society*, *57*(1), 289–300. https://doi.org/10.1111/j.2517-6161.1995.tb02031.x

Bolger, A. M., Lohse, M. & Usadel, B. (2014). Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformatics*, *30*(15), 2114–2120. https://doi.org/10.1093/bioinformatics/btu170

Carballeira, N. M. (2008). New advances in fatty acids as antimalarial, antimycobacterial and antifungal agents. *Progress in Lipid Research*, *47*(1), 50–61. https://doi.org/10.1016/j.plipres.2007.10.002

Cárdenas, P. A., Garcés, D., Prado-Vivar, B., Flores, N., Fornasini, M., Cohen, H., Salvador, I., Cargua, O. & Baldeón, M. E. (2020). Effect of Saccharomyces boulardii CNCM I-745 as complementary treatment of Helicobacter pylori infection on gut microbiome. *European Journal of Clinical Microbiology & Infectious Diseases*, *39*(7), 1365–1372. https://doi.org/10.1007/s10096-020-03854-3

Casillas-Vargas, G., Ocasio-Malavé, C., Medina, S., Morales-Guzmán, C., Valle, R. G. del, Carballeira, N. M. & Sanabria-Ríos, D. J. (2021). Antibacterial fatty acids: An update of possible mechanisms of action and implications in the development of the next-generation of antibacterial agents. *Progress in Lipid Research*, *82*, 101093. https://doi.org/10.1016/j.plipres.2021.101093

Champion, K. M., Helaszek, C. T. & White, B. A. (1988). Analysis of antibiotic susceptibility and extrachromosomal DNA content of Ruminococcus albus and Ruminococcus flavefaciens. *Canadian Journal of Microbiology*, *34*(10), 1109–1115. https://doi.org/10.1139/m88-196

Cui, C., Song, S., Yang, C., Sun, X., Huang, Y., Li, K., Zhao, S., Zhang, Y. & Deng, Y. (2019). Disruption of Quorum Sensing and Virulence in Burkholderia cenocepacia by a Structural Analogue of the cis-2-Dodecenoic Acid Signal. *Applied and Environmental Microbiology*, *85*(8), e00105-19. https://doi.org/10.1128/aem.00105-19

Czerucka, D., Piche, T. & Rampal, P. (2007). Review article: yeast as probiotics – Saccharomyces boulardii. *Alimentary Pharmacology & Therapeutics*, *26*(6), 767–778. https://doi.org/10.1111/j.1365-2036.2007.03442.x

Davies, J. & Davies, D. (2010). Origins and Evolution of Antibiotic Resistance. *Microbiology and Molecular Biology Reviews*, *74*(3), 417–433. https://doi.org/10.1128/mmbr.00016-10

Dhariwal, A., Junges, R., Chen, T. & Petersen, F. C. (2021). ResistoXplorer: a web-based tool for visual, statistical and exploratory data analysis of resistome data. *NAR Genomics and Bioinformatics*, *3*(1), lqab018. https://doi.org/10.1093/nargab/lqab018

Ewels, P., Magnusson, M., Lundin, S. & Käller, M. (2016). MultiQC: summarize analysis results for multiple tools and samples in a single report. *Bioinformatics*, *32*(19), 3047–3048. https://doi.org/10.1093/bioinformatics/btw354

Feng, J., Li, B., Jiang, X., Yang, Y., Wells, G. F., Zhang, T. & Li, X. (2018). Antibiotic resistome in a large-scale healthy human gut microbiota deciphered by metagenomic and network analyses. *Environmental Microbiology*, *20*(1), 355–368. https://doi.org/10.1111/1462-2920.14009

Forslund, K., Sunagawa, S., Kultima, J. R., Mende, D. R., Arumugam, M., Typas, A. & Bork, P. (2013). Country-specific antibiotic use practices impact the human gut resistome. *Genome Research*, *23*(7), 1163–1169. https://doi.org/10.1101/gr.155465.113

Francino, M. P. (2016). Antibiotics and the Human Gut Microbiome: Dysbioses and Accumulation of Resistances. *Frontiers in Microbiology*, *6*, 1543. https://doi.org/10.3389/fmicb.2015.01543

Getino, M., Sanabria-Ríos, D. J., Fernández-López, R., Campos-Gómez, J., Sánchez-López, J. M., Fernández, A., Carballeira, N. M. & Cruz, F. de la. (2015). Synthetic Fatty Acids Prevent Plasmid-Mediated Horizontal Gene Transfer. *MBio*, *6*(5), e01032-15. https://doi.org/10.1128/mbio.01032-15

Ghanbari, M., Klose, V., Crispie, F. & Cotter, P. D. (2019). The dynamics of the antibiotic resistome in the feces of freshly weaned pigs following therapeutic administration of oxytetracycline. *Scientific Reports*, *9*(1), 4062. https://doi.org/10.1038/s41598-019-40496-8

González, I., Cao, K.-A. L., Davis, M. J. & Déjean, S. (2012). Visualising associations between paired "omics" data sets. *BioData Mining*, *5*(1), 19. https://doi.org/10.1186/1756-0381-5-19

Heddini, A., Cars, O., Qiang, S. & Tomson, G. (2009). Antibiotic resistance in China—a major future challenge. *The Lancet*, *373*(9657), 30. https://doi.org/10.1016/s0140-6736(08)61956-x

Hoek, A. H. A. M. van, Mevius, D., Guerra, B., Mullany, P., Roberts, A. P. & Aarts, H. J. M. (2011). Acquired Antibiotic Resistance Genes: An Overview. *Frontiers in Microbiology*, *2*, 203. https://doi.org/10.3389/fmicb.2011.00203

Hu, Y., Yang, X., Qin, J., Lu, N., Cheng, G., Wu, N., Pan, Y., Li, J., Zhu, L., Wang, X., Meng, Z., Zhao, F., Liu, D., Ma, J., Qin, N., Xiang, C., Xiao, Y., Li, L., Yang, H., …

Zhu, B. (2013). Metagenome-wide analysis of antibiotic resistance genes in a large cohort of human gut microbiota. *Nature Communications*, *4*(1), 2151. https://doi.org/10.1038/ncomms3151

Huang, W.-C., Tsai, T.-H., Chuang, L.-T., Li, Y.-Y., Zouboulis, C. C. & Tsai, P.-J. (2014). Anti-bacterial and anti-inflammatory properties of capric acid against Propionibacterium acnes: A comparative study with lauric acid. *Journal of Dermatological Science*, *73*(3), 232–240. https://doi.org/10.1016/j.jdermsci.2013.10.010

Jonsson, V., Österlund, T., Nerman, O. & Kristiansson, E. (2016). Statistical evaluation of methods for identification of differentially abundant genes in comparative metagenomics. *BMC Genomics*, *17*(1), 78. https://doi.org/10.1186/s12864-016-2386-y

Levy, S. B., Marshall, B., Schluederberg, S., Rowse, D. & Davis, J. (1988). High frequency of antimicrobial resistance in human fecal flora. *Antimicrobial Agents and Chemotherapy*, *32*(12). https://doi.org/10.1128/aac.32.12.1801.

Li, H. (2013). Aligning sequence reads, clone sequences and assembly contigs with BWA-MEM. *ArXiv*.

Li, H., Handsaker, B., Wysoker, A., Fennell, T., Ruan, J., Homer, N., Marth, G., Abecasis, G., Durbin, R. & Subgroup, 1000 Genome Project Data Processing. (2009). The Sequence Alignment/Map format and SAMtools. *Bioinformatics*, *25*(16), 2078–2079. https://doi.org/10.1093/bioinformatics/btp352

Li, J., Rettedal, E. A., Helm, E. van der, Ellabaan, M., Panagiotou, G. & Sommer, M. O. A. (2019). Antibiotic Treatment Drives the Diversification of the Human Gut Resistome. *Genomics, Proteomics & Bioinformatics*, *17*(1), 39–51. https://doi.org/10.1016/j.gpb.2018.12.003

Lin, H. & Peddada, S. D. (2020). Analysis of microbial compositions: a review of normalization and differential abundance analysis. *Npj Biofilms and Microbiomes*, *6*(1), 60. https://doi.org/10.1038/s41522-020-00160-w

Modi, S. R., Lee, H. H., Spina, C. S. & Collins, J. J. (2013). Antibiotic treatment expands the resistance reservoir and ecological network of the phage metagenome. *Nature*, *499*(7457), 219–222. https://doi.org/10.1038/nature12212

Moré, M. I. & Swidsinski, A. (2015). Saccharomyces boulardii CNCM I-745 supports regeneration of the intestinal microbiota after diarrheic dysbiosis – a review. *Clinical and Experimental Gastroenterology*, *8*, 237–255. https://doi.org/10.2147/ceg.s85574

Murzyn, A., Krasowska, A., Stefanowicz, P., Dziadkowiec, D. & Łukaszewicz, M. (2010). Capric Acid Secreted by S. boulardii Inhibits C. albicans Filamentous Growth, Adhesion and Biofilm Formation. *PLoS ONE*, *5*(8), e12050. https://doi.org/10.1371/journal.pone.0012050

Nash, A. K., Auchtung, T. A., Wong, M. C., Smith, D. P., Gesell, J. R., Ross, M. C., Stewart, C. J., Metcalf, G. A., Muzny, D. M., Gibbs, R. A., Ajami, N. J. & Petrosino, J. F. (2017). The gut mycobiome of the Human Microbiome Project healthy cohort. *Microbiome*, *5*(1), 153. https://doi.org/10.1186/s40168-017-0373-4

Olekhnovich, E. I., Manolov, A. I., Samoilov, A. E., Prianichnikov, N. A., Malakhova, M. V., Tyakht, A. V., Pavlenko, A. V., Babenko, V. V., Larin, A. K., Kovarsky, B. A., Starikova, E. V., Glushchenko, O. E., Safina, D. D., Markelova, M. I., Boulygina, E. A., Khusnutdinova, D. R., Malanin, S. Y., Abdulkhakov, S. R., Abdulkhakov, R. A., … Ilina, E. N. (2019). Shifts in the Human Gut Microbiota Structure Caused by Quadruple Helicobacter pylori Eradication Therapy. *Frontiers in Microbiology*, *10*, 1902. https://doi.org/10.3389/fmicb.2019.01902

O'Neill, J. (2016). *Tackling drug-resistant infections globally: final report and recommendations. The Review on Antimicrobial Resistance*. Review on Antimicrobial Resistance. https://wellcomecollection.org/works/thvwsuba

Pais, P., Almeida, V., Yılmaz, M. & Teixeira, M. C. (2020). Saccharomyces boulardii: What Makes It Tick as Successful Probiotic? *Journal of Fungi*, *6*(2), 78. https://doi.org/10.3390/jof6020078

Paulson, J. N., Stine, O. C., Bravo, H. C. & Pop, M. (2013). Differential abundance analysis for microbial marker-gene surveys. *Nature Methods*, *10*(12), 1200–1202. https://doi.org/10.1038/nmeth.2658

Penders, J., Stobberingh, E. E., Savelkoul, P. H. M. & Wolffs, P. F. G. (2013). The human microbiome as a reservoir of antimicrobial resistance. *Frontiers in Microbiology*, *4*, 87. https://doi.org/10.3389/fmicb.2013.00087

Pereira, M. B., Wallroth, M., Jonsson, V. & Kristiansson, E. (2018). Comparison of normalization methods for the analysis of metagenomic gene abundance data. *BMC Genomics*, *19*(1), 274. https://doi.org/10.1186/s12864-018-4637-6

Peres-Neto, P. R. & Jackson, D. A. (2001). How well do multivariate data sets match? The advantages of a Procrustean superimposition approach over the Mantel test. *Oecologia*, *129*(2), 169–178. https://doi.org/10.1007/s004420100720

Quizhpe, A., Encalada, D., Encalada, L., Barten, F. & Velden, K. van der. (2017). Antibiotic use without prescription in Ecuadorian children according to their families' socioeconomic characteristics. *Médicas UIS*, *30*(2), 21–27. https://doi.org/10.18273/revmed.v30n2-2017002

Robinson, M. D., McCarthy, D. J. & Smyth, G. K. (2010). edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. *Bioinformatics*, *26*(1), 139–140. https://doi.org/10.1093/bioinformatics/btp616

Sabino, Y. N. V., Santana, M. F., Oyama, L. B., Santos, F. G., Moreira, A. J. S., Huws, S. A. & Mantovani, H. C. (2019). Characterization of antibiotic resistance genes in the species of the rumen microbiota. *Nature Communications*, *10*(1), 5252. https://doi.org/10.1038/s41467-019-13118-0

Sovran, B., Planchais, J., Jegou, S., Straube, M., Lamas, B., Natividad, J. M., Agus, A., Dupraz, L., Glodt, J., Costa, G. D., Michel, M.-L., Langella, P., Richard, M. L. & Sokol, H. (2018). Enterobacteriaceae are essential for the modulation of colitis severity by fungi. *Microbiome*, *6*(1), 152. https://doi.org/10.1186/s40168-018-0538-9

Suchodolski, J., Derkacz, D., Bernat, P. & Krasowska, A. (2021). Capric acid secreted by Saccharomyces boulardii influences the susceptibility of Candida albicans to fluconazole and amphotericin B. *Scientific Reports*, *11*(1), 6519. https://doi.org/10.1038/s41598-021-86012-9

Tacconelli, E., Angelis, G. D., Cataldo, M. A., Mantengoli, E., Spanu, T., Pan, A., Corti, G., Radice, A., Stolzuoli, L., Antinori, S., Paradisi, F., Carosi, G., Bernabei, R., Antonelli, M., Fadda, G., Rossolini, G. M. & Cauda, R. (2009). Antibiotic Usage and Risk of Colonization and Infection with Antibiotic-Resistant Bacteria: a Hospital Population-Based Study. *Antimicrobial Agents and Chemotherapy*, *53*(10), 4264–4269. https://doi.org/10.1128/aac.00431-09

Truong, D. T., Franzosa, E. A., Tickle, T. L., Scholz, M., Weingart, G., Pasolli, E., Tett, A., Huttenhower, C. & Segata, N. (2015). MetaPhlAn2 for enhanced metagenomic taxonomic profiling. *Nature Methods*, *12*(10), 902–903. https://doi.org/10.1038/nmeth.3589

Tsigalou, C., Konstantinidis, T., Stavropoulou, E., Bezirtzoglou, E. E. & Tsakris, A. (2020). Potential Elimination of Human Gut Resistome by Exploiting the Benefits of Functional Foods. *Frontiers in Microbiology*, *11*, 50. https://doi.org/10.3389/fmicb.2020.00050

Veloo, A. C. M., Baas, W. H., Haan, F. J., Coco, J. & Rossen, J. W. (2019). Prevalence of antimicrobial resistance genes in Bacteroides spp. and Prevotella spp. Dutch clinical isolates. *Clinical Microbiology and Infection*, *25*(9), 1156.e9-1156.e13. https://doi.org/10.1016/j.cmi.2019.02.017

Weiss, S., Xu, Z. Z., Peddada, S., Amir, A., Bittinger, K., Gonzalez, A., Lozupone, C., Zaneveld, J. R., Vázquez-Baeza, Y., Birmingham, A., Hyde, E. R. & Knight, R. (2017). Normalization and microbial differential abundance strategies depend upon data characteristics. *Microbiome*, *5*(1), 27. https://doi.org/10.1186/s40168-017-0237-y

Willmann, M., El-Hadidi, M., Huson, D. H., Schütz, M., Weidenmaier, C., Autenrieth, I. B. & Peter, S. (2015). Antibiotic Selection Pressure Determination through Sequence-Based Metagenomics. *Antimicrobial Agents and Chemotherapy*, *59*(12), 7335–7345. https://doi.org/10.1128/aac.01504-15

Willmann, M., Vehreschild, M. J. G. T., Biehl, L. M., Vogel, W., Dörfel, D., Hamprecht, A., Seifert, H., Autenrieth, I. B. & Peter, S. (2019). Distinct impact of antibiotics on the gut microbiome and resistome: a longitudinal multicenter cohort study. *BMC Biology*, *17*(1), 76. https://doi.org/10.1186/s12915-019-0692-y

World Health Organization. (2015). *Global Action Plan on Antimicrobial Resistance* (p. 45). World Health Organization. https://www.who.int/publications/i/item/9789241509763

Wright, G. D. (2007). The antibiotic resistome: the nexus of chemical and genetic diversity. *Nature Reviews Microbiology*, *5*(3), 175–186. https://doi.org/10.1038/nrmicro1614

Wu, G., Zhang, C., Wang, J., Zhang, F., Wang, R., Shen, J., Wang, L., Pang, X., Zhang, X., Zhao, L. & Zhang, M. (2016). Diminution of the gut resistome after a gut microbiota-targeted dietary intervention in obese children. *Scientific Reports*, *6*(1), 24030. https://doi.org/10.1038/srep24030

Yang, H.-T., Chen, J.-W., Rathod, J., Jiang, Y.-Z., Tsai, P.-J., Hung, Y.-P., Ko, W.-C., Paredes-Sabja, D. & Huang, I.-H. (2018). Lauric Acid Is an Inhibitor of Clostridium difficile Growth in Vitro and Reduces Inflammation in a Mouse Infection Model. *Frontiers in Microbiology*, *8*, 2635. https://doi.org/10.3389/fmicb.2017.02635

Zankari, E., Hasman, H., Cosentino, S., Vestergaard, M., Rasmussen, S., Lund, O., Aarestrup, F. M. & Larsen, M. V. (2012). Identification of acquired antimicrobial resistance genes. *Journal of Antimicrobial Chemotherapy*, *67*(11), 2640–2644. https://doi.org/10.1093/jac/dks261

CHAPTER 6. GENERAL CONCLUSIONS

- AMR is complex and must be approached comprehensively and from a One-health perspective.
- Human gut microbiota is an essential reservoir of ARGs and pathogenic bacteria. When commensal and pathogenic bacteria congregate in the same ecological niche, the opportunity for HGT among these bacteria increases.
- Metagenomics has emerged as a method to predict the complete resistance profile harbored by complex microbial communities such as gut microbiota.
- Although exposure to domestic animals is not able to modify the overall structure of the children's resistome, *mdf*(A), *tet*(O/W), and *tet*(32) genes that confer resistance to multidrug and tetracyclines, respectively, are significantly more abundant in children exposed to animals.
- There is a positive and significant correlation between the children's gut microbiota and resistome.
- The structure of the gut resistome of children between 3 months to 7 years old is temporally variable and tends to be more similar among children of 4 to 7 years old than among younger children.
- ARGs of clinical importance that confers resistance to fluoroquinolones, colistin, and broad-spectrum beta-lactams are transient in the children's gut.
- *E. coli* strains are highly variable over time within the gut of the same child and between children in the same community.
- There is no conclusive evidence that probiotics can protect or recover the gut microbiota structure during *H. pylori* eradication therapy.

- The supplementation with S. *boul*ardii CNCM I-745 during triple *H. pylori* eradication therapy significantly reduces the abundance of ARGs that confer resistance to lincosamides, tetracyclines, MLS-B, and beta-lactams.

CHAPTER 7. PROSPECTS

1. Shotgun metagenomics is a comprehensive method to study the microbiota (Tringe et al., 2005) and the resistome (Forslund et al., 2014) in complex communities such as those inhabiting the human intestine. However, this approach with short reads cannot resolve the genetic context of resistance genes, nor is it capable of reconstructing mobile genetic elements to study the mobile capacity of resistance genes (Maguire, 2020). Additionally, individual genomes can be reconstructed from metagenomic data using metagenome-assembled genome (MAG) binning methods (Kashaf et al., 2021; Nissen et al., 2021), but binning has its limitations, it is an indirect clustering method. Hi-C-based metagenomic deconvolution methods can indicate a sequence's origin to overcome these difficulties. The Hi-C proximity ligation method can reconstruct genomes, and MGE, and report on the genetic context of ARGs (Press et al., 2017; Stalder et al., 2019).

2. Possibly, the beneficial effects of *S. boulardii* CNCM I-745 in the modification of fecal resistome in patients treated with eradication therapy of *H. pylori* are due to the action of its structural and metabolic products with antimicrobial activity (Ansari et al., 2021; Murzyn et al., 2010; Suchodolski et al., 2021), or with the ability to modulate the microbiota, or the immune system (Pais et al., 2020). While metagenomics can outline the taxonomy and predict the functionality of a microbial community (Aguiar-Pulido et al., 2016; Quince et al., 2017), metabolomics can allow us to study the metabolites released by the microbiota or the probiotics in the luminal microenvironment (Aguiar-Pulido et al., 2016; Krautkramer et al., 2021). Metagenomics combined with

metabolomics can allow us to understand probiotic-gut microbiota-host interactions and work on measures to combat AMR.

References

Aguiar-Pulido, V., Huang, W., Suarez-Ulloa, V., Cickovski, T., Mathee, K. & Narasimhan, G. (2016). Metagenomics, Metatranscriptomics, and Metabolomics Approaches for Microbiome Analysis. *Evolutionary Bioinformatics*, *12s1*(Suppl 1), EBO.S36436. https://doi.org/10.4137/ebo.s36436

Ansari, F., Samakkhah, S. A., Bahadori, A., Jafari, S. M., Ziaee, M., Khodayari, M. T. & Pourjafar, H. (2021). Health-promoting properties of Saccharomyces cerevisiae var. boulardii as a probiotic; characteristics, isolation, and applications in dairy products. *Critical Reviews in Food Science and Nutrition*, 1–29. https://doi.org/10.1080/10408398.2021.1949577

Forslund, K., Sunagawa, S., Coelho, L. P. & Bork, P. (2014). Metagenomic insights into the human gut resistome and the forces that shape it. *BioEssays*, *36*(3), 316–329. https://doi.org/10.1002/bies.201300143

Kashaf, S. S., Almeida, A., Segre, J. A. & Finn, R. D. (2021). Recovering prokaryotic genomes from host-associated, short-read shotgun metagenomic sequencing data. *Nature Protocols*, *16*(5), 2520–2541. https://doi.org/10.1038/s41596-021-00508-2

Krautkramer, K. A., Fan, J. & Bäckhed, F. (2021). Gut microbial metabolites as multi-kingdom intermediates. *Nature Reviews Microbiology*, *19*(2), 77–94. https://doi.org/doi: 10.1038/s41579-020-0438-4

Murzyn, A., Krasowska, A., Stefanowicz, P., Dziadkowiec, D. & Łukaszewicz, M. (2010). Capric Acid Secreted by S. boulardii Inhibits C. albicans Filamentous Growth, Adhesion and Biofilm Formation. *PLoS ONE*, *5*(8), e12050. https://doi.org/10.1371/journal.pone.0012050

Nissen, J. N., Johansen, J., Allesøe, R. L., Sønderby, C. K., Armenteros, J. J. A., Grønbech, C. H., Jensen, L. J., Nielsen, H. B., Petersen, T. N., Winther, O. & Rasmussen, S. (2021). Improved metagenome binning and assembly using deep variational autoencoders. *Nature Biotechnology*, *39*(5), 555–560. https://doi.org/10.1038/s41587-020-00777-4

Pais, P., Almeida, V., Yılmaz, M. & Teixeira, M. C. (2020). Saccharomyces boulardii: What Makes It Tick as Successful Probiotic? *Journal of Fungi*, *6*(2), 78. https://doi.org/10.3390/jof6020078

Press, M. O., Wiser, A. H., Kronenberg, Z. N., Langford, K. W., Shakya, M., Lo, C.-C., Mueller, K. A., Sullivan, S. T., Chain, P. S. G. & Liachko, I. (2017). Hi-C deconvolution of a human gut microbiome yields high-quality draft genomes and reveals plasmid-genome interactions. *BioRxiv*, 198713. https://doi.org/10.1101/198713

Quince, C., Walker, A. W., Simpson, J. T., Loman, N. J. & Segata, N. (2017). Shotgun metagenomics, from sampling to analysis. *Nature Biotechnology*, *35*(9), 833–844. https://doi.org/10.1038/nbt.3935

Stalder, T., Press, M. O., Sullivan, S., Liachko, I. & Top, E. M. (2019). Linking the resistome and plasmidome to the microbiome. *The ISME Journal*, *13*(10), 2437–2446. https://doi.org/10.1038/s41396-019-0446-4

Suchodolski, J., Derkacz, D., Bernat, P. & Krasowska, A. (2021). Capric acid secreted by Saccharomyces boulardii influences the susceptibility of Candida albicans to fluconazole and amphotericin B. *Scientific Reports*, *11*(1), 6519. https://doi.org/10.1038/s41598-021-86012-9

Tringe, S. G., Mering, C. von, Kobayashi, A., Salamov, A. A., Chen, K., Chang, H. W., Podar, M., Short, J. M., Mathur, E. J., Detter, J. C., Bork, P., Hugenholtz, P. & Rubin, E. M. (2005). Comparative Metagenomics of Microbial Communities. *Science*, *308*(5721), 554–557. https://doi.org/10.1126/science.1107851

ANEXOS

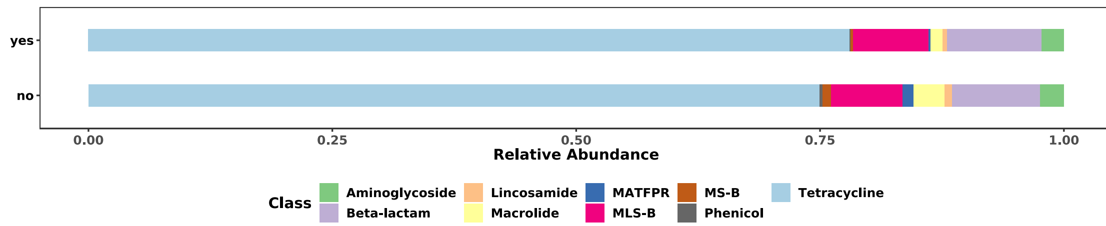
Anexo A. Chapter 2. Supporting information

Supplementary Table 1. Correlation between ARGs' mechanisms of action.

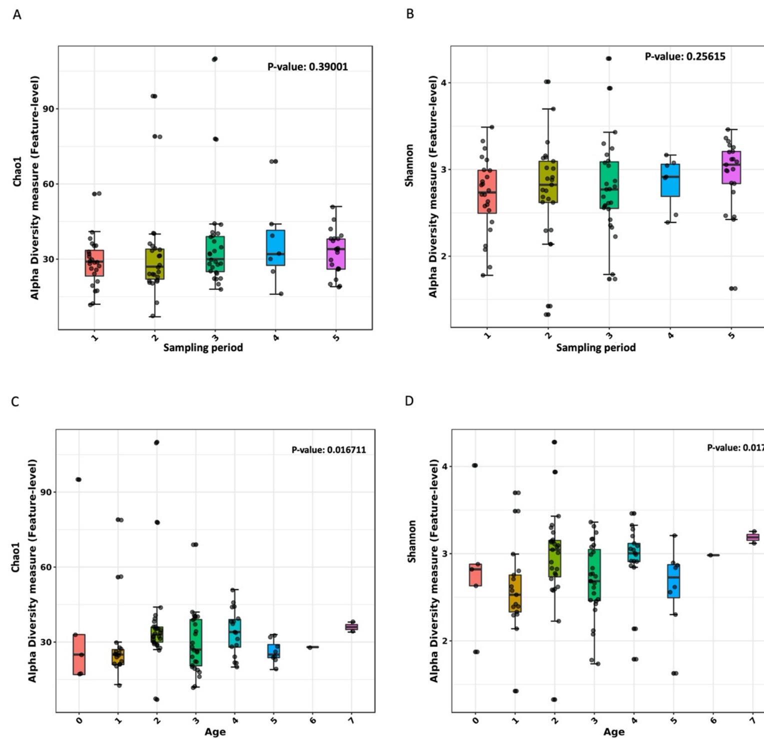
	Enzymatic inactivation	**Enzymatic modification**	**Increased efflux**	**Target modification**	**Target protection**
Enzymatic inactivation	1	-0.14507	0.018703	0.63103	0.41075
Enzymatic modification	-0.14507	1	0.096728	-0.19869	-0.12807
Increased efflux	0.018703	0.096728	1	0.047433	0.18062
Target modification	0.63103	-0.19869	0.047433	1	0.51835
Target protection	0.41075	-0.12807	0.18062	0.51835	1

Supplementary Table 2. Antimicrobial resistance genes (ARGs) identified in whole genomes of 3GCR-EC isolates

Sample	**Isolate ID**	**Antimicrobial resistance genes (ARGs)**
PR001	2018081420isolate5	*aph(3'')-Ib, aph(6)-Id,* ***bla***$_{\text{CTX-M-55}}$*, sul2, sul3*
PR004	2018091110isolate2	*aph(3'')-Ib, mcr.1.1, tet(A)*
PR013	2018091123isolate1	*aph(3'')-Ib,* ***bla***$_{\text{CTX-M-55}}$*,* ***bla***$_{\text{TEM-1B}}$*, fosA3, sul2, tet(A)*
	2018091123isolate2	*bla*$_{\text{CMY-2}}$*, dfrA12, mef(B), qnrS1, sul2, sul3*
	2018091123isolate3	*ant(3'')-Ia, aph(3'')-Ib, aph(6)-Id,* ***bla***$_{\text{CTX-M-55}}$*,* ***bla***$_{\text{TEM-1B}}$*, dfrA5, fosA3, sul2, sul3*
	2018091123isolate4	*aph(3'')-Ib,* ***bla***$_{\text{CTX-M-55}}$*,* ***bla***$_{\text{TEM-1B}}$*, dfrA5, fosA3, sul2, tet(A)*
	2018091123isolate5	*ant(3'')-Ia, aph(3'')-Ib, aph(6)-Id,* ***bla***$_{\text{CTX-M-55}}$*, dfrA5, fosA3, sul2, sul3, tet(A)*
PR017	201808148isolate1	***bla***$_{\text{CTX-M-27}}$*, qnrB19*
	201808148isolate2	***bla***$_{\text{CTX-M-27}}$*, qnrB19*
	201808148isolate3	***bla***$_{\text{CTX-M-27}}$
	201808148isolate4	*aadA5, dfrA17, qnrB19, qnrS1*
	201808148isolate5	*dfrA17, fosA3, sul3, tet(B)*
PR046	2018091810isolate1	***bla***$_{\text{CTX-M-15}}$*, qnrB19, sul3*
	2018091810isolate2	***bla***$_{\text{CTX-M-15}}$*, qnrB19, sul3*
	2018091810isolate3	***bla***$_{\text{CTX-M-15}}$*, qnrB19, sul3*
	2018091810isolate4	***bla***$_{\text{CTX-M-15}}$*, qnrB19, sul3*
	2018091810isolate5	***bla***$_{\text{CTX-M-15}}$*, qnrB19, sul3*
PR050	2018082119isolate1	*qnrB19*
PR066	201808217isolate2	No ARGs identified
PR067	2018080721isolate1	*aph(3'')-Ib,* ***bla***$_{\text{TEM-1B}}$*, dfrA8, qnrB19*
PR110	2018080715isolate1	*aph(3'')-Ib, aph(6)-Id,* ***bla***$_{\text{TEM-1B}}$*, mcr-1.1, qnrS1*
PR115	201808219isolate1	***bla***$_{\text{CTX-M-15}}$*, qnrS1*



Supplementary Figure 1. Composition of the children's fecal resistome. Cumulative relative abundance compared between antimicrobial consumption in proportions. Different colors refer to different ARGs grouped by antimicrobial classes.

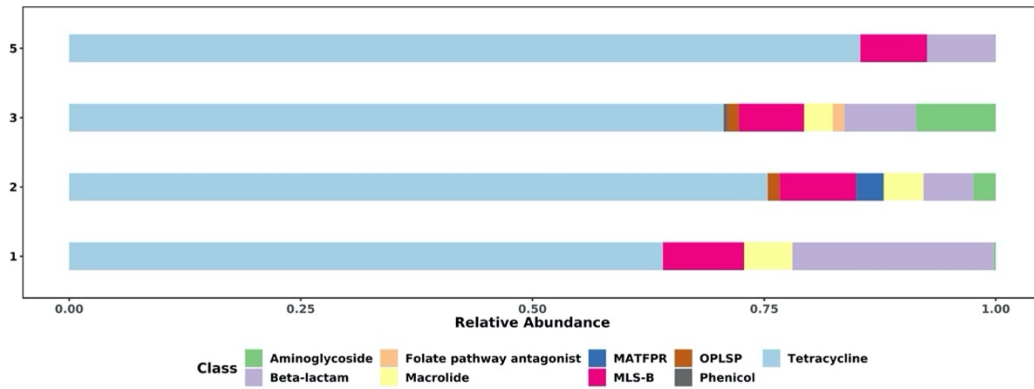


Supplementary Figure 2. Alpha diversity of the children's fecal resistome analyzed by cycle and age. Median values are indicated by central black horizontal lines; the 25th and 75th percentiles are indicated on the top and bottom side of the boxes, and the whiskers extend from each end of the box to the most extreme values.

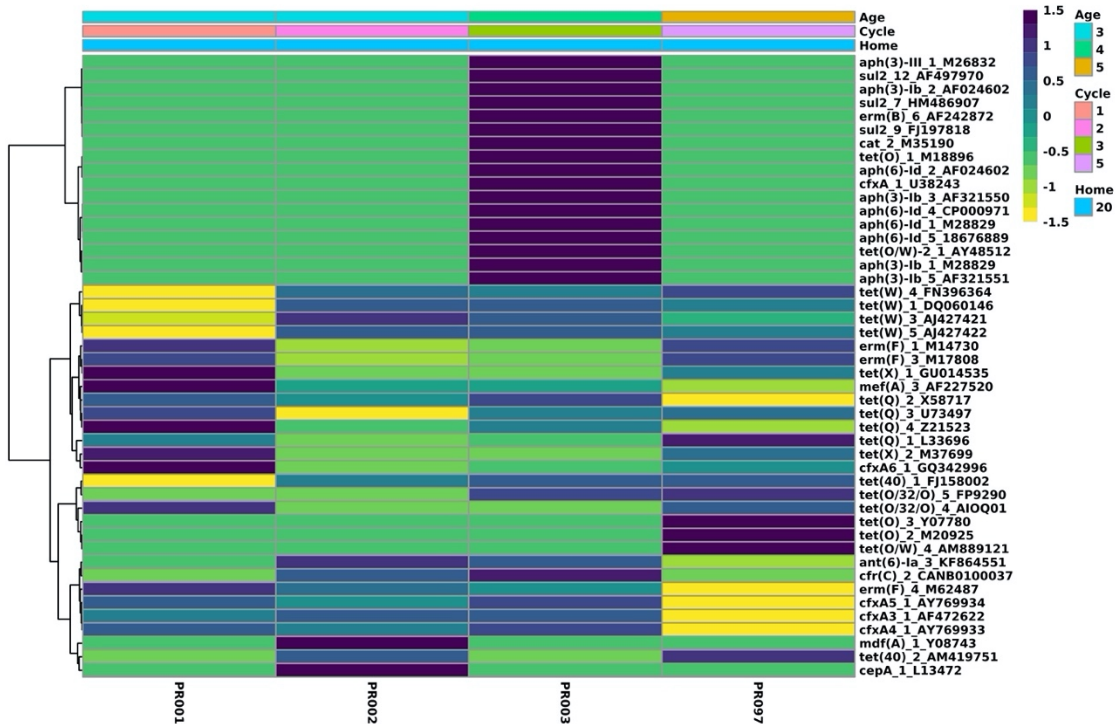
Supplementary Figures 3- 30. Temporal dynamics of the children's resistome. A) Relative abundance of fecal resistome compared by cycles. The relative abundance was evaluated in proportions. B) Heatmap and hierarchical clustering analysis. Rows are gene alleles, and columns are samples. Relative abundances were scaled by ranks (genes). Euclidean distances were used, and the grouping was by cycle.

Participant 20

A

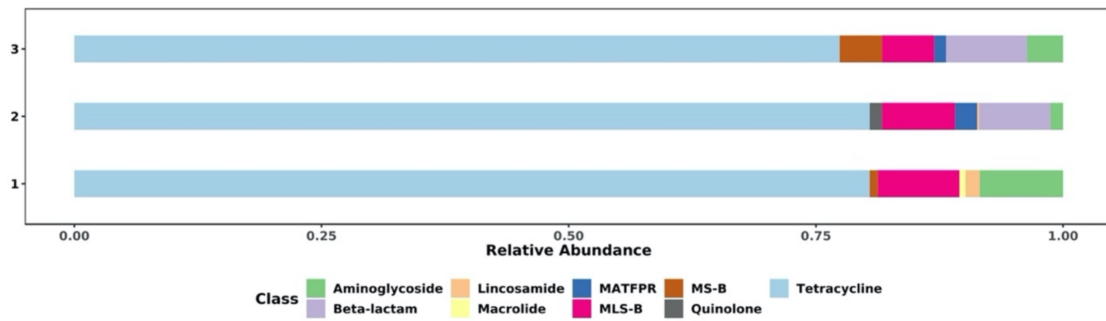


B

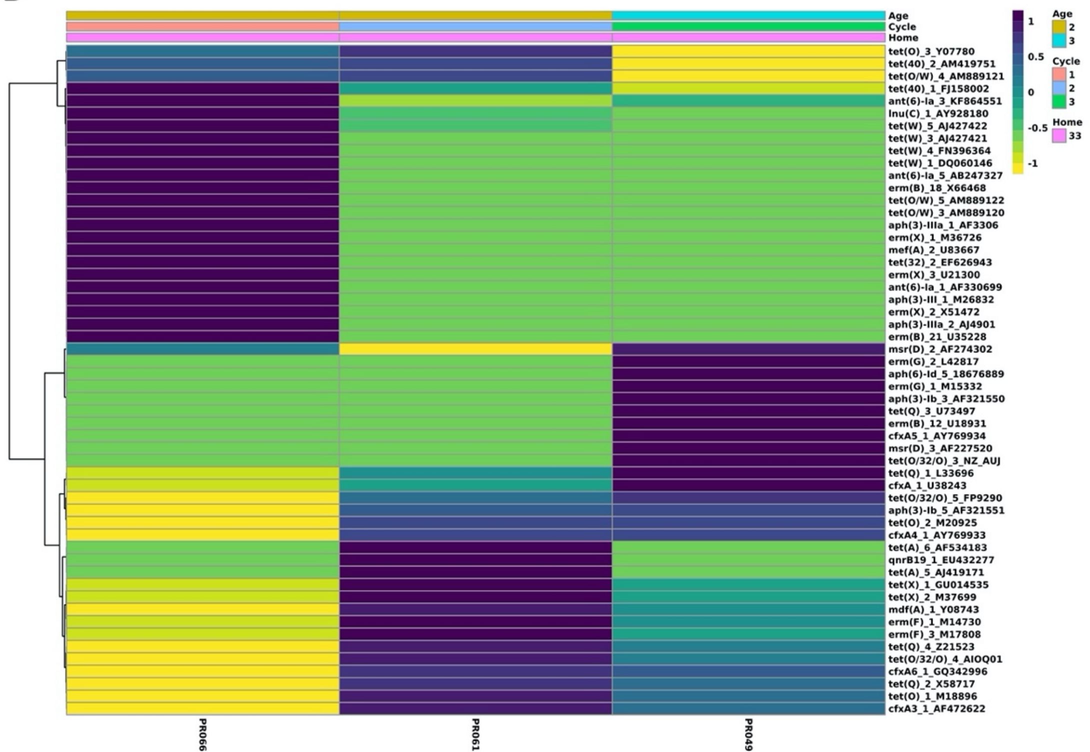


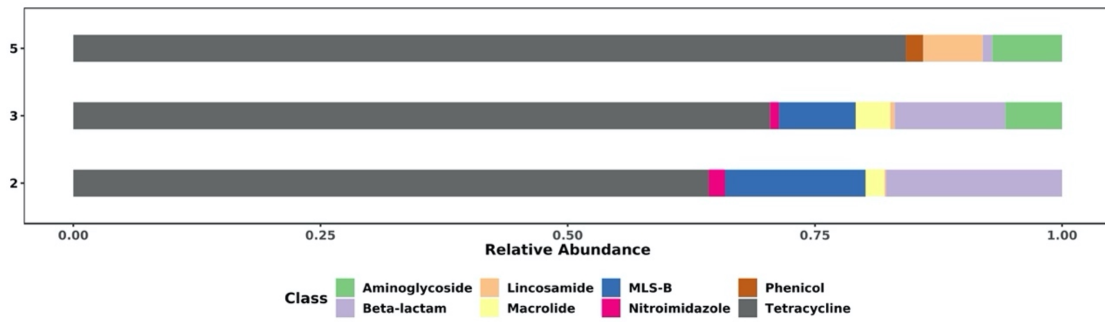
Participant 33

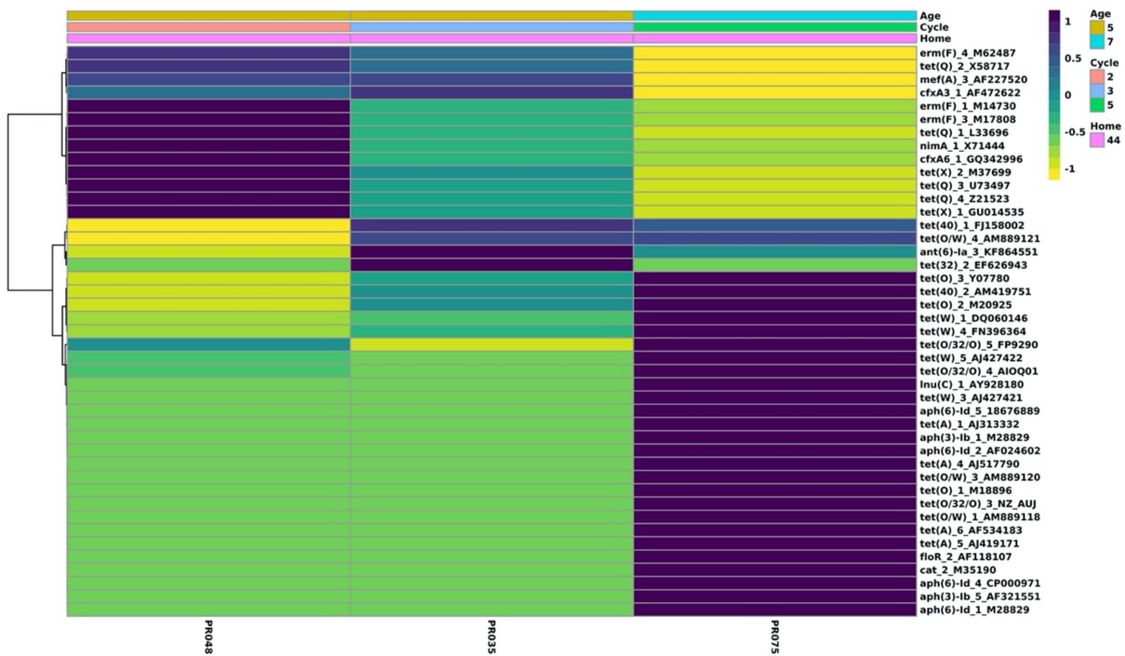
A



B

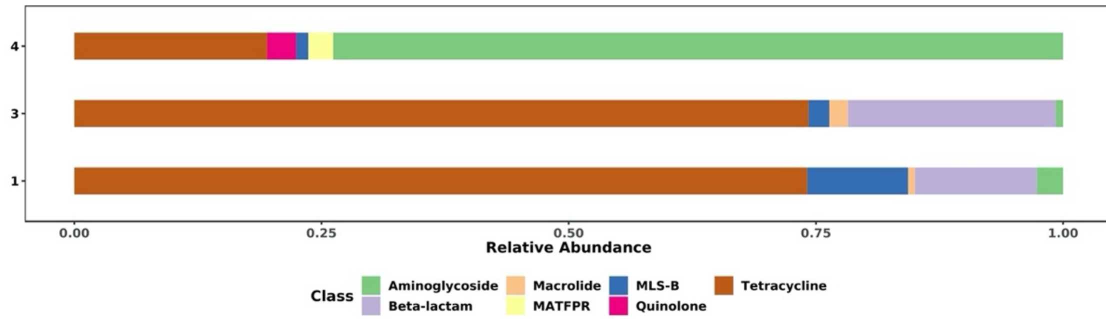



A
Participant 44
5
3
2
0.00
0.25
0.50
0.75
1.00
Relative Abundance
Class
Aminoglycoside
Beta-lactam
Lincosamide
Macrolide
MLS-B
Nitroimidazole
Phenicol
Tetracycline

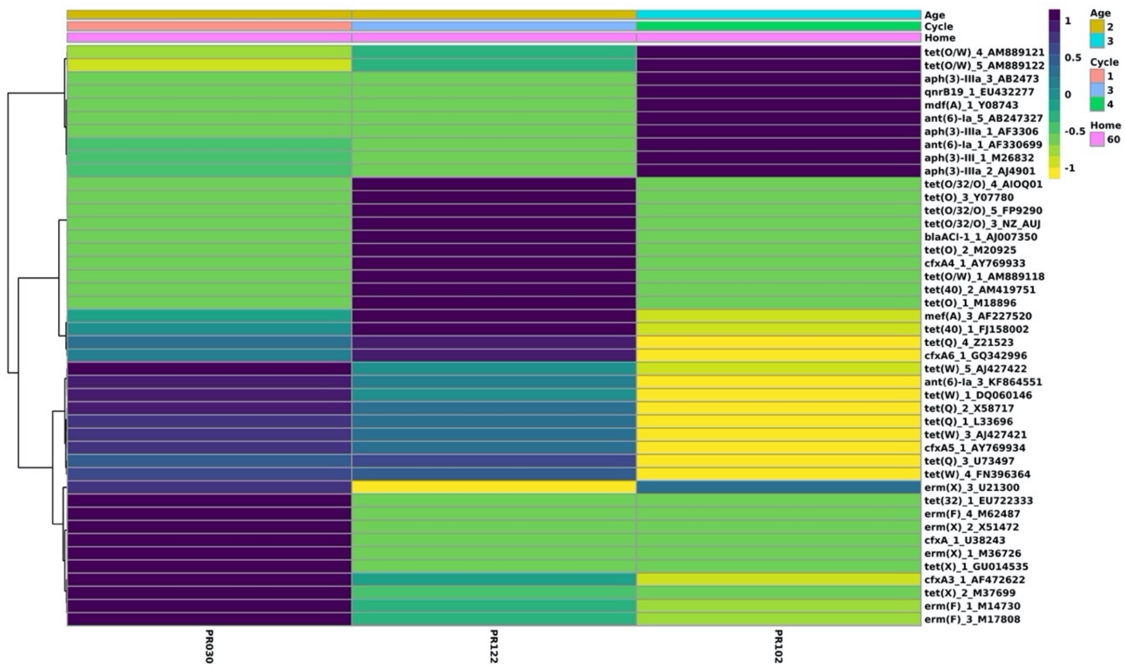

B
Age
Cycle
Home
erm(F)_4_M62487
tet(Q)_2_X58717
mef(A)_3_AF227520
cfxA3_1_AF472622
erm(F)_1_M14730
erm(F)_3_M17808
tet(Q)_1_L33696
nimA_1_X71444
cfxA6_1_GQ342996
tet(X)_2_M37699
tet(Q)_3_U73497
tet(Q)_4_Z21523
tet(X)_1_GU014535
tet(40)_1_FJ158002
tet(O/W)_4_AM889121
ant(6)-Ia_3_KF864551
tet(32)_2_EF626943
tet(O)_3_Y07780
tet(40)_2_AM419751
tet(O)_2_M20925
tet(W)_1_DQ060146
tet(W)_4_FN396364
tet(O/32/O)_5_FP9290
tet(W)_5_AJ427422
tet(O/32/O)_4_AIOQ01
lnu(C)_1_AY928180
tet(W)_3_AJ427421
aph(6)-Id_5_18676889
tet(A)_1_AJ313332
aph(3)-Ib_1_M28829
aph(6)-Id_2_AF024602
tet(A)_4_AJ517790
tet(O/W)_3_AM889120
tet(O)_1_M18896
tet(O/32/O)_3_NZ_AUJ
tet(O/W)_1_AM889118
tet(A)_6_AF534183
tet(A)_5_AJ419171
floR_2_AF118107
cat_2_M35190
aph(6)-Id_4_CP000971
aph(3)-Ib_5_AF321551
aph(6)-Id_1_M28829
PR048
PR035
PR075
1
0.5
0
-0.5
-1
Age
5
7
Cycle
2
3
5
Home
44

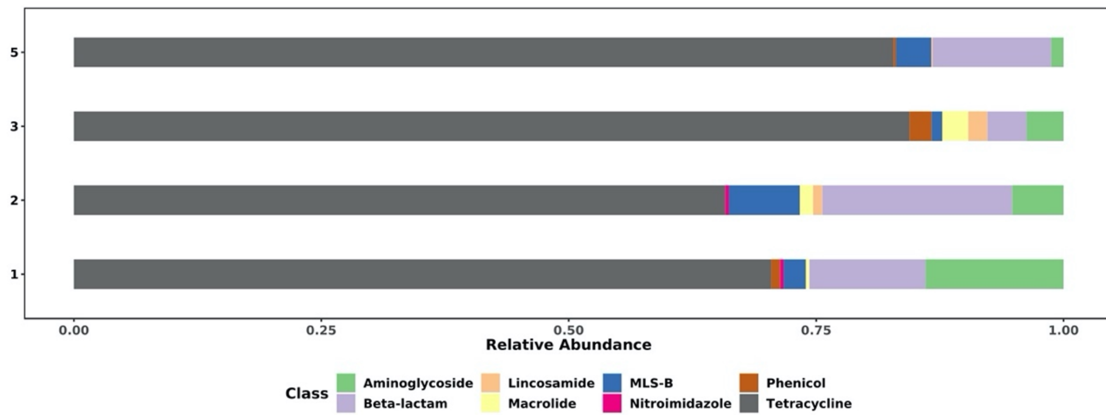
A

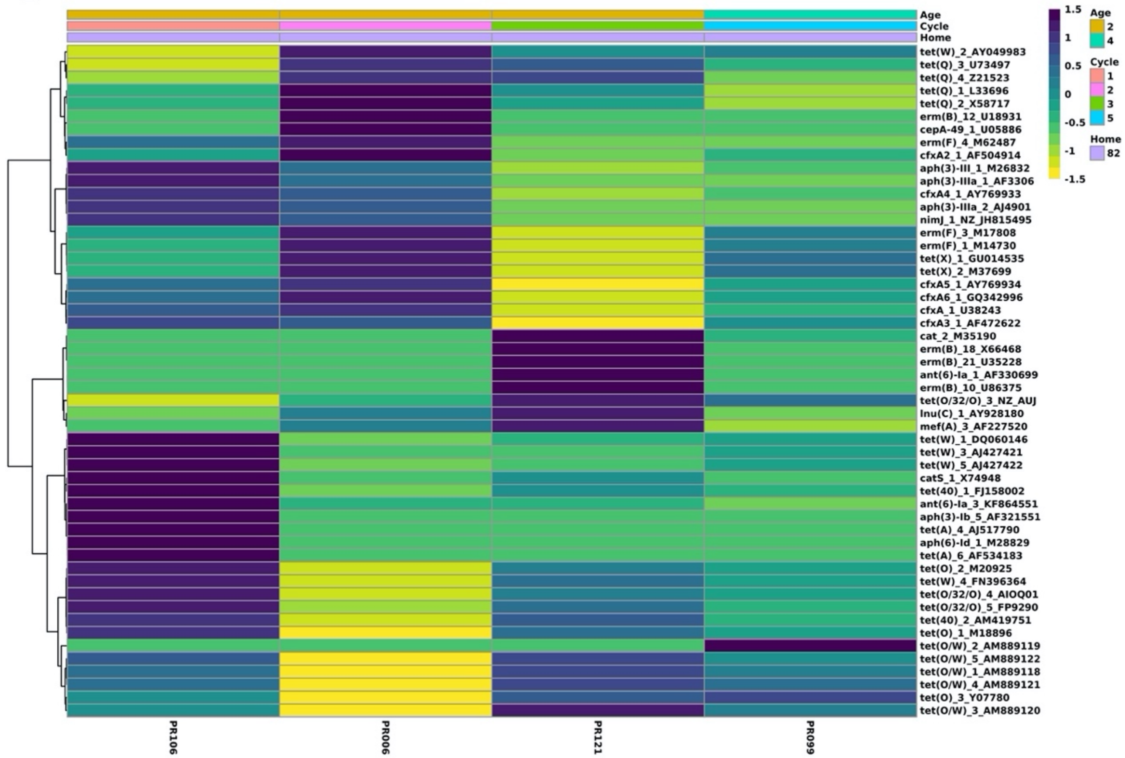
Participant 60



B

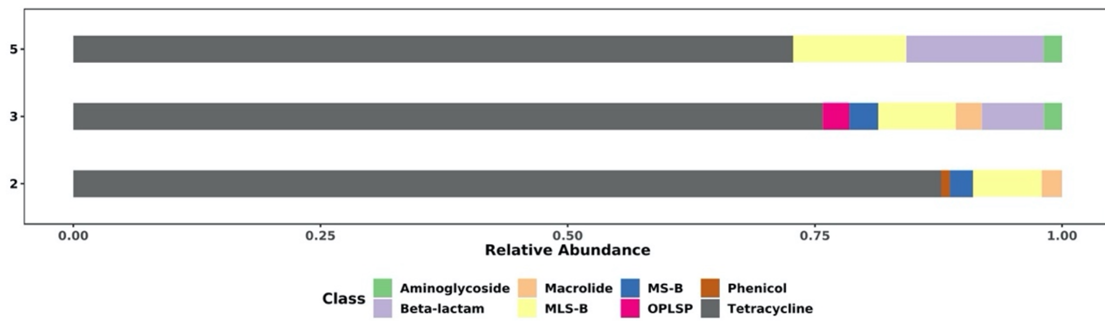



A
Participant 82
5
3
2
1
0.00
0.25
0.50
0.75
1.00
Relative Abundance
Class
Aminoglycoside
Beta-lactam
Lincosamide
Macrolide
MLS-B
Nitroimidazole
Phenicol
Tetracycline

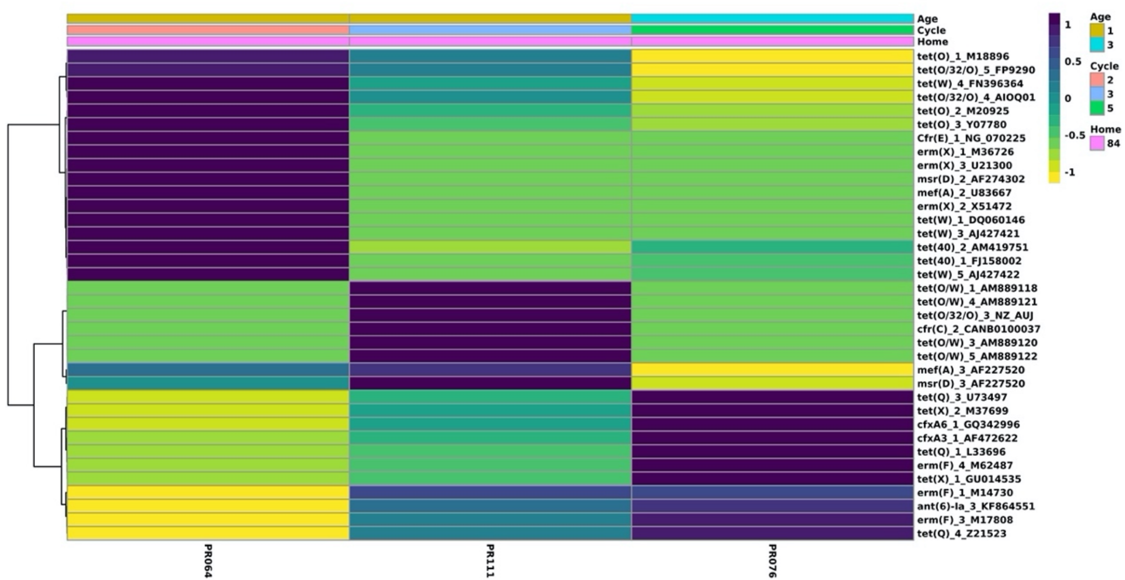

B
Age
Cycle
Home
tet(W)_2_AY049983
tet(Q)_3_U73497
tet(Q)_4_Z21523
tet(Q)_1_L33696
tet(Q)_2_X58717
erm(B)_12_U18931
cepA-49_1_U05886
erm(F)_4_M62487
cfxA2_1_AF504914
aph(3)-III_1_M26832
aph(3)-IIIa_1_AF3306
cfxA4_1_AY769933
aph(3)-IIIa_2_AJ4901
nimJ_1_NZ_JH815495
erm(F)_3_M17808
erm(F)_1_M14730
tet(X)_1_GU014535
tet(X)_2_M37699
cfxA5_1_AY769934
cfxA6_1_GQ342996
cfxA_1_U38243
cfxA3_1_AF472622
cat_2_M35190
erm(B)_18_X66468
erm(B)_21_U35228
ant(6)-Ia_1_AF330699
erm(B)_10_U86375
tet(O/32/O)_3_NZ_AUJ
lnu(C)_1_AY928180
mef(A)_3_AF227520
tet(W)_1_DQ060146
tet(W)_3_AJ427421
tet(W)_5_AJ427422
catS_1_X74948
tet(40)_1_FJ158002
ant(6)-Ia_3_KF864551
aph(3)-Ib_5_AF321551
tet(A)_4_AJ517790
aph(6)-Id_1_M28829
tet(A)_6_AF534183
tet(O)_2_M20925
tet(W)_4_FN396364
tet(O/32/O)_4_AIOQ01
tet(O/32/O)_5_FP9290
tet(40)_2_AM419751
tet(O)_1_M18896
tet(O/W)_2_AM889119
tet(O/W)_5_AM889122
tet(O/W)_1_AM889118
tet(O/W)_4_AM889121
tet(O)_3_Y07780
tet(O/W)_3_AM889120
PR106
PR006
PR121
PR099
1.5
1
0.5
0
-0.5
-1
-1.5
Age
2
4
Cycle
1
2
3
5
Home
82

A

Participant 84

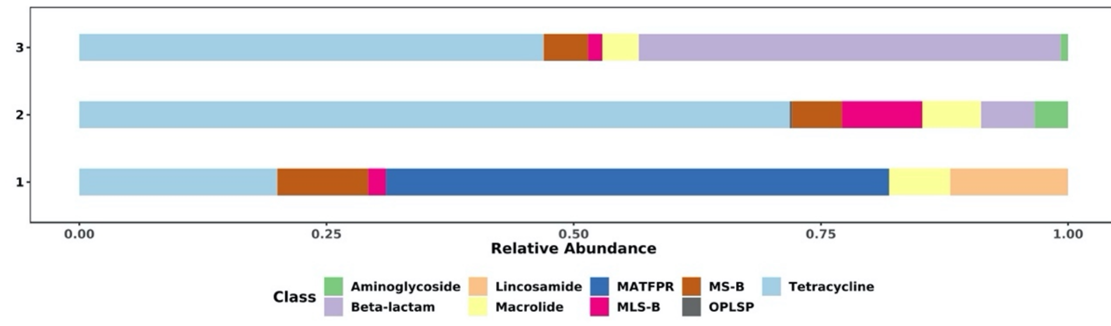


B

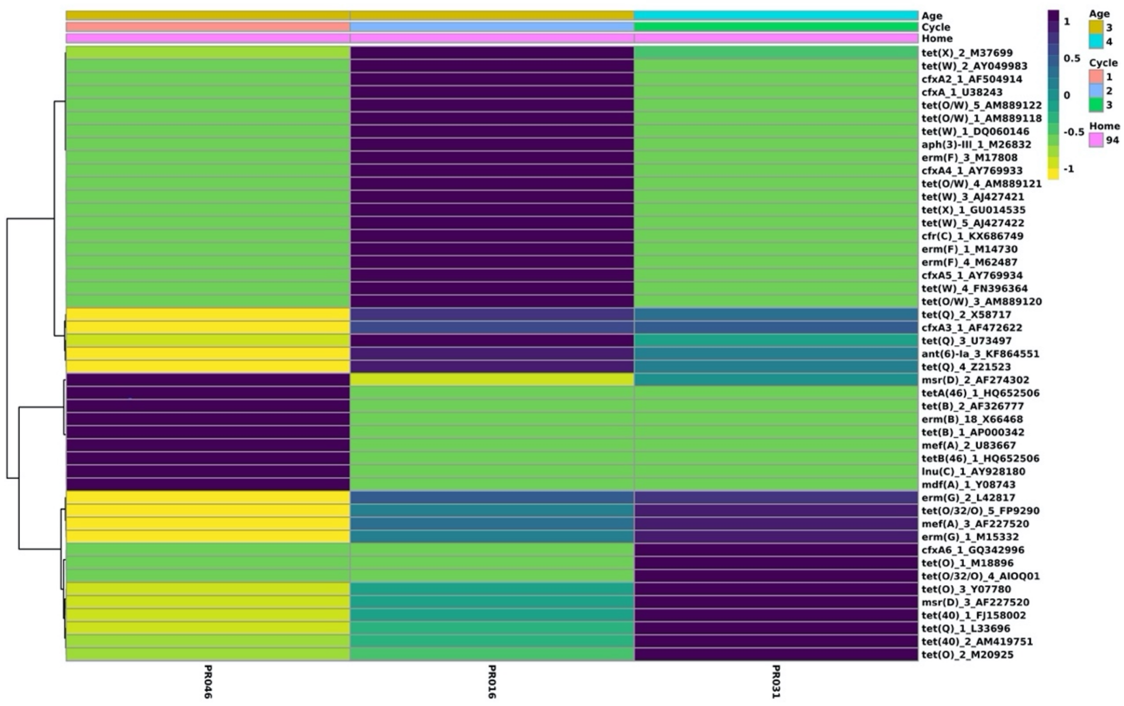


Participant 94

A

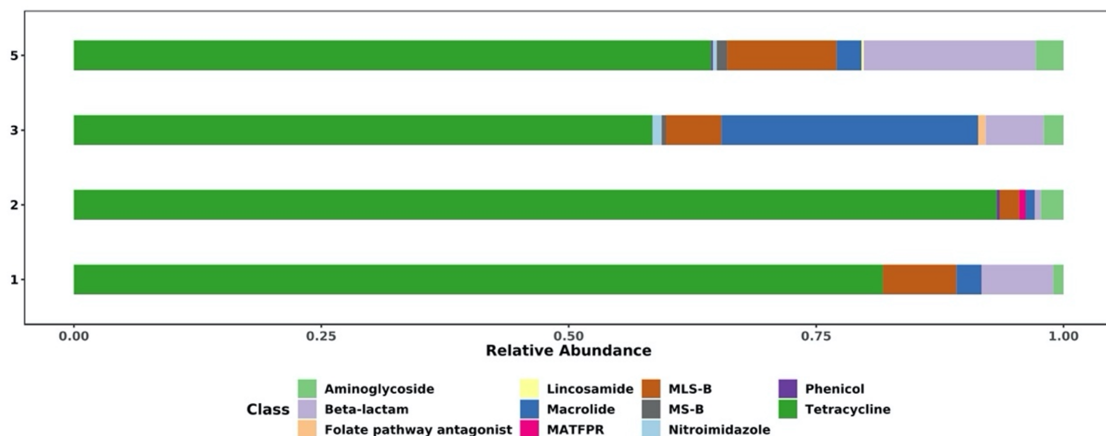


B

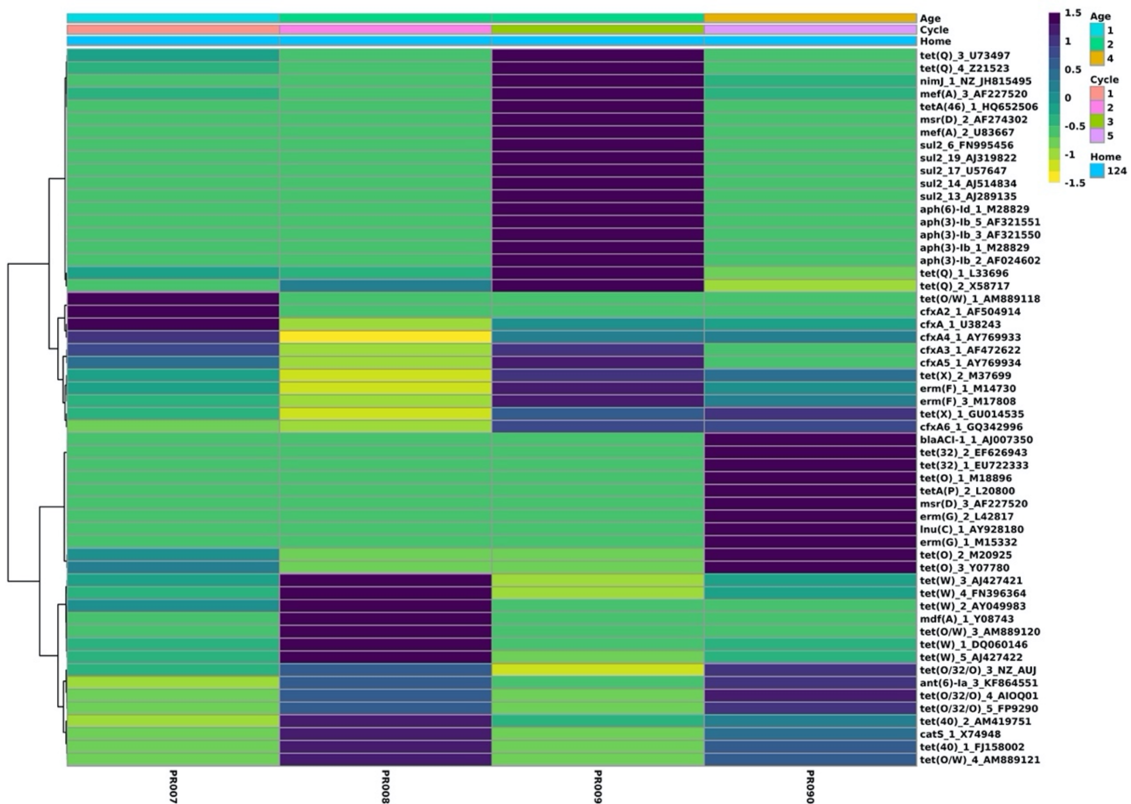


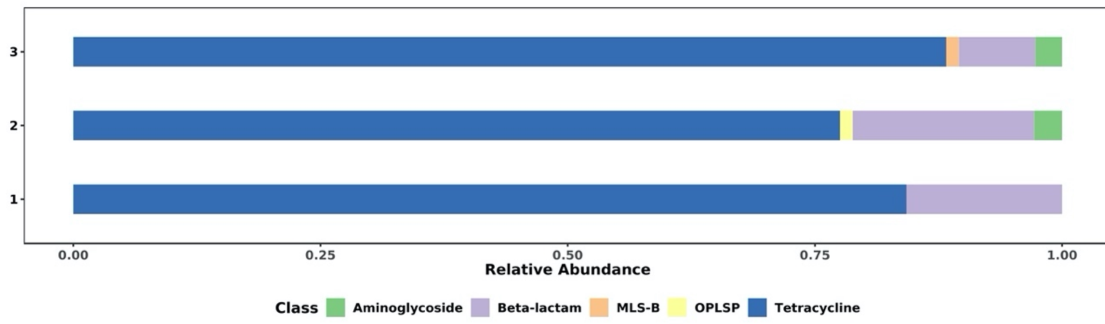
Participant 124

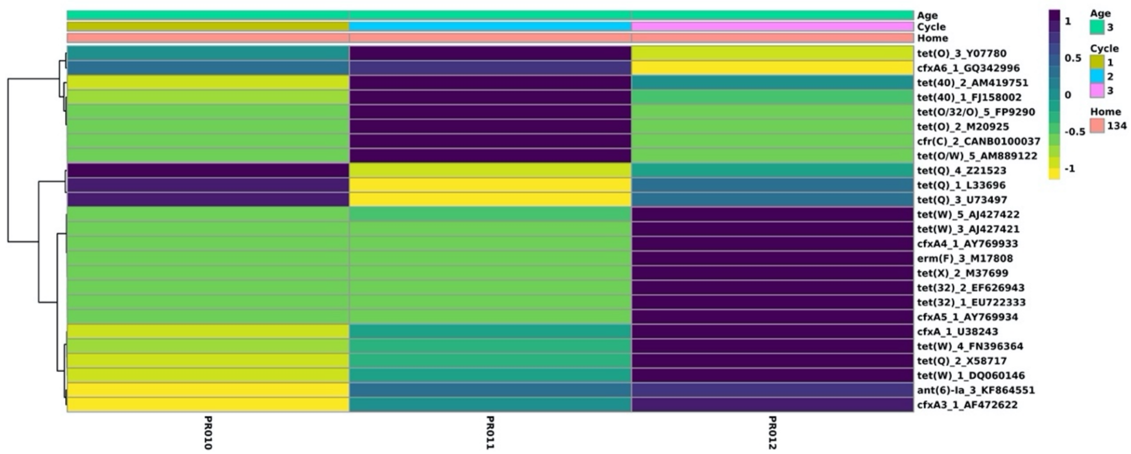
A



B

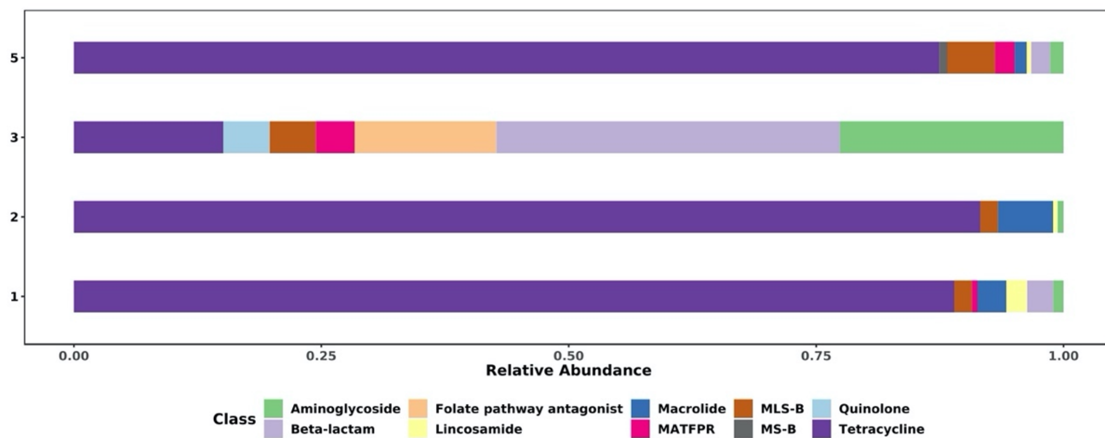



A
Participant 134
3
2
1
0.00
0.25
0.50
0.75
1.00
Relative Abundance
Class
Aminoglycoside
Beta-lactam
MLS-B
OPLSP
Tetracycline

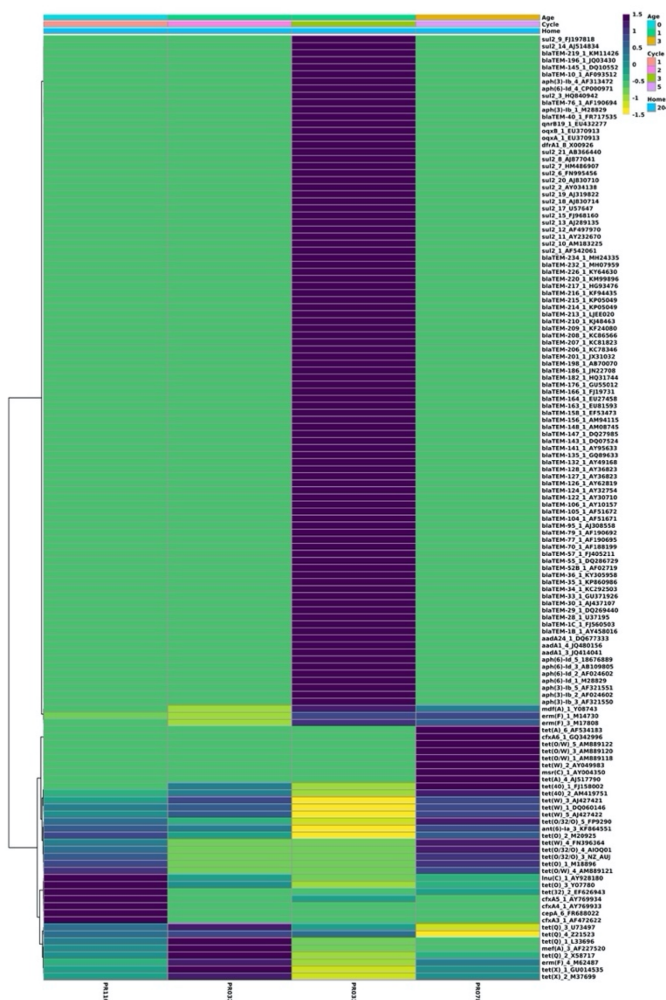

B
Age
Cycle
Home
tet(O)_3_Y07780
cfxA6_1_GQ342996
tet(40)_2_AM419751
tet(40)_1_FJ158002
tet(O/32/O)_5_FP9290
tet(O)_2_M20925
cfr(C)_2_CANB0100037
tet(O/W)_5_AM889122
tet(Q)_4_Z21523
tet(Q)_1_L33696
tet(Q)_3_U73497
tet(W)_5_AJ427422
tet(W)_3_AJ427421
cfxA4_1_AY769933
erm(F)_3_M17808
tet(X)_2_M37699
tet(32)_2_EF626943
tet(32)_1_EU722333
cfxA5_1_AY769934
cfxA_1_U38243
tet(W)_4_FN396364
tet(Q)_2_X58717
tet(W)_1_DQ060146
ant(6)-Ia_3_KF864551
cfxA3_1_AF472622
PR010
PR011
PR012
1
0.5
0
-0.5
-1
Age
3
Cycle
1
2
3
Home
134

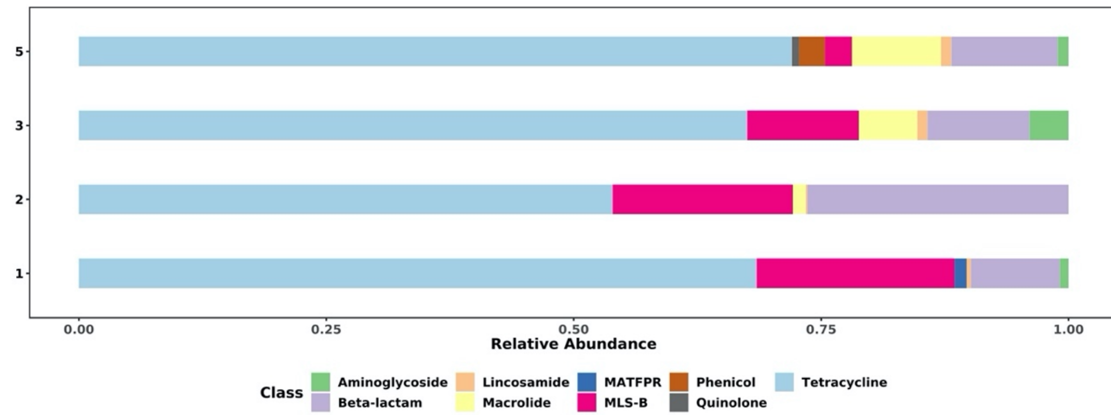
A

Participant 204



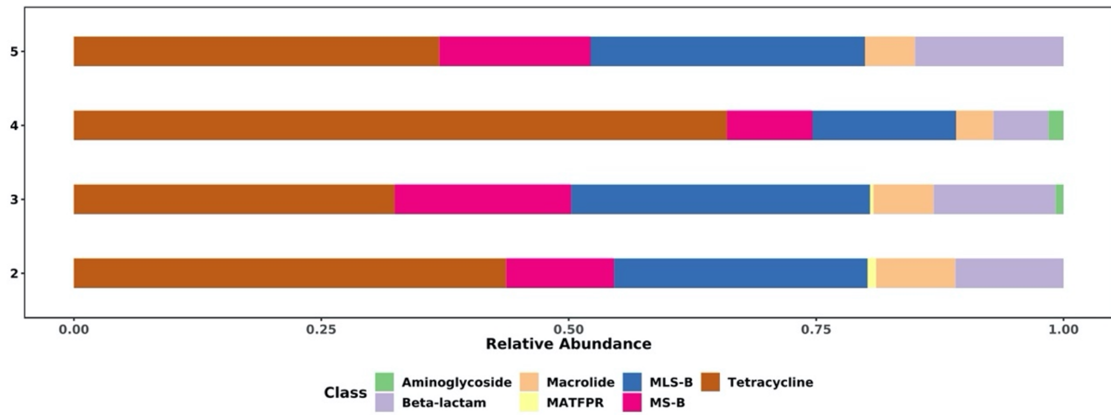
B



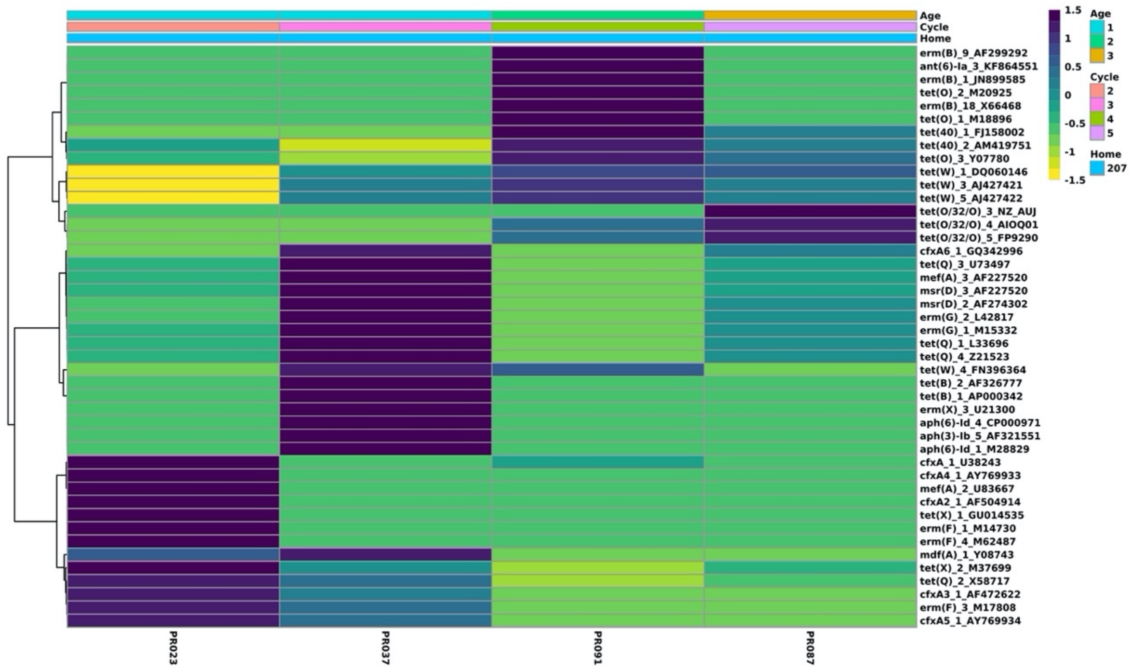

A
Participant 206
5
3
2
1
0.00
0.25
0.50
0.75
1.00
Relative Abundance
Class
Aminoglycoside
Beta-lactam
Lincosamide
Macrolide
MATFPR
MLS-B
Phenicol
Quinolone
Tetracycline

B
Age
Cycle
Home
tet(W)_3_AJ427421
tet(W)_1_DQ060146
tet(W)_4_FN396364
tet(W)_5_AJ427422
tet(O/W)_4_AM889121
tet(O/W)_5_AM889122
tet(W)_2_AY049983
erm(X)_1_M36726
tet(O/W)_3_AM889120
tet(O/W)_1_AM889118
erm(X)_3_U21300
mdf(A)_1_Y08743
erm(X)_2_X51472
erm(F)_1_M14730
erm(F)_3_M17808
cfxA6_1_GQ342996
erm(G)_1_M15332
erm(G)_2_L42817
ant(6)-Ia_3_KF864551
aph(6)-Id_1_M28829
erm(F)_4_M62487
tet(X)_1_GU014535
tet(X)_2_M37699
tet(Q)_2_X58717
cfxA3_1_AF472622
cfxA_1_U38243
tet(Q)_1_L33696
cfxA4_1_AY769933
cfxA5_1_AY769934
tet(Q)_3_U73497
tet(Q)_4_Z21523
tet(40)_1_FJ158002
tet(40)_2_AM419751
tet(O)_2_M20925
tet(O)_3_Y07780
tet(O)_1_M18896
tet(O/32/O)_5_FP9290
tet(C)_2_AY046276
tet(O/32/O)_4_AIOQ01
catS_1_X74948
tet(O/32/O)_3_NZ_AUJ
tet(32)_1_EU722333
tet(C)_3_AF055345
qnrB5_1_DQ303919
qnrB19_1_EU432277
erm(B)_12_U18931
cat_2_M35190
mef(A)_3_AF227520
lnu(C)_1_AY928180
cfxA2_1_AF504914
1.5
1
0.5
0
-0.5
-1
-1.5
Age
2
3
4
Cycle
1
2
3
5
Home
206
PR067
PR057
PR052
PR086

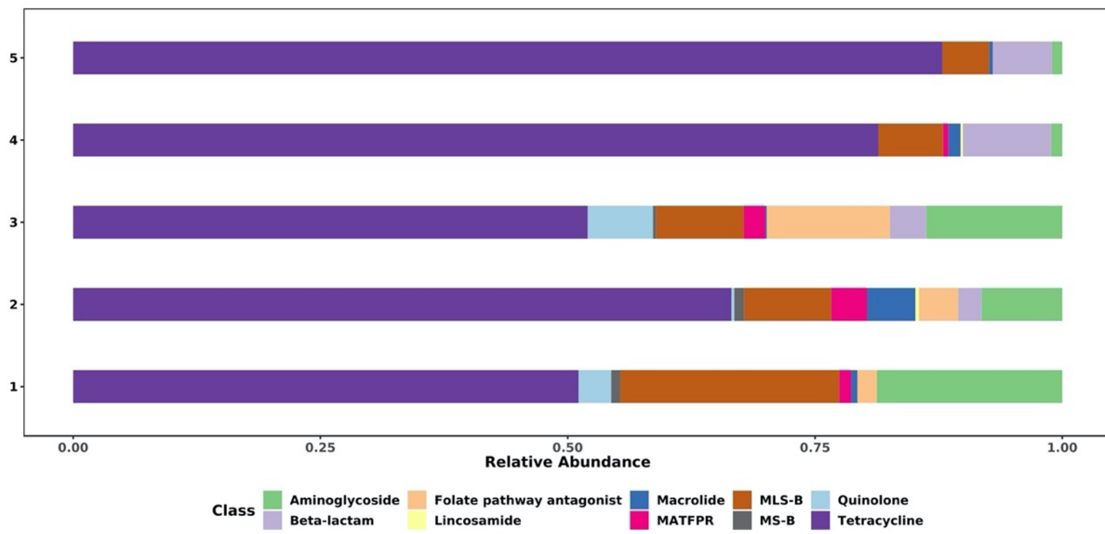
A



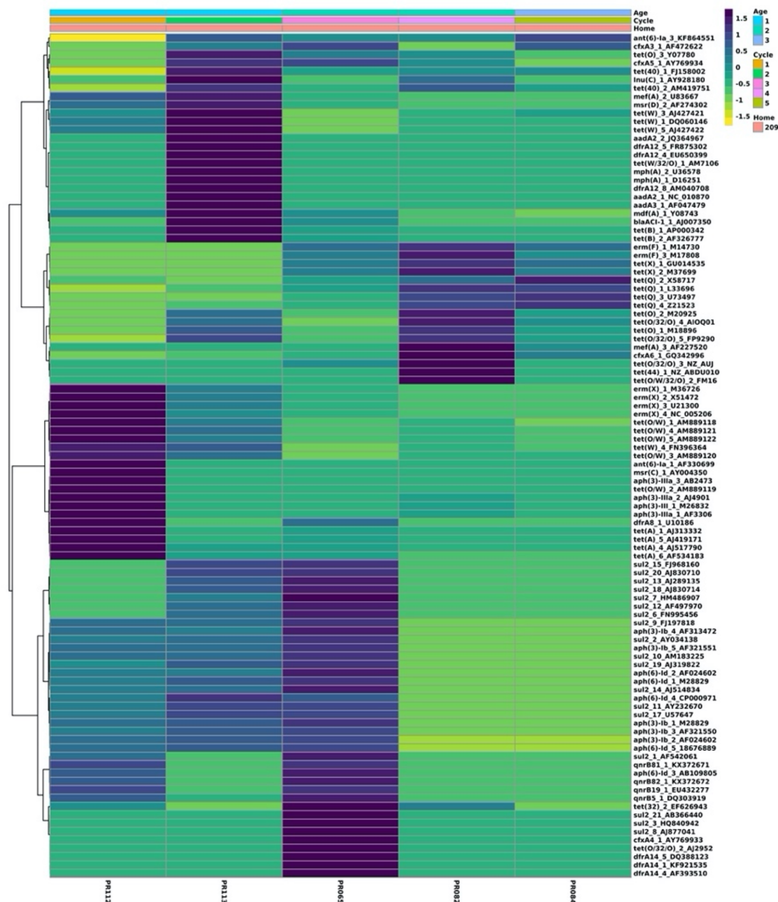
B

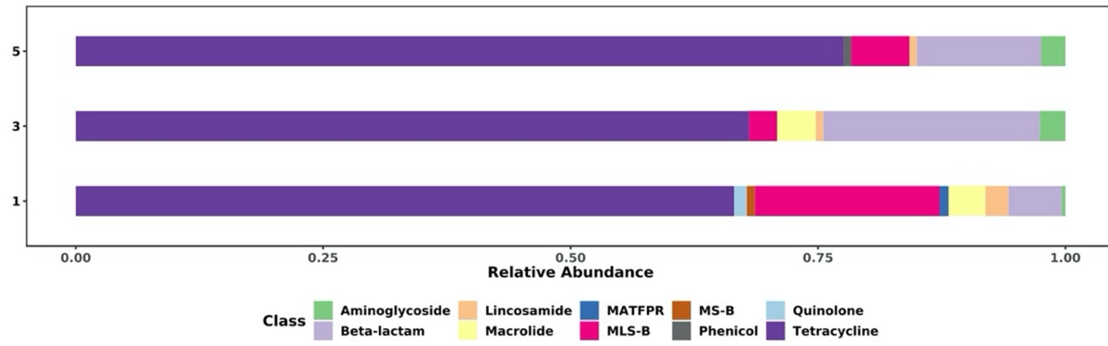


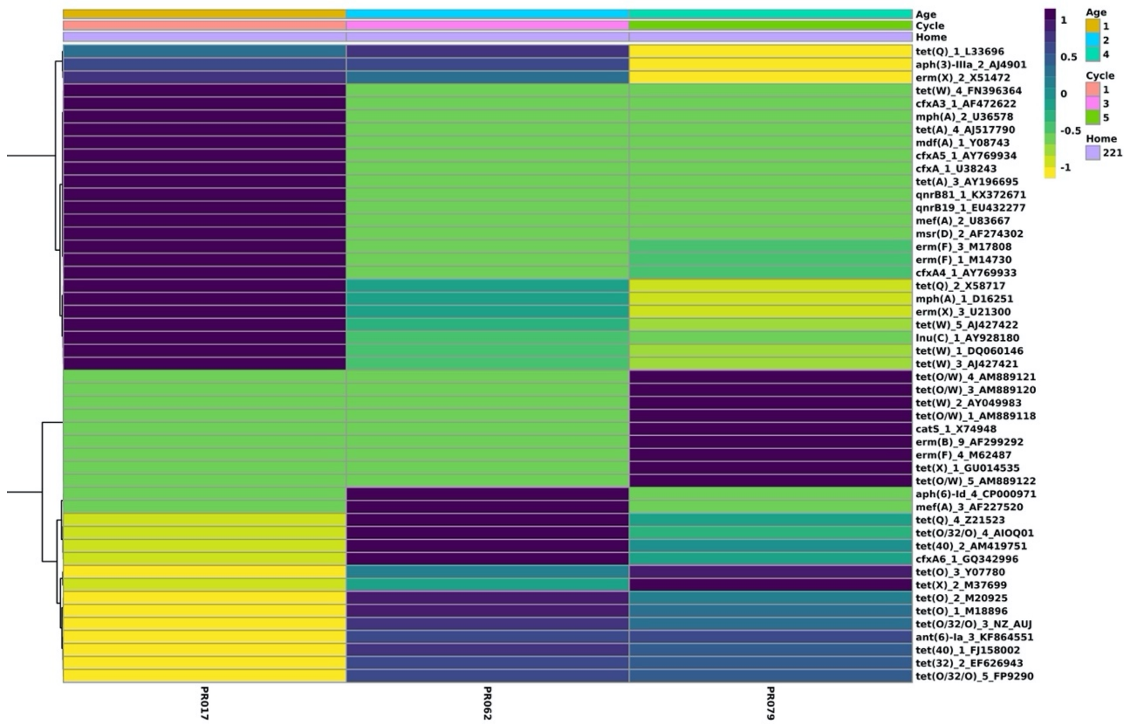
A

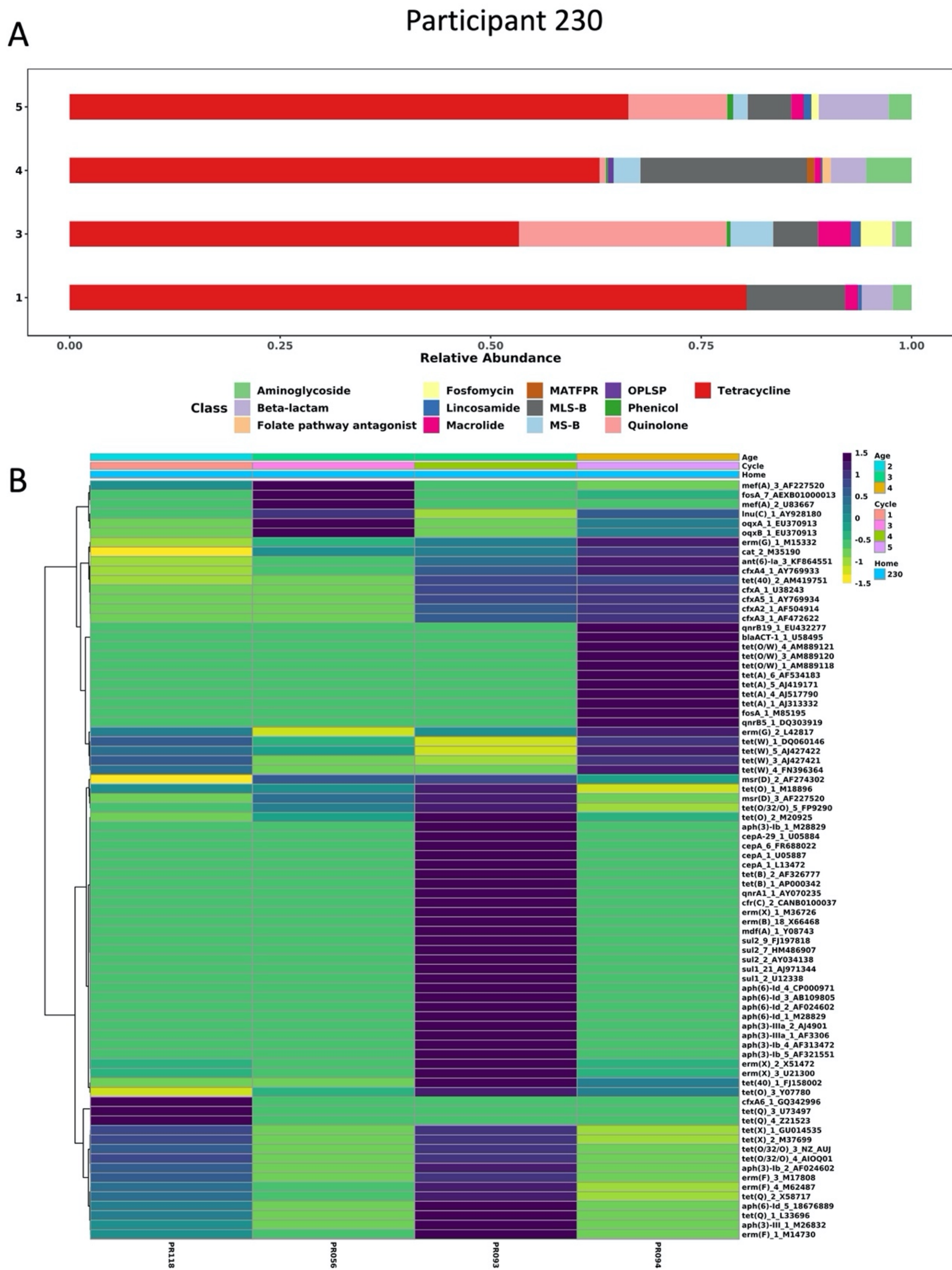


B




A
Participant 221
5
3
1
0.00
0.25
0.50
0.75
1.00
Relative Abundance
Class
Aminoglycoside
Beta-lactam
Lincosamide
Macrolide
MATFPR
MLS-B
MS-B
Phenicol
Quinolone
Tetracycline

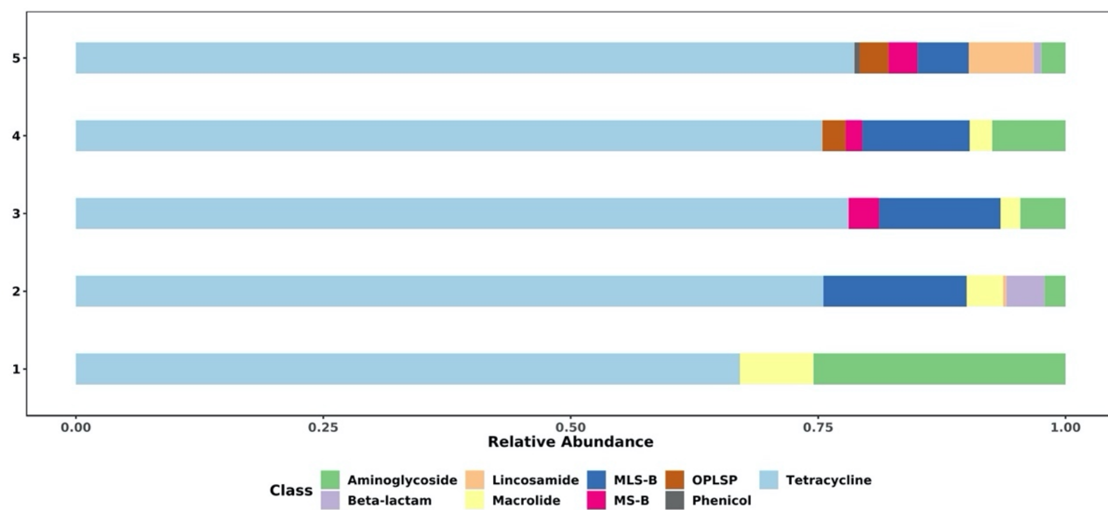

B
Age
Cycle
Home
tet(Q)_1_L33696
aph(3)-IIIa_2_AJ4901
erm(X)_2_X51472
tet(W)_4_FN396364
cfxA3_1_AF472622
mph(A)_2_U36578
tet(A)_4_AJ517790
mdf(A)_1_Y08743
cfxA5_1_AY769934
cfxA_1_U38243
tet(A)_3_AY196695
qnrB81_1_KX372671
qnrB19_1_EU432277
mef(A)_2_U83667
msr(D)_2_AF274302
erm(F)_3_M17808
erm(F)_1_M14730
cfxA4_1_AY769933
tet(Q)_2_X58717
mph(A)_1_D16251
erm(X)_3_U21300
tet(W)_5_AJ427422
lnu(C)_1_AY928180
tet(W)_1_DQ060146
tet(W)_3_AJ427421
tet(O/W)_4_AM889121
tet(O/W)_3_AM889120
tet(W)_2_AY049983
tet(O/W)_1_AM889118
catS_1_X74948
erm(B)_9_AF299292
erm(F)_4_M62487
tet(X)_1_GU014535
tet(O/W)_5_AM889122
aph(6)-Id_4_CP000971
mef(A)_3_AF227520
tet(Q)_4_Z21523
tet(O/32/O)_4_AIOQ01
tet(40)_2_AM419751
cfxA6_1_GQ342996
tet(O)_3_Y07780
tet(X)_2_M37699
tet(O)_2_M20925
tet(O)_1_M18896
tet(O/32/O)_3_NZ_AUJ
ant(6)-Ia_3_KF864551
tet(40)_1_FJ158002
tet(32)_2_EF626943
tet(O/32/O)_5_FP9290
PR017
PR062
PR079
1
0.5
0
-0.5
-1
Age
1
2
4
Cycle
1
3
5
Home
221



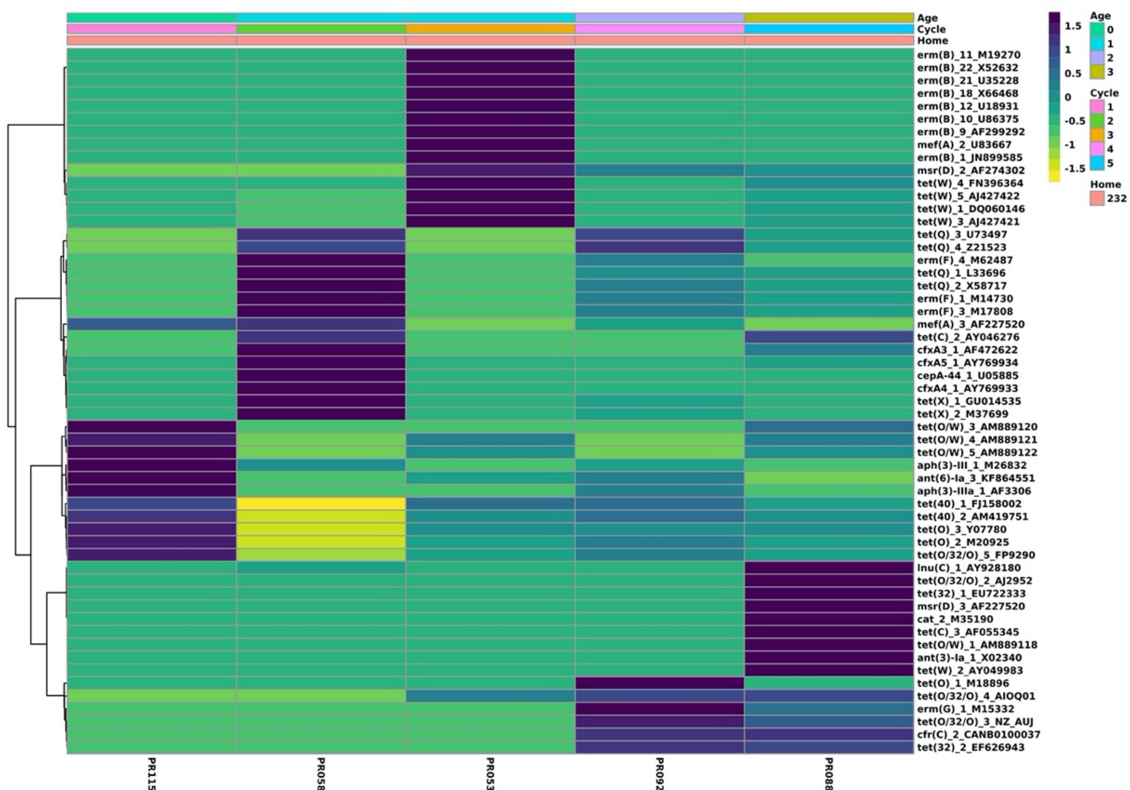
A
Participant 230
5
4
3
1
0.00
0.25
0.50
0.75
1.00
Relative Abundance
Class
Aminoglycoside
Beta-lactam
Folate pathway antagonist
Fosfomycin
Lincosamide
Macrolide
MATFPR
MLS-B
MS-B
OPLSP
Phenicol
Quinolone
Tetracycline
B
Age
Cycle
Home
mef(A)_3_AF227520
fosA_7_AEXB01000013
mef(A)_2_U83667
lnu(C)_1_AY928180
oqxA_1_EU370913
oqxB_1_EU370913
erm(G)_1_M15332
cat_2_M35190
ant(6)-Ia_3_KF864551
cfxA4_1_AY769933
tet(40)_2_AM419751
cfxA_1_U38243
cfxA5_1_AY769934
cfxA2_1_AF504914
cfxA3_1_AF472622
qnrB19_1_EU432277
blaACT-1_1_U58495
tet(O/W)_4_AM889121
tet(O/W)_3_AM889120
tet(O/W)_1_AM889118
tet(A)_6_AF534183
tet(A)_5_AJ419171
tet(A)_4_AJ517790
tet(A)_1_AJ313332
fosA_1_M85195
qnrB5_1_DQ303919
erm(G)_2_L42817
tet(W)_1_DQ060146
tet(W)_5_AJ427422
tet(W)_3_AJ427421
tet(W)_4_FN396364
msr(D)_2_AF274302
tet(O)_1_M18896
msr(D)_3_AF227520
tet(O/32/O)_5_FP9290
tet(O)_2_M20925
aph(3)-Ib_1_M28829
cepA-29_1_U05884
cepA_6_FR688022
cepA_1_U05887
cepA_1_L13472
tet(B)_2_AF326777
tet(B)_1_AP000342
qnrA1_1_AY070235
cfr(C)_2_CANB0100037
erm(X)_1_M36726
erm(B)_18_X66468
mdf(A)_1_Y08743
sul2_9_FJ197818
sul2_7_HM486907
sul2_2_AY034138
sul1_21_AJ971344
sul1_2_U12338
aph(6)-Id_4_CP000971
aph(6)-Id_3_AB109805
aph(6)-Id_2_AF024602
aph(6)-Id_1_M28829
aph(3)-IIIa_2_AJ4901
aph(3)-IIIa_1_AF3306
aph(3)-Ib_4_AF313472
aph(3)-Ib_5_AF321551
erm(X)_2_X51472
erm(X)_3_U21300
tet(40)_1_FJ158002
tet(O)_3_Y07780
cfxA6_1_GQ342996
tet(Q)_3_U73497
tet(Q)_4_Z21523
tet(X)_1_GU014535
tet(X)_2_M37699
tet(O/32/O)_3_NZ_AUJ
tet(O/32/O)_4_AIOQ01
aph(3)-Ib_2_AF024602
erm(F)_3_M17808
erm(F)_4_M62487
tet(Q)_2_X58717
aph(6)-Id_5_18676889
tet(Q)_1_L33696
aph(3)-III_1_M26832
erm(F)_1_M14730
1.5
1
0.5
0
-0.5
-1
-1.5
Age
2
3
4
Cycle
1
3
4
5
Home
230
PR118
PR056
PR093
PR094

A

Participant 232

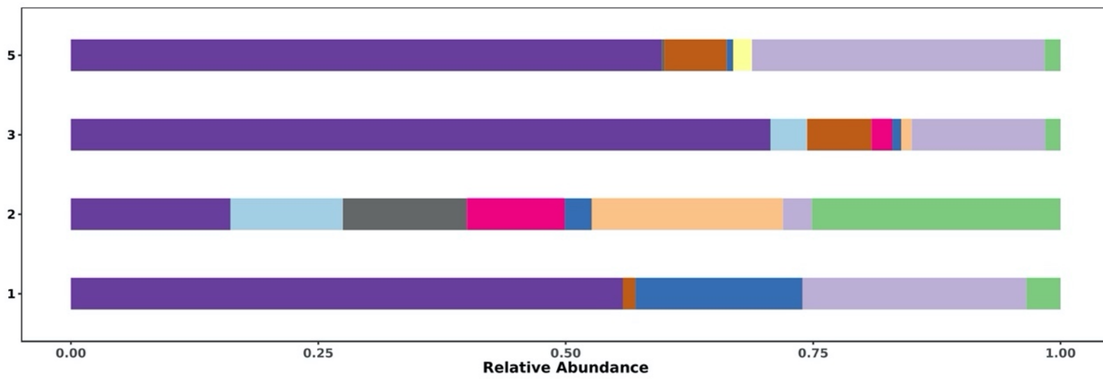


B



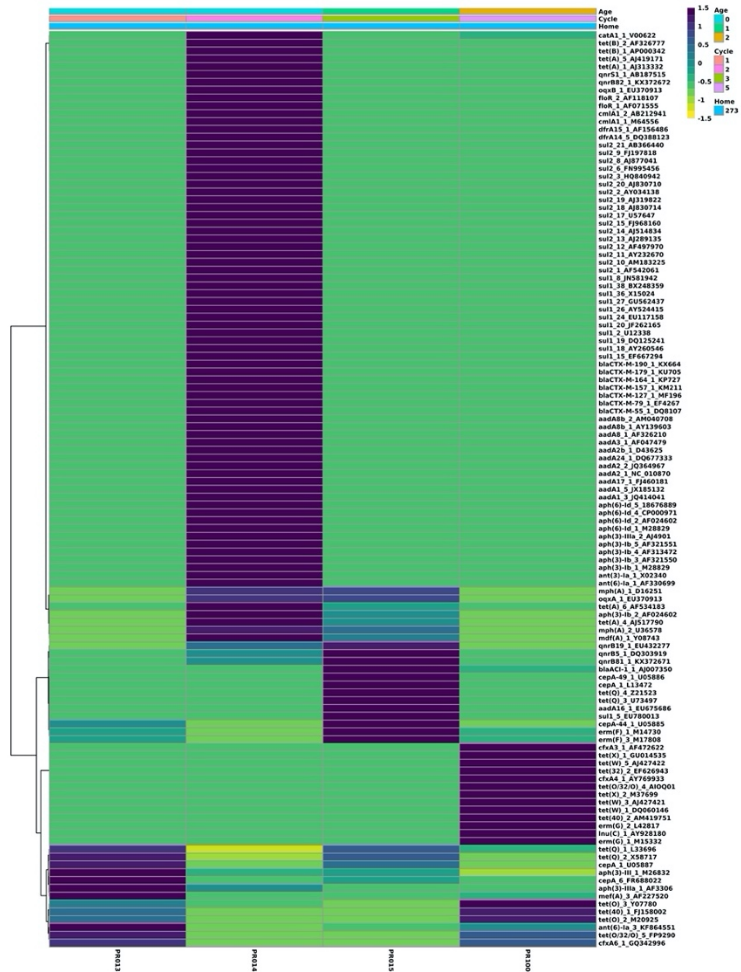
A

Participant 273



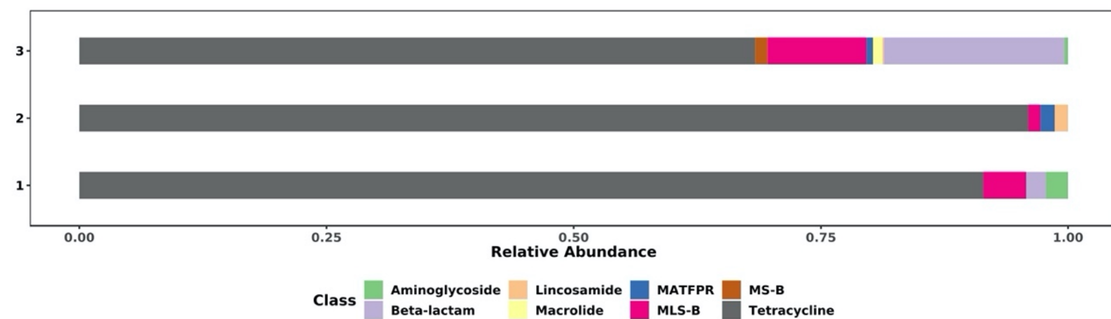
Class: Aminoglycoside, Beta-lactam, Folate pathway antagonist, Lincosamide, Macrolide, MATFPR, MLS-B, Phenicol, Quinolone, Tetracycline

B

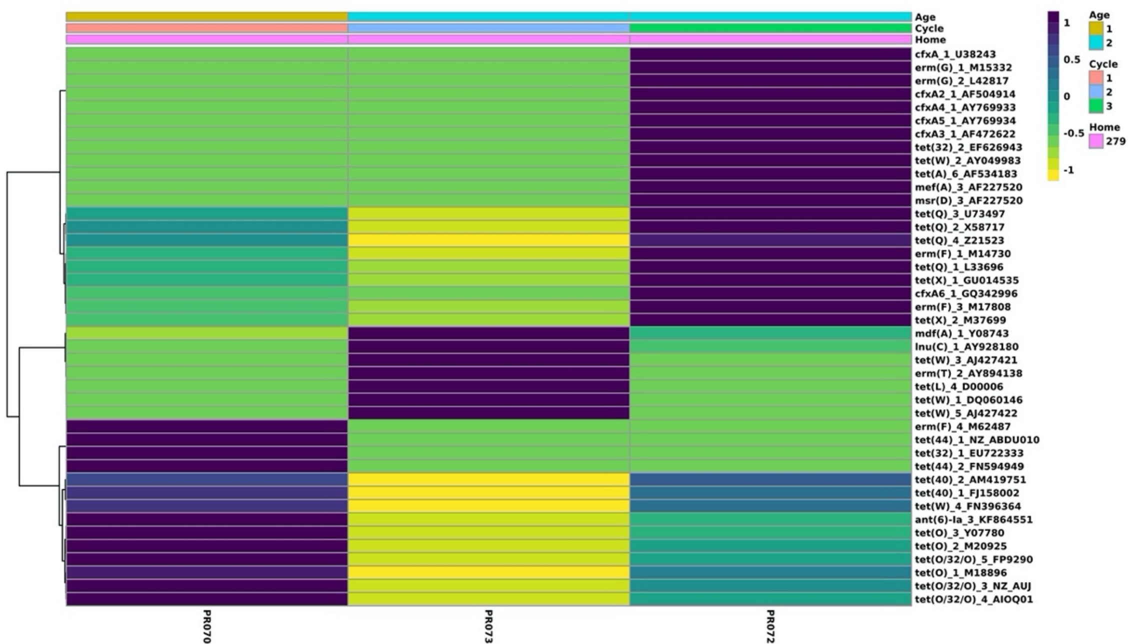


A

Participant 279

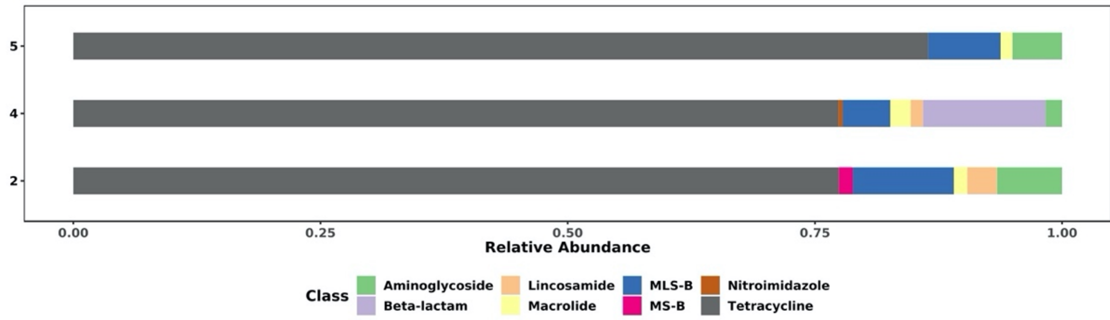


B

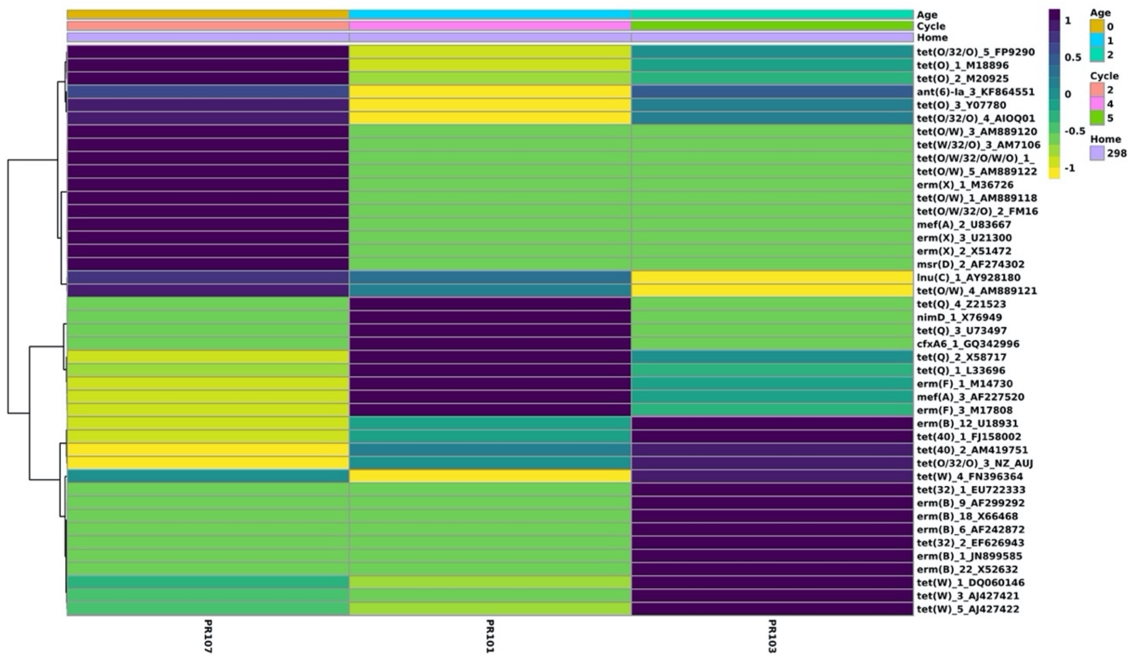


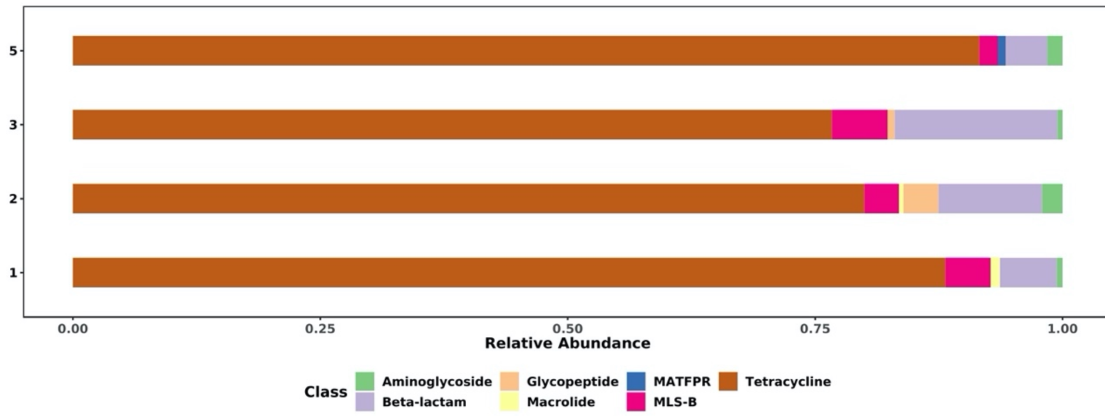
A

Participant 298

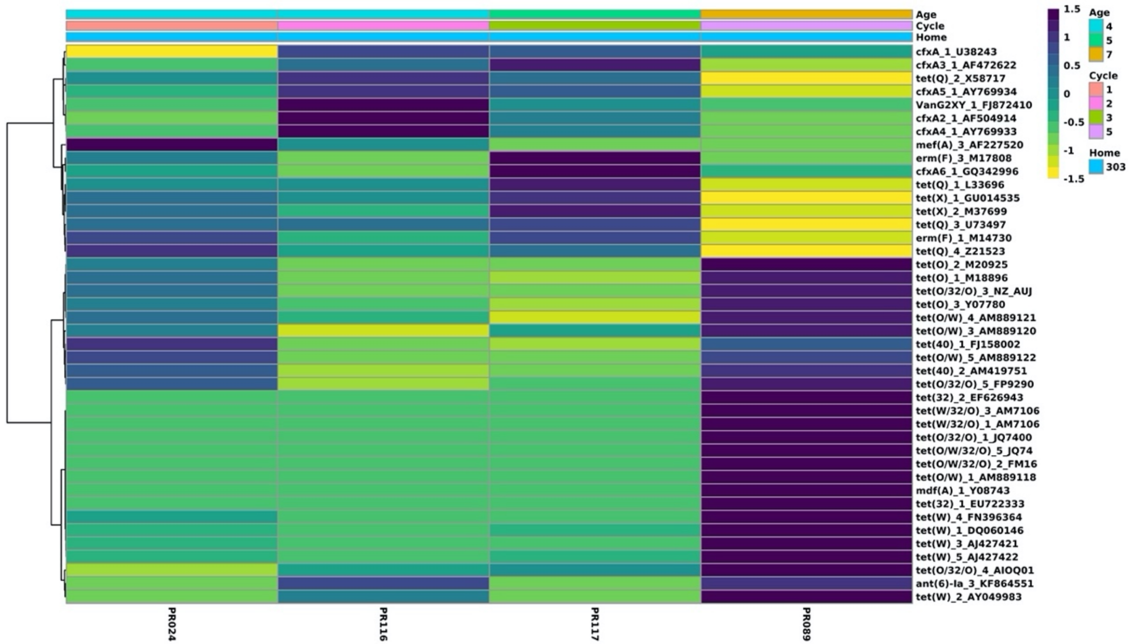


B





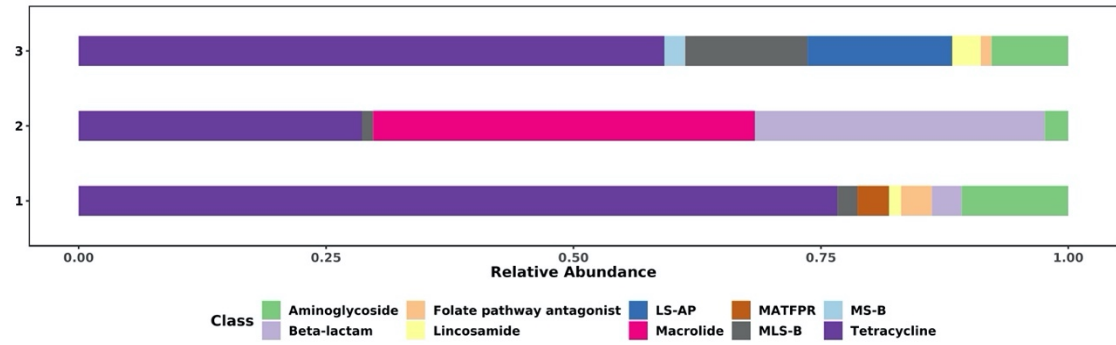
A
Participant 303
5
3
2
1
0.00
0.25
0.50
0.75
1.00
Relative Abundance
Class
Aminoglycoside
Beta-lactam
Glycopeptide
Macrolide
MATFPR
MLS-B
Tetracycline



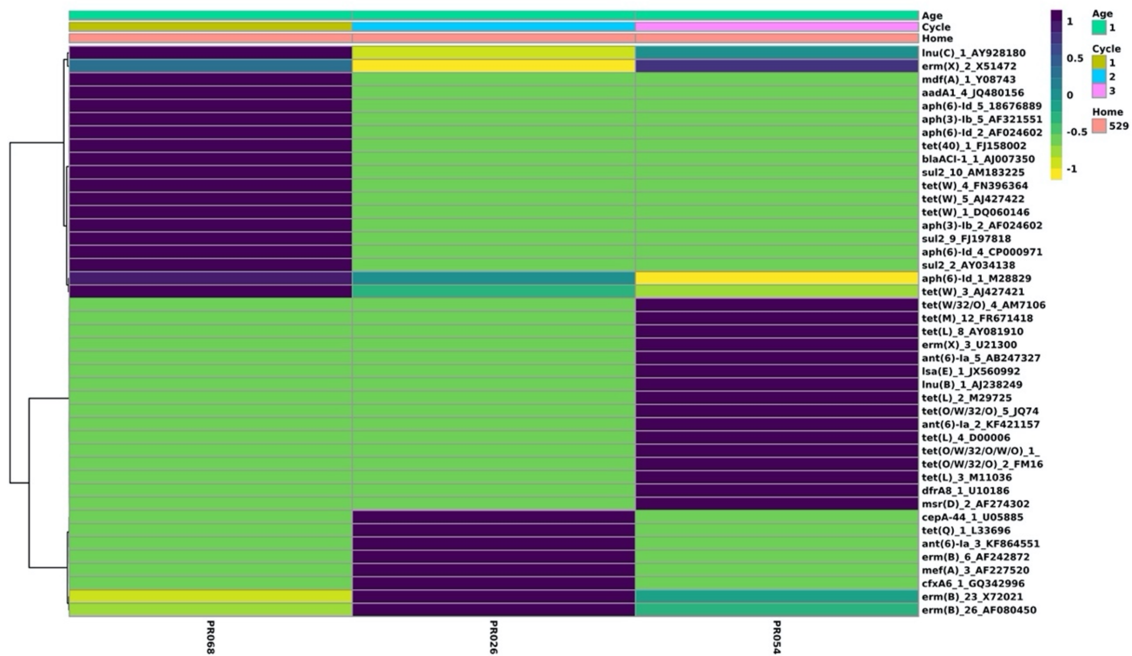
B
Age
Cycle
Home
cfxA_1_U38243
cfxA3_1_AF472622
tet(Q)_2_X58717
cfxA5_1_AY769934
VanG2XY_1_FJ872410
cfxA2_1_AF504914
cfxA4_1_AY769933
mef(A)_3_AF227520
erm(F)_3_M17808
cfxA6_1_GQ342996
tet(Q)_1_L33696
tet(X)_1_GU014535
tet(X)_2_M37699
tet(Q)_3_U73497
erm(F)_1_M14730
tet(Q)_4_Z21523
tet(O)_2_M20925
tet(O)_1_M18896
tet(O/32/O)_3_NZ_AUJ
tet(O)_3_Y07780
tet(O/W)_4_AM889121
tet(O/W)_3_AM889120
tet(40)_1_FJ158002
tet(O/W)_5_AM889122
tet(40)_2_AM419751
tet(O/32/O)_5_FP9290
tet(32)_2_EF626943
tet(W/32/O)_3_AM7106
tet(W/32/O)_1_AM7106
tet(O/32/O)_1_JQ7400
tet(O/W/32/O)_5_JQ74
tet(O/W/32/O)_2_FM16
tet(O/W)_1_AM889118
mdf(A)_1_Y08743
tet(32)_1_EU722333
tet(W)_4_FN396364
tet(W)_1_DQ060146
tet(W)_3_AJ427421
tet(W)_5_AJ427422
tet(O/32/O)_4_AIOQ01
ant(6)-Ia_3_KF864551
tet(W)_2_AY049983
PR024
PR116
PR117
PR089
1.5
1
0.5
0
-0.5
-1
-1.5
Age
4
5
7
Cycle
1
2
3
5
Home
303

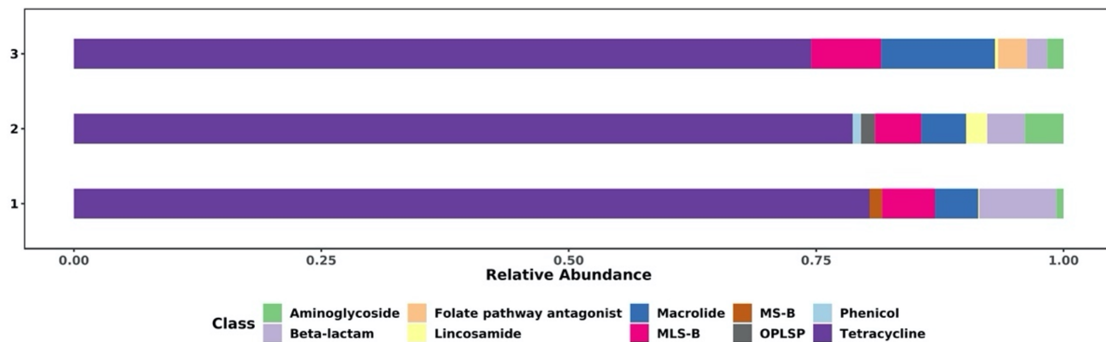
A

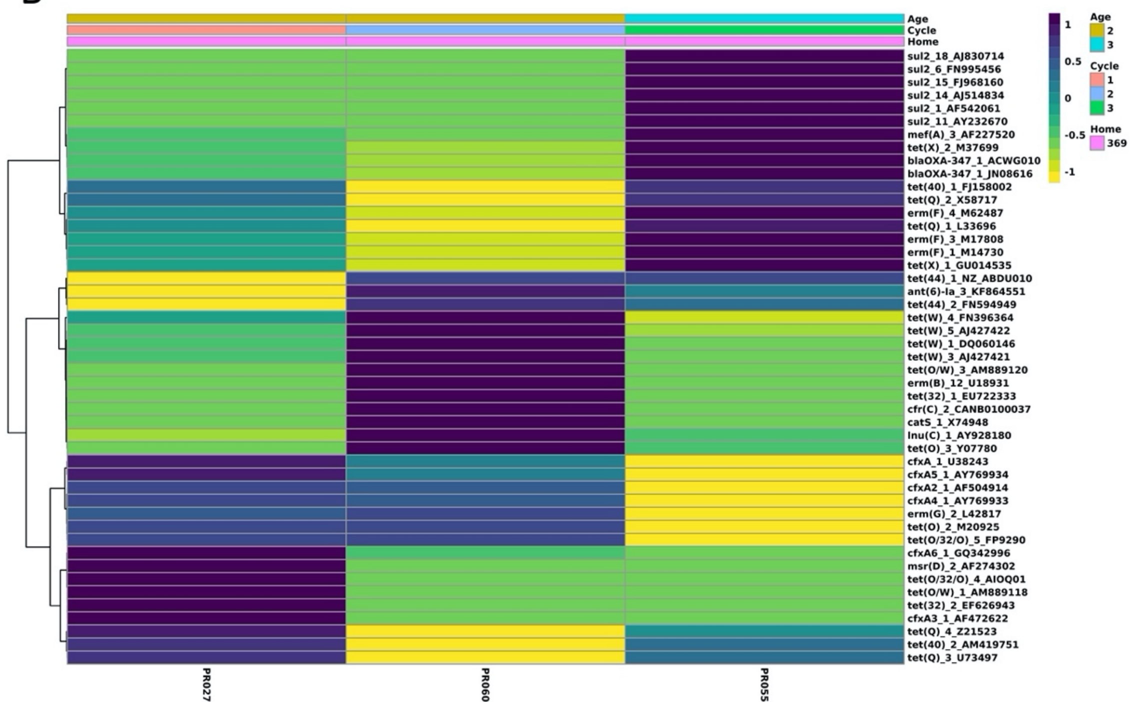
Participant 329



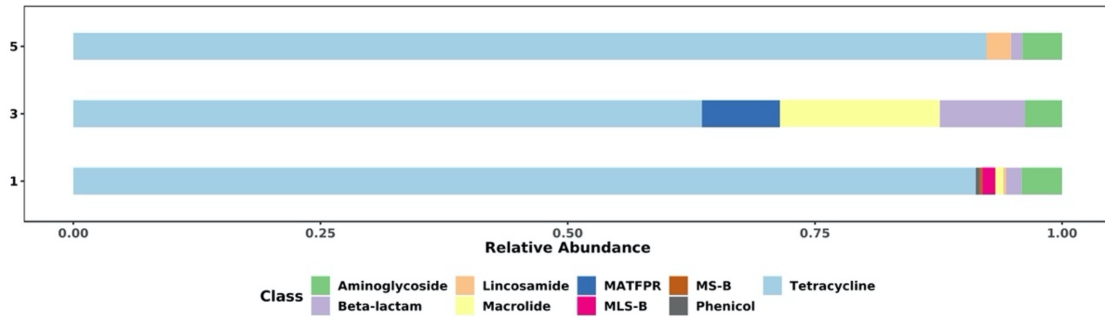
B



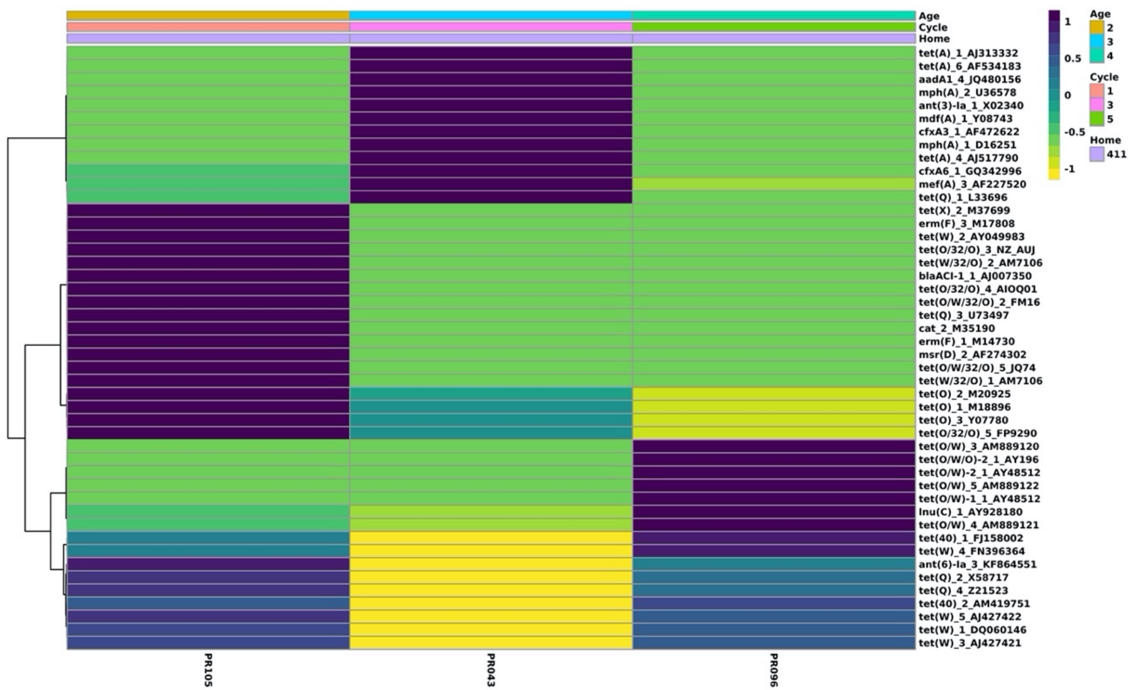

A
Participant 369
3
2
1
0.00
0.25
0.50
0.75
1.00
Relative Abundance
Class
Aminoglycoside
Beta-lactam
Folate pathway antagonist
Lincosamide
Macrolide
MLS-B
MS-B
OPLSP
Phenicol
Tetracycline


B
Age
Cycle
Home
sul2_18_AJ830714
sul2_6_FN995456
sul2_15_FJ968160
sul2_14_AJ514834
sul2_1_AF542061
sul2_11_AY232670
mef(A)_3_AF227520
tet(X)_2_M37699
blaOXA-347_1_ACWG010
blaOXA-347_1_JN08616
tet(40)_1_FJ158002
tet(Q)_2_X58717
erm(F)_4_M62487
tet(Q)_1_L33696
erm(F)_3_M17808
erm(F)_1_M14730
tet(X)_1_GU014535
tet(44)_1_NZ_ABDU010
ant(6)-Ia_3_KF864551
tet(44)_2_FN594949
tet(W)_4_FN396364
tet(W)_5_AJ427422
tet(W)_1_DQ060146
tet(W)_3_AJ427421
tet(O/W)_3_AM889120
erm(B)_12_U18931
tet(32)_1_EU722333
cfr(C)_2_CANB0100037
catS_1_X74948
lnu(C)_1_AY928180
tet(O)_3_Y07780
cfxA_1_U38243
cfxA5_1_AY769934
cfxA2_1_AF504914
cfxA4_1_AY769933
erm(G)_2_L42817
tet(O)_2_M20925
tet(O/32/O)_5_FP9290
cfxA6_1_GQ342996
msr(D)_2_AF274302
tet(O/32/O)_4_AIOQ01
tet(O/W)_1_AM889118
tet(32)_2_EF626943
cfxA3_1_AF472622
tet(Q)_4_Z21523
tet(40)_2_AM419751
tet(Q)_3_U73497
PR027
PR060
PR055
1
0.5
0
-0.5
-1
Age
2
3
Cycle
1
2
3
Home
369

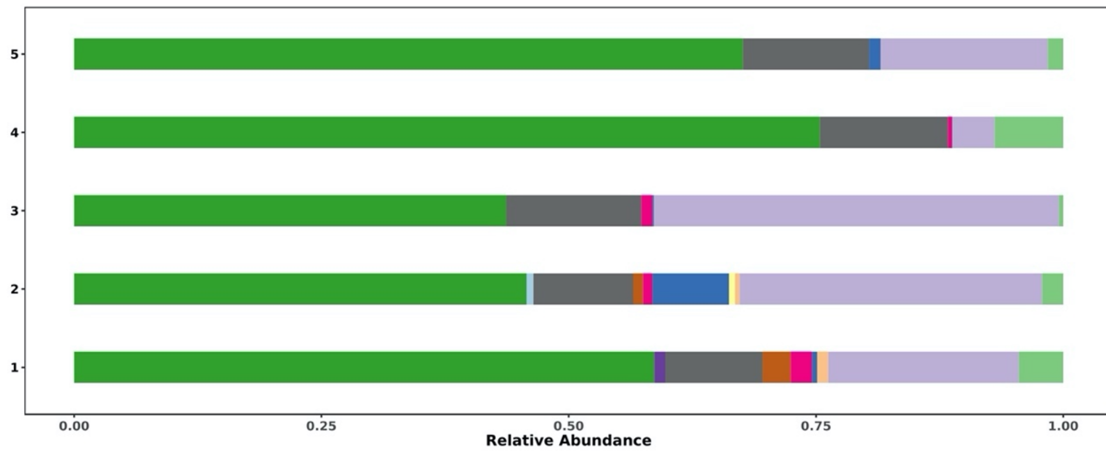
A



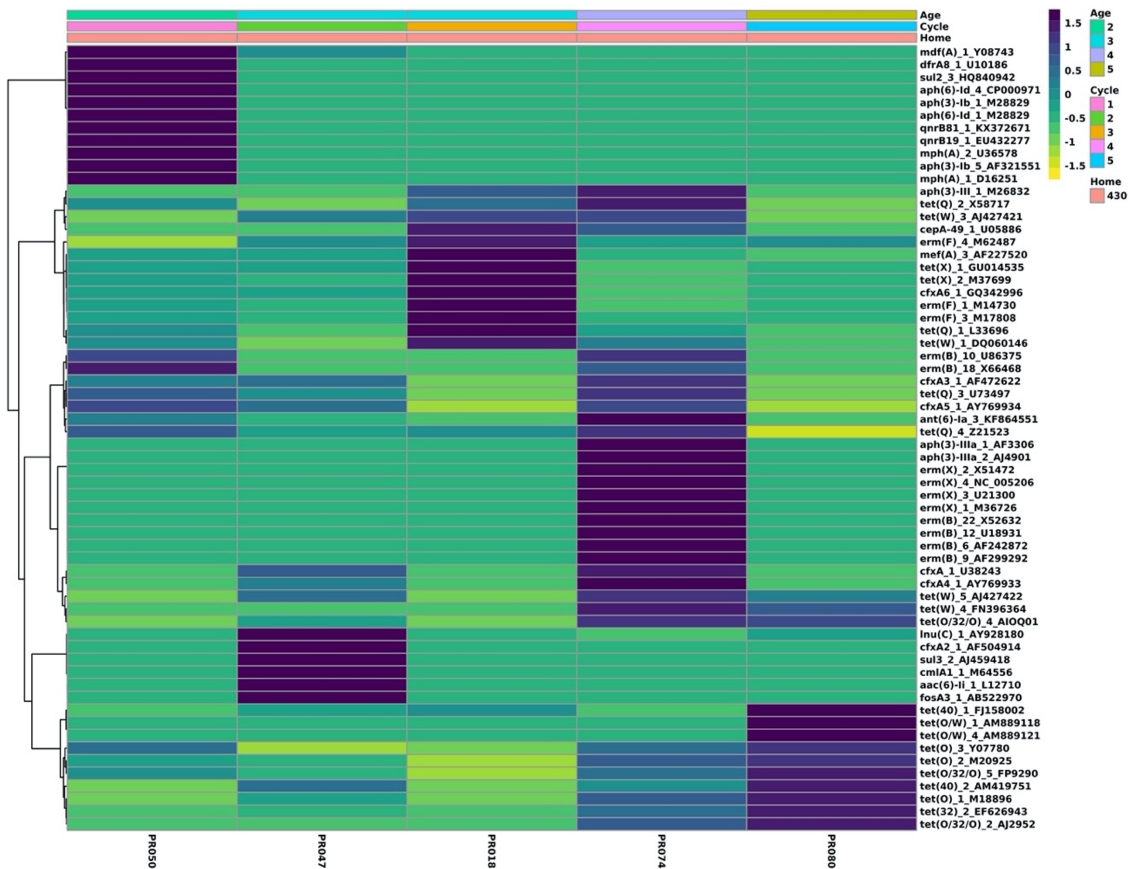
B

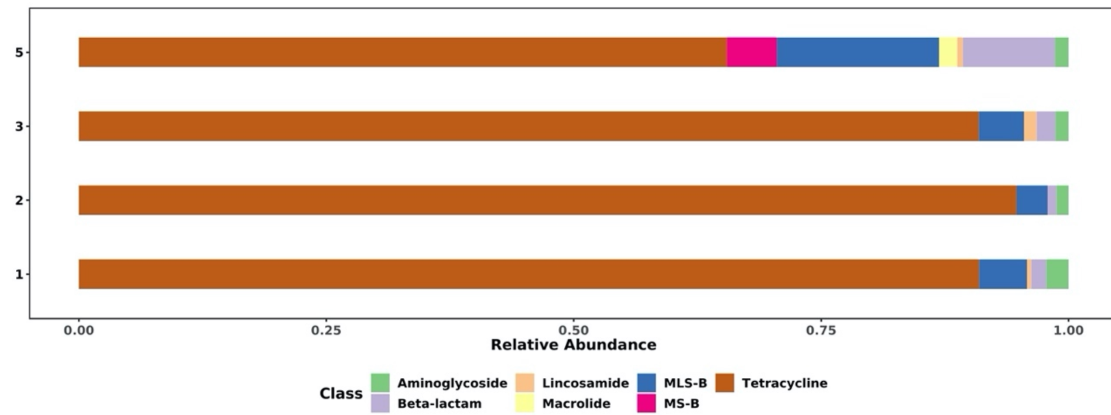


A

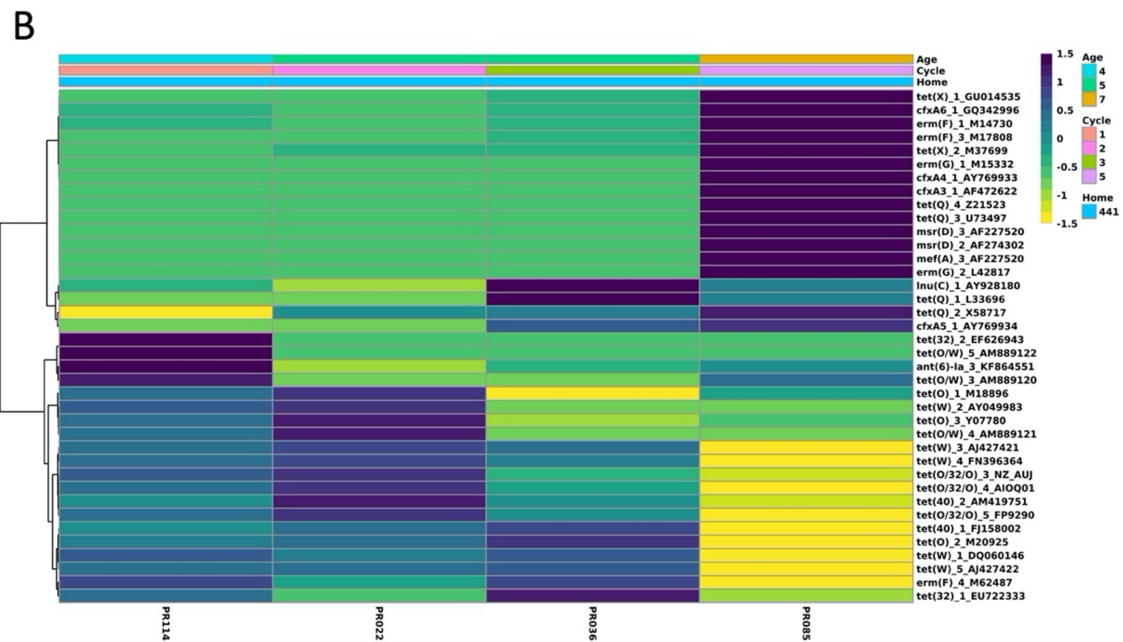


B





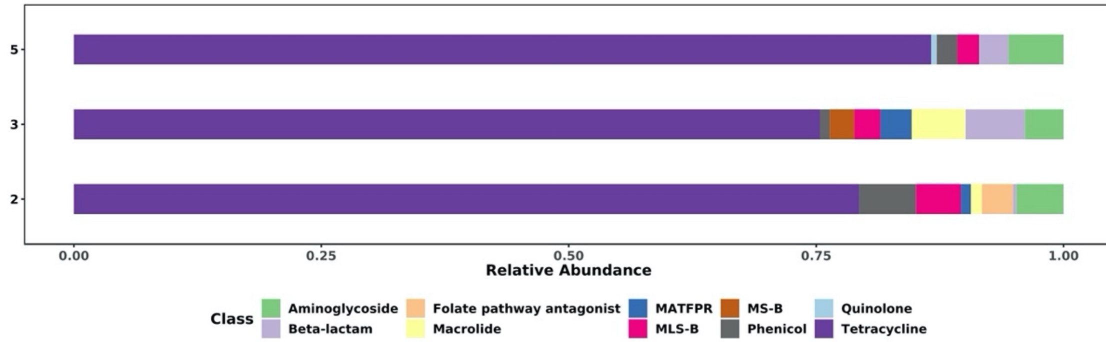
A
Participant 441
5
3
2
1
0.00
0.25
0.50
0.75
1.00
Relative Abundance
Class
Aminoglycoside
Beta-lactam
Lincosamide
Macrolide
MLS-B
MS-B
Tetracycline



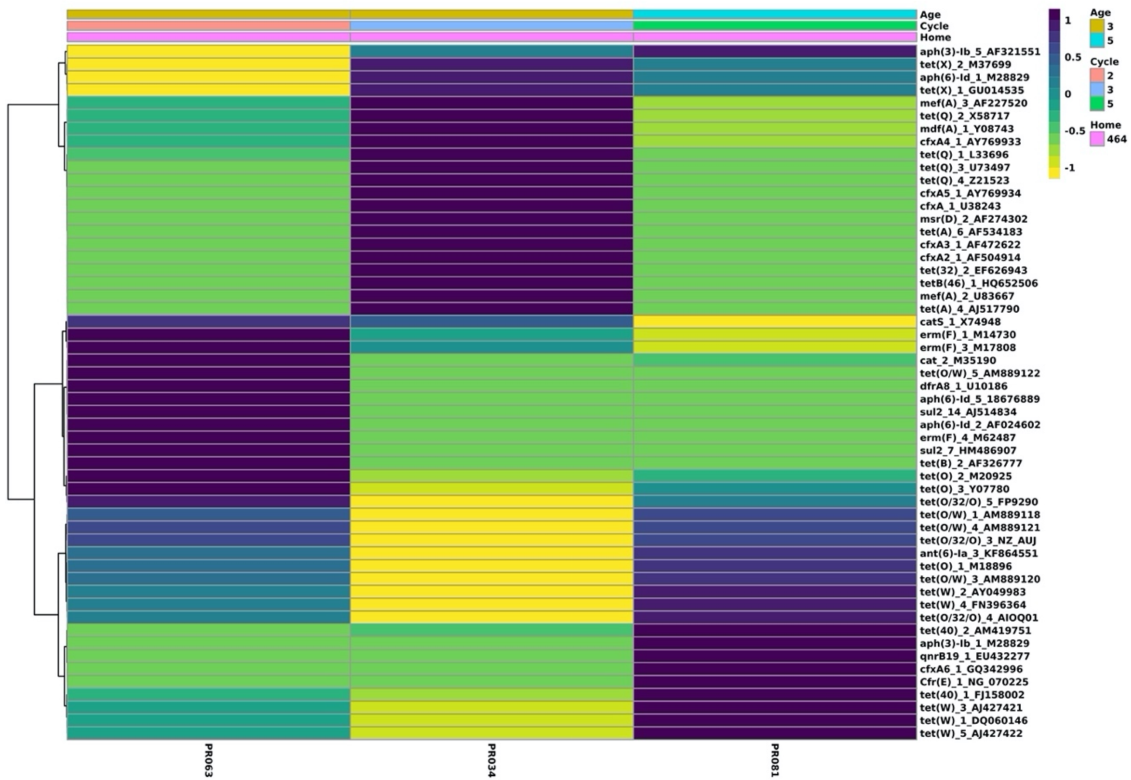
B
Age
Cycle
Home
tet(X)_1_GU014535
cfxA6_1_GQ342996
erm(F)_1_M14730
erm(F)_3_M17808
tet(X)_2_M37699
erm(G)_1_M15332
cfxA4_1_AY769933
cfxA3_1_AF472622
tet(Q)_4_Z21523
tet(Q)_3_U73497
msr(D)_3_AF227520
msr(D)_2_AF274302
mef(A)_3_AF227520
erm(G)_2_L42817
lnu(C)_1_AY928180
tet(Q)_1_L33696
tet(Q)_2_X58717
cfxA5_1_AY769934
tet(32)_2_EF626943
tet(O/W)_5_AM889122
ant(6)-Ia_3_KF864551
tet(O/W)_3_AM889120
tet(O)_1_M18896
tet(W)_2_AY049983
tet(O)_3_Y07780
tet(O/W)_4_AM889121
tet(W)_3_AJ427421
tet(W)_4_FN396364
tet(O/32/O)_3_NZ_AUJ
tet(O/32/O)_4_AIOQ01
tet(40)_2_AM419751
tet(O/32/O)_5_FP9290
tet(40)_1_FJ158002
tet(O)_2_M20925
tet(W)_1_DQ060146
tet(W)_5_AJ427422
erm(F)_4_M62487
tet(32)_1_EU722333
1.5
1
0.5
0
-0.5
-1
-1.5
Age
4
5
7
Cycle
1
2
3
5
Home
441
PR114
PR022
PR036
PR085

A

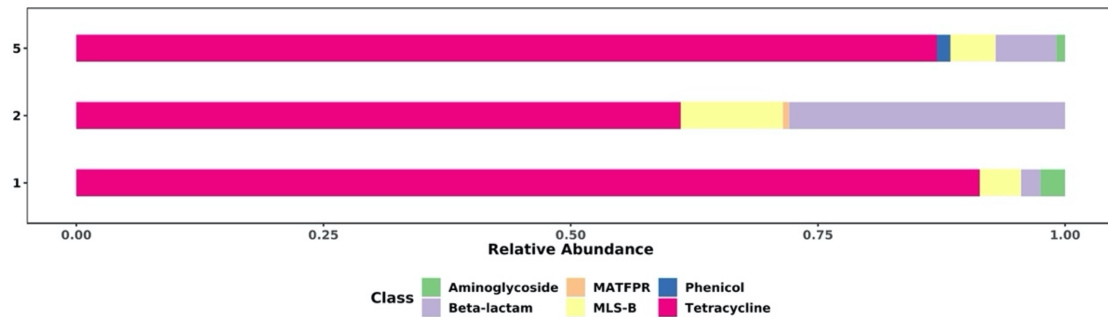
Participant 464



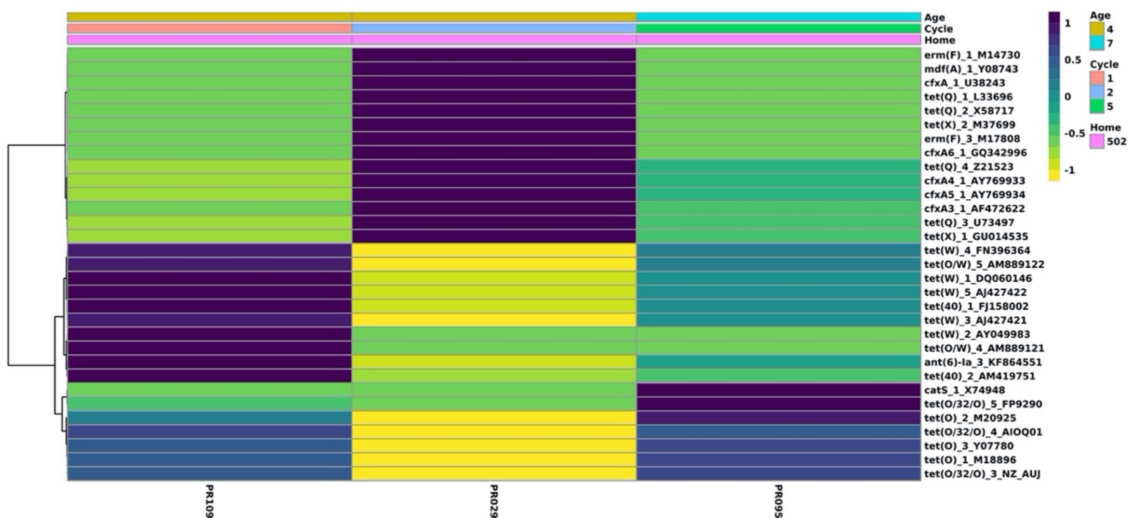
B



A

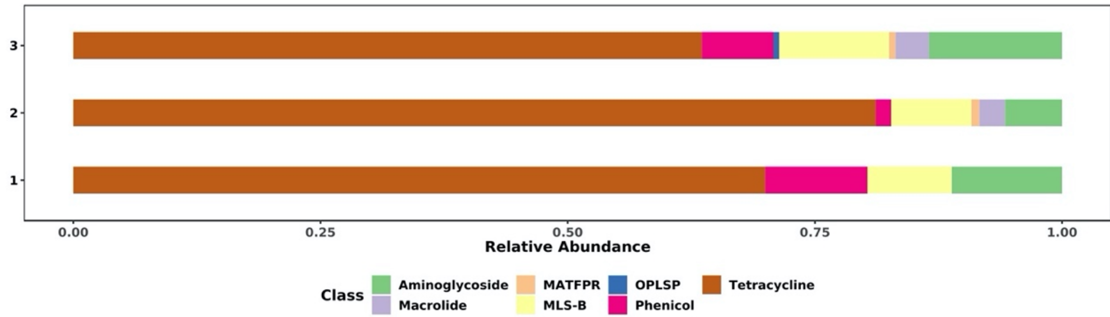


B

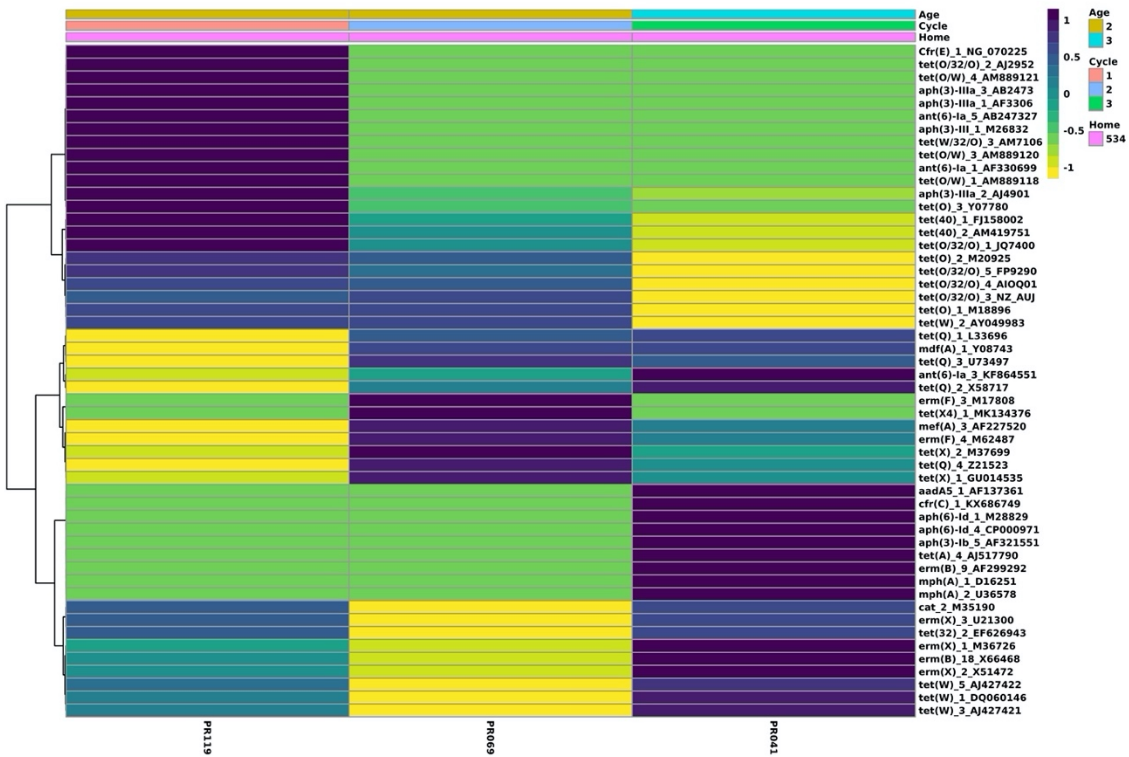


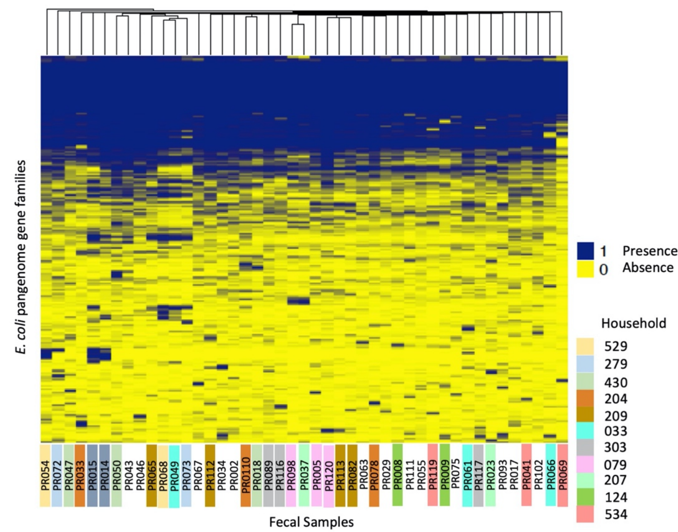
A

Participant 534



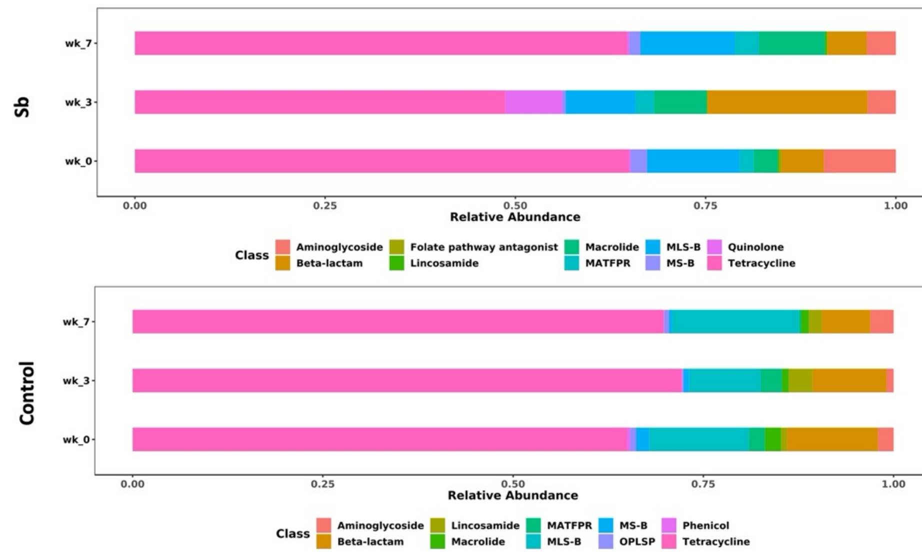
B



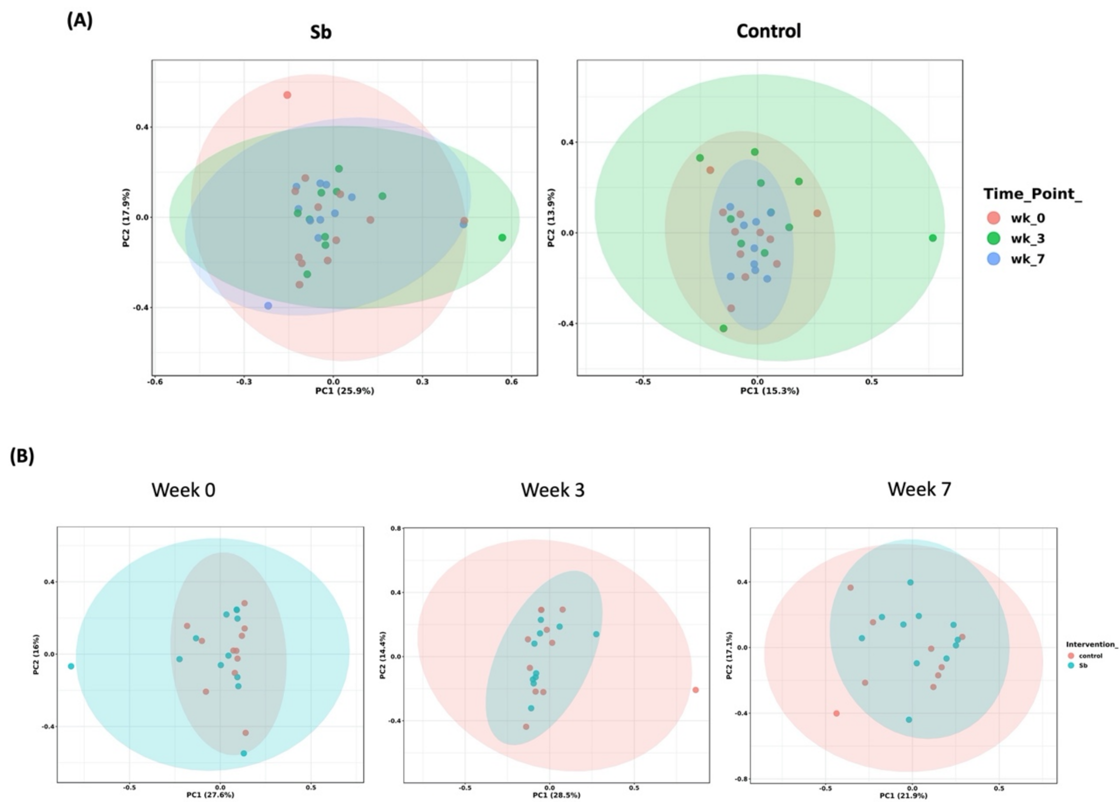


Supplementary Figure 31. E. coli profiling from 45 metagenome samples. Heatmap and hierarchical clustering analysis. Each column represents a dominant strain in each sample and the same colors represent the same children (household). Each row represents a gene family from E. coli pangenome (200 reference genomes not included in this figure). Euclidean distances were used, and the grouping was by samples.

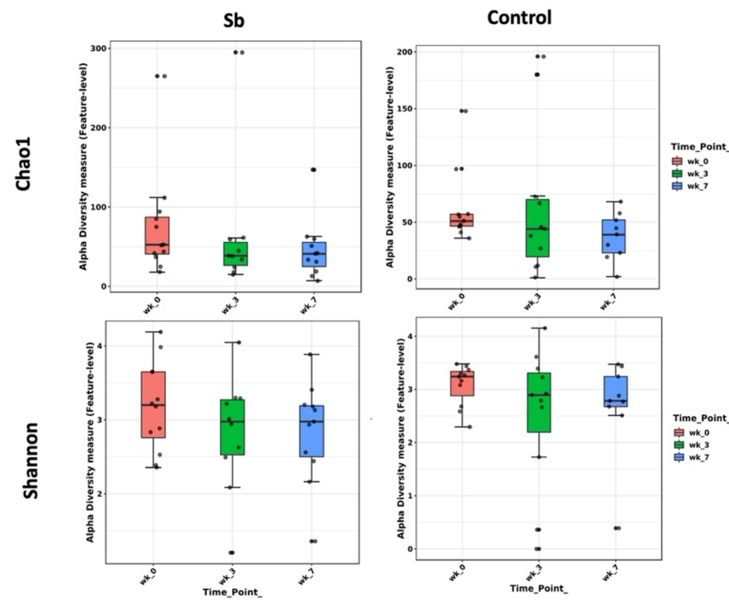
Anexo B. Chapter 4. Supporting information



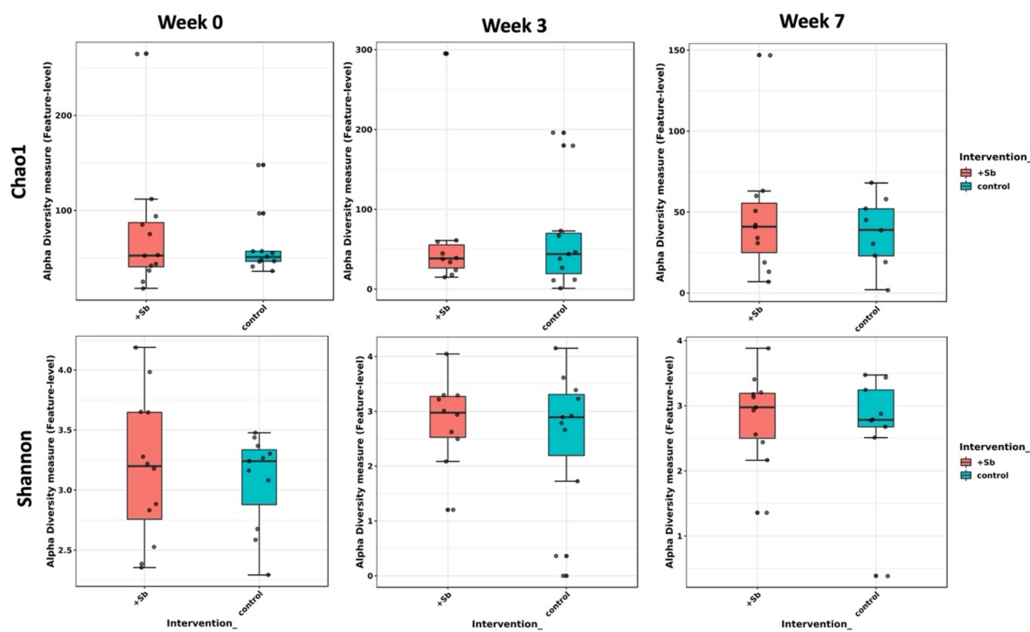
Supplementary Figure 1. Relative abundance of ARGS in *S. boulardii* (*Sb*) and control treatment groups. Macrolide, lincosamide, Streptogramin B (MLS-B); Macrolide, Aminoglycoside, Tetracycline, Fluoroquinolone, Phenicol, Rifamycin (MATFPR); Macrolide, Streptogramin B (MS-B); Oxazolidinone, Phenicol, Lincosamide, Streptogramin A, Pleuromutilin (OPLSP).



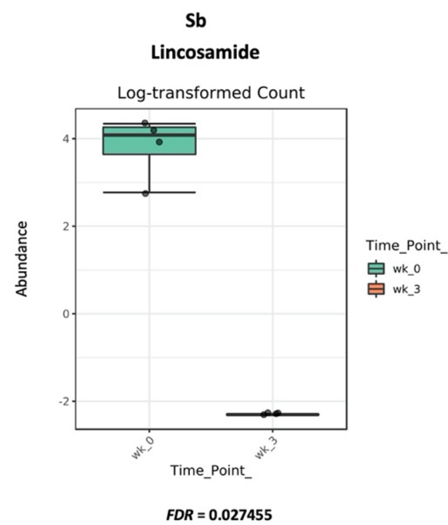
Supplementary Figure 2. Fecal resistome composition of ARGs among (**A**) interventions and (**B**) time points based on ordination analysis. PCA plots using PERMANOVA test, P> 0.05.



Supplementary Figure 3. Alpha diversity of the fecal resistome in *S. boulardii* and control treatment groups. Median values are indicated by central black horizontal lines; the 25th and 75th percentiles are indicated (boxes), and the whiskers extend from each end of the box to the most extreme values.



Supplementary Figure 4. Comparison of alpha diversity of antibiotic resistant genes between *S. boulardii* and control treatment groups. Median values are indicated by central black horizontal lines; the 25th and 75th percentiles are indicated (boxes), and the whiskers extend from each end of the box to the most extreme values.



Supplementary Figure 5. Differential abundance of ARGs based on pairwise comparison before (week0) and after treatments (week 3). Median values are indicated by central black horizontal lines; the 25th and 75th percentiles are indicated (boxes), and the whiskers extend from each end of the box to the most extreme values.