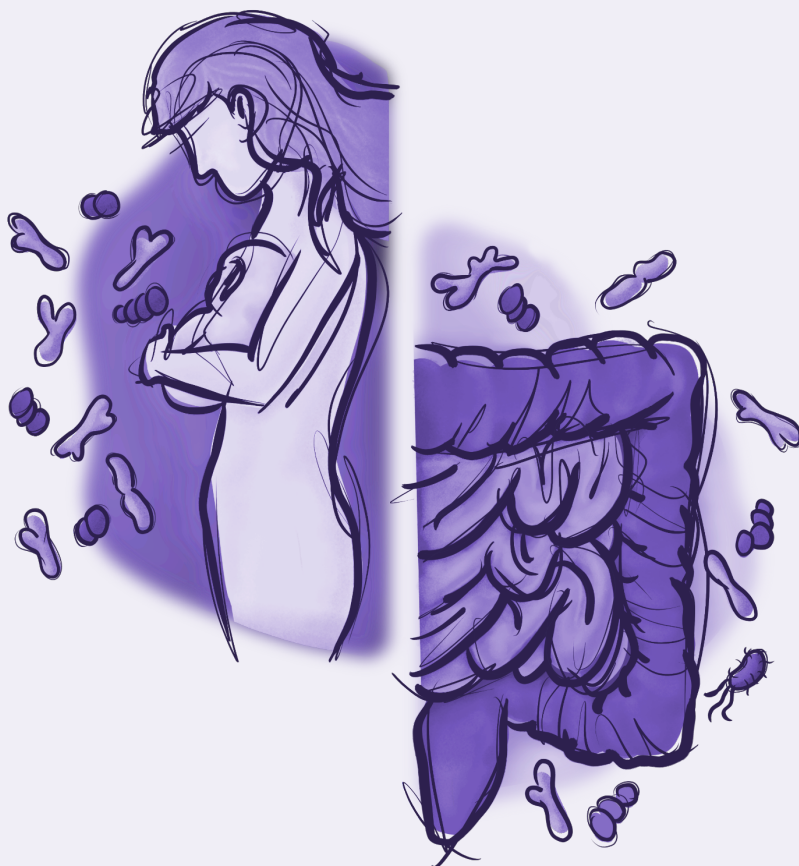


Antibiotic Resistance in Early Life: Deciphering the Influence of Perinatal Factors, Breastfeeding, and Gut Microbiota on Infant's Resistome

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No encontraba el momento de escribir esto, quizás porque significa que la tesis está casi terminada y que el final de esta etapa está cada vez más cerca. Ni la más extensa de las tesis podría resumir lo que han sido estos últimos cuatro años, sobre todo porque nada está a la altura de toda la gente que ha formado parte de este camino. Pero aquí va, por escrito, todo mi agradecimiento.

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RESUMEN

El uso de antibióticos en las primeras etapas de la vida influye en el desarrollo de la microbiota intestinal del lactante y, al mismo tiempo, contribuyen a la aparición de resistencias antimicrobianas, con importantes implicaciones para la salud global. El resistoma intestinal -el conjunto de genes de resistencia a antibióticos (ARGs) presentes en la microbiota- se establece típicamente durante este periodo crítico, a menudo como resultado de la transmisión vertical materna o de exposiciones ambientales y dietéticas. El principal objetivo de esta tesis doctoral es caracterizar la evolución del resistoma intestinal infantil durante el primer año de vida, identificar los factores clave que modulan su configuración y explorar el papel de la dieta -en particular, la lactancia materna y la composición de los oligosacáridos de la leche humana (HMOs)- como una estrategia natural para limitar la adquisición y transmisión de genes de resistencia.

Se llevó a cabo un análisis longitudinal de la dinámica temporal de 45 genes de resistencia seleccionados, cribados a partir de un panel inicial de 384 ARGs, utilizando una plataforma de qPCR de alta resolución en muestras fecales de lactantes a los 7 días, 1, 2 y 4 meses de edad. Este análisis se acompañó de la caracterización de la microbiota intestinal mediante secuenciación del gen 16S rRNA, y se evaluó el impacto de variables como el modo de parto y el tipo de lactancia. Se observó un aumento progresivo en la diversidad y abundancia de ARGs durante los primeros meses de vida, especialmente genes asociados a aminoglucósidos, betalactámicos y macrólidos, mientras que los elementos genéticos móviles (MGEs) disminuyeron con el tiempo. Los lactantes nacidos por cesárea y aquellos alimentados con fórmula presentaron un perfil de resistencia más amplio y diverso, particularmente en los primeros meses de vida.

Se exploró la relación entre la abundancia del género *Bifidobacterium* y la carga total de ARGs en la microbiota intestinal de lactantes de 7 días y 1 mes de edad. Mediante el análisis combinado de secuenciación 16S rRNA y qPCR dirigida

a genes de resistencia específicos, se identificó una asociación inversa entre la abundancia de *Bifidobacterium* y los niveles de ARGs, lo que sugiere que este género bacteriano actúa como modulador clave del resistoma infantil y contribuye a reducir la colonización por cepas resistentes.

Para evaluar de forma más profunda la evolución conjunta del resistoma y la microbiota intestinal durante el primer año de vida, se realizó un análisis metagenómico en muestras fecales de una cohorte de más de 200 lactantes. Se confirmaron los hallazgos previos, y se observó que la lactancia materna promueve perfiles microbianos más estables y con menor carga de ARGs, incluso en lactantes nacidos por cesárea o expuestos a antibióticos. Este estudio también permitió identificar rutas de transmisión vertical de ARGs y puso de relieve la importancia de la lactancia materna en los primeros meses como una ventana de oportunidad para influir en el desarrollo del resistoma.

También se analizó la influencia de la composición individual de los HMOs en la leche humana sobre el resistoma intestinal del lactante. Mediante la caracterización de los HMOs en muestras de leche y su correlación con datos metagenómicos del resistoma en muestras fecales maternas, infantiles y de leche humana, se observó que ciertos perfiles de HMOs -especialmente aquellos presentes en madres secretoras- desempeñan un papel modulador del resistoma, reduciendo la carga de genes de resistencia en lactantes nacidos por cesárea.

Finalmente, se evaluó la capacidad de cepas bacterianas aisladas del entorno materno-infantil para degradar HMOs y sus perfiles de resistencia a antibióticos. Se aislaron cepas de bifidobacterias y lactobacilos de muestras fecales materno-infantiles y de leche humana, y se analizaron mediante secuenciación genómica completa, ensayos de crecimiento con HMOs y pruebas de susceptibilidad a antibióticos. Se encontró que *Bifidobacterium bifidum* (*B. bifidum*) y *Bifidobacterium longum subsp. infantis* (*B. infantis*) mostraron una alta capacidad de utilización de HMOs y baja resistencia a antibióticos, mientras que otras especies como *Bifidobacterium animalis subsp. lactis* (*B. lactis*) presentaron

resistencia a múltiples antibióticos sin capacidad de degradar HMOs. Estos resultados subrayan el papel clave de la lactancia materna en la promoción de la colonización por bacterias beneficiosas especializadas en la utilización de HMOs y con bajos perfiles de resistencia antibiótica. Al mismo tiempo, destacan la vulnerabilidad de estas cepas a la exposición a antibióticos durante los primeros meses de vida, lo cual podría comprometer su establecimiento y función en el ecosistema intestinal.

En conjunto, los resultados de esta Tesis Doctoral amplían nuestro conocimiento sobre el resistoma intestinal infantil y demuestran la influencia de factores perinatales, ambientales y nutricionales en su desarrollo. Los hallazgos refuerzan la importancia de prácticas clínicas como fomentar la lactancia materna, minimizar el uso innecesario de antibióticos durante el periodo perinatal y monitorizar la microbiota como estrategia de salud pública para reducir la propagación de resistencias. Asimismo, se abren nuevas líneas de investigación centradas en el uso de compuestos dietéticos como los HMOs o intervenciones microbianas dirigidas para prevenir la expansión del resistoma desde los primeros días de vida.

SUMMARY

Antibiotics in early life influence the development of the infant gut microbiota and, at the same time, contribute to the emergence of antimicrobial resistance, with significant implications for global health. The intestinal resistome - the collection of antibiotic resistance genes (ARGs) present in the microbiota - is typically established during this critical period, often as a result of maternal vertical transmission or environmental and dietary exposure. The main objective of this doctoral thesis is to characterize the evolution of the infant gut resistome during the first year of life, identify the key factors modulating its configuration, and explore the role of diet -particularly breastfeeding and the composition of human milk oligosaccharides (HMOs)- as a natural strategy to limit the acquisition and transmission of resistance genes.

A longitudinal analysis was conducted on the dynamics of 45 selected resistance genes, screened from an initial panel of 384 ARGs, using a high-resolution qPCR platform on fecal samples from infants at 7 days, 1, 2, and 4 months of age. This analysis was accompanied by characterization of the gut microbiota through 16S rRNA gene sequencing, and the impact of variables such as mode of delivery and feeding type was assessed. A progressive increase in the diversity and abundance of ARGs was observed during the first months of life, especially genes associated with aminoglycosides, beta-lactams, and macrolides, while mobile genetic elements (MGEs) decreased over time. Infants born by cesarean section (C-section) and those fed with formula displayed a broader and more diverse resistance profile, particularly in the early stages.

The relationship between the abundance of the genus *Bifidobacterium* and the total ARG load in the infant gut microbiota was explored in infants of 7 days and 1 month of age. Through combined 16S rRNA sequencing and targeted qPCR analysis of specific resistance genes, an inverse association was identified between *Bifidobacterium* abundance and ARG levels, suggesting that this bacterial genus

acts as a key modulator of the infant resistome and contributes to reducing colonization by resistant strains.

To further assess the joint evolution of the resistome and gut microbiota during the first year of life, shotgun metagenomic analysis was performed on fecal samples from a cohort of over 200 infants. Previous findings were confirmed, and it was observed that breastfeeding promotes more stable microbial profiles with lower ARG loads, even in infants born by C-section or exposed to antibiotics. This study also enabled the identification of vertical transmission routes of ARGs and highlighted the importance of breastfeeding in the early months as a window of opportunity to influence resistome development.

The influence of the individual composition of HMOs in human milk on the infant gut resistome was also analyzed. By profiling HMOs in milk samples and correlating them with metagenomic resistome data from maternal-infant feces and human milk samples, it was observed that certain HMO profiles - particularly those present in secretor mothers- play a modulatory role in the resistome, reducing resistance gene load in infants born by C-section.

Finally, the capacity of bacterial strains isolated from the maternal-infant environment to degrade HMOs and their antibiotic resistance profiles was evaluated. Strains of bifidobacteria and lactobacilli were isolated from human milk and maternal-infant fecal samples, and analyzed via whole genome sequencing, growth assays using HMOs, and antibiotic susceptibility testing. It was found that *Bifidobacterium bifidum* (*B. bifidum*), and *Bifidobacterium longum subsp. Infantis* (*B. infantis*) exhibited high capacity for HMO utilization and low antibiotic resistance, while other species such as *Bifidobacterium animalis subsp. lactis* (*B. lactis*) showed resistance to multiple antibiotics without the ability to degrade HMOs. These results underscore the key role of breastfeeding in promoting colonization by beneficial bacteria specialized in HMO utilization with low antibiotic resistance profiles. At the same time, they highlight the vulnerability of these strains to

antibiotic exposure during the early months of life, which could compromise their establishment and function in the intestinal ecosystem.

Overall, the results of this Doctoral Thesis expand our understanding of the neonatal intestinal resistome and demonstrate the influence of perinatal, environmental, and nutritional factors in its development. The findings reinforce the importance of clinical practices such as promoting breastfeeding, minimizing unnecessary antibiotic use during the perinatal period, and monitoring microbiota as a public health strategy to reduce the spread of resistance. Furthermore, new research avenues are opened, focused on the use of dietary compounds such as HMOs or targeted microbial interventions to prevent the expansion of the resistome from the earliest days of life.

RESUM

Els antibiòtics en les primeres etapes de la vida influeixen en el desenvolupament de la microbiota intestinal del lactant i, alhora, contribueixen a l'aparició de resistències als antimicrobians, amb importants implicacions per a la salut global. El resistoma intestinal —el conjunt de gens de resistència a antibiòtics (ARGs) presents a la microbiota— s'estableix típicament durant aquest període crític, sovint com a resultat de la transmissió vertical materna o per exposicions ambientals i dietètiques. L'objectiu principal d'aquesta tesi doctoral és caracteritzar l'evolució del resistoma intestinal del lactant durant el primer any de vida, identificar els factors clau que modulen la seva configuració i explorar el paper de la dieta -en particular, la lactància materna i la composició dels oligosacàrids de la llet humana (HMOs)- com a estratègia natural per limitar l'adquisició i la transmissió de gens de resistència.

Es va dur a terme una anàlisi longitudinal de la dinàmica de 45 gens de resistència seleccionats, triats d'un panell inicial de 384 ARGs, utilitzant una plataforma de qPCR d'alta resolució en mostres fecals de lactants als 7 dies, 1, 2 i 4 mesos d'edat. Aquesta anàlisi es va complementar amb la caracterització de la microbiota intestinal mitjançant la seqüenciació del gen 16S rRNA, i es va avaluar l'impacte de variables com el mode de part i el tipus d'alimentació. Es va observar un augment progressiu en la diversitat i abundància dels ARGs durant els primers mesos de vida, especialment de gens associats a aminoglucòsids, betalactàmics i macròlids, mentre que els elements genètics mòbils (MGEs) van disminuir amb el temps. Els lactants nascuts per cesària i aquells alimentats amb fórmula presentaven un perfil de resistència més ampli i divers, especialment en les primeres etapes.

Es va explorar la relació entre l'abundància del gènere *Bifidobacterium* i la càrrega total d'ARGs en la microbiota intestinal de lactants de 7 dies i 1 mes d'edat. Mitjançant l'anàlisi combinada de la seqüenciació 16S rRNA i qPCR dirigida a gens de resistència específics, es va identificar una associació inversa entre

l'abundància de *Bifidobacterium* i els nivells d'ARGs, suggerint que aquest gènere bacterià actua com a modulador clau del resistoma infantil i contribueix a reduir la colonització per soques resistents.

Per aprofundir en l'evolució conjunta del resistoma i la microbiota intestinal durant el primer any de vida, es va realitzar una anàlisi metagenòmica shotgun en mostres fecals d'una cohort de més de 200 lactants. Es van confirmar els resultats previs, i es va observar que la lactància materna promou perfils microbians més estables i amb una menor càrrega d'ARGs, fins i tot en lactants nascuts per cesària o exposats a antibiòtics. Aquest estudi també va permetre identificar rutes de transmissió vertical dels ARGs i va destacar la importància de la lactància materna durant els primers mesos com una finestra d'oportunitat per influir en el desenvolupament del resistoma.

També es va analitzar la influència de la composició individual dels HMOs a la llet humana sobre el resistoma intestinal del lactant. Mitjançant la caracterització dels HMOs en mostres de llet i la seva correlació amb dades metagenòmiques del resistoma en mostres fecals maternes, infantils i de llet humana, es va observar que certs perfils d'HMOs -especialment aquells presents en mares secretadores- tenen un paper modulador del resistoma, reduint la càrrega de gens de resistència en lactants nascuts per cesària.

Finalment, es va avaluar la capacitat de soques bacterianes aïllades de l'entorn maternoinfantil per degradar HMOs i els seus perfils de resistència als antibiòtics. Es van aïllar soques de bifidobacteris i lactobacils de mostres fecals maternoinfantils i de llet humana, i es van analitzar mitjançant seqüenciació genòmica completa, assaigs de creixement amb HMOs i proves de susceptibilitat als antibiòtics. Es va trobar que *Bifidobacterium bifidum* (*B. bifidum*) i *Bifidobacterium longum subsp. infantis* (*B. infantis*) mostraven una alta capacitat per utilitzar HMOs i baixa resistència als antibiòtics, mentre que altres espècies com *Bifidobacterium animalis subsp. lactis* (*B. lactis*) presentaven resistència a múltiples antibiòtics sense capacitat de degradar HMOs. Aquests resultats

subratllen el paper clau de la lactància materna en la promoció de la colonització per bacteris beneficiosos especialitzats en la utilització d'HMOs i amb baixos perfils de resistència als antibiòtics. Al mateix temps, destaquen la vulnerabilitat d'aquestes soques a l'exposició als antibiòtics durant els primers mesos de vida, fet que podria comprometre el seu establiment i funció en l'ecosistema intestinal.

En conjunt, els resultats d'aquesta Tesi Doctoral amplien el nostre coneixement sobre el resistoma intestinal neonatal i demostren la influència de factors perinatals, ambientals i nutricionals en el seu desenvolupament. Les troballes reforcen la importància de pràctiques clíniques com promoure la lactància materna, minimitzar l'ús innecessari d'antibiòtics durant el període perinatal i monitorar la microbiota com a estratègia de salut pública per reduir la propagació de resistències. A més, s'obren noves línies de recerca centrades en l'ús de compostos dietètics com els HMOs o intervencions microbianes dirigides per prevenir l'expansió del resistoma des dels primers dies de vida.

INTRODUCTION

GENERAL INTRODUCTION

Antibiotics discovery in the mid-20th century revolutionized medicine, representing one of the greatest triumphs in human healthcare. Since then, widespread use of antibiotics drastically reduced mortality from bacterial infections, transforming once-lethal diseases into manageable conditions. Beyond their therapeutic role, antibiotics have also played a pivotal role in enabling complex medical procedures, including surgeries, chemotherapy, and organ transplants, by preventing and managing infections. However, the efficacy of antibiotics is increasingly linked with the global rise in antibiotic resistance, which threatens to reverse decades of progress in infectious disease control.

Antibiotic resistance occurs when bacteria develop the ability to survive exposure to antibiotics that would kill them. This phenomenon is a natural evolutionary process but has been significantly accelerated by anthropogenic factors, such as the overuse and misuse of antibiotics in human medicine, agriculture, and veterinary practices being a global challenge. The human and environmental microbiota serve as a vast reservoir of ARGs, playing a crucial role in the emergence, evolution, and dissemination of antimicrobial resistance (AMR). These genes are present in commensal, pathogenic, and environmental bacteria, contributing to the spread of resistance across ecosystems.

One of the most concerning aspects of antibiotic resistance is its impact on vulnerable populations, particularly infants. Early-life acquisition of antibiotic resistances can occur through vertical transmission from mothers during birth and lactation or through environmental and dietary sources. Infants are particularly susceptible due to their immature immune systems and developing gut microbiota. The infant gut microbiome, a dense and dynamic microbial community, acts as a reservoir of antibiotic resistance genes (ARGs) collectively referred to as the gut resistome. The acquisition and establishment of the infant resistome are influenced by numerous factors, including mode of delivery, feeding practices, maternal antibiotic use, and environmental exposures.

In this context, human milk plays a crucial role and it is the main postnatal link between mothers and infants. While it provides essential nutrients, immunological components, oligosaccharides and beneficial bacteria, it may serve as a protective and preventive strategy for antibiotic resistance acquisition in early life. Nonetheless, it can also serve as a vector for the transfer of ARMs. Similarly, factors such as antibiotic use during pregnancy and the neonatal period can disrupt the delicate balance of the infant gut microbiota, favoring the proliferation of resistant strains. Understanding the mechanisms and pathways underlying these processes is crucial for developing strategies to mitigate AR in early life.

This thesis focuses on the early-life gut microbiota and resistome, with an emphasis on maternal-infant interactions and external influencing factors. The gut microbiota represents a critical interface between the host and its environment, contributing to immune development, nutrient metabolism, and defense against pathogens. However, it also acts as a reservoir for ARGs, facilitating their dissemination to potentially pathogenic microorganisms. The research examines the factors shaping the infant gut resistome during the first year of life, a critical window for microbial colonization and immune programming. This work aims to provide a comprehensive understanding of the early life resistome and identify potential interventions to control the spread of antibiotic resistances in infants.

The thesis starts with an extensive state-of-the art, presenting current knowledge on antibiotic resistance in early life. This includes a discussion on maternal-infant transmission of resistances, factors influencing the resistome, and methodologies for studying it. This introduction expands and updates three review articles and a Chapter of a book recently published by the author of this thesis¹⁻⁴.

Then, the thesis is structured into five Chapters. In **Chapter 1** we describe the evolution and dynamics of ARGs in the gut microbiota during the first four months of life, accounting for the influence of factors such as delivery mode and feeding mode. In this Chapter, a high-throughput quantitative approach is applied to describe the evolution of selected ARGs in a subset of infants from the MAMI cohort.

In **Chapter 2** we present the role of *Bifidobacterium* genus abundance in modulating the neonate microbiota and, consequently, the abundance of antibiotic resistances. Here, the microbial profile using 16S rRNA sequencing of a subset of 7-days and 1 month old infants from the MAMI cohort was studied, and several different ARGs were identified by qPCR. **Chapter 3** is an extension of Chapter 1 and Chapter 2 in terms of the methodology, the number of participants and the factors studied. It encompasses a longitudinal metagenomic study of over 200 infants during the first year of life. The role of *Bifidobacterium* genus on shaping infant's resistome is here confirmed, and the influence of breastfeeding practices on decreasing ARGs load, and correcting the effects of mode of delivery, are presented. In **Chapter 4** we demonstrate the potential benefits of breastfeeding on ameliorating infant's acquisition of resistances during the first month of life, and the direct influence of human milk oligosaccharides (HMOs) composition on the resistome of lactating newborns. Finally, results from **Chapter 5** reinforce the essential role of breastfeeding in shaping a healthy infant gut microbiome by supporting the growth of HMO-utilizing beneficial bacteria, while also highlighting the potential disruption caused by antibiotic exposure during this critical developmental period. At the end, we will discuss the results of the studies presented in this thesis in relation to the current literature and formulate future perspectives for next studies.

ANTIBIOTIC RESISTANCE: RISKS AND CHALLENGES

The problem and rise of resistance to antibiotics

One of the most significant health achievements of the twentieth century, which marked a complete shift in the existing therapeutic paradigm, was the discovery and routine administration of antibiotic compounds for the treatment of bacterial infections ⁵. However, in recent years, there has been an increase in bacteria resistant to these antibiotics, which seriously threatens this achievement, making antibiotic resistance one of the greatest challenges for the global healthcare system.

Antibiotic resistance occurs when microorganisms resist the effects of antibiotics, making them no longer effective in treating bacterial infections. This occurs as bacteria evolve and develop new strategies to counteract the impact of antibiotics, leading to the rise of antibiotic-resistant microorganisms (ARM)⁶. Resistance to antibiotics is a natural evolutionary process for microorganisms, but its progression is accelerated by the selective pressure exerted by the excessive use of antibacterial drugs⁷. Inadequate prescription, self-consumption, over-consumption and prolonged use of antibiotics in humans and animals have affected the microbiota equilibrium, resulting in an increased risk of encountering health issues in the future, and causing a global crisis of antibiotic resistance⁸. Indeed, the creation of a new antibacterial drug is soon followed by the emergence of a resistance to it. ARMs are widespread and can be easily transmitted from person to person or from animals to humans, including through consumption of animal-derived food⁹. Accordingly, the World Health Organization (WHO) and the US Centers for Disease Control and Prevention (CDC) classify ARMs as a threat to human health worldwide¹⁰, and one of the three most important public health threats of the 21st century¹¹. To address this challenge, a comprehensive global action plan has been put in place to ensure that prevention and treatment of infectious diseases are carried out using safe and effective medicines¹¹. In Spain, the “Plan Nacional frente a la Resistencia a los Antibióticos (PRAN)”, aims to reduce the risk of selection and spread of antibiotic resistance and, consequently, reduce the impact of this problem on the health of people and animals, sustainably preserving the efficacy of existing antibiotics. Despite its importance, ARM infections are currently underreported, but it is estimated that there are more than 2.8 million AMR infections and up to 35,000 deaths per year in the United States alone, with an estimated derived cost of more than 4.7 billion dollars¹². In Europe, more than 33,000 deaths are attributed to acquired ARM infections each year, representing \$1.5 billion in direct and indirect costs¹³. Furthermore, the risk of spreading antibiotic-resistant pathogens is particularly severe in newborns as their gut microbiota and immune system are not as mature and robust as those of adults. The worldwide problem of antibiotic resistance results in approximately 214,000

yearly newborn fatalities from septic infections, highlighting its devastating impact on infant health¹⁴.

Bacteria that develop resistance to antibiotics possess specific ARGs, which enable them to overcome the effects of these drugs. When subjected to antibiotic pressure, bacterial populations that were once sensitive may develop resistance, thereby enhancing their survival. For this reason, it is vital to identify the most commonly used clinical antibiotics, understand their mechanisms of action (**Box 1**), and clarify the range of bacteria they target (**Table 1**).

Box 1. Mechanisms of antibiotic resistance.

Antibiotic modification: the most common resistance mechanism involves bacterial enzymes that chemically modify antibiotic molecules, rendering them ineffective before they reach their target. Key enzymatic reactions include:

- **Acetylation, phosphorylation, and adenylation** (notably targeting aminoglycosides, chloramphenicol, and lincosamides).
- **β -lactamase activity**, which hydrolyzes the β -lactam ring, deactivating antibiotics like penicillins and cephalosporins.

Prevention of antibiotic from reaching its target

- **Altering porins** (membrane channels), limiting access for hydrophilic antibiotics such as β -lactams and fluoroquinolones.
- **Activating efflux pumps**, which expel antibiotics from the cell, reducing their intracellular concentration. These pumps are often encoded on mobile genetic elements and affect multiple antibiotic classes.

Modification of antibiotic target sites: resistance can also result from structural changes at the antibiotic's target, making the drug ineffective. Notable examples include MRSA and VRE

Global cellular adaptation: Some bacteria develop resistance through complex metabolic and regulatory changes, without acquiring new resistance genes. For example, certain *S. aureus* strains with intermediate vancomycin resistance exhibit altered metabolism (e.g., enhanced fatty acid usage, reduced glutamate availability) and upregulation of genes involved in cell wall synthesis.

Table 1. Main groups of antibiotics.

Class	Antibiotic	Mechanism of action	Antimicrobial spectrum
Beta-lactams	Penicillin (amoxicillin, ampicillin, piperacillin)	Inhibits the synthesis of peptidoglycan that forms the bacterial cell wall.	Effective against gram-positive bacteria. Broad-spectrum penicillin also effective against gram negatives.
	Cephalosporins (ceftazidime, ceftriaxone)		Broad therapeutic range and generally lack activity against enterococci, <i>Listeria monocytogenes</i> , and cloxacillin-resistant staphylococci. The adverse reactions are similar to those of penicillins.
	Monobactams (aztreonam)		Effective against gram-negative aerobic bacteria.
	Carbapenems (imipenem, meropenem, ertapenem)		Effective against both gram-positive, gram-negative and anaerobic bacteria, possess the broadest spectrum of action known.
	Beta-lactamase inhibitors (clavulanic acid, tazobactam)		Enhance activity when used with broad-spectrum penicillins by irreversibly binding to beta-lactamases.
Aminoglycosides	Streptomycin, neomycin, kanamycin (amikacin, kanamycin, tobramycin), gentamicin, spectinomycin	Binds to the bacterial 30S ribosomal subunit, causing inhibition of protein synthesis or the production of defective proteins.	Primarily effective against aerobic gram-negative bacilli.
Chloramphenicol	Chloramphenicol	Inhibits protein synthesis (binding to the 50S ribosomal subunit) and are bacteriostatic.	Effective against gram-positive and gram-negative bacteria, including anaerobic bacteria, <i>Chlamydomphila</i> , <i>Mycoplasma</i> and <i>Rickettsia</i> . Due to high toxicity, it should only be used when there is no effective alternative.
Glycopeptides	Vancomycin	Inhibits the synthesis of the bacterial cell wall.	Very active against gram-positive cocci. Commonly used to treat methicillin-resistant <i>Staphylococcus aureus</i> (MRSA).
Lincosamides	Clindamycin, lincomycin	Interferes with protein synthesis by binding to the bacterial 50S ribosomal subunit.	Used primarily for severe infections caused by anaerobes and gram-positive bacteria (except enterococci). Their spectrum of action extends to strains of streptococci resistant to macrolides due to an efflux pump mechanism.

Macrolides	Erythromycin	Interferes with protein synthesis by binding to the bacterial 50S ribosomal subunit.	Structural modifications at the macrolide binding site on the ribosome, the existence of active efflux pumps, and development of inactivating enzymes (very rare).
Quinolones	Nalidixic acid, ciprofloxacin	Inhibits bacterial DNA gyrase, an enzyme necessary for the correct folding of the DNA double helix.	Nalidixic acid is active against gram negatives. Fluoroquinolones have a broader antibacterial spectrum and better pharmacokinetic conditions for use in systemic infections.
Sulphonamides	Cotrimoxazole	Inhibits folic acid synthesis.	Indicated only for the treatment of urinary infections, some forms of acute gastroenteritis, and other specific infections. Its initially broad spectrum has been significantly reduced due to the emergence of resistance.
Tetracyclines	Tetracycline, tigecycline	Inhibits protein synthesis. They interfere with the protein synthesis of susceptible microorganisms by binding to the 30S subunit of the ribosome and preventing its interaction with tRNA.	Initially had a broad spectrum which promoted frequent use and the emergence of resistance; thus, they are usually not the antibiotics of choice for infections by gram positives and negatives today.
Nitroimidazoles	Metronidazole, tinidazole, benznidazole	Penetration of the cell cytoplasm through passive diffusion.	Effective against anaerobic bacteria. Under strict anaerobic conditions, effective against <i>E. coli</i> , <i>Proteus</i> and <i>Klebsiella</i> .
Oxazolidinones	Linezolid, tedizolid	Inhibition of protein synthesis by binding to the 50S ribosomal subunit and blocking the formation of the 70S initiation complex.	Activity is primarily against gram-positive organisms, including cocci (staphylococci, streptococci, enterococci, and pneumococci), and strains resistant to penicillin, methicillin, and vancomycin.
Rifamycin	Rifabutin, rifampin, and rifaximin	Binding to the subunit b of RNA polymerase responsible for transcribing bacterial DNA into RNA.	Broad spectrum with activity against <i>Staphylococcus</i> spp., <i>S. pyogenes</i> , <i>Neisseria</i> spp., <i>H. influenzae</i> , <i>Campylobacter jejuni</i> , <i>H. pylori</i> , <i>C. trachomatis</i> , both tuberculous and non-tuberculous mycobacteria, <i>Aspergillus</i> spp., and <i>Toxoplasma gondii</i> .
Others	fusidic acid, bacitracin, gramicidin, tyrothricin, bedaquiline, delamanid, daptomycin, fosfomycin, isoniazid, pyrazinamide, ethambutol, mupirocin, nitrofurantoin, polymyxins and trimethoprim		

Adapted from Guide-ABE ¹⁵

The resistome and types of antibiotic resistances

All types of ARGs (acquired and intrinsic resistance genes), their precursors, and some potential resistance mechanisms within microbial communities that require evolution or alterations in the expression context to confer resistance, is known as the antibiotic **resistome** ¹⁶.

It is important to distinguish between intrinsic, acquired and phenotypic antibiotic resistance to determine how the resistance develops (**Figure 1**). Intrinsic resistance is naturally present in the microorganism; it is chromosomically encoded and is connected to its physiology ¹⁶. The intrinsic resistance is conferred by several elements that contribute to antibiotic resistance, independent of earlier exposure to antibiotics, and can only be vertically transmitted¹⁷. The acquired resistance is based on mutations in the bacterial chromosomal DNA and on the acquisition of mobile resistance genes, by either horizontal or vertical gene transfer. Acquired resistance ARGs are taxa non-specific. The strategies that bacteria use to develop acquired resistance are encoded on plasmids and may be classified into four mechanisms: 1) decreased permeability of the cell wall to antibiotics; 2) modification of enzymes to inactivate antibiotics; 3) drug target site changes; and 4) efflux pumps that remove antibiotics from the cell ¹⁸. Adaptative or phenotypic resistance refers to a transient situation in which a susceptible bacterial population becomes resistant to antibiotics¹⁷. The elements contributing to this phenotype are a part of the intrinsic resistome that are only unveiled under specific growing conditions. Phenotypic bacterial resistance appears in three categories: 1) persistence, defined as when bacterial subpopulation is not killed by a given antibiotic under conditions in which the bulk of the population is inhibited ¹⁹; 2) biofilm formation ²⁰; and 3) swarming, which consists in the formation of hyper-flagellated cells in nutrient-rich environments, allowing the colonization of such environments and reduce the susceptibility to antibiotics ¹⁷.

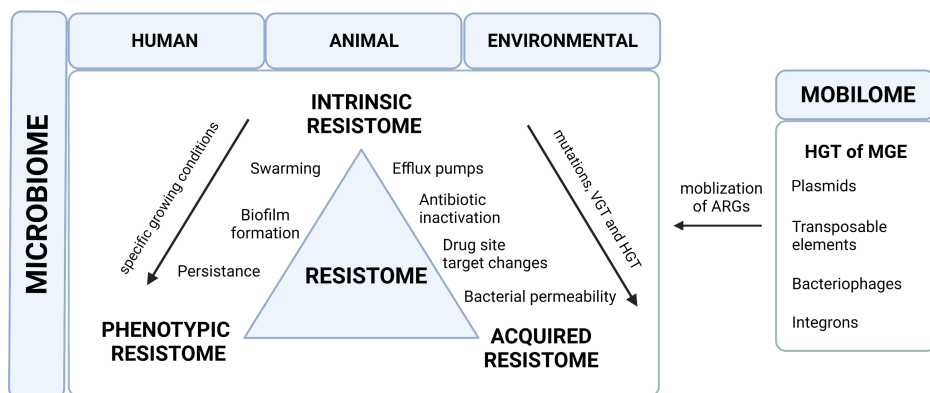


Figure 1. A schematic overview of the human resistome and mobilome. MGE: mobile genetic elements; HGT: horizontal gene transfer; VGT: vertical gene transfer; ARG: antibiotic genetic elements. Figure from Samarra *et al.* ¹.

Acquisition of resistance to antibiotics

Bacteria are widely known to genetically adapt to withstand various environmental challenges, such as exposure to antibiotics that could threaten their survival. Those bacteria that occupy the same ecological niche as antimicrobial-producing organisms have developed mechanisms to resist the effects of these harmful antibiotic molecules. As a result, their intrinsic resistance allows them to become resistant. Bacteria use two strategies to overcome the effects of an antibiotic: 1) mutations in genes and 2) horizontal gene transfer (HGT).

Mutations

This phenomenon is based on the emergence of a resistant mutant in a susceptible population, which will resist the effect of the antibiotic and predominate over the rest of the bacteria through four different mechanisms: 1) modifications of the antimicrobial target (decreasing the affinity for the drug; see below), 2) a decrease in the drug uptake, 3) activation of efflux mechanisms to extrude the harmful molecule, or 4) global changes in important metabolic pathways via modulation of regulatory networks ⁵.

In some cases, chromosomal point mutations can confer simultaneous resistance to multiple antibiotics from different families, i.e., beta-lactams, chloramphenicol, trimethoprim, and tetracyclines. The cause of this

multiresistance, which occurs particularly frequently in some enterobacteria such as *Klebsiella*, *Enterobacter*, and *Serratia*, is usually a mutation in the genes encoding for porins²¹.

Horizontal gene transfer

Gene transfer between bacteria allows for the rapid and extensive dissemination of genetic information in both gram-positive and gram-negative bacteria. It is characterized as "horizontal" because it occurs independently of reproduction processes. The microbial communities present in an ecological niche interact within this ecosystem, facilitating the exchange of genetic material. This exchange can occur through mechanisms such as transduction, conjugation and transformation enabling the transfer of ARGs between different bacterial species (Figure 2). Briefly, during transformation, DNA enters the interior of a competent recipient cell; in transduction, DNA travels from one cell to another via bacteriophages; and conjugation involves the exchange of material between two contacting cells through plasmids. There are also particles, known as **fusion vesicles**²², that are structurally similar to bacteriophages but produced by the bacterial cells themselves. These fusion vesicles allow for exchange without cell contact.

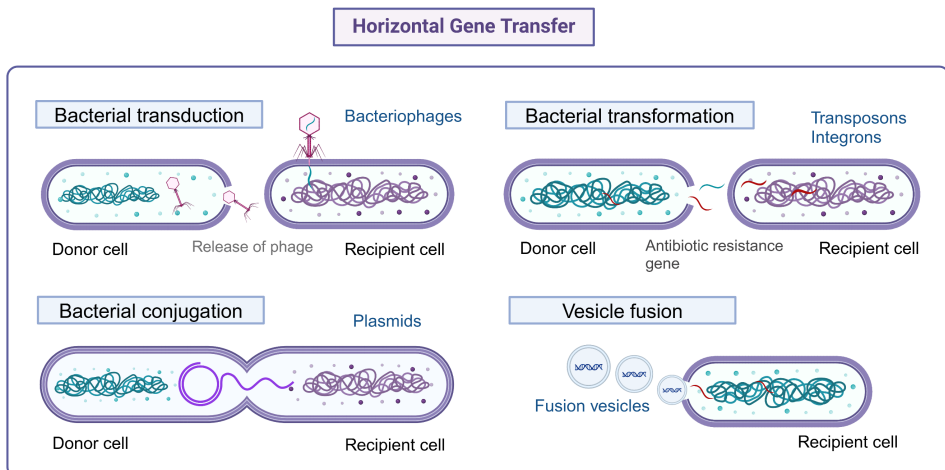


Figure 2. Horizontal gene transmission mechanisms: transduction, transformation, conjugation and vesicle fusion. Figure adapted with permission from Bernabeu *et al.* ³.

The mobilome

As mentioned before, apart from transformation, the other two processes require special components: a plasmid for conjugation and a bacteriophage for transduction. Therefore, in these two processes, the genetic environment of the genes involved in the HGT, possibly intrinsic ARGs, may alter the efficacy of gene mobility²³. Is in this scenario where mobility genes take part and facilitate the transferability of ARGs, by the action of MGEs. Regarding mobile genes, a loss or gain of horizontal gene transfer through mobile genetic elements, related to selection pressure-driven changes, may facilitate or impede antibiotic resistance²⁴. Thus, the mobile resistome is known as **mobilome**²⁵.

The mobilome was first defined as all mobile genetic elements in a cell, which are able to move around within or between genomes²⁶. MGEs include plasmids, transposable elements, bacteriophages and integrons²⁷. Plasmids are extrachromosomal replicons that carry genes encoding functions for the survival of the bacteria, such as ARGs or virulence factors²⁸. Conjugative plasmids include genes that encode proteins required for plasmid transfer. The most prominent are group F (IncF) plasmids, commonly found within the *Enterobacteriaceae* family²⁹. Transposable elements, including insertion sequences (ISs), carry genes that code for proteins essential to their mobility. When two ISs flank an accessory gene, such as an ARG, they form a transposon. Unlike plasmids, transposable elements are usually integrated into the host chromosome³⁰. Bacteriophages are viruses that replicate within bacterial and archaeal hosts. Although their role in the dissemination of ARGs remains uncertain, emerging evidence suggests that phages carrying ARGs may transfer them at frequencies comparable to those of plasmids³¹. Finally, integrons themselves are not MGE, but are often embedded with transposons and plasmids allowing them to be mobilized³². Individual integrons consist of three main parts: an integrase (*int1*) gene that helps in the integration of specific gene cassettes; an attachment (*att1*) site into which the gene cassettes are integrated; and the common promoter (Pc) for expression of the gene cassettes^{33,34}.

INFANT GUT MICROBIOTA AND RESISTOME

Early-life microbiome succession in the infant gut ecosystem

The human intestinal microbiome is a diverse community of microorganisms, with the colon harboring the highest density. The significance of the gut microbiota for human health has gained remarkable attention in recent years, becoming a major focus of biomedical research. A growing body of evidence demonstrates that disruptions in the composition and function of the gut microbiota — often referred to as dysbiosis — are closely associated with an increased risk of developing non-communicable diseases (NCDs), such as obesity, type 2 diabetes, allergies, and neurodevelopmental disorders^{35–38}. Notably, the prevalence of many of these NCDs is rising sharply among children, raising concerns about the long-term health implications of early-life microbial imbalances³⁹. Understanding the development and stability of the gut microbiome during infancy and childhood is therefore crucial.

The evolution and assembly of the gut microbiota in infants occurs in three distinct stages: the developmental phase, spanning from birth to approximately 14 months; the transitional phase, covering ages 15 to 30 months; and the stable phase, which begins around 31 months and continues onward ⁴⁰ (Figure 3). Initial microbial colonization begins at birth, when the neonate is exposed to microorganisms originating from the maternal vagina, gut, and the surrounding environment. Several studies have detected bacteria in the neonate's first-pass feces, known as meconium, with species from the phyla Pseudomonadota and Bacillota, particularly members of the *Enterobacteriaceae* and *Enterococcaceae* families, as well as other genera including *Bifidobacterium*, *Bacteroides* and *Prevotella* ^{41,42}.

During vaginal delivery, neonates are exposed to maternal microbiota from the vagina, but also from feces and skin, leading to the transmission of specific maternal microbial strains. In these cases, the maternal gut microbiota plays a dominant role in shaping the infant's early microbial community as they are adapted

to persist in the infant gut for months, playing a vital role in gut immunity and homeostasis^{43–45}. In contrast, infants born via C-section are less exposed to maternal microbiota and then, more frequently colonized by bacteria from environmental sources. As a result, during the first weeks of life, the gut microbiota composition differs significantly between vaginally delivered and C-section-born infants^{43,44,46}.

The initial microbial assembly just after birth is marked by increasing diversity and richness, progressing from first-pass fecal samples to the 7-day neonatal microbiota. This early colonization is mainly driven by key pioneer bacteria such as *Bifidobacterium* (**Box 2**) and *Bacteroides*, but also by other microbes including *Escherichia coli* (*E. coli*), *Clostridiales*, *Klebsiella* as well as members of other kingdoms such as yeast, fungi, viruses and phages^{47,48}.

During the transitional phase, taxa abundance remains highly variable, and the neonatal microbiota is believed to undergo two critical adaptation periods⁴⁹. The first adaptation occurs as a result of lactation. Newborns and infants are fed human milk or formula (or fortifier) exclusively for the first months of life. During formula feeding, the gut microbiota changes – bifidobacteria and lactobacilli usually promoted during breastfeeding decrease-, and clostridia, enterobacteria, and enterococci become more common^{50–52}. The second adaptation is initiated within the weaning period- the introduction of solid foods-, which promotes the establishment of an adult-like microbiota characterized by an increased abundance of species belonging to the *Bacillota* and *Bacteroidota* phyla^{45,53}. At this point, the gut microbiota reaches a stable and mature composition that typically continues into adulthood. As infants grow, microbial diversity within the gut increases, while interindividual variation decreases, leading to a more consistent and adult-like microbial profile.^{45,54}

Box 2. A closer look into the *Bifidobacterium* genus in early life.

Bifidobacterium spp. are essential microbial pioneers in the early development of the infant gut microbiota, particularly in breastfed infants, where they can comprise up to 80% of the total gut microbial community during the first months of life. Colonization typically begins shortly after birth, with *Bifidobacterium* acquired through maternal transmission and environmental exposure. This colonization is strongly influenced by mode of delivery, feeding practices, antibiotic exposure, and gestational age.

Their ability to metabolize HMOs provides them with a competitive advantage, allowing them to colonize and thrive in the neonatal gut. The predominant species in the infant gut include *B. infantis*, *B. breve*, *B. bifidum*, and *B. longum*, which are specifically adapted to utilize HMOs.

This genus contributes to critical physiological processes, including:

- digestion of complex carbohydrates
- production of SCFA
- modulation of gut barrier function



Recent studies have highlighted that *Bifidobacterium*, particularly in healthy infants, tend to harbor fewer ARGs compared to other gut bacteria. Conversely, reduced *Bifidobacterium* colonization has been linked to increased colonization by opportunistic and potentially resistant pathogens such as *Enterobacteriaceae* and *Clostridioides difficile*. Their dominance in early life has been associated with a lower overall resistome burden, suggesting a potential protective role against the early establishment of antimicrobial resistance in the infant gut.

Early-life factors such as delivery mode, feeding practices, antibiotic exposure, and environmental influences can have lasting effects on the microbial ecosystem and, by extension, on the child's risk of developing chronic diseases. Antibiotic use significantly alters the gut microbiota by reducing microbial diversity and selectively promoting the growth of resistant strains⁵⁵. The selective pressure imposed by antibiotics can lead to the proliferation of ARGs within the gut, contributing to the development and spread of multidrug-resistant organisms. In this context, microbiome research is emerging as a vital tool not only for addressing challenges like antibiotic resistance, but also for designing preventive strategies aimed at preserving microbial health from the earliest stages of life (**Figure 3**).

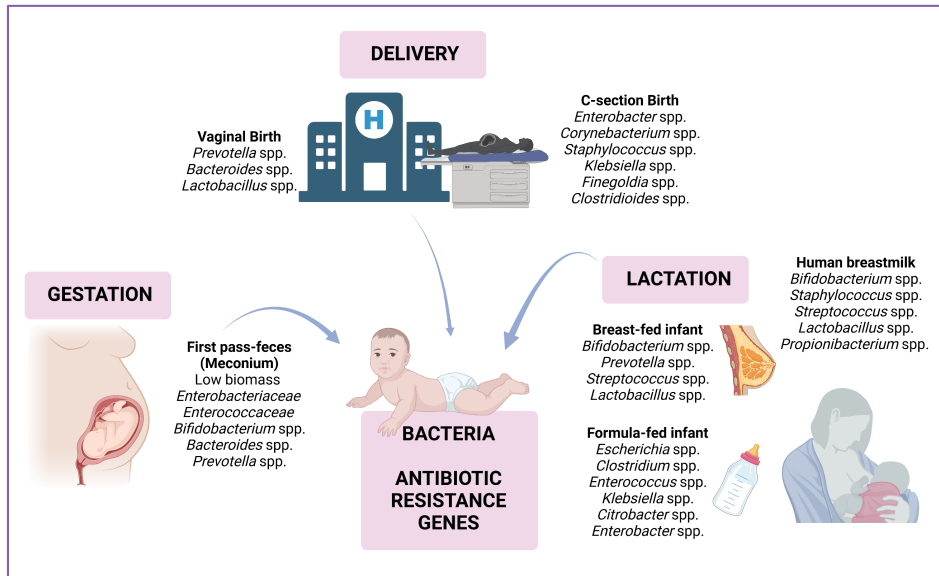


Figure 3. Early microbial contact from conception to first year of life. Modified from Samarra *et al.*²

The maternal-infant gut resistome

The composition of the gut microbiota is a key factor in determining the presence of ARGs, as most of these genes are carried by specific taxa. The development of the infant's resistome is linked to the evolution of the microbial composition, which contributes to the dynamics and stabilization of the infant resistome to adulthood^{26,45}. Metagenomic shotgun sequencing has revealed the high levels of ARGs in the gut microbiome of infants compared to adults, even when not exposed to antimicrobials^{54,56–58} (Table 2). This is likely due to the main role of Pseudomonadota in the infant microbiota during the first months of life, being later replaced by other bacterial phyla. Further, infant resistome analyses at different time points have elucidated higher relative abundances of ARGs in younger infants in comparison to older children^{45,59}. There is evidence that an increased presence of MGEs in infant feces may facilitate the transmission of ARGs among and between species⁵⁶. In the context of mother-infant transfer, it has been seen that the acquisition of the mobilome in early stages of life may be due to maternal influence

and/or family-specific environment, with the latter being the most important influence at 6 months of life⁶⁰.

Studies employing culture-based or metagenomics methods have shown that Gammaproteobacteria are major carriers of ARGs, while Bacillota is also enriched in pregnant women and can carry ARGs^{61,62}. The most abundant and unique ARGs in the infant gut belong to *Escherichia*, *Klebsiella*, *Citrobacter*, and *Enterobacter* species⁶³. On the contrary, *Bifidobacterium* spp. are negatively correlated with antibiotic resistance load⁶⁴. Members of this genus are relevant commensals that dominate the breastfed infant gut microbiome, primarily due to their ability to use HMOs and other carbohydrates efficiently^{65,66}. *Bifidobacterium* species are less likely to possess ARGs than other taxa such as *Streptococcus*, *Staphylococcus*, *Enterococcus* and *Bacteroides* spp.^{67,68}.

Although load and variety of antibiotic resistance may vary between groups of infants according to different early-life factors, most predominant ARGs confer resistance to beta-lactams, tetracyclines, macrolides, aminoglycosides and quinolones⁶⁹.

Beta-lactamases are commonly used during pregnancy and labor, resistances to this type of antibiotic have appeared in infant fecal samples^{63,69} (**Table 2**). Most beta-lactamases genes are extended-spectrum beta-lactamases (ESBLs), that can be grouped in: TEM beta-lactamases (class A), OXA beta-lactamases (class D), SHV beta-lactamases (class A) and CTX-M beta-lactamases (class A)⁷⁰. ESBL-producing Enterobacteriaceae (ESBL-E), including *E. coli* and *Klebsiella pneumoniae*, are a public health concern as they can potentially contribute to the neonatal mortality, and they are ranked as a priority among pathogens in need of new treatment by the WHO⁷¹. These multidrug-resistant bacteria cause a mortality rate of more than 70% in infants as they are widely spread in the gut microbiome⁷².

Genes conferring resistance to tetracycline (*tet* genes) have been reported to be the most abundant among infant fecal samples and are therefore the most representative resistant genes in the infant resistome⁷³. The *tet* genes are the most frequently shared ARGs between mothers and infants, indicating that they are easily transmitted from the mother to the newborn^{63,74–76}. Although this antibiotic is not recommended during pregnancy and early life due to potential dental staining effects, it is still extensively used in animals and, therefore, the environment and the diet may be sources of *tet* genes without direct exposure to the antibiotic⁷⁶.

Macrolides are commonly used against infections in infants, such as trachoma, pertussis and *Campylobacter enteritis*^{77,78} (Table 2). Clindamycin and erythromycin are used to treat infections in skin and tissues produced by staphylococci. Nevertheless, the expression of macrolide-lincosamide-streptogramin B resistance (MLSB) can reduce the efficacy of these antibiotics. The accumulation of macrolide resistance genes in the gut microbiome of newborns could present future consequences for their health. Klassert *et al.*⁶⁹ found that genes for macrolide resistance (*mefA*, *ermB*, and *ermC*) are the most widely distributed among mother-infant pairs and have the greatest likelihood of being transmitted vertically⁷⁹.

Aminoglycosides, usually combined with a beta-lactam, are widely used for neonatal sepsis (Table 2). Antibiotics such as gentamicin, streptomycin, kanamycin, neomycin are examples of aminoglycosides. It has been identified in the infant gut microbiota the presence of genes for acetylation, phosphorylation and adenylation conferring aminoglycoside resistance⁸⁰. The *aac(6')-aph(2'')*, *aadE* and *aph(3')-I* genes are the most detected ARGs against aminoglycosides during the first year of life⁷⁰.

Quinolone antibiotics are synthetic and widely used in medicine (Table 2). Like other antibiotics, resistance to quinolones has emerged due to their frequent use, and it is now common in some bacterial pathogens. Although quinolones are rarely used in paediatrics, the presence of ciprofloxacin-resistant *E. coli* has been

found in the feces of infants⁸¹. Additionally, when mothers are prescribed quinolones, there is an increased risk of their offspring acquiring community-acquired, quinolone-resistant *E. coli*⁸².

Table 2. Antibiotics commonly used during the perinatal period.

Antibiotic	Class of antibiotic	Target infection
Penicillins	β-lactams	Group B streptococci infections, respiratory infections, urinary tract and skin infections, septicaemia.
Cephalosporin		Respiratory tract, urinary tract and skin infections, gonorrhoea.
Carbapenems		Gram-negative bacterial infections of the urinary tract, kidney, gastrointestinal tract, respiratory tract, and urogenital and pelvic area.
Azithromycin, erythromycin	Macrolides	Respiratory, intestine, gynaecological, urogenital and skin infections, chlamydia infections and syphilis.
Ciprofloxacin, norfloxacin	Quinolones	Serious gram-negative bacterial infections.
Clindamycin	Lincomycin	Respiratory, skin, blood, female reproductive organ, and internal organ infections.
Gentamycin, streptomycin	Aminoglycosides	Pyelonephritis, urinary tract infection, gram-negative bacterial infections.
Metronidazole	Nitroimidazole	Infections affecting the reproductive system, gut, skin, heart, bones, joints, lungs, blood, and nervous system.
Vancomycin	Glycopeptide	Colitis, <i>C. difficile</i> –induced diarrhea, gram-positive bacterial infections.
Trimethoprim	Dyaminopyrimidines	Urinary tract infections, pneumonia, diarrhea.

Adapted from Samarra *et al*⁷.

Factors shaping the infant's gut microbiome and resistome

The gut microbial community is highly dynamic during infancy and is, therefore, influenced by both host-related and environmental factors that can alter the microbiota's composition and functionality. Understanding these dynamics is crucial for investigating how the infant gut resistome is established (**Figure 4**).

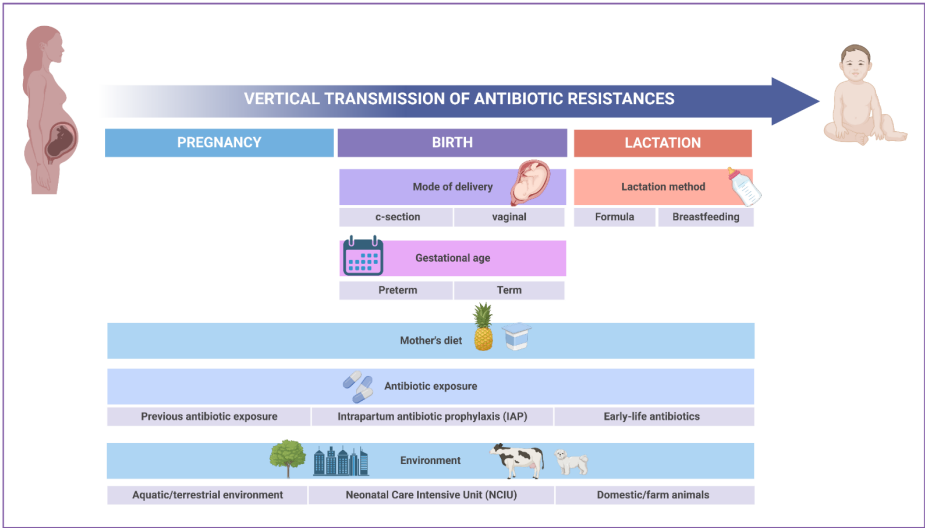


Figure 4. Factors influencing antibiotic resistance acquisition and vertical transmission. Figure from Samarra *et al*¹. **Annex Table 1** presents recent studies investigating the infant gut resistome and transfer of ARGs. The table includes studies investigating the infant resistome and applying shotgun metagenomics and selected articles that have looked at ARG transfer, regardless of which methods were used, in the last 5 years.

Mode of delivery

Women who undergo a C-section receive a single dose of penicillin or a first-generation cephalosporin as a preventive measure, rather than other types of antibiotics, and that they should be administered 20 minutes before skin incision⁸³. For vaginal deliveries, the WHO advocates the use of a single dose of intravenous amoxicillin and clavulanic acid administered as soon as possible after birth and no more than 6 hours after birth⁸⁴. Although not extensively studied, infants born by C-section are currently exposed to antepartum antibiotics via the umbilical cord, as these antibiotics rapidly cross the placenta. This exposure to antibiotics, together with the above-mentioned lack of contact with maternal vaginal microbiota, C-section infants have a lower diversity and richness of microbiota, mainly characterized by the presence of skin- and environmental-associated microbes^{45,85,86}. The gut microbiota of vaginal-delivered infants differs from those born via C-section over the first year of life, with higher levels of *Bifidobacterium* spp. and lower levels of *Klebsiella* and *Enterococcus* genus^{86,87}. Moreover, C-section

delivery has been associated with reduced human milk microbiota diversity and richness^{88–90}. Therefore, as delivery mode shapes maternal-infant and milk microbiota, it can also influence the maternal, infant and milk resistome⁹¹.

It has been seen that C-section delivered infants harbor larger proportions of ARGs and a distinct resistome composition than vaginal born infants^{63,92–94}. ARGs have been found in neonates within hours of birth, indicating that colonization of the newborns with ARMs can occur at birth or soon after, likely through contact with the mother's vagina and skin or from the hospital environment⁹⁵. As reviewed by Patangia *et al.*⁷⁵, some studies have shown that ARMs can be present in a mother's vagina and be transferred to her offspring. These include *E. coli* resistant to ampicillin and cefotaxime-resistant and multidrug-resistant *E. coli*^{96–98}, penicillin-resistant *Streptococcus*⁹⁹, and tetracycline-resistant bacteria including *Prevotella*, *Lactobacillus*, *Ureaplasma*, *Gardnerella*, *Corynebacterium* and *Staphylococcus* spp. carrying *tetM*, *tetO* and *tetW* genes¹⁰⁰.

Interestingly, the delivery mode exerts a stronger influence on the infant resistome than antibiotics administered in C-section deliveries, but the choice of antibiotic mattered in vaginally delivered infants exposed to intrapartum antibiotics¹⁰¹. Moreover, the effect of delivery mode on individual ARGs seems to be strong during the first 6 months of life¹⁰¹, and persists till 1 year of age⁵⁹. However, longitudinal studies exploring the long-term persistence of this influence remain scarce. Additionally, there is no definitive evidence regarding the impact of delivery mode on the ARG load in human milk and its role in shaping the maternal-infant resistome. Further research is required to elucidate how factors such as delivery mode, IAP, gestational age, maternal diet, and other variables collectively influence early-life antibiotic resistance.

Finally, it has been reported that maturation of gut resistome was driven by infants' changing carbohydrate metabolism¹⁰². The introduction of solid foods, commonly referred to as weaning, represents a pivotal phase in microbial colonization. During this period, the composition of the microbiota undergoes rapid changes, leading to enhanced functional diversity. This diversity supports the metabolism of compounds introduced through an adult-like diet¹⁰³. Despite some

evidence of dietary structure influencing ARG abundance in early life¹⁰², direct evidence connecting dietary transitions during infancy to resistome dynamics remains limited. Further research is required to clarify how dietary profiles shape the resistome during this critical developmental stage.

Gestational age

Premature infants (<37 weeks of gestation) are of particular concern as they often undergo prolonged and early antibiotic treatment soon after birth, which can significantly alter their gut microbiome¹⁰⁴. As a result, antibiotic treatment depletes symbiotic gut bacteria and enriches the amount of ARGs^{105,106}. Premature newborns are disadvantaged compared to term infants, as they display a lower diversity of the gut microbiota, delayed colonization with *Bifidobacterium* and *Bacteroides*^{107,108}, and increased abundance of bacteria with pathogenic potentials, such as *Enterobacter* spp., *Escherichia* spp. and *Enterococcus* spp., which are related with hospital infections and in neonatal intensive care units (NICUs)^{57,109,110}. In addition, preterm infants are often delivered via C-section, which also promotes the acquisition of antibiotic-resistant microorganisms¹¹¹.

Early antibiotics exposure

The impact of antibiotic exposure on an infant's resistome is not limited to direct administration to the infant; it also occurs during critical stages such as pregnancy, delivery, and lactation. Didier *et al.*¹¹² demonstrated that antibiotic use during the antenatal period can elevate the risk of antibiotic-resistant infections. Similarly, Li *et al.*¹¹³ found that perinatal antibiotic administration disrupted the transfer of maternal bacteria to infants during the critical early stages of microbial colonization. Antibiotic exposure increased the prevalence of horizontally transmitted bacteria from the environment, including harmful strains frequently associated with hospital settings. This effect was most pronounced within the first 3 to 7 days post-birth. Additionally, offspring of mothers who received antibiotics during the third trimester exhibited higher levels of genes conferring resistance to tetracycline, macrolides, and MLSB⁹⁴. Offspring of mothers exposed to beta-lactam

antibiotics during pregnancy or labor showed a significantly increased presence of *bla* genes⁷⁰.

Intrapartum antimicrobial prophylaxis (IAP) is a common clinical procedure during delivery that is used to prevent early-onset Group-B-streptococci (GBS) infections and in cases of pre-labour rupture of membranes or C-section deliveries. It is present in more than 30% of labours and is by far the most common exposure way to antibiotics in the perinatal and neonatal stages^{114,115}. The effect of this treatment on the gut microbiome has gained attention the last decade and several articles have demonstrated an impact of IAP on the gut microbiota development. Several common findings highlighted the increment of *Enterobacteriaceae* and the reduction of strict anaerobes such as the genus *Bifidobacterium* or *Bacteroides*¹¹⁶. Data accumulated during the last years suggest that IAP may also have lasting and significant effects on the infant's resistome and mobilome. Two seminal studies demonstrated the impact of IAP on the prevalence of ARGs and mobile elements in vaginally delivered full-term babies, showing also a beneficial effect of breastfeeding on the ARGs burden^{56,117}. In a longitudinal study, the importance of antibiotic exposure on the resistome diminished with time since treatment, but some ARGs persisted more than six months after major antibiotic treatments⁶³. Most of the current studies concluded that the selective pressure exerted by IAP favours the presence of different bacteria carrying ARGs, most of them species belonging to Pseudomonadota phyla or *Staphylococcus* genus¹¹⁶. In addition, IAP has also been associated with increments of ARGs in other body microbiomes, such as the nasopharynx and oral microbiota¹¹⁶. Still, there are some studies that haven't found any or little association between the use of specific antibiotics and ARG profiles^{94,106}.

Antibiotic exposure in preterm infants or due to neonatal infections has been observed to increase the abundance of ARG¹¹⁸, specifically, beta-lactam and tetracycline resistance, and decrease the diversity of the resistome^{119,120}, as well as a dramatic increase in the number of opportunistic pathogens carrying multidrug efflux pump encoding genes⁹². Additionally, infants can inherit residual effects of

past and present antibiotic use of the mother. Patangia *et al.* found that even before being exposed to antibiotics, newborns carry ARMs acquired from their mothers and the environment⁷⁵. In relation, one study has indicated that antibiotic use by breastfeeding mothers had no overall significant influence on ARGs in the gut of exclusively breastfed children at 1 year of age¹¹⁸. The long-term effects of antibiotic exposure in infants remain poorly understood, largely due to the infrequency of prolonged treatments in this population. However, given the documented short-term impacts of antibiotic exposure on the infant microbiome and resistome, further research is essential to comprehensively understand these consequences and their potential long-term implications.

Environmental factors: One Health perspective

In order to understand ARG transmission, the development of the human gut resistome in early life and the crossover between mother and offspring resistome, it is necessary to refer to the One-Health perspective (Human-Animal-Environment)¹²¹. There is increasing concern that the use of antibiotics in food-producing and pet animals may be a source of antimicrobial resistance to humans¹²². In this sense, animal and environmental resistome may have a direct effect on the establishment and development of the human resistome. Indeed, having pets or animals at home has been found to influence the growth and development of gut bacteria^{123–128} and, therefore, this may affect the ARG carriage through the impact on the microbiota and the potential transference of strains harbouring ARGs. Although antibiotic usage in animals is more and more regulated, antibiotics are still given to animals to treat disease and to not only prevent infection but also the propagation of disease to other animals¹²⁹. Similar to humans, antibiotics in animals cause changes in gut bacterial composition: a decrease in the species of *Lactobacillus*, *Bifidobacterium*, and *Ruminococcus* and a rise in *E. coli* levels were observed, leading to alterations in ARG levels¹³⁰. In addition, the animal resistome is not only developed in antibiotic-treated animals; wild animals also have ARGs¹³¹

Humans can be exposed to ARGs from animals by direct contact with them but also by consuming vegetables that have been cultivated on soil fertilized with manure and swine farm wastewater, or by consuming animal meat. Both manure and wastewater from livestock farms have been observed to contain residues of antibiotics and resistant microorganisms, such as *Pseudomonadota*, *Bacillota*, *Actinomycetota* and *Bacteroidota*¹³². Downstream, ARGs and antibiotic residues have been detected in vegetables grown in manure-amended soils, which then may reach human microbiome promoting horizontal gene transfer¹³³. Moreover, the process of dressing a carcass may result in contamination with ARMs, which can come from sources such as the animal's skin and gut contents, the hands of the workers, and the slaughter environment¹³¹. Therefore, it is important to consider that all ecological niches harbouring microbial communities will contain ARGs, including aquatic environments and terrestrial environments (agricultural sites, wastewater plants and hospital environments such as the NCIU)^{134,135}. Therefore, the One-Health initiative must be brought to the forefront of ARG research.

Early life breastfeeding practices

Breastfeeding provides numerous benefits for both infants and mothers. It is considered the main postnatal link between mothers and infants, including the transfer of maternal immune cells and antibodies, and it serves as a consistent source of bacteria for the newborn's gut, being the genus *Lactobacillus*, *Bifidobacterium*, *Staphylococcus* and *Streptococcus* the most dominant genera in a woman's milk¹³⁶. Feeding mode significantly outweighs other factors in shaping the gut microbiome and resistome¹³⁷. Infants who are exclusively breastfed have a more varied gut microbiome and fewer antibiotic-resistant bacteria, whereas formula feeding can cause changes in the gut environment leading to a simpler microbiome dominated by bacteria carrying antibiotic resistance genes^{59,60,92,94}. As reported by Pärnänen *et al*¹⁰⁵, formula feeding is associated with an increase in the presence of antibiotic-resistant genes and a decrease in the presence of bacteria that are typically found in a healthy gut microbiome. The families of obligate anaerobic bacteria, including *Veillonellaceae*, *Bifidobacteriaceae*,

Lachnospiraceae, *Clostridiaceae*, and *Porphyromonadaceae*, were reduced in formula-fed infants. On the other hand, the populations of facultative anaerobic and aerotolerant bacteria, such as *Enterobacteriaceae*, *Enterococcaceae* and *Staphylococcaceae* increased in these infants. Formula-fed neonates had clinically significant ARGs that could potentially lead to methicillin resistance in *Staphylococcus aureus* and ESBL phenotypes.

Nonetheless, human milk could also be a source of antibiotic-resistant bacteria. Studies have shown the presence of antibiotic-resistant strains of *Staphylococcus*, *Streptococcus*, *Acinetobacter*, *Enterococcus*, and *Corynebacterium* in human milk^{56,138}. Multi-drug-resistant profiles have also been detected in these species¹³⁹. Antibiotic resistant bacteria found in human milk have been linked to the administration of certain antibiotics to mothers during lactation¹³⁹. Multi-drug resistant strains of *Streptococcus* and *Staphylococcus* have been detected to be transferred to infants through breastfeeding^{140–142}. Metagenomic analyses have highlighted the high levels of ARGs and MGEs in breastmilk and the resemblance of the infant resistome to the mother's milk resistome⁵⁶. Pärnänen *et al.*⁵⁶ showed that infants shared gut resistomes and mobilomes with their own mother's milk microbiota with 70% of the ARGs detected in milk present in infant feces. Therefore, a mother can impact the infant's gut resistome by transmitting resistant bacteria through her milk, although the resistance genes can also come from other parts of her body or external sources. Additional research is necessary to examine the transfer of antibiotic resistance from a mother to her infant, as it can affect the proper establishment of the gut microbiota and resistome.

Finally, it has been reported that maturation of gut resistome was driven by infants' changing carbohydrate metabolism¹⁰². The introduction of solid foods, commonly referred to as weaning, represents a pivotal phase in microbial colonization. During this period, the composition of the microbiota undergoes rapid changes, leading to enhanced functional diversity. This diversity supports the metabolism of compounds introduced through an adult-like diet¹⁰³. Despite some evidence of dietary structure influencing ARG abundance in early life¹⁰², direct evidence connecting dietary transitions during infancy to resistome dynamics

remains limited. Further research is required to clarify how dietary profiles shape the resistome during this critical developmental stage.

THE IMPORTANCE OF HUMAN MILK IN EARLY LIFE

Human milk is considered the optimal source of nutrition for all infants. Its composition includes a complex mixture of macronutrients—such as lipids, proteins, carbohydrates, and vitamins—as well as a variety of bioactive molecules, including growth factors, hormones, cytokines, and antimicrobial agents. This rich blend not only stimulates infant growth but also plays a critical role in defending against pathogens, promoting immune development, and nurturing the commensal gut microbiota¹⁴³. Therefore, the comprehensive benefits of human milk warrant dedicated exploration to fully understand its complex roles and mechanisms.

Human milk bioactive composition

The composition of human milk is influenced by various factors, including the maternal age, dietary habits, physical activity, ethnicity, geographic region, secretor status, and lactation stage^{144–147}. Lipids serve as the main energy source in human milk, primarily occurring as triacylglycerols, alongside smaller amounts of diacylglycerols, monoacylglycerols, phospholipids, cholesterol, and over 200 different fatty acids¹⁴⁸. The protein content of human milk is highly diverse and fulfill various biological roles, such as providing essential amino acids, facilitating enzymatic digestion, generating antimicrobial peptides, and supporting the infant's immune system and gut microbiota¹⁴⁹. The most abundant carbohydrates in human milk are lactose and the HMOs. Lactose and HMOs are the predominant carbohydrates in human milk. While lactose is efficiently digested in the small intestine, HMOs resist digestion and reach the infant's colon, where they carry out multiple beneficial roles, as discussed in the following section^{143,145,150}.

In humans, secretory IgA (sIgA) is the most predominant Ig in human milk, followed by IgM and IgG. sIgA acts as the primary protective agent of human milk as it has a major role in preventing attachment of enteropathogens to gut epithelium in breastfed infants¹⁵¹. In addition, lactoferrin, lactadherin, and mucin are key

immunological components of human milk that contribute to innate immunity. Cytokines are multifunctional glycoproteins essential for the healthy immune system development in newborns¹⁵².

Finally, human milk has also its own microbiome, which is characterized by the presence of the genus *Staphylococcus*, *Streptococcus*, *Lactobacillus*, *Pseudomonas*, *Bifidobacterium*, *Corynebacterium*, *Enterococcus*, *Acinetobacter*, *Rothia*, *Cutibacterium*, *Veillonella*, and *Bacteroides*¹⁵².

Human milk oligosaccharides

HMOs are a diverse group of free glycans composed of over 200 structural variations, all derived from five core monosaccharides: D-glucose (Glc), D-galactose (Gal), N-acetylglucosamine (GlcNAc), L-fucose (Fuc), and sialic acid (Sia)¹⁵³. All HMOs are built upon a lactose (Gal β 1-4Glc, Lac) core, which can be extended through β 1-3 or β 1-6 linkages, forming either a type 1 chain (Gal β 1-3GlcNAc) or a type 2 chain (Gal β 1-4GlcNAc). Additional modifications with fucose (Fuc) and sialic acid (Sia) result in oligosaccharides ranging from 3 to 22 sugar units in length^{154,155} (**Figure 5**).

Based on their structural modifications, HMOs can be classified into three main groups. Fucosylated HMOs, which constitute approximately 35–50% of all HMOs, are formed when lactose is modified by α -1,2 or α -1,3 linkages, producing molecules such as 2'-fucosyllactose (2'FL) and 3-fucosyllactose (3-FL). Sialylated HMOs, accounting for around 12–14% of total HMOs, contain sialic acid (Neu5Ac) linked via α -2,3 or α -2,6 bonds, leading to the formation of 3'-sialyllactose (3'SL) and 6'-sialyllactose (6'SL). Lastly, neutral non-fucosylated HMOs, making up about 42–55% of all HMOs, include structures like lacto-N-tetraose (LNT) and lacto-N-neotetraose (LNnT), which are formed when lacto-N-biose (Gal β 1-3GlcNAc, LNB, type 1 chain) or N-acetylgalactosamine (Gal β 1-4GlcNAc, GalNAc, type 2 chain) is linked to the lactose core. These molecules can further extend into larger oligosaccharides such as lacto-N-hexaose (LNH) and lacto-N-neohexaose (LNnH) through β 1-6 linkages. The degree of polymerization in HMOs can vary significantly,

ranging from 3 to 32 sugar units, with type 1 chain HMOs being more abundant than type 2 chains^{154,155}.

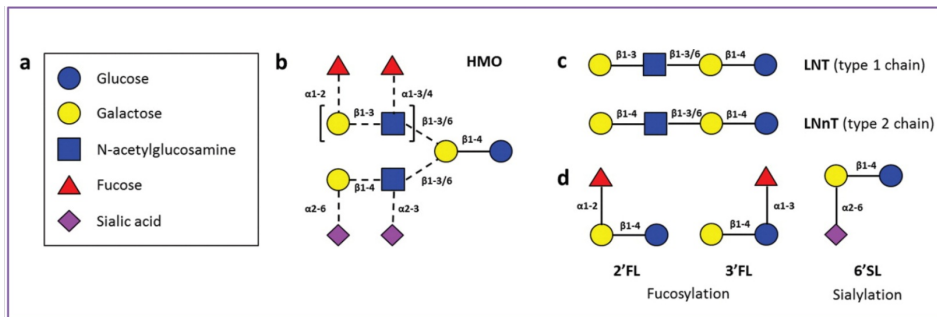


Figure 5. Structural diversity of HMOs. (a) Core monosaccharide components of HMOs; (b) Representative glycosidic linkages formed between these sugars; (c) Diagrammatic illustration of type 1 and type 2 chain structures; (d) Examples of HMOs modified with fucose (fucosylated) and sialic acid (sialylated) residues. Adapted from Akkerman *et al*¹⁵⁶.

The significant interindividual variability in HMO structural diversity among women is influenced by genetic polymorphisms in the Secretor (*Se*) and Lewis (*Le*) blood group genes¹⁵⁷ (Table 3). Therefore, nursing mothers are classified into four secretory types: *Se+Le+*, *Se+Le-* and *Se-Le+* for secretor phenotypes, and *Se-Le-* for non-secretor phenotypes, and the ratio of these four types varies depending on the geopolitical situation or donor's ethnicity¹⁵⁸. The expression of *Se* and *Le* alleles determines a mother's capacity to produce specific glycotransferases in the mammary gland: α -1,2-fucosyltransferase (*FUT2*) and α -1,3/4-fucosyltransferase (*FUT3*). Mothers with functional forms of both enzymes can synthesize a full range of fucosylated HMOs. However, an inactive *FUT2* gene prevents the production of α -1,2-fucosylated HMOs, such as 2'FL and lacto-N-fucopentaose I (LNFP I), while an inactive *FUT3* gene limits the synthesis of α -1,3- and α -1,4-fucosylated HMOs, including LNFP II and LNFP III. In European populations, the distribution of the resulting four milk groups is approximately 69% *Se+Le+*, 20% *Se-Le+*, 9% *Se+Le-*, and 1% *Se-Le-*¹⁵⁹. HMOs are highly concentrated in colostrum, reaching levels of 20–24 g/L, while their concentration decreases to 7–14 g/L in mature milk by day 30 of lactation¹⁶⁰. Secretor mothers produce higher concentrations of HMOs compared to non-secretor mothers (*Se+Le+* > *Se-Le+* ~ *Se+Le-* > *Se-Le-*)¹⁶¹.

Table 3. Characteristics of the principal HMOs secreted by women. Maternal genotype is determined by the Secretor (Se) and Lewis (Le) blood group genes.

Oligosaccharide	Abbreviation	Type	Charge	Secreted by
2'-Fucosyllactose	2'FL	Fucosyl	Neutral	Se+
3-Fucosyllactose	3-FL	Fucosyl	Neutral	All
Lacto-N-fucopentaose I	LNFP I	Fucosyl	Neutral	Se+
Lacto-N-fucopentaose II	LNFP II	Fucosyl	Neutral	Le+
Lacto-N-fucopentaose III	LNFP III	Fucosyl	Neutral	All
Lacto-N-difucohexaose I	LNDFH I	Fucosyl	Neutral	Se+Le+
Lacto-N-difucohexaose II	LNDFH II	Fucosyl	Neutral	Le+
Difucosyllactose	DFLac	Core	Neutral	Se+
Lacto-N-tetraose	LNT	Core	Neutral	All
Lacto-N-neo-tetraose	LNnt	Core	Neutral	All
3'-Sialyllactose	3'SL	Sialyl	Acidic	All
6'-Sialyllactose	6'SL	Sialyl	Acidic	All
Sialyl-lacto-N-tetraose a	LST a	Sialyl	Acidic	All
Sialyl-lacto-N-tetraose b	LST b	Sialyl	Acidic	All
Sialyl-lacto-N-tetraose c	LST c	Sialyl	Acidic	All
Disialyllacto-N-tetraose	DSLNT	Sialyl	Acidic	All

Table adapted from Kunz *et al.*¹⁵⁷

Differences in milk, maternal and offspring's gut microbiota composition based on mother's secretor status have been documented. Notably, *Bifidobacterium* spp. have been found to establish earlier and more frequently in infants fed by secretor mothers compared to non-secretor mothers¹⁶², likely due to the influence of secretor status on the composition of HMOs in human milk, as previously discussed¹⁶³. However, recent studies have reported no significant association between the *FUT2* genotype and fecal microbial composition in adults¹⁶⁴.

Beneficial effects of HMOs

HMOs act as prebiotic substrates for specific gastrointestinal microbes. The capacity to metabolize HMOs by bacteria is highly species-specific, with certain species demonstrating superior proficiency in metabolizing these complex carbohydrates due to the presence of a large array of carbohydrate-active enzymes (CAZymes) and glycan transporters encoded in their genomes^{165–168}. Among these,

Bifidobacterium species are dominant, comprising 50–90% of the gut microbiota in breastfed infants. Their prevalence is largely attributed to their ability to efficiently import and metabolize HMOs, facilitated by specialized HMO-related transporters, carbohydrate-binding proteins, and glycoside hydrolases (GHs)^{169,170} especially *Bifidobacterium* species like *B. infantis*, which are equipped with specialized membrane transporters and GHs for efficient HMO metabolism^{171,172}

Cross-feeding interactions further support microbial diversity, where breakdown products from one species promote the growth of others, such as *Bifidobacterium breve* (*B. breve*) benefiting from metabolites produced by *B. infantis*¹⁷³. Additionally, *Lactobacillus* and *Bacteroides* species, along with *Akkermansia muciniphila*, have demonstrated the ability to metabolize HMO-derived components^{174–176}

Beyond shaping microbial communities, HMOs provide protection against pathogens. By mimicking host cell surface glycans, they act as soluble decoy receptors, preventing pathogen adhesion and biofilm formation^{177–179}. Fermentation of HMOs also produces organic acids that lower intestinal pH and inhibit harmful bacteria¹⁸⁰. On a cellular level, HMOs modulate intestinal epithelial function by altering gene expression and glycosylation patterns, reducing pathogen binding and influencing cell proliferation and differentiation^{181,182}. They also play an immunomodulatory role by regulating cytokines and receptor expression in mucosal tissues, aiding immune development and reducing inflammation^{183,184}.

Importantly, HMOs contribute to brain development by serving as a key source of Sia, a nutrient essential for neural connectivity and synaptic plasticity via the formation of gangliosides and polysialylated¹⁸⁵. Additionally, by supporting a balanced microbiome, HMOs influence the gut-brain axis, indirectly affecting cognitive functions through the production of short-chain fatty acids and neurotransmitters³⁷

Can human milk protect against antibiotic resistances?

Building on the extensive health benefits of HMOs previously discussed, intriguing research is their potential role in mitigating antibiotic resistance acquisition in infants. High concentrations of HMOs in the infant gut may reduce susceptibility to colonization by ARM through several interconnected mechanisms, previously mentioned. As a preferred substrate for *Bifidobacterium* species, HMOs enable these beneficial microbes to dominate the infant gut microbiota, thereby inhibiting the establishment and proliferation of ARM and enteric pathogens. For instance, *B. infantis* efficiently metabolizes HMOs, producing acidic by-products that lower intestinal pH. This acidic environment is hostile to *Enterobacteriaceae*, a family that includes several critical antibiotic resistance threats, such as ESBL-producing strains and multidrug-resistant *Salmonella* and *Shigella*. Furthermore, *Bifidobacterium* species exhibit a relatively low prevalence of transferable ARGs

Little is known about the effects of differential microbial and HMO composition of human milk, influenced by the mother's secretor status, on the offspring's resistome. Investigating how variations in maternal secretor status impact the interplay between HMOs, the gut microbiome, and ARG transmission could reveal novel insights into personalized strategies for reducing antibiotic resistance.

HEALTH IMPLICATIONS AND STRATEGIES OF EARLY-LIFE RESISTOME MODULATION

Antibiotic resistance in infants is a significant public health concern, with serious implications for their health and development. Infants are particularly vulnerable due to their developing immune systems and the potential for infections to have both short- and long-term health consequences. This concern is particularly acute in neonates, where resistance is associated with increased mortality and extended hospitalizations, especially in cases involving Gram-negative bacteria such as *E. coli* and *Klebsiella* species¹⁸⁶. Preterm infants are particularly at risk, with a high prevalence of antibiotic resistance genes even without direct antibiotic exposure.

This highlights the importance of early intervention strategies to modulate the neonatal microbiome and limit the propagation of resistant bacteria¹⁸⁷. Apart from strategies that involve Antimicrobial stewardship programs (ASPs)¹⁸⁸, recent studies have focused on developing new approaches to ameliorate the antibiotic resistance load in the human microbiome, but further research into new mechanisms to reduce the resistance load, especially in infants, are needed.

Diet

Diet is significantly linked with the composition of microbiota, as it not only nourishes it but can also alter it, and, presumably, influence the resistome. Several studies have pointed the influence of maternal diet on their offspring gut microbiome, elucidating the role of the diet on the transfer of antibiotic resistances to the infant during delivery or lactation^{189–191}. Recently, studies have started to explore the impact of diet on the dissemination of ARGs in the intestinal microbiota. For instance, it has been reported that a diverse, fibre-rich diet induces lower abundance of ARGs in human gut¹⁹². A diet high in sugar, fat, and protein can alter bacterial composition and diversity and increase the permeability of bacterial membranes, resulting in gut inflammation and changes to gut bacteria and its by-products. These changes result in the amplification and transfer of exogenous ARGs in the gut¹⁹³. Therefore, diet seems to be a potential key factor on both acquisition and transmission of antibiotic resistances in all stages of life but more knowledge is needed to develop dietary strategies that would help to ameliorate antibiotic resistances in the mother-infant environment.

Biotics

It's becoming more than obvious that prebiotics and probiotics might have an important role in the restoration of dysbiosis and gut resilience. In this context, the utilization of probiotics appears to be a highly hopeful strategy in this field, either through simultaneous use with antibiotics to prevent disruption or by taking advantage of their benefits to heal the altered microbiome after antibiotics have caused harm. The presence of *Bifidobacterium infantis* EVC001 in the infant gut

microbiome has been observed to decrease the amount of ARGs and the types of bacteria that carry and potentially spread them among individuals¹⁹⁴. In premature babies, probiotic supplementation may increase protection against harmful bacteria and reduce the negative impact of antibiotics on the microbiome and resistome of the gut¹⁹⁵. A different study demonstrated that administering probiotics to preterm infants during hospitalization after birth decreased the gut microbiome's resistance gene diversity and prevented its persistence¹⁹⁶.

On the other hand, prebiotics, which are not made from live bacteria, are unlikely to lead to an increase in resistance genes. Prebiotics might be a valuable resource in the battle against antibiotic resistance and infection, although their effectiveness has yet to be confirmed¹⁹⁷. Further studies about the use of prebiotics during pregnancy and lactation to prevent antibiotic resistance transfer to offspring is needed.

Synbiotics are formulations that combine prebiotics and probiotics, allowing them to exert both indirect effects by providing metabolic precursors for SCFA production and direct modulation of the gut microbiota by introducing beneficial microbial strains. Research on the role of synbiotics in modulating multidrug-resistant organism colonization remains limited. One study found that synbiotic administration did not significantly alter microbial antibiotic resistance compared to standard treatment¹⁹⁸, while another reported an unexpected increase in *Candida* species colonization in the synbiotic group, though this was resolved after discontinuation of the intervention¹⁹⁹.

Microbiota-targeted restoration interventions

Fecal microbiota transplantation (FMT) has proven to be an effective strategy for decolonizing the gut from clinically relevant ARGs and an effective prevention strategy for recurrences of *Clostridioides difficile* infections^{200,201}. This procedure helps restore the gut microbiota to a composition more similar to that of a healthy individual, thereby reducing the presence of potential pathogens or lowering them to very minimal levels. However, the restoration of the gut

microbiome is not completed within the first few days after transplantation; instead, it may take months to reach a stable and balanced state. Although some resistance genes—particularly those present on plasmids—may persist, the overall burden of resistance genes is significantly reduced through FMT²⁰². Despite these promising results, resistome modulation through FMT remains an area of preliminary research, and there is currently no consensus regarding its benefits and risks in humans. From a safety perspective, expanding donor sample screening to include not only antibiotic-resistant organisms but also specific ARGs could be advisable.

Similarly, limited studies have evaluated the efficacy of vaginal seeding in restoring the gut microbiome of C-section-born infants, although evidence suggests that the procedure facilitates the partial restoration of key gut microbial communities²⁰³. However, vaginal seeding does not fully replicate the natural microbial transfer that occurs during vaginal birth²⁰⁴, highlighting the need for standardized protocols for both research and clinical applications. Further studies are required to establish clear links between the microbial changes induced by vaginal seeding and their potential clinical benefits. Moreover, few studies have specifically focused on the impact of vaginal seeding on the infant resistome, leaving an important gap in understanding its role in modulating ARG load.

THE STUDY OF THE RESISTOME: FROM CULTURING TO SEQUENCING TECHNOLOGIES

Distinct approaches have been employed to characterize ARGs in the human microbiome, including both culture-based and culture-independent methods. With the advancement of next-generation sequencing (NGS) and bioinformatics tools, research on the resistome has expanded significantly, particularly in both human and animal digestive systems (**Figure 6**).

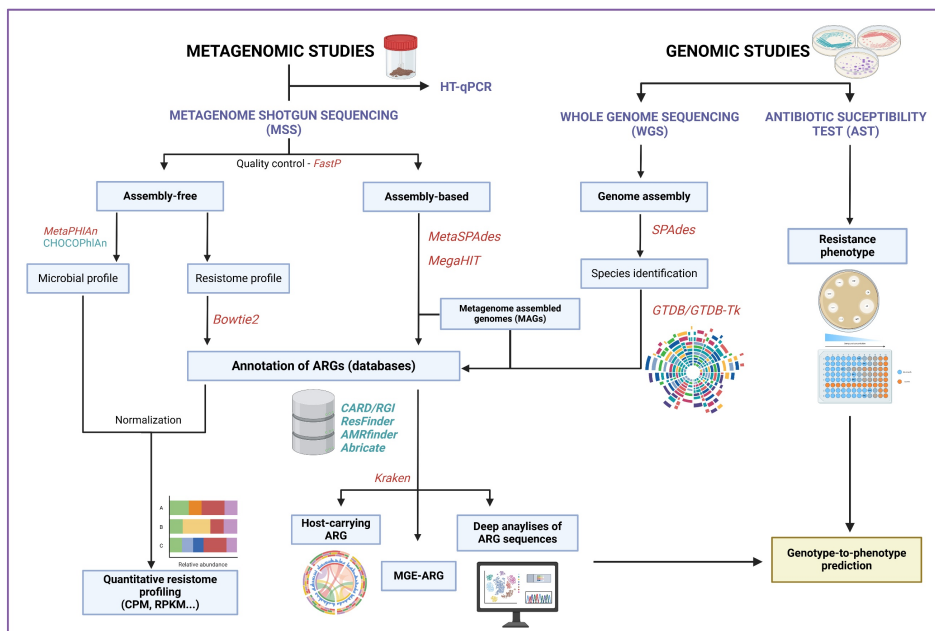


Figure 6. Overview of resistome study methodologies. This figure illustrates the principal methodologies for analyzing the resistome, detailing both culture-dependent approaches like culturomics and culture-independent techniques such as whole genome sequencing and shotgun metagenomics. The diagram highlights the steps involved in each methodology. It also includes the most commonly used bioinformatic tools and databases, which facilitate the identification and annotation of antibiotic resistance genes. *Created in BioRender.* <https://BioRender.com/mozupte>.

Culture-Based Approach

Culturomics is a culture-dependent method for studying complex microbial ecosystems like the human microbiota. This approach has been applied to identify ARGs in agroecosystems, emphasizing the importance of isolating individual ARG-carrying microbes to determine multi-antimicrobial resistance phenotypes²⁰⁵. Cultivating human or environmental samples in antibiotic-containing media has been a common technique for isolating bacteria, with a focus on the human microbiome. Antimicrobial susceptibility testing, both phenotypic and genotypic, is used to identify antibiotic-resistant bacteria. However, this method is time-consuming and has limitations, as it mainly detects easily cultivated bacteria from families like *Enterobacteriaceae* and *Enterococcaceae*, missing many ARGs present in other bacterial groups²⁰⁶. While useful for complementing

metagenomics, this approach is limited to cultivable bacteria and cannot identify unknown or distant elements²⁰⁷.

Non-Culture-Based Approach

Non-culture-based methods offer various techniques for studying the resistome, including high-throughput quantitative polymerase chain reaction (HT-qPCR), Whole Genome Sequencing (WGS) and Metagenome Shotgun Sequencing (MSS).

Polymerase Chain Reaction (PCR)-based approaches

Besides the sequencing technologies, quantitative polymerase chain reaction (qPCR) is the most used culture-independent technique to study the resistome. qPCR is a targeted molecular technique that enables the detection and quantification of specific genes, provided that their nucleotide sequences are known, and appropriate oligonucleotide primers can be designed. This method has been widely used for years to assess the prevalence of selected ARGs across various environmental and clinical reservoirs^{208,209}. It offers a significant advantage over sequencing by providing relative abundance data for each detected gene. Additionally, HT-qPCR can be customized to target specific ARGs of interest, making it a powerful tool for studying clinically relevant or ecologically significant resistance genes with high sensitivity and precision²¹⁰.

The advancement of WGS and the growing number of identified ARGs have facilitated the development of HT-qPCR, enabling the simultaneous analysis of hundreds of ARGs in a single run. However, this method requires the design of specific primers for each target gene, and the data obtained from each reaction are inherently limited to preselected genes. As a result, HT-qPCR is particularly valuable when used in combination with sequencing approaches, providing both targeted quantification and comprehensive resistome profiling.

Multiplex PCR-based amplicon sequencing can also be used to detect ARGs in complex samples, though current studies often target a limited set of genes²¹¹. This method employs targeted amplification to enrich AMR genes in

metagenomes, enabling rapid, high-throughput detection. Despite the limited gene panel, the method shows promise for large-scale AMR surveillance and could be extended to bacterial identification and virulence profiling in clinical settings.

Whole Genome Sequencing

WGS provides a comprehensive view of bacterial genomes, including any ARGs they carry. It allows for the detection of resistance genes directly from bacterial genomes. While WGS can detect ARGs in collected isolates, it does not always capture the complexity of the resistome in vivo, which is influenced by a dynamic microbial population in the human body.

The predominant sequencing technology used in bacterial WGS has been the Illumina platform, with Pacific Biosciences (PacBio) platforms also widely used. Recently, there has been a notable increase in the use of Nanopore platforms for generating WGS data²¹². A key consideration in selecting a sequencing platform involves balancing the number of strains to be sequenced against the comprehensiveness of each genome assembly. Long-read sequencing technologies, such as those from Pacific Biosciences and Oxford Nanopore, produce reads over 10 kb in length, which improves the assembly and mapping of genomic data. This method is particularly valuable for studying the genomic context of ARGs, as it can span repetitive regions that short-read sequencing cannot²¹³. Additionally, single-cell sequencing allows for the examination of the genomic, transcriptomic, and metabolomic functions of individual cells. This technique is particularly useful for studying non-culturable organisms and complex microbial populations. While the high cost of single-cell sequencing has limited its use, it is becoming more accessible and is increasingly applied to microbiome research²¹⁴.

A WGS-based analysis of antibiotic resistance typically follows a structured workflow. It begins with genome assembly, followed by species identification and strain subtyping. Next, the process involves annotation of genes and mutations linked to antibiotic resistance, enabling the identification of resistance determinants. Finally, post-analysis steps focus on understanding evolutionary

processes, such as the emergence, dissemination, and adaptation of resistance genes within microbial populations.

1) Assembly (-free) analyses

The assembly process in genomic workflows typically includes read pre-processing, assembly, and optionally, the refinement of these assemblies. For bacterial genomes, commonly utilized assembly tools are SPAdes²¹⁵ for short-read sequencing, and Flye²¹⁶ as well as miniasm/minipolish²¹⁷ for long-read sequencing. The quality of the assembly, characterized by the generation of contigs with minimal gaps, low error rates, and circularized plasmids, is dependent on various elements such as the genomic structure, sequencing approach, and the methods used for assembly. In some instances, particularly with clinical samples, the assembly phase is omitted (referred to as assembly-free or read-based analysis) to expedite the computational aspects. The primary goals in these scenarios are to rapidly perform taxonomic identification, subtyping, and predict antibiotic resistance at the genomic level soon after sequencing is completed²¹⁸.

2) Species identification

Species-level identification serves as the foundation for several downstream processes, including strain subtyping, selection of appropriate guidelines for antibiotic susceptibility testing, identification of mutations linked to antibiotic resistance, and conducting epidemiological and evolutionary analyses. Definitive species identification is typically achieved by calculating pairwise average nucleotide identity (ANI) values relative to a curated panel of reference genomes. The selection of reference genomes for this step can be guided by prokaryotic type strain repositories, such as gcType²¹⁹ and EzBioCloud²²⁰. For high-throughput scenarios involving numerous genomes, locally executable tools like GTDB-Tk²²¹ or user-developed in-house pipelines may be more suitable.

3) Annotation of antibiotic resistance genes

Some of the most popular and well-maintained databases that feature original curation include the Comprehensive Antibiotic Resistance Database (CARD)²²², ResFinder²²³, DeepARG²²⁴, and AMRfinder²²⁵. These databases are part of the annotation process, which typically involves sequence alignment searches and filtering based on reliable cutoffs. For instance, the Resistance Gene Identifier (RGI) tool associated with CARD employs blast bit score cutoffs that are manually curated to optimize the distinction of each entry against the NCBI non-redundant (nr) protein database²²². Similarly, AMRfinder utilizes bit score cutoffs specifically curated for each profile hidden Markov model (HMM) developed from reference ARG families²²⁵. DeepARG extends this approach by assessing each blast alignment—where a metagenomic read is matched to a reference ARG—calculating the probability of a true-positive detection using pre-trained deep learning models²²⁴. Additionally, tools like ABRICATE combine multiple independent databases and provide a unified script for streamlined annotation, making the process more accessible and efficient²²⁶.

4) Prediction of antibiotic resistances: from genotype to phenotype

Recent advances in artificial intelligence (AI) have significantly enhanced the capabilities of machine learning (ML) in predicting antibiotic resistance^{227,228}. These advancements allow for the effective integration of both phenotypic and genotypic data, providing a more comprehensive and accurate assessment of resistance patterns. Machine learning models can now analyse complex datasets that combine clinical data, genetic sequences, and resistance outcomes, identifying patterns and predictors that were previously obscured. The integration of diverse data types has led to the development of robust predictive models that are not only more precise but also adaptable to new strains and resistance mechanisms as they emerge. As ML continues to evolve, its role in combating antibiotic resistance through smarter, data-driven predictions is becoming increasingly important.

Metagenome shotgun sequencing (MSS)

Shotgun metagenomics enables the study of the environmental microbiome by sequencing all DNA present in a sample. This approach uses next-generation sequencing technologies to analyse complex microbial communities and identify unknown genetic elements²²⁹. To analyse the resistome, shotgun sequencing involves either direct mapping of sequence reads to reference databases or assembly into contigs followed by annotation²³⁰. This method requires high-quality data and robust bioinformatic pipelines for reliable results.

The computational workflows for shotgun metagenomic sequencing share several similarities with WGS-based workflows, particularly in steps such as assembly, species identification, and gene annotation. However, a key distinction in metagenomic analysis is its focus on quantitative profiling in addition to simply cataloguing ARGs present in a sample.

Assembly-free metagenomics allows for the quantitative profiling of reference ARGs in samples without the need for sequence assembly. By employing tools like blastx or other equivalent alignment methods, researchers can directly assess the relative abundance of each ARG by using raw reads as input. It's important to note, however, that the relative abundance indicated by the number of reads aligned to each ARG can be influenced by factors such as the ARG's length and the sequencing depth of the sample. To address these potential biases, it is crucial to implement appropriate normalization techniques to ensure accurate representations of ARG abundances in the metagenomic data.

Incorporating an assembly step (assembly-based metagenomics) in the workflow enables a broad range of downstream analyses, such as detailed characterization of ARG sequences, taxonomic identification of ARG-carrying hosts, and the examination of associations between ARGs and MGEs. However, a key challenge of assembly-based approaches is the potential loss of information, especially when dealing with complex microbiomes or low-throughput sequencing data. In addition, the assembly step is the most computationally intensive and

time-consuming part of the assembly-based metagenomic workflow, often becoming a bottleneck in data analysis. The choice of assembly tools and parameters is frequently constrained by available computational resources. Among the widely used assemblers in metagenomic studies are MEGAHIT²³¹ and metaSPAdes²³², both optimized for handling large and complex datasets. Once assembly is complete and contigs are generated, the next crucial steps in antibiotic resistome analysis involve gene prediction and annotation of ARGs. The tools and databases used for WGS-based ARG analysis are generally applicable to metagenomic studies, facilitating the identification and characterization of resistance genes.

After cataloging ARG sequences from contigs, the next steps in metagenomic analysis involve quantitative profiling of ARGs and their taxonomic assignment at the contig level, both of which are unique features of metagenomic workflows. The relative abundance of each ARG within a sample's metagenome is typically determined by assessing the coverage depth of the corresponding contig. If coverage depth is not provided in the assembler's output, it must be calculated separately by mapping raw sequencing reads back to the assembled contigs, ensuring accurate quantification of ARGs in the microbial community.

To link ARGs to the taxonomy of their microbial hosts, classification tools such as Kraken²³³ can be used to assign taxonomic labels to input sequences. Kraken operates by identifying exact k-mer matches between query sequences and reference genomes that are linked to a taxonomic database. However, the accuracy of these assignments varies based on contig length and sequence characteristics, leading to differing levels of taxonomic resolution—ranging from species-level identification to broader classifications at the phylum level.

From metagenomic assembly it is also possible to obtain metagenome assembled genomes (MAGs). The process begins with the assembly of sequencing reads into scaffolds, which are then clustered into candidate MAGs based on factors such as tetranucleotide frequencies, relative abundances, complementary

marker genes, taxonomic alignments, and codon usage patterns²³⁴. To ensure reliability, only MAGs with high completeness and minimal contamination are selected for further taxonomic classification and gene prediction²³⁵. This approach allows researchers to reconstruct microbial genomes from complex metagenomic datasets, providing deeper insights into microbial diversity and function. Downstream analyses designed to annotate MAGs, gene prediction, gene functional annotations and taxonomic classification and profiling are similar to those for WGS.

Box 3. Factors affecting resistome analysis.

Choice of Analytical Approach

- Read-based methods: Faster and computationally efficient but limited by lack of positional and contextual gene information.
- Assembly-based methods: Allow for reconstruction of genomes or large contigs, offering greater resolution of gene context and regulation, though they are resource-intensive and slower to process [248].

Reference database selection: the precision of ARG detection is highly dependent on the database used. Databases must be comprehensive and frequently updated to capture newly discovered resistance variants.

Alignment and detection parameters: parameters such as identity score, alignment coverage, and detection thresholds must be carefully optimized, as they can significantly alter the results. Gene-specific cutoffs help reduce false positives and improve detection sensitivity.

Sequencing depth and sampling strategy: the phylogenetic composition of the microbiota influences resistome content, and some ARGs may remain undetected in shallow sequencing. Comprehensive sequencing depth and balanced sampling across groups are critical for robust analysis.

Normalization strategies: proper normalization ensures comparability across samples. Common methods include normalization to total read count or normalization to 16S rRNA gene copy number.

FILLING THE GAPS: THE KEY QUESTIONS GUIDING THIS RESEARCH

Given the growing global concern over antibiotic resistance and the critical need to understand its early-life determinants, investigating the development of microbial communities and their resistome during infancy is essential. Despite significant advances in microbiome research, gaps remain in understanding the mechanisms that shape the infant gut resistome. Addressing these gaps is crucial for developing strategies to prevent the spread of resistance from the earliest stages of life. Key factors such as antibiotic exposure, the evolving gut microbiome and resistome, breastfeeding practices, and human milk components must be explored to inform early-life interventions aimed at mitigating antimicrobial resistance.

In this thesis, we explore key challenges in understanding the development of the gut resistome during an infant's first year of life, by trying to answer the following questions:

- 1- How does the acquisition, development, and establishment of the gut resistome occur during the first year of life?**
- 2- What role does the *Bifidobacterium*-driven microbiota play in shaping the infant's gut resistome?**
- 3- Which perinatal factors influence the development and assembly of the infant resistome?**
- 4- How does breastfeeding and specific human milk bioactive components modulate an infant's resistome?**
- 5- How do the HMO metabolism and antibiotic susceptibility of bacteria, specifically bifidobacteria and lactobacilli, influence the development of the infant gut resistome?**

OBJECTIVES



The principal objective of this thesis is to deepen our understanding of the acquisition and dynamics of the infant gut resistome during the first year of life, accounting for the influence of pre-and postnatal factors, with a special focus on mode of delivery, antibiotic exposure and feeding type. Furthermore, we aim to explore the mechanisms underlying the relationship between gut microbiota and antibiotic resistance during the infant's lactation period.

The specific objectives of the work, which will be addressed in each Chapter, are:

- I. To delineate the **assembly of the infant gut resistome** during the first year of life and to identify the **microbes** and **factors** that shape its dynamics through the analysis mother-infant dyads, using different quantitative and qualitative approaches.
- II. To study the influence of the presence of ***Bifidobacterium*** on early gut microbiota and the acquisition of antibiotic resistances in neonates of 7 days and 1 month of age.
- III. To ascertain the **perinatal factors** that may contribute to initial microbiota composition and antibiotic resistance acquisition.
- IV. To identify the potential **protective** role of **breastfeeding** over antibiotic resistance acquisition in early life, and the influence of **human milk bioactive compounds**.
- V. Explore the **metabolism of HMOs** by **bifidobacteria** and **lactobacilli** isolated from human milk and mother-infant dyads, as well as their antibiotic resistance profiles, to provide more evidence on the **potential role of human milk on early life microbiome and resistome establishment**.

STUDY DESIGN AND PARTICIPANTS

The findings presented in this thesis are part of the observational prospective longitudinal MAMI study (“Maternal Microbes”). The MAMI study is a mother-infant birth cohort based in the Spanish Mediterranean region, in which participants were enrolled during late pregnancy and followed up until the child reaches six years of age²³⁶ (Figure 7).

Participants

Recruitment started in 2016 and the six-year follow-up was completed early 2022. Written informed consent was obtained from all the participants. Women were enrolled either during pregnancy and/or within 1st week after birth. The study was approved by Ethical Committee of Hospital Clínico Universitario de Valencia and CSIC. The trial was registered on the ClinicalTrial.gov platform (number NCT03552939).

A total of 250 mother-infant pairs were initially recruited. The inclusion criteria required the mother to be older than 18 years old, healthy pregnancy and to be able to understand written and spoken Spanish. The exclusion criteria were non-compliance with any of the inclusion criteria, use of medications or drugs, suffering chorioamnionitis or health complications during the gestational period, as well as experiencing any chronic disease or taking medication for any chronic pathology.

Data and sample collection

At the time of delivery, clinical staff collected various biological samples, including feces, oral swabs, saliva, amniotic fluid, placenta, and urine, directly in the delivery room. Subsequent sample collection at later time points was either obtained by collaborators from primary healthcare units or self-collected by participating mothers following detailed verbal and written instructions. All samples were sent to biobank, and then, biological samples were managed and stored in sterile cryovials under specific standardized protocols at “Biobanco para la Investigación Biomédica y en Salud Pública de la Comunidad Valenciana (IBSP-CV)”.

Perinatal data including maternal-neonatal clinical information were recorded alongside sample collection during each visit. These data were obtained from clinical records maintained by healthcare professionals in hospitals and primary health centers. Information regarding pregnancy and delivery was extracted from the intrapartum medical records by the medical staff and gynecology team, while infant health outcomes were documented and provided by the pediatric team.

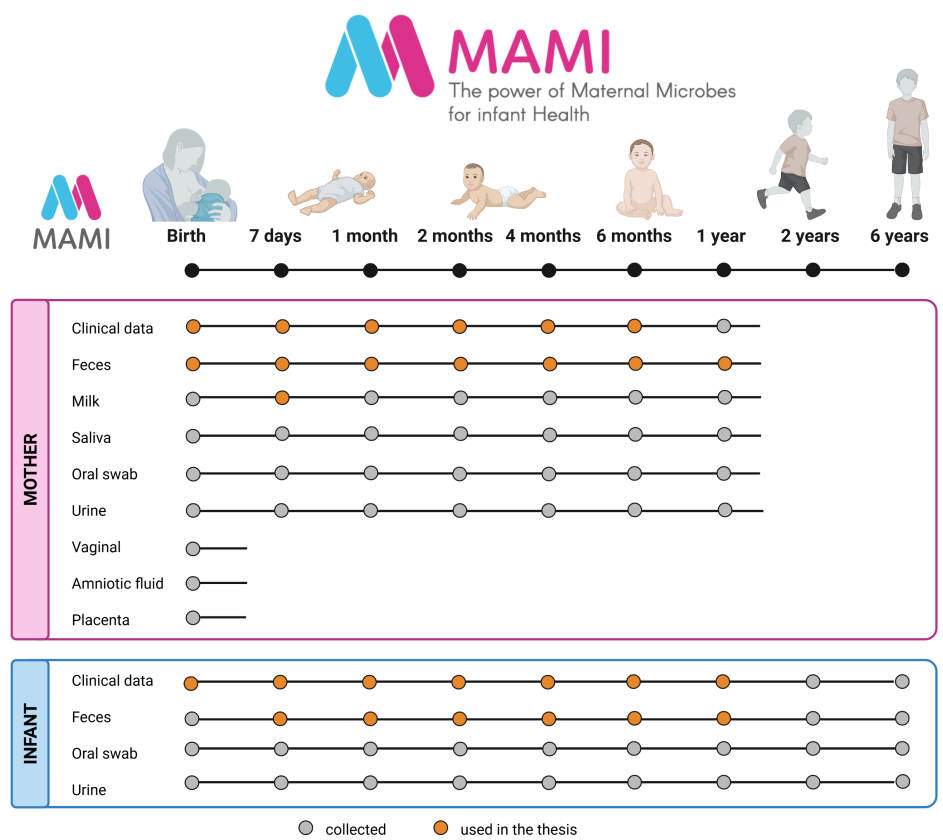
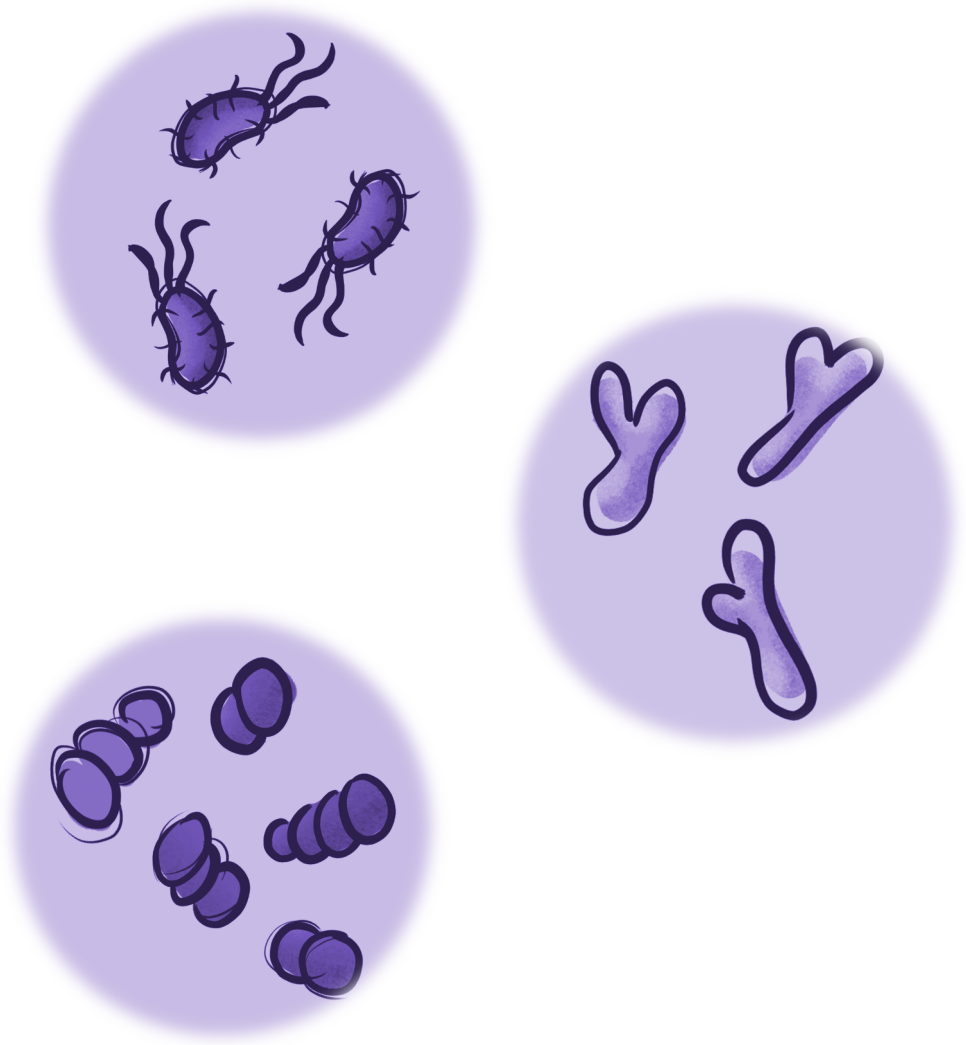


Figure 7. Scheme of the MAMI cohort. Collection of mother-infant samples from pregnancy and birth through to the 6-year postpartum follow-up. Grey filled circles represent all samples collected at each timepoint, while orange circles highlight those specifically used in the present thesis. *Created in BioRender (<https://BioRender.com/ox0c6vk>).*

RESULTS



CHAPTER I

CHAPTER I

Unravelling the Evolutionary Dynamics of Antibiotic Resistance Genes in the Infant Gut Microbiota During the First Four Months of Life

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My contribution to this research article was:

Conceptualization. Perform the experiments: DNA extraction, quantification, and purification. Data analysis and curation, statistical analysis and interpretation. Writing-original draft preparation.

1. Abstract

BACKGROUND: Alongside microbiota development, the evolution of the resistome is crucial in understanding the early-life acquisition and persistence of antibiotic resistance genes (ARGs). Therefore, the aim of this study is to provide a comprehensive view of the evolution and dynamics of the neonatal resistome from 7 days to 4 months of age using a high-throughput qPCR platform.

METHODS: In the initial phase, a massive screening of 384 ARGs using a high-throughput qPCR in pooled healthy mother-infant pairs feces from the MAMI cohort was carried out to identify the most abundant and prevalent ARGs in infants and in mothers. This pre-analysis allowed for later targeted profiling in a large number of infants in a longitudinal manner during the first 4 months of life. 16S rRNA V 3-V 4 amplicon sequencing was performed to assess microbial composition longitudinally. Potential factors influencing the microbiota and ARGs in this period were also considered, such as mode of birth and breastfeeding type.

RESULTS: Following the massive screening, the Top 45 abundant ARGs and mobile genetic elements were identified and studied in 72 infants during their first months of life (7 days, 1, 2, and 4 months). These genes were associated with resistance to aminoglycosides, beta-lactams and tetracyclines, among others, as well as integrons, and other mobile genetic elements. Changes in both ARG composition and quantity were observed during the first 4 months of life: most ARGs abundance increased over time, but mobile genetic elements decreased significantly. Further exploration of modulating factors highlighted the effect on ARG composition of specific microbial genus, and the impact of mode of birth at 7 days and 4 months. The influence of infant formula feeding was observed at 4-month-old infants, who exhibited a distinctive resistome composition.

CONCLUSIONS: This study illustrates the ARG evolution and dynamics in the infant gut by use of a targeted, high-throughput, quantitative PCR-based method. An increase in antibiotic resistance over the first months of life were observed with a fundamental role of delivery mode in shaping resistance profiles. Further, we

highlighted the influence of feeding methods on the resistome development. These findings offer pivotal insights into dynamics of and factors influencing early-life resistome, with potential avenues for intervention strategies.

2. Introduction

Antibiotic resistance, a complex and pressing global health crisis, continues to challenge our ability to combat infectious diseases effectively. The emergence and transfer of antibiotic resistance genes within bacterial populations have brought attention to the concept of the "resistome," a vast reservoir of genetic elements responsible for conferring resistance to antibiotics¹. Antibiotic resistance continues to challenge our ability to combat infectious diseases as once-effective antimicrobial agents become increasingly ineffective, leading to longer hospital stays, higher mortality rates, and escalating healthcare costs². This becomes particularly dangerous in early stages of life, where the acquisition of antibiotic resistances has short- and long-term consequences on the infant health.

Infancy is a critical period characterized by rapid microbial acquisition and colonization of the gut with potential impacts on health outcomes³. During early life, the gut microbiota is highly dynamic and variable, influenced by a multitude of intrinsic and extrinsic factors⁴⁻⁶. Among these factors, antibiotic exposure, either through maternal transmission or direct administration, is recognized as a significant driver of antibiotic resistance gene dissemination and selection within the infant gut microbiome^{7,8}. Other factors, such as breastfeeding practices and mode of delivery have also been associated with the establishment of the infant gut resistome⁹⁻¹¹. Consequently, deciphering the dynamics of the infant gut resistome during the lactation period has become a matter of importance, as it sets the stage for continued microbial colonization and immune development in early life.

Despite variations in the abundance and diversity of antibiotic resistance among different infant groups due to varying early-life factors, prevailing resistance genes primarily encode for proteins that provide resistance against beta-lactams, tetracyclines, macrolides, aminoglycosides, and quinolones¹². Infant resistome

analyses at different time points have elucidated greater relative abundances of antibiotic resistance genes (ARGs) in younger infants compared to older children^{13–16}. Thus, the development of the infant resistome is linked to the evolution of the microbial composition, contributing to the long-term dynamics and stabilization of the resistome into adulthood¹⁷.

The potential risks posed by antibiotic resistant bacteria in samples can be estimated quantitatively with high-throughput qPCR. This technique is widely used to determine the presence and abundance of multiple ARGs in environmental microbiota and in various types of samples^{18–22}. High-throughput qPCR can be easily customized to target specific ARGs of interest, allowing us to focus on clinically relevant or ecologically significant resistance genes²³.

Hence, quantitatively studying the evolution of the infant gut resistome is crucial for understanding the early-life acquisition and persistence of antibiotic resistances and the role of lactation and other factors on these resistances. For this reason, the aim of this research is to provide a comprehensive overview of the evolution and dynamics of the infant resistome, from 7 days to 4 months of age, using a high-throughput qPCR platform. By deepening our knowledge of the infant gut resistome, we can pave the way for targeted interventions aimed at mitigating the spread of antibiotic resistance during this critical developmental period.

3. Materials and methods

3.1. Study design and cohort

A total of 32 mother-infant pairs from the MAMI birth cohort²⁴ participated in this study, taking a total of 72 infant fecal samples collected at 7 days, 1 month, 2 months and 4 months of age (**Figure I-1**). Maternal-infant clinical data including gender, mode of delivery, lactation practice (exclusive breastfeeding or artificial lactation, which included both mixed and exclusive formula-feeding) and duration, antibiotic exposure, gestational age, and anthropometric measures were collected (**Table I-1**).

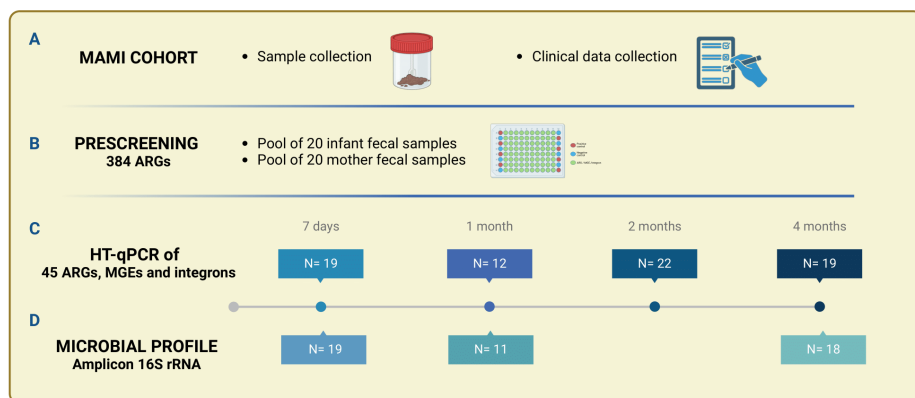


Figure I-1. Flowchart of the study designed. A total of 32 mother-infant pairs were recruited for this study, from which clinical data and fecal samples were collected. In the initial phase, a massive screening of 384 ARGs in pooled infant and maternal feces from the MAMI cohort was carried out to identify the most abundant and prevalent ARGs. This pre-analysis allowed for later targeted profiling in a large number of infants in a longitudinal manner during the first 4 months of life. 16S rRNA V 3-V 4 amplicon sequencing was performed to assess microbial composition longitudinally. Exact number of samples for each analysis (N) are shown in the illustration.

Table I-1. Characteristics of study participants.

Mother characteristics		Total (N=32)
Gestational age (weeks)		39.5 [39-40]
Weight gain over the pregnancy (kg)		11.46 ±3.891
Pre-gestational BMI (kg m ⁻²)		23.658 ±3.597
Antibiotic consumption during pregnancy (%)		9 (28.12%)
Infant characteristics		
Gender: female (%)		32 (44.44%)
Birth mode: vaginal birth (%)		47 (65.27%)
Duration of breastfeeding (months)		6 [3-12]
Exclusive breastfeeding (%)		
	7 days	27 (84.37%)
	1 month	19 (59.37%)
	2 months	18 (56.25%)
	4 months	17 (53.12%)
Antibiotic exposure (%)		
	At birth	13 (40.62%)
	7 days	3 (9.37%)
	1 month	2 (6.25%)
	2 months	1 (3.12%)
	4 months	1 (3.12%)
BMIZ		
	At birth	(-)0.265 ±1.058
	1 month	(-)0.500 ±0.924
	6 months	1.103 ±0.309

Categorical variables are expressed as positive cases-prevalence and (percentage, %). Normally distributed data are presented as mean ± standard deviation (SD) and non-normal

data as median and interquartile range [IQR]. BMIZ, body mass index z-score. Infant body length and weight was only recorded at moment of birth, 1 month and 6 months of age. N=19 of 7 days old infants, N=12 of 1 month old infants, N=22 of 2 months old infants and N=19 of 4 months old infants.

3.2. Neonatal biological samples and DNA extraction and quantification

The neonatal samples were collected as described previously²⁵ in sterile containers by the parents at home using detailed instructions (**Figure I-1A**). Stools were collected at seven days, and one, two and four months after birth and immediately stored at -20°C . Within 24 hours of collection, samples were taken to Primary Health Centers (during scheduled pediatric visits). There they were stored at -80°C until analysis.

Total DNA was extracted, purified, and quantified as described in Selma-Royo *et al*⁵. Briefly, DNA was extracted from 50-100mg of fecal material using the Master-Pure DNA extraction Kit (Epicentre, Madison, WI, USA) following the manufacturer's instructions with the following modifications: treatment with lysozyme (20 mg/ mL) and mutanolysin (5 U/mL) for 60 min at 37°C and a preliminary step of cell disruption with 3- μm diameter glass beads for 1 min at 6 m/s by a FastPrep 24-5G Homogenizer bead beater (MP Biomedicals). Purification of the DNA was performed using a DNA Purification Kit (Macherey-Nagel, Duren, Germany) according to the manufacturer's instructions. DNA concentration was measured using Qubit® 2.0 Fluorometer (Life Technology, Carlsbad, CA, USA) for further analysis.

3.3. Screening of the Top antibiotic resistance genes in the maternal-infant gut

A pool DNA from of 20 infant fecal samples (1 ng) taken at 2 months of age and a pool of DNA from their respective mothers (1 ng) at the same timepoint were used to pre-screen for the positive detection of 384 previously validated ARGs using the SmartChip Real-Time PCR (Resistomap, Finland) (**Figure I-1B** and **Supplementary Table I-1**)²⁶. The 16S rRNA gene was used as a positive control and to normalize the abundances of detected genes in fecal samples. Within the chip, each primer set was designed to target sequence diversity within a gene to assess the gene's variants in the resistome. Therefore, each gene was analyzed

independently. Briefly, SmartChip has 5184 reaction wells with a volume of 100 nL. Each 100 nL reaction is comprised of 1 × SmartChip TB Green Gene Expression Master Mix (TakaraBio), nuclease-free PCR-grade water, 300 nM of each primer and a DNA template of 2 ng/μL. The chips are filled using the SmartChip Multisample Nanodispenser (Takara Bio). qPCR cycling conditions were maintained and initial data processing was done as previously described²⁷. qPCR reactions were performed with three technical replicates with a limit of detection of cycle threshold (CT) = 27²⁷⁻²⁹. A gene was considered present in a sample if it was detected in at least two of the three replicates. The mean CT values of the replicates for each reaction was used to calculate the ΔCT values (ΔCT = CT detected gene – CT 16S rRNA gene), and the 2–ΔCT method was used to calculate the relative abundances of the detected gene relative to the 16S rRNA gene in each sample²⁹. The 45 most abundant and representative ARGs, MGEs and integrons present in the infant gut of our cohort were intentionally chosen for targeted quantification for this study to guarantee their consistent detection across all samples.

3.4. Gut microbiota profiling by specific 16S rRNA amplicon sequencing

We also carried out the microbiota profiling of a subset of 48 infant fecal samples by amplification of the V 3-V 4 regions of the 16S rRNA gene (**Figure I-1D**). Amplicons were obtained with PCR amplification using barcoded conventional primers (341F 5'-CCTACGGGNGGCWGCAG-3' and 806R 5'GGACTACNNGGTATCTAAT-3') with a 466bp fragment length. Amplicons were checked with a Bioanalyzer DNA 1000 chip and libraries were sequenced using a 2×250 bp paired-end kit on a MiSeq-Illumina platform (FISABIO sequencing service, Valencia, Spain). The 63754 (±33733520) mean sequences with good quality were obtained from samples. Overall, these sequences were clustered into 87 ZOTUs (±14). Bacterial diversity analysis was done using raw reads, which were quality controlled and filtered (Quality: 25 and length: 125 bp) using trimGalore (v 0.6.4_dev; <https://github.com/FelixKrueger/TrimGalore>). The paired-end reads with a minimum overlap of 30 bp were joined using Fastq-join³⁰. Sequences were trimmed of primers and distal bases, and singletons were removed with USEARCH

v11 31. zOTUs (zero-radius operational taxon units) mapping to the human genome (GRCh38) using the Burrow–Wheeler Aligner in Deconseq v0.4.3 were filtered out. The resulting reads were denoised and chimeras were filtered with UNOISE³². Taxonomic assignment of zOTUs was performed in QIIME2 v2018.2³³ using the QIIME2 feature classifier plugin³⁴ and the Ribosomal Database Project (RDP 2.12)³⁵. The zOTUs were aligned with MAFFT³⁶ to then make a phylogenetic tree with FASTTREE 37 that was midpoint-rooted.

3.5. Statistical analysis

All statistical analyses were performed with R version 3.6.0, and Figures were drawn with the “ggplot 2” R package³⁸. Variables are presented as the number (percentage) or mean (standard deviation, SD), as appropriate. Statistical analyses for comparison of clinical and perinatal characteristics included the use of non-parametric Mann–Whitney U tests or Fishers exact test and Pearson χ^2 tests for continuous and categorical variables, respectively, to compare different groups of infants. Normality of the data was evaluated with Shapiro–Wilk tests.

Antibiotic resistance genes statistical tendency through the multiple timepoints was calculated with Wilcoxon test. The Shannon index for alpha diversity analyses of the relative abundances of the genes was calculated using the *diversity* function of the “vegan” R package³⁹. Principal Component Analysis (PCA) was conducted to study the composition of ARGs between all timepoints and to confirm the correlation between environmental factors and ARG composition, using the ‘factoextra’⁴⁰ and ‘FactoMineR’⁴¹ R packages. We tried to identify groups of variables whose balance was more associated with the response variable, using the R package MaAsLin2⁴² which is a complete R package to efficiently determine multivariable associations through general linear models that can accommodate most modern study designs.

Calculations of microbial richness (Observed, Chao 1 and ACE) and evenness indices (Shannon and Simpson) were done using the “phyloseq” R package⁴³. Beta diversity was characterised by Principal Coordinate Analysis (PCoA)

conducted by plotting the Bray-Curtis distance matrix of log transformed zOTU counts for each timepoint separately. We filtered the zOTU data using the *filter_taxa* function in the “phyloseq” package, and only zOTUs present in at least 10% of samples were retained. The *adonis* permutational test was used to evaluate overall differences in microbiota structure between the timepoints with the “vegan” R package³⁹. We also fitted a Poisson (log-linear regression) generalized lineal mixed model (GLMM) using the “mvabund” package⁴⁴ to assess the association of the Top 10 most abundant taxa individually adjusted by time, mode of delivery and mode of lactation, on the antibiotic resistance gene composition. For all methods, p-values were adjusted for multiple comparisons using False Discovery Rate (FDR) based on Benjamini–Hochberg (BH)⁴⁵.

4. Results

4.1. Top antibiotic resistances in the maternal-infant gut (pre-screening)

In this study, we identified a set of ARGs with the highest values in the maternal-infant gut from a pre-screening analysis. A total of 211 ARGs were identified in the infant’s gut, while only 135 were detected in the mothers’ gut. The overall antibiotic resistance abundance was higher in 2-month-old infants than in their mothers, and statistically significant in the case of MGE (0.008 ± 0.032 in infant vs 0.0005 ± 0.003 in mothers, $p < 0.0001$) and aminoglycosides (0.0018 ± 0.007 in infants vs 0.0006 ± 0.001 , $p < 0.0001$; **Table I-2**).

Table I-2. Results of the antibiotic resistance genes detected in the pre-screening of the mother and infant fecal sample pools.

		Genes detected (%)	Relative abundance	p-value
Integrans (N=4)	Infant	100.00	0.043 [0.021-0.121]	0.859
	Mother	75.00	0.004 [0.004-0.042]	
MGE (N=48)	Infant	60.41	0.008 [0.001-0.038]	<0.0001*
	Mother	43.75	0.0005 [0.0002-0.003]	
Betalactams (N=54)	Infant	46.29	0.0006 [0.0002-0.003]	0.397
	Mother	18.51	0.0004 [0.0002-0.001]	
MLSB	Infant	53.19	0.001 [0.0005-0.007]	0.871

(N=47)	Mother	29.78	0.003 [0.0002-0.009]	
Aminoglycoside (N=60)	Infant	58.33	0.001 [0.0003-0.007]	0.035*
	Mother	41.66	0.0006 [0.0002-0.001]	
MDR (N=39)	Infant	64.10	0.001 [0.0008-0.0204]	0.201
	Mother	35.89	0.002 [0.0001-0.003]	
Sulfonamide (N=6)	Infant	100.00	0.004 [0.0007-0.01]	0.322
	Mother	66.66	0.0004 [0.0001-0.001]	
Phenicol (N=22)	Infant	45.45	0.0004 [0.0002-0.002]	0.943
	Mother	18.18	0.0005 [0.0001-0.001]	
Tetracycline (N=26)	Infant	69.23	0.008 [0.002-0.0171]	0.216
	Mother	53.84	0.0009 [0.0002-0.0131]	
Quinolone (N=11)	Infant	54.54	0.001 [0.0005-0.003]	0.401
	Mother	18.18	0.0006 [0.0003-0.0008]	
Other (N=17)	Infant	52.94	0.0017 [0.0002-0.0119]	0.710
	Mother	41.17	0.005 [0.002-0.008]	
Vancomycin (N=24)	Infant	33.33	0.001 [0.0008-0.002]	0.008
	Mother	20.83	0.0001 [0.0001-0.0001]	
Trimethoprim (N=17)	Infant	47.05	0.0002 [0.0001-0.0003]	-
	Mother	0.00	-	

Normally distributed data are presented as mean $\pm$ standard deviation (SD) and non-normal data as median and interquartile range [IQR]. Pearson's-Chi-square test was used for categorical variables, and Mann-Whitney U test (or Fisher's exact test) was used for continuous variables, as appropriate, for calculating statistical significance, which are marked with a *. $p < 0.05$ was considered statistically significant.

The 45 most abundant ARGs, MGEs and integrons present in the infant gut were intentionally chosen for this study to guarantee their consistent detection across all samples (Table I-3). The targeted genes we included in the customized SmartChip were the total 16S rRNA gene, specific genes for the Bacteroides and Bacillota phyla, and the 45 antibiotic ARGs, MGEs, integrons, and other genes associated with antibacterial compounds. Notably, genes from different functional groups appeared in this set of 45 genes, highlighting the diverse genetic elements and mechanisms at play in the early infant gut (Supplementary Table I-2).

Table I-3. List of antibiotic resistance genes measured in the infant fecal samples.

Antibiotic class	ARG
Integrons (N=1)	<i>int1_1</i>
MGE (N=3)	<i>IS 26_1</i> , <i>tnpA_1</i> , <i>ISEcp1</i>

MLSB (N=6)	<i>ermX_2, mphA, ermF, oleC, mefA, ermB_3</i>
Quinolone (N=3)	<i>qepA, qnrS_1, qnrB</i>
Aminoglycosides (N=6)	<i>aph 4-ib, strB, aac(6')-Ib_1, aacC2, apmA, aadA7</i>
MDR (N=4)	<i>mdtH, czcA, pcoA, tolC_2</i>
Betalactams (N=10)	<i>blaCTX-M, blaTEM, blaOXY, penA, blaACT, blaSHV11, blaSFO, pbp, cfxA, blaOXA48</i>
Other (N=2)	<i>bacA, mcr1</i>
Phenicol (N=3)	<i>mdtL, cmlV, catA1</i>
Vancomycin (N=3)	<i>vanA, vanHB, vanB_1</i>
Tetracycline (N=4)	<i>tetO_2, tetA_2, tetW</i>

4.2. Early-life antibiotic resistance composition changes over time

The diversity of antibiotic resistance genes was found to increase significantly through time, going from a Shannon index of 1.81 ± 0.842 in 7-day-old infants, to 1.969 ± 0.612 at 1 month of age, 2.468 ± 0.495 at 2 months, and 2.397 ± 0.312 at 4 months ($p < 0.05$ between all timepoints). The antibiotic resistance gene composition was also compared between all timepoints (**Figure I-2**) and differed significantly between 7 days to 4 months of age ($p = 0.024$). Indeed, MGEs were highly correlated to 7-day-old samples ($p < 0.001$), whereas ARGs conferring resistance to quinolones, vancomycin and aminoglycosides were significantly correlated to 4-month-old samples ($p < 0.001$). The evolution of each individual ARG over time was reported (**Figure I-3** and **Supplementary Figure I-1**) and detailed results are described below.

Aminoglycosides

Aminoglycoside evolution was tested by quantifying the relative abundances of the *aacC2*, *aadA7*, *apmA* and *strB* genes (**Figure I-3A**). The relative abundance of the aminoglycoside resistant gene *aadA7* shows a significant increase from 7 days to 2 months of age (3.20% to 19.44%; $p < 0.01$) and 4 months (31.11%; $p < 0.001$). At 2 and 4 months of age, *aadA7* has a significantly higher relative abundance than the other aminoglycoside resistance genes ($p < 0.05$). Moreover, at 2 months, the *strB* is more abundant than *aacC2* gene (2.15% and 0.43%; $p = 0.004$).

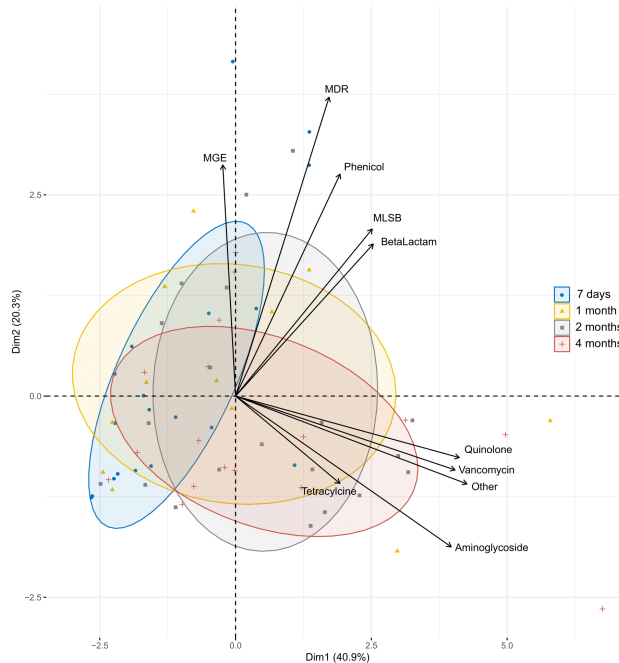


Figure I-2. Antibiotic resistance composition changes over time. Principal Coordinate Analysis (PCA) biplot depicting the relationship between the composition of antibiotic resistances and time. Blue ellipses represent 7-days old infants, yellow ellipses the 1-month old , grey ellipses the 2-months old, and red ellipses the 4-months old infants. The black arrows are the group of ARGs that best explain the differences between the sample groups. Angles between the arrows represent correlations; acute angles represent positive correlations and obtuse angles represent negative correlations.

Beta-lactams

A total of 9 ARGs conferring resistance to antibiotics were measured (**Figure I-3B**). A significant increase from 7 days to 2 months and to 4 months of age was observed for *blaOXA48* (0.04% to 0.45%; $p < 0.01$, and to 0.56%; $p < 0.01$), *blaACT* (0.44% to 1.77%; $p < 0.01$, and to 14.04%; $p < 0.01$) and *blaOXY* (2.52% to 4.98%; $p < 0.05$, and to 7.41%; $p < 0.001$). The *penA* resistance gene showed a significant increase from 7 days to 2 months (3.33% to 6.94%; $p < 0.05$) but then decreases at 4 months (4.94%; $p < 0.01$), although it keeps statistically higher compared to 7 days abundance. Both 2- and 4-month-old infants have higher amounts of the *blaOXY* gene (7.98% and 8.41%, respectively) than *blaOXA 48* (0.45%; $p = 0.003$, and 0.56%; $p = 0.002$), *cfxA* (0.92%; $p = 0.001$ and 1.41%; $p = 0.016$) and *pbp* (0.89%; $p = 0.012$ and

1.14%; $p=0.024$), whereas *penA* is in higher amounts than *blaOXA48* ($p=0.006$ and $p=0.015$) and *cfxA* ($p=0.003$ and $p=0.032$). The resistant gene *cfxA* has a lower abundance in 2-month-old infant guts than *blaSHV11* (3.59%; $p=0.007$), *blaSFO* (1.60%; $p=0.015$), and *blaACT* (1.77%; $p=0.013$). Finally, 2-month-old infants had higher abundances of *penA* than *pbp* ($p=0.015$), and *blaSHV11* than *blaOXA48* ($p=0.04$).

Integrans

Integron abundance in infants' guts was measured by quantifying the *int 1* integron, which showed an increase from 7 days (10.47%) to 1 month of age (36.12%), and then a decrease at 2 (24.84%) and 4 months of age (22.84%), but no statistical differences were found between time points (Figure I-3C).

Multi drug resistances (MDR)

czcA, *mdtH*, *pcoA* and *tolC2* were measured to assess the evolution of multi-drug resistant genes. *mdtH* increased significantly from 7 days (11.20%) to 2 months of age (17.22%; $p<0.001$) but then decreased when infants reached 4 months of age (12.77%; $p<0.01$). This gene is highly more abundant than *tolC2* and *pcoA* genes in 2- and 4-month-old infants (1.23%; $p=0.06$ and 1.77%; $p=0.004$ for 2 months, and 0.44%; $p<0.0004$ and 1.09%; $p=0.002$, for 4 months, respectively for each gene). Finally, 2-month-old infants had lower amounts of *pcoA* than *czcA* (3.53%; $p=0.015$) in their gut (Figure I-3D).

MGE

Transposase *tnpA 1* and insertion sequences *IS 261* and *ISEcp 1* were measured as representative for the MGE group (Figure I-3E). All three of these genes showed a decrease from 7 days to 4 months of age, with *ISEcp 1* significant between 7 days and 2 months (6.09% and 4.58%; $p<0.01$) and between 7 days and 4 months (3.36%; $p<0.01$).

Macrolide-lincosamide-streptogramin B (MLSB)

The six macrolide-lincosamide-streptogramin antibiotic resistance genes measured were: *ermB3*, *ermF*, *ermX2*, *mefA*, *mphA* and *oleC* (Figure I-3F). Similar

to other groups of antibiotics, we observed an increase in the abundance of *mphA* and *oleC* genes from 7 days to 2 months of age (3.14% to 11.02%; $p < 0.01$ and 0.18% to 1.20%; $p < 0.001$, respectively). The same pattern was observed from 7 days to 4 months of age (3.14% to 11.02%; $p < 0.001$ for *mphA*, 0.18% to 4.52%; $p < 0.01$ for *oleC*, and 3.14% to 11.02%; $p < 0.001$ for *ermB3*). This increase longer-range increase was also observed for the *ermB3* gene (from 0.53% at 7 days to 1.76% at 4 months of age; $p < 0.05$). *ermX2* was significantly more abundant at 2 and 4 months of age (23.97% and 59.18%, respectively) than *oleC* ($p = 0.0005$ and $p = 0.009$, respectively for each timepoint), *mefA* ($p = 0.0002$ and $p = 0.006$), *ermF* (1.48%; $p = 0.0004$ and 1.28%; $p = 0.0006$) and *ermB3* (0.59%; $p = 0.0002$ and 1.76%; $p = 0.003$). *mphA* was also more abundant than *oleC* and *mphA* in 2- and 4-month-old infants. *ermF* amounts at 4 months of age were significantly lower compared to the *oleC* gene.

Other ARGs

bacA and *mcr 1* were highly relevant in the initial pre-screening (Figure I-3G). Both showed a significant increase from 7 days to 2 and 4 months of age: *bacA* increased from 2.01% to 6.31% ($p < 0.05$) and then to 14.66% ($p < 0.05$), whereas *mcr 1* increased from 0.74% to 1.53% ($p < 0.05$) and then decreased to 1.06% ($p < 0.05$).

Phenicol

To quantify phenicol-resistant genes, *catA1*, *cmlV* and *mdtL* were measured (Figure I-3H). The comparison between their abundance in each timepoint showed that at both 2 and 4 months of age, *catA1* (0.37% and 0.23%) was significantly lower than *mdtL* (3.96% and 2.75%; $p = 0.003$ and $p = 0.002$ for each time) and *cmlV* (3.06% and 4.44%; $p = 0.001$ and $p = 0.006$). The individual genes did not show any time-related fluctuations between sampling points ($p > 0.05$).

Quinolone

Quinolone-resistant genes *qepA* and *qnrB* showed a significant increase from 7 days (6.55% and 0.17%, respectively) to 2 months (23.44%; $p = 0.01$ and 0.26%; $p = 0.05$) and then again at 4 months of age (28.26%; $p = 0.01$ and 0.31%;

$p=0.01$). *qnrS1* was significantly lower in abundance than *qepA* and *qnrB* at all timepoints ($p<0.05$) (**Figure I-3I**).

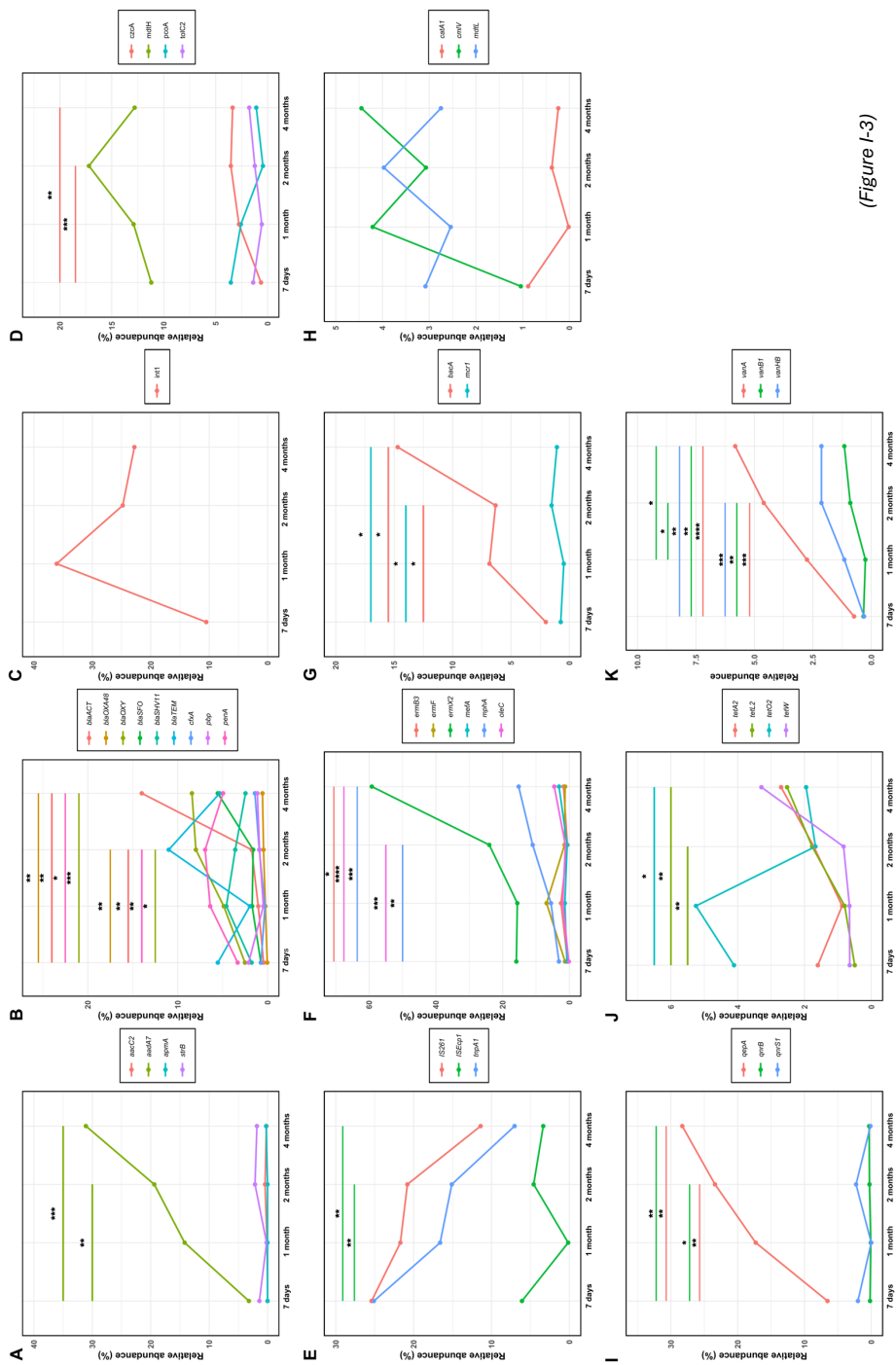
Tetracycline

Antibiotic resistance to tetracycline was measured by the quantification of *tetA2*, *tetL2*, *tetO2*, and *tetW* genes (**Figure I-3J**). The *tetL* 2 gene increased significantly from 7 days (0.49%) to 2 months (1.77%; $p=0.01$) and then again at 4 months (2.51%; $p=0.01$). On the contrary, the *tetO2* gene decreased in abundance from 7 days (4.11%) to 4 months (1.95%; $p=0.05$).

Vancomycin

The *vanA*, *vanB* and *vanHB* genes, conferring resistance to vancomycin, increased significantly over time (**Figure I-3K**). *vanA* increased from 0.73% at 7 days of age to 4.60% at 2 months ($p=0.001$) and to 5.82% at 4 months ($p=0.0001$). Similarly, *vanHB* increased significantly from 7 days (0.30%) to 2 months (2.13%; $p=0.001$) and to 4 months of age (2.12%; $p=0.01$). Finally, *vanB1* gene increased significantly from 7 days (0.33%) to 2 months (0.89%; $p=0.01$), from 1 months (0.25%) to 2 months of age ($p=0.05$), from 1 months to 4 months (1.15%; $p=0.05$) and from 7 days to 4 months ($p=0.01$). At the end, the abundance of *vanA* is higher than *vanB1* ($p=0.039$).

► **Figure I-3. Evolution of antibiotic resistance genes relative abundance.** Tendency plots representing the antibiotic resistance genes evolution from 7 days to 4 months of age of each ARGs measured, grouped by antibiotic resistance to: **(A)** Aminoglycosides, **(B)** Beta-lactams, **(C)** Integrans, **(D)** MDR, **(E)** MGE, **(F)** MLSB, **(G)** Others, **(H)** Phenicol, **(I)** Quinolone, **(J)** Tetracycline and **(K)** Vancomycin. Statistical differences are marked as following: * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$.



(Figure I-3)

4.3. Factors that modulate antibiotic resistance though time

Mode of delivery and antibiotic exposure at birth

An infant's mode of delivery exerted a strong influence on antibiotic resistance composition in 7-day-old infants, resulting in a significantly different antibiotic resistance composition for infants born vaginally or with C-section procedures (**Figure I-4A-D**). Hence, at 7 days of age most of the antibiotic resistances are associated with C-section birth ($p < 0.001$). The class of ARGs most contributive to the first PC component was MDRs, followed by vancomycins, MLSBs, others, quinolones and phenicol. MGEs and genes conferring resistances to beta-lactamases, contributed mostly to the second PC component.

In 1-month-old infants, we observed that most of the ARGs, except for MGEs, were positively associated to C-section birth. Vancomycin-related genes and aminoglycoside-related ones were the main contributors to the ARG variation in our study population depending on mode of delivery ($p < 0.001$).

Similarly, at 2 months of age, infants born by C-section were also associated with the majority of the antibiotic resistances ($p < 0.001$) with the exception of tetracyclines, which were associated with vaginally birth. However, this pattern was reverted at 4-months of age, where again most antibiotic resistances were associated with C-section delivery, following the order of vancomycin, quinolone, others, aminoglycosides, beta-lactams, MLSB and tetracyclines ($p < 0.001$ for all). MDR-, phenicol- and MGE- related genes made a greater contribution to the second component ($p < 0.001$) and their association with C-section was slightly lower.

Infant feeding type

Infant feeding type (maternal breastfeeding or artificial lactation, which included mix and formula) was also found to influence the ARG profile in infants (**Figure I-4E-H**). At 7 days of age, vancomycin, MDR, other, MLSB, quinolone, and phenicol ARGs were highly associated with infants who received infant formula-feeding ($p < 0.001$). This pattern was maintained at 1 month of age, where beta-

lactam- and tetracycline-resistant genes also gained importance in artificially fed infants. However, at this timepoint MGE are associated to breastfed infants. At 2 months of age genes classified as quinolones, vancomycin, aminoglycosides and others were strongly correlated to infants who received artificial lactation. Finally, 4-month-old infants also showed a significant contribution between artificial lactation ($p < 0.001$) and most ARGs, namely aminoglycoside-, vancomycin-, quinolone- and beta-lactam-resistant genes.

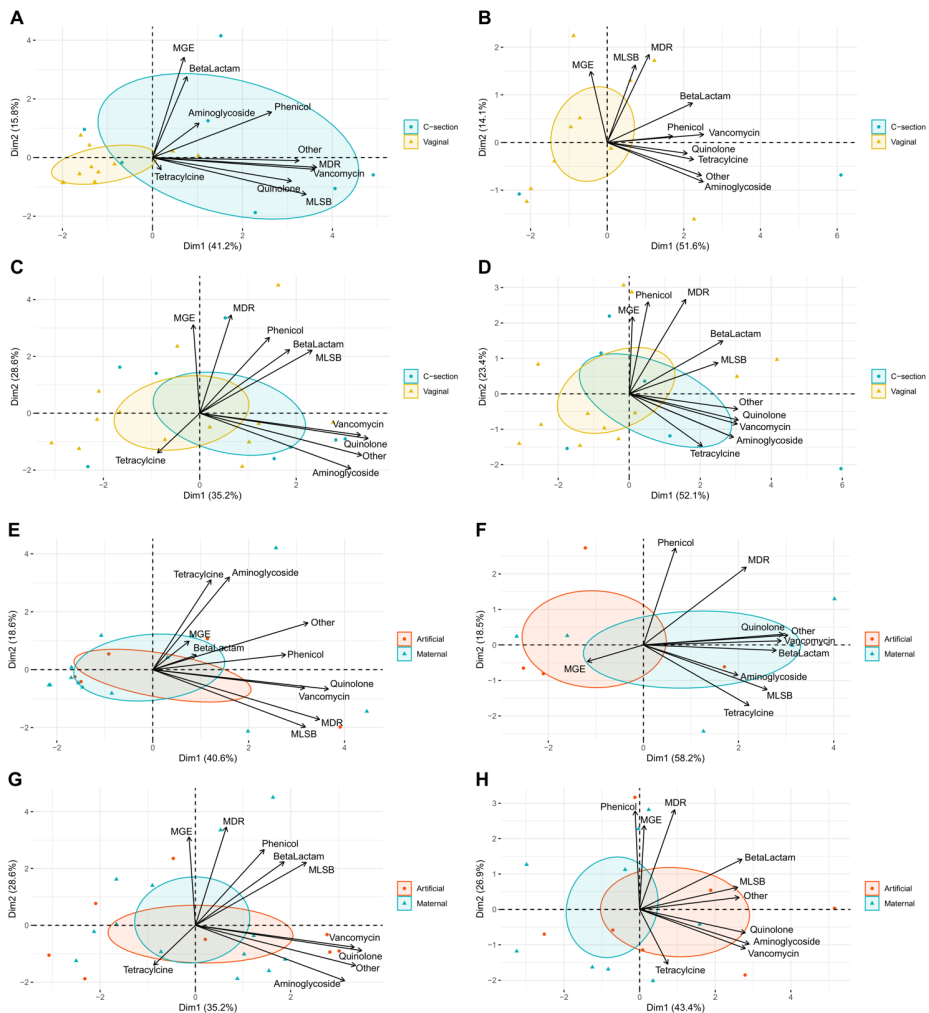


Figure I-4. Association between antibiotic resistances and external factors. Principal Component Analysis (PCA) biplot depicting the relationship between the composition of antibiotic resistances and mode of delivery (A-D) and lactation (E-H) at: 7 days (A and E), 1

month (B and F), 2 months (C and G) and 4 months (D and H) of age. For delivery mode, blue ellipses represent the infants born by C-section and yellow ellipses the infants born vaginally. For lactation, red ellipses represent the infants who received artificial lactation and blue ellipses the exclusive breastfed infant. The black arrows are the group of ARGs that best explain the differences between the sample groups. Angles between the arrows represent correlations; acute angles represent positive correlations and obtuse angles represent negative correlations.

In addition, the MaAsLin approach allowed us to find specific associations (Figure I-5) when controlling for infant age and other variables (lactation and delivery). We observed an association of longitudinal samples with the establishment of resistance genes (very strong and rapid changes with respect to time). It is complex to have a marker associated since there are many changes associated with time and more in concert with the samples of 1, 2 and 4 months. Even though, we can find markers positively associated with delivery, such as MGE_tnpA_1, and also associated with lactation, such as aminoglycoside_aacC 2, Beta-Lactam_blaTEM and MGE_IS 26_1.

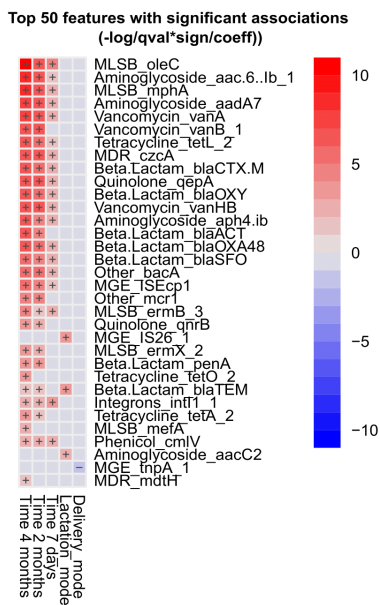


Figure I-5. Top 50 features with significant associations with external factors and time. Delivery_mode includes C-section and vaginal delivery. Lactation_mode includes exclusive breastfeeding and artificial lactation. Only significant associations ($p < 0.05$) are shown.

Environmental factors, microbiota and ARGs

Alpha and beta diversity analyses and taxa composition of microbiota are reported in **Supplementary Figure I-2**. Beta diversity analyses showed that microbial composition was significantly different between timepoints (7 days vs.4 months: $p=0.006$), and 1 month vs 4 months: $p=0.006$). The GLMM explained the associations between the Top 10 most abundant microbial genus, adjusted by environmental factors, on the antibiotic resistance gene load (**Table I-4**). For the time categories, 1 and 4 months of age showed significant p-values ($p=0.001$ and 0.003 , respectively), indicating evidence that age has an effect on the ARGs composition. Variables related to breastfeeding and vaginal delivery also have significant p-values. From the 10 most abundant genera, *Bifidobacterium* ($p=0.042$), *Streptococcus* ($p=0.002$), and *Bacteroides* ($p=0.018$) showed an influence on the ARG composition, meaning that the abundance of these species plays a crucial role on the composition of antibiotic resistances. The interaction between all environmental factors studied showed that breastfeeding and 4 months of age have a strong interaction and influence on the ARGs composition, followed by the same interaction with the addition of vaginal delivery, and the interaction of only that age and mode of delivery. Similarly, vaginal mode of delivery and 1 month of age also show a significant influence, higher than the interaction of that age and breastfeeding. These results highlight the importance of time on ARG composition among the other factors but elucidate that vaginal mode of delivery and breastfeeding can also drive the ARG composition, though likely in a time-related manner.

Table I-4. Generalized lineal mixed model (GLMM) to explain the associations between the microbiota and environmental factors on the antibiotic resistance gene load.

Variable	Wald value	p-value	Statistics
Time			
Time_4months	1.551	0.003	**
Time_1month	0.821	0.001	***
Microbiota (Top 10 genus)			
<i>Bifidobacterium</i> spp.	2.528	0.042	*
<i>Streptococcus</i> spp.	3.060	0.002	**
<i>Bacteroides</i> spp.	2.297	0.018	*

Environmental factors			
Lactation_Maternal	0.855	0.015	*
Delivery_Vaginal	0.582	0.005	**
Interactions			
Time_4months --- Lactation_Maternal	1.393	0.001	***
Time_4months --- Lactation_Maternal -	0.438	0.009	**
Time_4months --- Delivery_Vaginal	0.366	0.007	**
Time_1month --- Delivery_Vaginal	0.332	0.001	***
Lactation_Maternal --- Delivery_Vaginal	0.319	0.016	*
Time_1month --- Lactation_Maternal	0.221	0.018	*

Wald test is applied to assess the significance of the coefficients obtained with the GLMM. Only significant associations are shown. Significance codes: (*): $p < 0.05$; (**): $p > 0.001$; (***): $p > 0.0001$. N=19 of 7 days old infants, N=11 of 1 month old infants and N=18 of 4 months old infants.

5. Discussion

In this study, we investigated the dynamic evolution of antibiotic resistance genes in the early-life gut microbiome of infants, shedding light on ARG evolution as well as the factors influencing them. Our findings reveal a significant shift in the ARGs profile from 7 days to 4 months of age, indicating a complex trajectory of resistance development in the infant gut. This observation highlights the novelty of our research, as it underscores the importance of understanding the resistome dynamics in early life, a critical period for microbiome development.

One novel aspect of our study is the use of a high-throughput, quantitative approach to measure and quantify ARGs, providing a high-resolution view of ARG abundance. This approach offers advantages over other methods, such as amplicon sequencing or shotgun metagenomics, as it allows for precise quantification of specific resistance genes⁴⁶. This precision is particularly valuable when monitoring changes in ARG abundance over time and in response to different factors. The same high-throughput qPCR approach employed in this study has until now, mostly been used for environmental resistome analyses, such as those of aquatic environments^{47,48}, wastewater treatment plants⁴⁹, and soil and manure^{50,51}, and, to a lesser extent, in farm and wild animal gut resistomes^{52–54}.

The examination of several genes in our study revealed novel information that enhances our understanding of the evolution of the infant resistome, shedding

light on previously unexplored aspects of this important subject. We have observed that most antibiotic resistances increase through time, as 4-month category had a higher value, suggesting that the effect of age on the ARGs composition is more pronounced at that moment.

Comparing our findings with existing literature, we note several key insights. Firstly, our study highlights the emergence of mobile genetic elements (MGEs) as prominent contributors to ARGs in the early infant gut, particularly at 7 days of age. This finding aligns with previous research emphasizing the role of MGEs in disseminating resistance genes^{55–57}. Our study found that MGE were higher in infants than in mothers in agreement with previous studies⁵⁸. In addition, despite limited data on MGE, we also found higher MGE at 7 days compared to the later time points. This observation is also in agreement with a recent study where they found higher MGEs at 7 days compared with later time points using a metagenomic approach⁵⁹. Research has indicated that during the initial stages of life, the presence of mobile genetic elements could be attributed to maternal factors or the specific family environment, which has the greatest impact by the time a child reaches 6 months of age⁶⁰. In contrast, beta-lactam resistance genes exhibit complex patterns, with some genes increasing in abundance over time, while others experience shifts. These trends reflect the intricate dynamics of ARGs in response to selective pressures⁶¹.

Abundance patterns of ARGs associated with quinolones, vancomycin, and aminoglycosides were significantly increased with 4-month-old samples. It has been described that the prescription of quinolones to mothers increases the risk of their offspring acquiring community-acquired, quinolone-resistant *E. coli*⁶². Vancomycin is prescribed during the perinatal period for treating colitis, *C. difficile*–induced diarrhea, and gram-positive bacterial infections⁶³. Aminoglycosides, usually combined with a beta-lactam, are widely used for neonatal sepsis, and genes for acetylation, phosphorylation and adenylation conferring aminoglycoside resistance have been identified in infant gut microbiota⁶⁴. However, the percentage of infants of our study that were exposed to antibiotics was very low, and none of

them suffered from sepsis or colitis. All this consistently supports the notion that antibiotic exposure, either directly or indirectly, influences the infant resistome, and also that antibiotic resistances can occur even in the absence of antibiotic exposure.

Our study highlights the significant increase of tetracycline resistance over time. The resistance gene *tet* has been reported to be the most abundant and representative resistance genes in the infant gut resistome, possibly due to its prevalence in various bacteria species, such as Firmicutes and Bacteroidetes^{65,66}. It has also been reported that vertical transmission from the mother can be responsible for the acquisition of these resistance in infants⁶⁷⁻⁶⁹. Although tetracyclines are not used during pregnancy and early life, they are still extensively used in animals and, therefore, the environment and the diet may be sources of *tet* genes without direct exposure to the antibiotic^{67,70}. As a result, infants face a significant increase of tetracycline resistance over time.

Multidrug resistance in bacteria occurs by accumulation of ARGs on plasmids or transposons. Macrolide–lincosamide–streptogramin B (MLSB) is a significant multidrug-resistant phenotype usually related to staphylococci as it leads to methicillin-resistant *Staphylococcus aureus* (MRSA). Neonatal MRSA infections not only have high mortality and morbidity rates but also have long-term adverse effects on neonates⁷¹. The macrolide resistance gene *ermB* has previously been found to be prevalent in the infant gastrointestinal tract⁷², correlating with our findings. Most *ermB* carriers turned out to be *Enterococcus* spp., and *Klebsiella* spp., from which some pathogenic species are considered multi-drug resistant bacteria. This study also reports that the *mcr1* gene, which confers resistance to colistin, increases significantly in the infant gut over time, and it has been previously identified in multidrug resistant *Salmonella enterica*, which causes acute diarrhea⁷³.

Our analysis also considers the influence of factors like mode of delivery and lactation on the resistome. The results presented in our study showed a

significant difference in ARG composition in 7-day-old infants based on the mode of delivery. The alteration of the early gut microbiome due to delivery mode may contribute to differences in ARG composition, and some studies have elucidated the presence of ARGs in C-section born babies⁷⁴⁻⁷⁶. Moreover, the association of MDR genes with infants born via C-section is particularly noteworthy. A study by Yassour *et al.* (2016) found that C-section delivery was associated with a higher risk of the infant gut microbiome being enriched with opportunistic pathogens carrying MDR genes⁷⁷. This aligns with our findings, where MDR genes were prominent in C-section-born infants at 7 days of age. The differential association of tetracycline resistance genes in 2-month-old infants is of interest, as it suggests that factors beyond the mode of delivery may also contribute to ARG acquisition.

The influence of mode of lactation on ARG composition in infants, as demonstrated in our study, adds another layer to the complexity of early-life microbiota and resistance gene dynamics. Previous research has shown that breastfed infants exhibit a distinct gut microbiome and resistome profile^{57, 78, 59} compared to formula-fed infants. Our findings are consistent with this observation, as we noted differences in ARG composition based on mode of lactation, particularly at 4 months of age. The prevalence of vancomycin, MDR, and other ARGs in artificially fed infants is concerning. These genes confer resistance to antibiotics that are crucial for treating various infections. The temporal changes observed in the association of specific ARGs with mode of lactation raise important questions about the mechanisms underlying these shifts.

The varying contributions of different ARGs at different time points emphasize the dynamic nature of early-life gut microbiota and its susceptibility to external influences. One-health approach methodologies are essential for deciphering the complex resistome structure in the microbiomes of humans, animals, and the environment. Further research is needed to explore the mechanisms behind these fluctuations and their potential long-term consequences.

Gut microbiota profiling was performed in a subset of our population to address the possible influence on the antibiotic resistance load. Interestingly, *Bifidobacterium* spp. showed the strongest effect on ARG composition, shedding light into the important role of these genus on the variation of antibiotic resistances in early life. In some studies, the negative correlation between *Bifidobacterium* abundance and ARG load has been described in infants from 7 days to 4 months of age⁸⁰. Moreover, it has been found that *Bifidobacterium* genus themselves are less likely to possess ARGs than other taxa such as *Enterococcus*, *Streptococcus*, *Staphylococcus*, and *Bacteroides* genus^{81, 82}. It is important to consider that as the infant grows, their microbiome diversifies, acquiring a more complex phylogenetic structure. This increased microbial diversity is accompanied by an increase in the diversity of the resistome. Although these findings are important, further metagenomic approaches should be performed in order to specifically assess the influence of the different bacterial taxa in the early infant gut on the abundance of resistances.

While this study has provided valuable insights into the relationship between environmental factors and the composition of ARGs, several limitations should be considered. The sample size in this study was limited to 72 samples from specific geographic locations, potentially restricting the generalizability of the findings. Moreover, our study population did not have a representative group of infants exposed to antibiotics during their early-life, and this was beneficial to study the more targeted effect of lactation; it would be interesting for next approaches to include this factor.

The high-throughput methodology employed to quantify ARGs was robust; however, it was confined to a specific set of ARGs. This may have overlooked certain aspects or variations in other ARGs and low abundance ARGs have been missed. Despite these limitations, this study serves as a foundational exploration into understanding the interplay between environmental factors and ARG composition. Future research could address these limitations by expanding sample diversity, employing multi-omics approaches for ARG characterization, and conducting

longer longitudinal studies to unravel the temporal dynamics of ARGs in different environmental settings.

6. Conclusion

Our investigation into the dynamics of the early-life gut resistome in infants has illuminated the intricate and evolving landscape of antibiotic resistance genes during this critical developmental phase. Our study provides valuable insights into the evolution of the infant gut resistome and the use of qPCR-based ARG quantification, alongside comprehensive analysis of factors like mode of delivery and lactation, contributes to our understanding of how resistance genes develop and change over time. The significant shifts in resistance gene composition over time and the differences according to infant feeding type emphasize the need for a nuanced understanding of early-life resistance dynamics. These findings have important implications for strategies aimed at mitigating antibiotic resistance in infants and underscore the need for continued research in this vital area of microbiome science.

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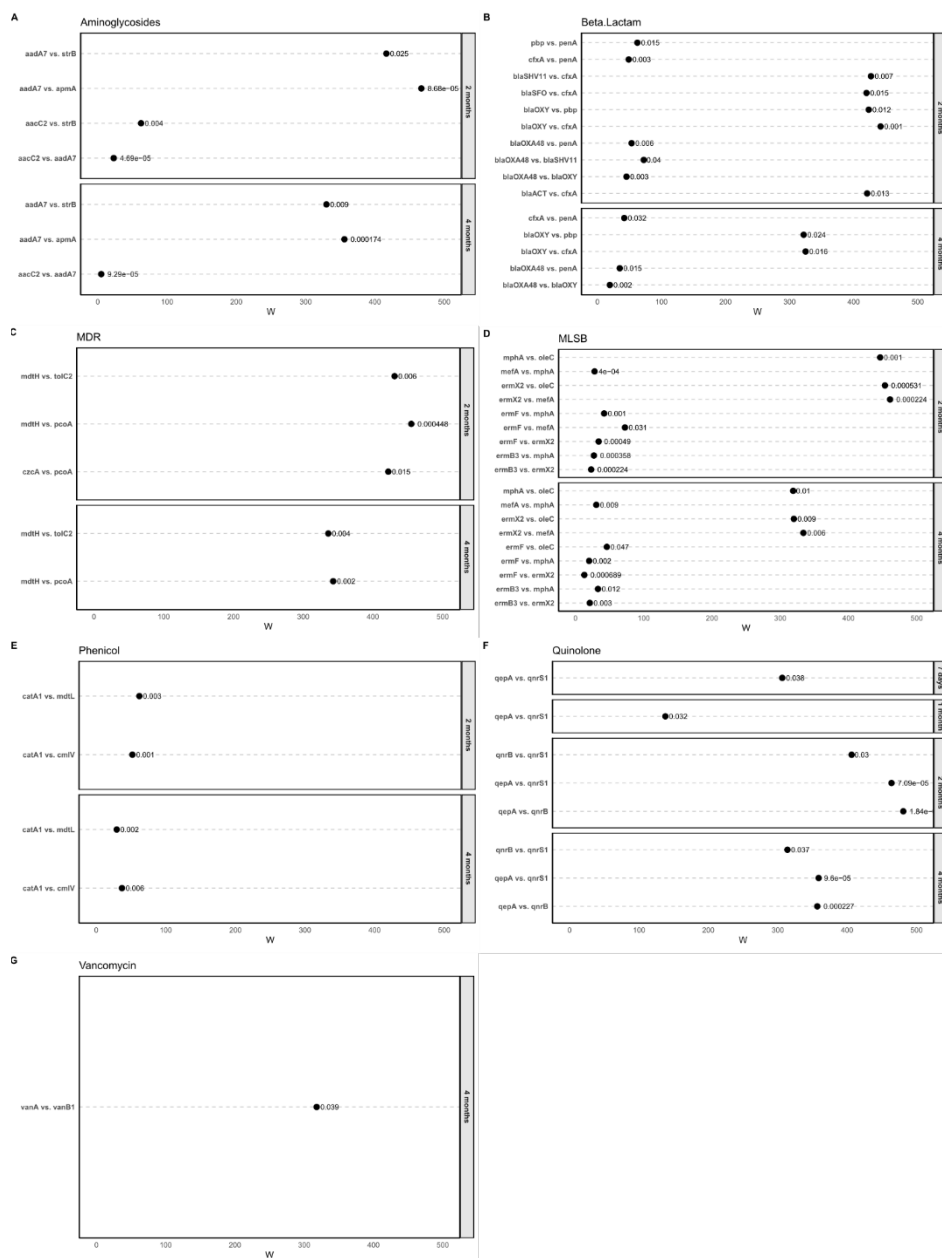
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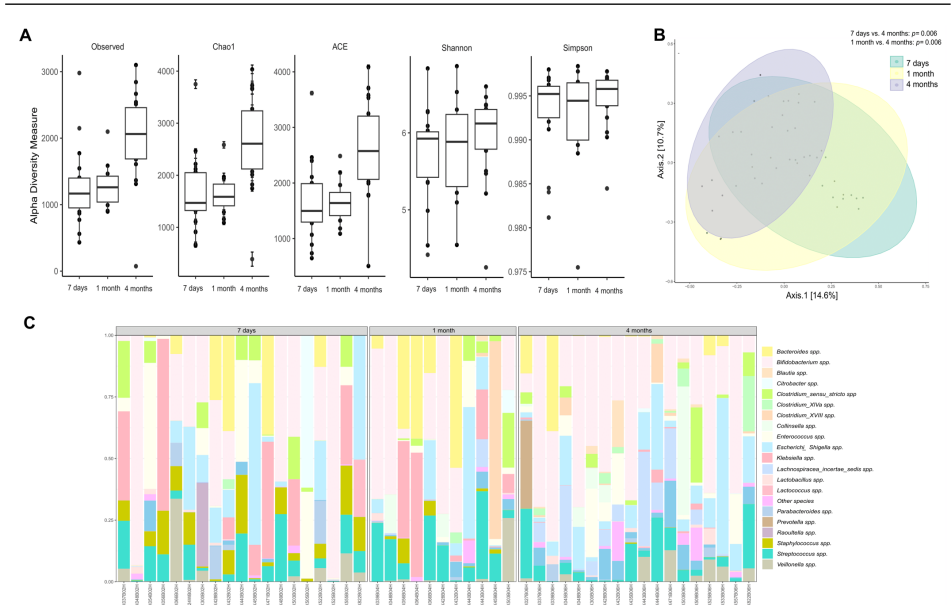
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8. Supplementary materials



Supplementary Figure I-1. Statistical differences between ARGs. Wilcoxon statistic value (W) between between ARGs. (A) Aminoglycosides, (B) Beta-lactams, (C) Integrans, (D) MDR, (E) MGE, (F) MLSB, (G) Others, (H) Phenicol, (I) Quinolone, (J) Tetracycline and (K) Vancomycin. Only ARGs of the same antibiotic group with significantly different relative abundances are represented. p-value is indicated next to each dot.



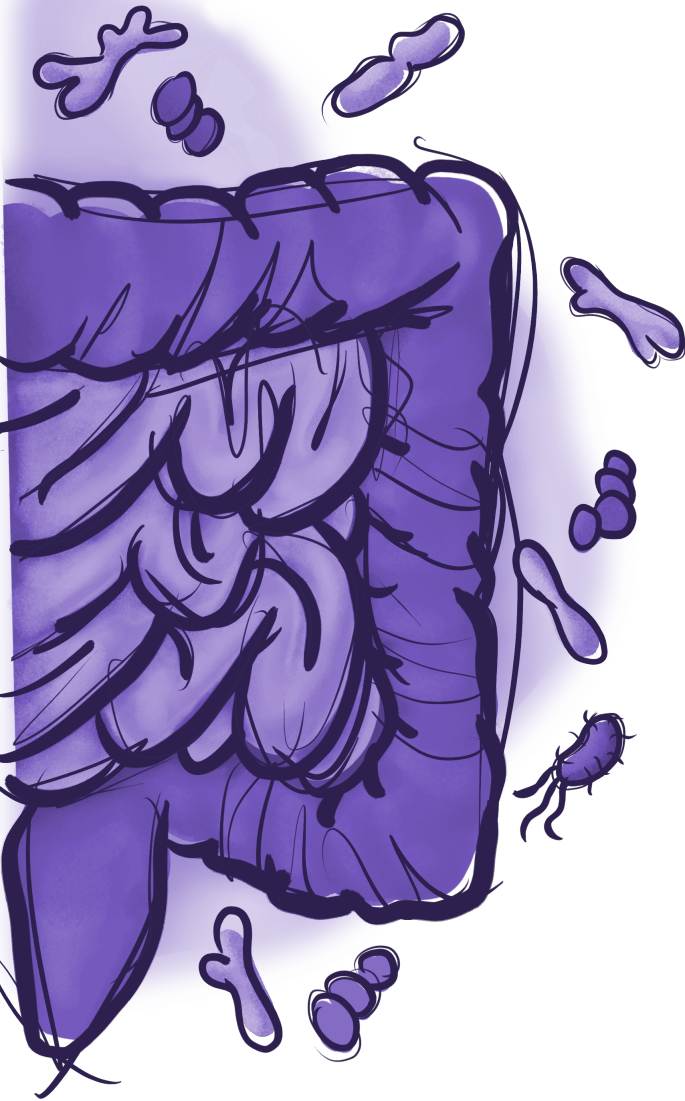
Supplementary Figure I-2. Microbiota analysis of the study population. A) Alpha diversity measures of observed, Chao 1, ACE, Shannon and Simpson indexes. B) Beta-diversity analysis performed with a bray-distance based PCoA of the microbial composition, coloured by the age of infants. Significant differences were found between time of 7 days and time 1 month ($p=0.003$) and 4 months ($P=0.009$). C) Taxaplot representing the composition of the study population. **Supplementary Table 3** shows the genus relative abundance of the 21 most abundant taxa in each sample. N=19 of 7 days old infants, N=11 of 1 month old infants and N=18 of 4 months old infants.

Supplementary Table I-1. (See Annex Chapter I)

Supplementary Table I-2. Copy numbers of the 48 ARGs, MGE and integrons quantified by HT-qPCR.

Supplementary Table I-3. Genus relative abundance of the 21 most abundant taxa in each sample.

Given their considerable size, these tables (Supplementary Table I-2 and I-3) are available in the supplementary materials hosted in the *Annals of Clinical Microbiology and Antimicrobials* journal repository, accessible via the following DOI: 10.1186/s12941-024-00725-z



CHAPTER II

CHAPTER II

The Role of *Bifidobacterium* genus in Modulating the Neonate Microbiota: Implications for Antibiotic Resistance Acquisition in Early Life

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My contribution to this research article was:

Conceptualization. Perform the experiments: DNA extraction, quantification, and purification, qPCR preparation. Data analysis and curation, statistical analysis and interpretation. Writing-original draft preparation.

1. Abstract

Resistance to antibiotics in newborns is a huge concern as their immune system is still developing, and infections and resistance acquisition in early life have short and long-term consequences for their health. *Bifidobacterium* species are important commensals capable of dominating the infant gut microbiome and are known to be less prone to possess antimicrobial resistance genes than other taxa that may colonize infants. We aimed to study the association between *Bifidobacterium*-dominated infant gut microbiota and the antibiotic resistance gene load in neonates, and to ascertain the perinatal factors that may contribute to the antibiotic resistance acquisition.

Two hundred infant fecal samples at 7 days and 1 month of age from the MAMI birth cohort were included in the study and for whom maternal-neonatal clinical records were available. Microbiota profiling was carried out by 16S rRNA amplicon sequencing, and targeted antibiotic resistance genes (ARGs) including *tetM*, *tetW*, *tetO*, *blaTEM*, *blaSHV* and *ermB* were quantified by qPCR.

Infant microbiota clustered into two distinct groups according to their *Bifidobacterium* genus abundance: high and low. The main separation of groups or clusters at each time point was performed with an unsupervised non-linear algorithm of k-means partitioning to cluster data by timepoints based on *Bifidobacterium* genus relative abundance. Microbiota composition differed significantly between both groups, and specific bifidobacterial species were enriched in each cluster. Lower abundance of *Bifidobacterium* in the infant gut was associated with a higher load of antibiotic resistance genes. Our results highlight the relevance of *Bifidobacterium* genus in the early acquisition and establishment of antibiotic resistance in the gut. Further studies are needed to develop strategies to promote a healthy early colonization and fight against the spread of antibiotic resistances.

2. Introduction

Antibiotic resistance in infants is a growing concern. Early exposure to antibiotics can lead to the development of antibiotic-resistant bacteria and disrupt the delicate balance of the gut microbiome, having consequences for future infant health, immune development and metabolism. In the early stages of life, antibiotic acquired resistance can result from the horizontal transfer of genes between bacteria, vertical transmission from the mother at birth or during lactation, and/or antibiotic exposure of the infant¹.

The gut microbiota composition is the main source of most antibiotic resistance genes (ARGs) that are carried by a limited number of taxa. Members of the family Enterobacteriaceae, such as *Escherichia*, *Klebsiella*, *Citrobacter*, and *Enterobacter*, responsible for many perinatal infections, contribute the most abundant and unique ARGs in the infant gut². *Bifidobacterium* are negatively correlated with antibiotic resistance load³; these species are important commensals capable of dominating the gut microbiome of breastfed infants, in part because of their capacity to use human milk oligosaccharides (HMOs) and other carbohydrates efficiently^{4,5}. *Bifidobacterium* species are also less likely to possess ARGs than other taxa such as *Enterococcus*, *Streptococcus*, *Staphylococcus* and *Bacteroides* genus^{6,7}. It has also been reported that the resistance genes detected in anaerobes (such as *Bifidobacterium* or *Lactobacillus*) are distinct from the ARGs found in pathogenic bacteria that grow aerobically^{8,9}, which suggest that horizontal gene transfer between commensals and pathogens is more unlikely than the transfer of ARGs between pathogens^{10,11}.

The infant gut microbiota changes significantly during the first days and weeks of life, with external factors, such as breastfeeding, mode of delivery, antibiotic exposure, and diet, exerting no influences on the composition and diversity of the infant gut microbiota, which in turn affects the resistome¹². Understanding these influences is crucial for developing interventions and strategies aimed at promoting a healthy gut microbiota and mitigating the risks associated with the emergence and spread of antibiotic resistance in infants. Our

objective was to study the influence of the presence of *Bifidobacterium* spp. on the early gut microbiota and the acquisition of antibiotic resistance gene load in neonates from 7 days to 1 month old. Further, we examined which perinatal factors may contribute to the initial microbiota composition and antibiotic resistance acquisition.

3. Materials and methods

3.1. Study design and volunteers

A total of 200 infant fecal samples collected during the first month of life of healthy term (37 weeks or more) infants from the MAMI birth cohort¹³, recruited during 2015-2017, were included in this observational study (**Supplementary Figure II-1**). Maternal-infant clinical records including body mass index (BMI), gestational age, neonate gender, birth weight, mode of delivery, type of lactation (maternal lactation, referring to breastfeeding; mixed lactation, referring to combination of both breastfeeding and formula-feeding; and exclusive formula-feeding) and antibiotic exposure of the infant were collected. All participants received oral and written information about the study, and written consent was obtained. The study was approved by Ethical Committee of Hospital Clínico Universitario de Valencia and CSIC. The trial was registered on the ClinicalTrial.gov platform (number NCT 03552939).

3.2. Neonatal biological samples and DNA extraction and quantification

Samples were collected at two time points (7 days and 1 month after birth) in sterile containers by the parents at home following detailed instructions. They were immediately stored at -20 °C, transported within 24 h to primary health centres and stored at -80 °C until analysis. Sample management and processing, as well as Total DNA was extraction, purification and concentration were performed as described previously¹⁴.

3.3. Quantification of antibiotic resistance genes and *Bifidobacterium* spp. by targeted qPCR

To analyse the antibiotic resistance gene load in infants' gut, a subset of samples from each time point (7 days: n=71, 1 month: n=66) was used, and the presence and abundances (log copies/ μ l) of each ARG were compared between the two timepoints and the two *Bifidobacterium* relative abundance clusters. Quantifications of the total number of copies of genes conferring resistance to tetracycline (*tetM*, *tetO* and *tetW*), beta-lactam (*blaTEM* and *blaSHV*) and erythromycin (*ermB*) were performed using described primers (**Supplementary Table II-1**) by qPCR in a LightCycler 480 real-time PCR system (Roche Technologies, Basel, Switzerland). The quantified ARGs were chosen according to their importance and high prevalence in infant resistome as described elsewhere^{2,3,15-18}. Each reaction mixture of 10 μ L was composed of 5 μ L SYBR GreenPCR Master Mix (Roche Technologies, Basel, Switzerland), 0.25 μ L of each of the primers at a concentration of 10 μ M, 3.5 μ L of distilled water and 1 μ L of DNA. The amplification conditions were 35 cycles of: initial denaturation at 95°C for 5 min denaturation at 95°C for 30s, specific annealing temperature for each primer pair¹⁹ for 30s and a final extension step of 72°C for 30s. The qPCR results were calculated as the antibiotic resistance gene copies per sample (log copies/ μ l). *Bifidobacterium* genus total counts were also determined by targeted quantitative PCR by the same quantitative system based on the amplification of specific 16S rRNA gene region. Melting curves were also assessed to test the specificity of the reaction. For all qPCR measurements, standard curves for the specific targeted gene were generated using Ct values and the calculated gene copy numbers were determined based on the fragment amplification length.

3.4. Gut microbiota profiling by specific 16S rRNA amplicon sequencing

The microbiota characterization was performed by amplification of the V 3-V 4 regions of the 16S rRNA. Amplicons were obtained with PCR amplification using barcoded conventional primers (341F 5'-CCTACGGGNGGCWGCAG-3' and 806R 5'-GGACTACNNGGTATCTAAT-3') with a 466 bp fragment length. Amplicons were

checked with a Bioanalyzer DNA 1000 chip and libraries were sequenced using a 2×250 bp paired-end kit on a MiSeq-Illumina platform (FISABIO sequencing service, Valencia, Spain). Bacterial diversity analysis was done using raw reads, which were quality controlled and filtered (Quality; 25 and length; 125 bp) using TrimGalore (v 0.6.4_dev; <https://github.com/FelixKrueger/TrimGalore>).

The paired-end reads with a minimum overlap of 30 bp were joined using Fastq-join²⁰. Sequences were trimmed of primers and distal bases, and singletons were removed with USEARCH v 11²¹. zOTUs (zero-radius operational taxon units) mapping to the human genome (GRCh 38) using the Burrow–Wheeler Aligner in Deconseq v 0.4.3 were filtered out. The resulting reads were denoised and chimeras were filtered with UNOISE 3²². Taxonomic assignment of zOTUs was performed in QIIME 2 v 2018.2²³ using the QIIME 2 feature classifier plugin²³⁷ and the Ribosomal Database Project (RDP)²⁵. The zOTUs were aligned with MAFFT²⁶ to then make a phylogenetic tree with FASTTREE²⁷ that was midpoint rooted. The Bayesian LCA-based Taxonomic Classification Method (BLCA)²⁸ was used to assign zOTUs to species that were only assigned to *Bifidobacterium* genus with QIIME 2 (assignment of 80% confidence). The database used was a custom database, taking the reference sequences of all *Bifidobacterium* species from the SILVA database²⁹. We filtered the zOTU data using filter_taxa function of “*phyloseq*” package, and only zOTUs present in at least 10% of samples were retained.

3.5. Bioinformatic and statistical analyses

All statistical analyses were performed with R version 3.6.0, and Figures were drawn with the “*ggplot 2*” R package³⁰. Categorical variables are expressed as positive cases-prevalence and (percentage, %). Normally distributed data are presented as mean ± standard deviation (SD) and non-normal data as median and interquartile range [IQR]. Pearson’s-Chi-square test was used for categorical variables, and Mann-Whitney U test or Fisher’s exact test were used for continuous variables, as appropriate, for calculating statistical significance. Statistical analyses were performed equally for both paired and unpaired data points, as the

majority of the population was paired in both timepoints, and confirmation analyses confirmed there were no differences (data not shown).

The main separation of groups or clusters at each time point was performed with an unsupervised non-linear algorithm of k-means partitioning to cluster data by timepoints based on *Bifidobacterium* genus relative abundance. The elbow method and average silhouette method were performed using in the “factoextra” R package³¹ were used to determine the optimal number of clusters and k-means clustering of the data was performed with the “stats” R core package. Optimal centres of k-means were established as 2 clusters, which corresponded with high and low *Bifidobacterium* relative abundance (**Supplementary Figure II-2**).

Venn diagrams were generated to assess the core and unique microbiota, at the zOTU level, for the two clusters at each time point separately, using the R package “VennDiagram”³². Calculations of richness (Observed, Chao 1 and ACE) and evenness indices (Shannon and Simpson) were done using the “phyloseq” R package³³.

Beta diversity was characterized by Principal Coordinate Analysis (PCoA) conducted by plotting the Bray-Curtis distance matrix of log transformed zOTU counts for each timepoint separately. The Adonis permutational test was used to evaluate overall differences in microbiota structure between the k-mean-categorized groups with the “vegan” R package³⁴. The same analysis was performed according to the mode of delivery and antibiotic exposure of the study participants. A Mann-Whitney U test in the “rstatix” R package³⁵ was performed on the log transformed abundances of genera to identify significantly different genera between the two clusters. Phylogenetic trees based on zOTUs assigned to *Bifidobacterium* genus were constructed using “plot_tree” function of “phyloseq” package.

Multivariate Redundancy Analysis (RDA) was conducted to confirm the correlation between ARGs and microbiota with respect to the clusters of *Bifidobacterim* abundance. Linear discriminant analysis effect size (LEfSe) was used to identify microbial genera significantly enriched in each cluster. An LDA

score ($\log_{10}$) >3 was considered significant. Spearman rank correlation coefficients were estimated to determine the linear association between the 20 Top genera and the number of copies of ARGs.

The effect size and significance of each covariate were determined using the “*envfit*” function in the “*vegan*” package comparing the difference in the centroids of each group relative to the total variation. Ordination was performed using NMDS based on Bray–Curtis dissimilarity. The significance value was determined based on 999 permutations. The squared correlation coefficient (R^2) for each variable was calculated and scaled as a vector. In total, 6 covariates with known associations to gut microbiome development in neonates, infants, and children were included in the ‘*envfit*’ analysis and the grouping used within each variable is presented in **Table II-1**. Specifically, we tested hierarchical clustering based on k-means according to *Bifidobacterium* relative abundance (high or low), mode of delivery, mode of lactation, antibiotic exposure during pregnancy, antibiotic exposure at birth, and direct antibiotic exposure of the infants. MaAsLin2³⁶ was used to build general linear models for longitudinal data to efficiently determine the multivariate association between factors and taxonomy in both clusters while accounting for potentially confounding covariates. MaAsLin2 relies on general linear models to accommodate most modern epidemiological study designs, including cross-sectional and longitudinal. In this case, the analysis with MaAsLin2 was a longitudinal analysis, so the first added variable was Time (7 days and 1 month). The following variables were added depending on the strength of each variable seen in the other analyses, so we added “kmeans_ab 02” (Low vs High), “Delivery_mode” (C-section vs vaginal) and “Lactation” (maternal vs. mixed vs. formula). No more variable groups or other variables were added. The command runs MaAsLin 2 on the data, running a multivariable regression model to test for the association between microbial species abundance versus Time, kmeans_ab 02, Delivery_mode and Lactation. Finally, the input data were transformed into a log format.

For all methods, p-values were adjusted for multiple comparisons using False Discovery Rate (FDR) based on Benjamini–Hochberg (BH)³⁷. Normality of the data was evaluated with Shapiro–Wilk tests.

4. Results

4.1. *Bifidobacterium* dominance associates with a specific microbial composition

Hierarchical clustering revealed two significantly distinct ($p < .01$) microbial clusters at each time point, characterized by a high (54.38% for 7 days; 61.72% for 1 month) and low (11.26% for 7 days; 17.08% for 1 month) relative abundance of *Bifidobacterium* members (**Figure II-1**). 71.43% of infants maintained this classification according to *Bifidobacterium* relative abundance through time (from 7 days to 1 months).

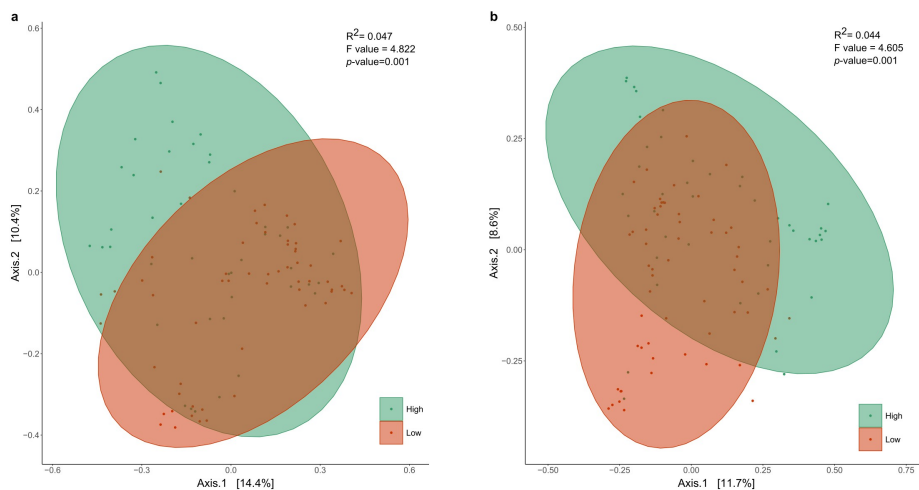


Figure II-1. Principal coordinates analysis of the hierarchical clustering of *Bifidobacterium* relative abundance of the infants at (A) 7 days of age and (B) 1 month of age, based on Bray-Curtis distances regarding the microbial community composition ($p < 0.001$; ADONIS pairwise test). Samples are grouped by hierarchical clustering of *Bifidobacterium* relative abundance in intestinal microbiota. Orange ellipses represent the high-*Bifidobacterium* groups and blue ellipses represent low-*Bifidobacterium* groups. **Supplementary Figure II-2 provides the optimal number of clusters found by the elbow and average silhouette methods of K-means partitioning clustering algorithm.**

Clinical characteristics of the participating infants and their respective mothers at both timepoint according to *Bifidobacterium* cluster are shown in **Table II-1**. Gender was distributed similarly between the two clusters. Regarding the mode of delivery, vaginal-born infants corresponded to 90.47% and 81.39% of the neonates clustered as high- *Bifidobacterium* at 7 days and 1 month, respectively. Most of the infants participating in the study were breastfed and no differences with other breastfeeding practices were found between clusters. Finally, antibiotic exposure was recorded during the gestation period until 1 month of age. 42.10% of infants clustered as low- *Bifidobacterium* at 7 days of age were exposed to antibiotics at delivery, significantly more than the high- *Bifidobacterium* infants ($p=.0008$). According to maternal characteristics, no statistical differences were found according to the *Bifidobacterium* cluster.

Table II-1. Characteristics of study participants.

	7-days-old infants (N=99)			1-month-old infants (N=101)		
	High (N=42)	Low (N=57)	p- value	High (N=43)	Low (N=58)	p- value
Gender						
Female	17 (40.47%)	32 (56.14%)	.181	21 (48.83%)	31 (53.44%)	.797
Male	25 (59.52%)	25 (43.38%)		22 (51.16%)	27 (46.55%)	
Delivery mode						
C-section	4 (9.52%)	21 (36.84%)	.004*	8 (18.6%)	17 (29.31%)	.317
Vaginal	38 (90.47%)	36 (63.15%)		35 (81.39%)	41 (70.68%)	
Clinical data						
Weight (kg)	3.38 ±0.68	3.30 ±0.45	.147	4.28 ±0.49	3.99 ±0.75	.527
Lactation mode						
Maternal	39 (92.85%)	50 (87.71%)	.843	30 (69.76%)	46 (79.31%)	.536
Mixed	0	1 (1.75%)		6 (13.95%)	6 (10.34%)	

Formula	3 (7.14%)	6 (10.52%)		7 (16.27%)	6 (10.34%)	
Antibiotic exposure						
At birth	4 (9.52%)	24 (42.10%)	.0008*	10 (23.25%)	18 (31.04%)	.523
During first 7 days	3 (7.14%)	9 (15.7%)	.321	6 (13.95%)	5 (8.62%)	.598
During 1st month	1 (2.38%)	5 (8.77%)	.372	3 (6.97%)	5 (8.62%)	1
Pregnancy data						
Gestational age (weeks)	38.88 (39-41)	39.38 (39-40)	.070	38.55 (38.25-40)	39.74 (39-40)	.180
Weight gain over the pregnancy (kg)	11.88 ±4.09	12.47 ±4.52	.397	13.02 ±3.99	12.56 ±5.21	.018
Pre-gestational BMI (kg m ⁻²)	22.33 ±4.99	23.99 ±3.57	.089	23.34 (4.75)	22.38 (3.28)	.07
Antibiotic consumption during pregnancy	7 (16.6%)	10 (17.5%)	.070	11 (25.58%)	19 (32.75%)	.575

Categorical variables are expressed as positive cases-prevalence and percentatge (%). Normally distributed data are presented as mean ± standard deviation (SD) and non-normal data as median and interquartile range [IQR]. Pearson's-Chi-square test was used for categorical variables, and Mann-Whitney U test or Fisher's exact test were used for continuous variables, as appropriate, for calculating statistical significance. Asterisks (*) indicate a significant difference ($p < .05$) between two groups. BMI: Body Mass Index.

To assess if confounding variables might influence clustering, beta diversity was also compared across other metadata variables, separating the infants by their mode of delivery in combination with the two different clusters, corresponding to the *Bifidobacterium* relative abundance (**Supplementary Table II-2**). For both time points, vaginal-born infants clustered in two groups according to the *Bifidobacterium* relative abundance (Vaginal_High and Vaginal_Low) with significantly different bacterial communities ($p = .006$). The microbiota of C-section_Low infants was significantly different from Vaginal_Low and Vaginal_High infants ($p < .05$), both at 7 days and 1 month of age. Finally, in 1-month-old infants, C-section_High infants clustered separately from Vaginal_Low infants ($p = .036$). No

differences between C-section-High and C-section Low were found at either time point.

The impact of antibiotic exposure microbial composition in the context of *Bifidobacterium* clustering was also examined (**Supplementary Table II-3**). Both high and low- *Bifidobacterium* groups of infants that did not receive antibiotics had different gut microbial compositions ($p = .012$ and $p = .006$ for 7 days and 1 month of age). Infants with a low abundance of *Bifidobacterium* have a significantly different microbial composition at 7 days of age depending on if they have received antibiotics or not ($p = .012$), which is not observed in high- *Bifidobacterium* cluster.

4.2. Microbiota is shaped by *Bifidobacterium* spp. abundance

In both time points, the percentage of the core zOTUs was 35.1%, while 48.8% (7 days) and 50.5% (1 month) are unique for the Low cluster and 16.1% (7 days) and 14.1% (1 month) were unique for the High cluster, signifying that the two groups, harbour different inhabitant niches, independent of time.

Infants in the High cluster showed lower but not statistically significant microbial diversity and richness at 7 days and 1 month of age (**Supplementary Table II-4**). The observed diversity of *Bifidobacterium* species between the two clusters at the two sampling times was significantly different ($p < .001$ for both times): the high- *Bifidobacterium* group had more diversity of *Bifidobacterium* spp. than the low- *Bifidobacterium* group (**Supplementary Figure II-3**).

Differences in relative abundance of the 8 most abundant genera (more than 5% of relative abundance) were calculated according to zOTUs counts (**Figure II-2A and B** and **Supplementary Table II-5 and 6**). *Bifidobacterium* relative abundance was 54.38% and 61.72% in the High-clustered samples of 7 days and 1 month respectively, and 11.26% and 17.08% of the Low-clustered samples. *Bifidobacterium* genus was quantified in 7 days old infant samples at 6.93 (3.71) log copies/ μ L (median (IQR)) for Low cluster and 7.71 (7.03) log copies/ μ L (median (IQR)) for High cluster. For 1-month samples, 7.08 (4.53) and 7.53 (6.14) log copies/ μ L (median (IQR)) were quantified in Low and High groups, respectively.

In 7-days old infants, *Streptococcus*, and *Escherichia/Shigella* spp. predominated in the low- *Bifidobacterium* group, appearing in higher relative abundances than the *Bifidobacterium* species, although no statistical differences were found between the two groups. Seven-day-old infants in the low- *Bifidobacterium* group had a significantly higher relative abundance of *Staphylococcus* ($p < .01$), *Enterococcus* ($p < .05$), and *Klebsiella* ($p < .05$), compared to the high- *Bifidobacterium* group. *Veillonella* and *Bacteroides* showed the same pattern but without statistical differences. On the other hand, low- *Bifidobacterium* 1-month-old infants had also higher relative abundance of *Klebsiella* ($p < .05$), whereas *Collinsella* appeared in higher proportions in the high- *Bifidobacterium* group, though differences between groups were not statistically significant. Similar to 7-day-old infants, *Staphylococcus*, *Enterococcus*, and *Veillonella* appeared in higher abundances in the low- *Bifidobacterium* group, but with no statistically significant difference compared to the high- *Bifidobacterium* group. Tendency analysis of the microbiota also showed that *Bacteroides* spp. was more abundant in high- *Bifidobacterium* infant than in low- *Bifidobacterium* infants at 1 month of age ($p < .001$) (**Supplementary Table II-5** and **6**).

LEfSe, performed on samples from 7-day-old infants, showed that *Klebsiella* ($p = .031$) and *Clostridium* ($p = .0036$) were significant characterized in the Low cluster and *Streptococcus* ($p = .024$), and *Corynebacterium* ($p = .0378$) characterized the High cluster. For the 1-month-old infant samples, *Streptococcus* ($p = .011$), *Enterococcus* ($p = .042$), *Rothia* ($p = .038$) and *Actinomyces* ($p = .0021$) were enriched in the High cluster, and *Bacteroides* ($p = .0009$), *Finnegoldia* ($p = .009$) and *Gemella* ($p = .045$) were enriched in the Low cluster (**Figure II-2C** and **D**).

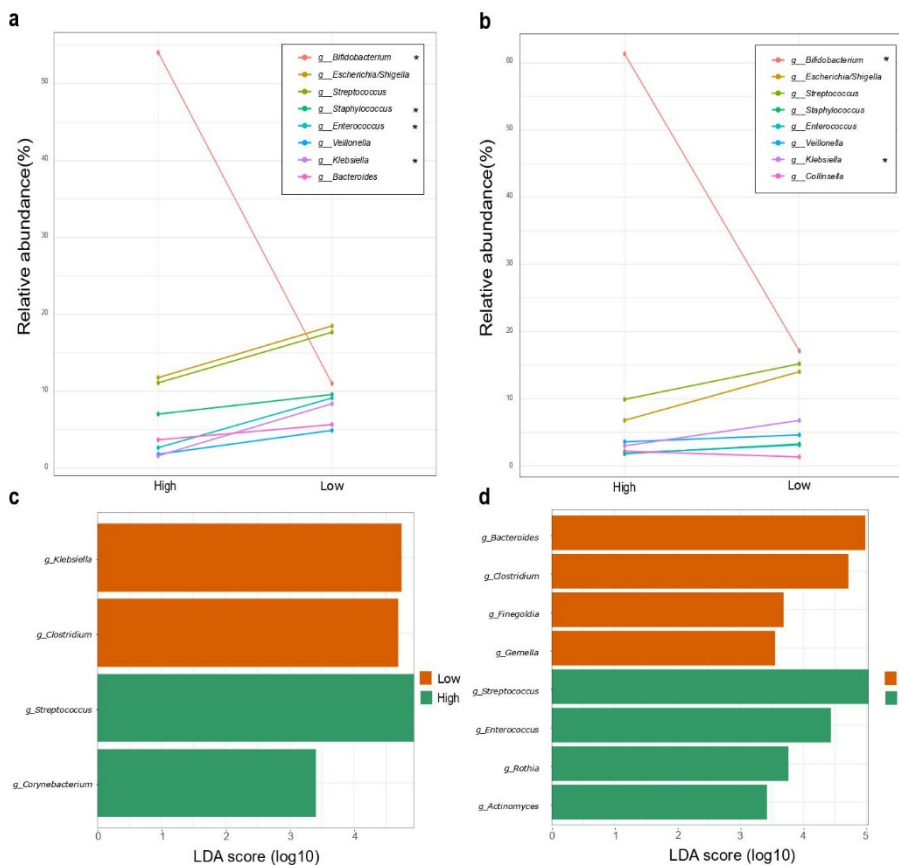


Figure II-2. Microbial composition according to *Bifidobacterium*-abundance groups. Differences in relative abundance (%) of the 8 most abundant genera in fecal microbiota (more than 5% of relative abundance) among the first 7 days (A) and 1 month of life (B). Asterisks (*) indicate a significant difference between groups based on Mann-Whitney U of the log-transformed relative abundances. **Supplementary Table II-s 5 and 6** provide genus abundances and statistical analyses of the groups for the two timepoints. Linear discriminant analysis (LDA) Effect Size (LEfSE) plot of taxonomic biomarkers identified in the gut microbiota of infants of 7 days (C) and 1 month (D) of age, according to *Bifidobacterium* dominance cluster (High and Low coloured in green and orange, respectively). The threshold for the logarithmic discriminant analysis (LDA) score was 3 and $p < .05$ for Wilcoxon test was considered significant.

The Bayesian LCA-based Taxonomic Classification Method for *Bifidobacterium* spp. showed that, in both timepoints, *Bifidobacterium longum* (High:46.96-50.72%; Low:46.09-56.24%, for 7 days and 1 month, respectively) was the most abundant species of *Bifidobacterium* in infants (Figure II-3A and B). Relative abundance of *B. dentium* (High:3.95-10.66%; Low:13.35%-14.48%) was

significantly more abundant in 1-month-old infants in the low- *Bifidobacterium* group according to LEfSE analysis (**Figure II-3C**), whereas an unclassified species of *Bifidobacterium* was enriched in the high- *Bifidobacterium* group. This species, together with *B. breve*, appeared in higher amounts in the high- *Bifidobacterium* cluster of infants in both timepoints, but without statistical significance. On the other hand, *B. adolescentis*, *B. animalis* and *B. scardovii* were more abundant in low- *Bifidobacterium* infants of both ages, but no significant differences were found. The phylogenetic tree of *Bifidobacterium* species confirmed that, in both timepoints, there were unique zOTUs assigned for specific *Bifidobacterium* spp. (**Supplementary Figure II-3**).

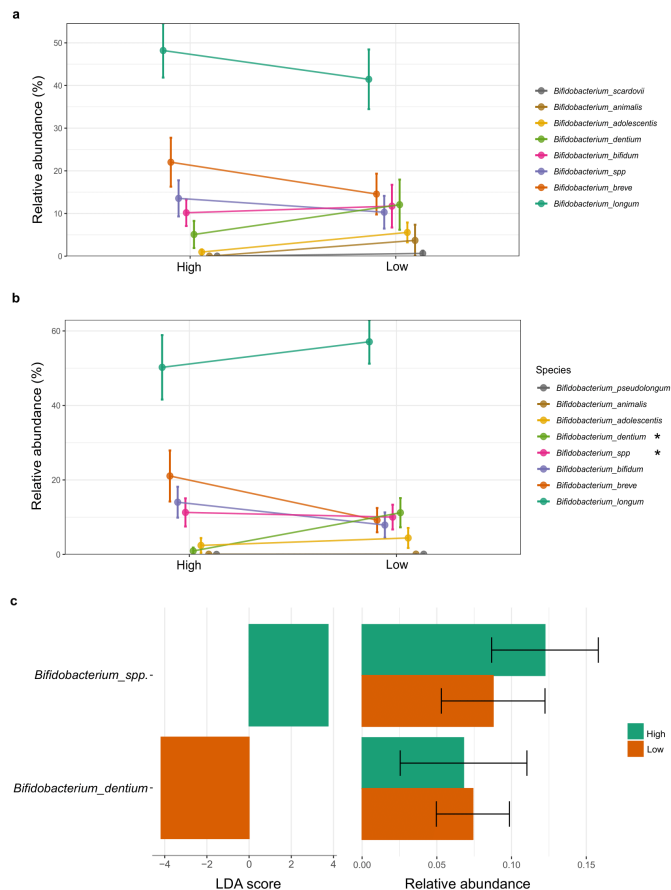


Figure II-3. *Bifidobacterium* spp. relative abundance according to *Bifidobacterium* relative abundance group. Differences in relative abundance (%) of species of *Bifidobacterium* identified in fecal microbiota among the **(A)** first 7 days and **(B)** 1 month of

life. **(C)** Linear discriminant analysis (LDA) Effect Size (LEfSE) plot of *Bifidobacterium* species identified in the gut microbiome of 1-month-old infants and their relative abundances. Species bacterial traits found at *Bifidobacterium* species level according to *Bifidobacterium* dominance cluster (High and Low coloured in green and orange, respectively). The threshold for the logarithmic discriminant analysis (LDA) score was 3 and $p < .05$ for Wilcoxon test was considered significant.

4.3. Influence of environmental factors on *Bifidobacterium* relative abundance and specific microbial associations

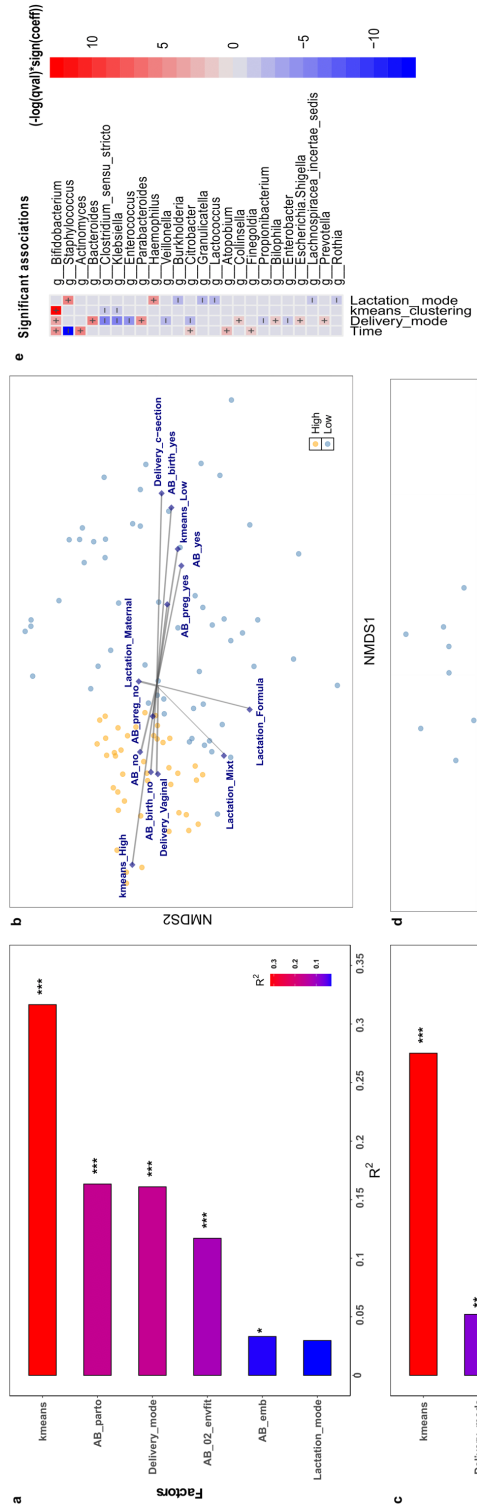
Analysis with ‘envfit’ confirmed that the high-low *Bifidobacterium* relative abundance clustering, based on k-means hierarchical clustering method, is the best approach to explain the dissimilarities in gut microbial composition according to *Bifidobacterium* relative abundance, as it was the factor that explained the largest amount of variance in both timepoints ($R^2=0.316$, $p < .001$ at 7 days ; $R^2=0.275$, $p < .001$ at 1 month) (**Figure II-4A** and **C**). In 7-day-old infants, antibiotic exposure at the moment of birth ($R^2=0.163$, $p < .001$) was the second most important categorical factor, followed by mode of delivery ($R^2=0.161$, $p < .001$). C-section procedures imply the use of antibiotics during delivery, so it is expected that both variables have similar contributions. Accordingly, vaginal-delivery and absence of antibiotic exposure at birth were associated with the high- *Bifidobacterium* group (**Figure II-4B**). Overall antibiotic exposure during the first 7 days of life (including moment of birth till 7 days of age) was the next most important factor ($R^2=0.117$, $p < .001$), followed by mode of lactation, which was not statistically significant ($R^2=0.045$, $p > 0.05$), and the use of antibiotics by the mother during pregnancy ($R^2=0.033$, $p < .05$).

Delivery mode was the second most important environmental factor contributing to the different microbial composition at 1 month of life ($R^2=0.0521$, $p < .01$) (**Figure II-4C**). Vaginal birth was associated with high- *Bifidobacterium* infants (**Figure II-4D**). Lactation mode played a crucial role in the gut composition ($R^2=0.051$, $p < .05$), even more than antibiotic exposure at birth ($R^2=0.046$, $p < .05$). Breastfeeding (both, exclusive breastfeeding and mixed practises) contributed to the high- *Bifidobacterium* cluster, while infant formula-feeding was associated with the low- *Bifidobacterium* group. Antibiotic exposure of the mother during pregnancy

and of the infant during the first month of life were the least contributing factors, without statistical significance, ($R^2=0.033$, $p>0.05$ and $R^2=0.001$, $p<.05$, respectively).

Significant associations between microbial genera and environmental variables were assessed (**Figure II-4E**). The *Bifidobacterium* genus was most strongly and positively correlated with k-means clustering, followed by time of sampling and delivery mode. Hence, these results support the previous analysis, confirming that the microbial composition of our population differentiates into two groups due mainly to *Bifidobacterium* relative abundance. Moreover, *Clostridium* and *Klebsiella* species correlate negatively to this clustering. The variable time shows a significant positive correlation with *Actinomyces*, *Citrobacter*, *ATop obium* and *Finegoldia*, while it is negatively correlated with *Staphylococcus*. Mode of delivery is positively associated with *Bacteroides*, *Parabacteroides*, *Collinsella*, *Bilophila*, *Escherichia/Shigella* and *Prevotella*, and mode of lactation, is positively correlated with *Staphylococcus* and *Haemophilus* genus.

► **Figure II-4. Environmental factors contributing to *Bifidobacterium*-specific microbiota.** Significance and explained variance of 6 microbiota covariates modelled by 'envfit' across all data types for each time point: for (A) 7 days old and for (C) 1 month old. Horizontal bars show the amount of variance (R^2) explained by each covariate in the model as determined by 'envfit'. The groups within each covariate are detailed in **Table II-1**. Covariates are gradient-coloured based on R^2 . Significant covariates (false discovery rate (FDR) $p < .05$) are represented in bold. Asterisks (*) denotes the significant covariates at each time point: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. kmeans: hierarchical clusterization based on k-means according to *Bifidobacterium* relative abundance; AB_birth: antibiotic exposure at birth; AB_pregnancy: antibiotic exposure of the mother during pregnancy; AB_exposure: antibiotic exposure of the infant during his life; Delivery_mode: mode of delivery; Lactation_mode: mode of lactation. Dissimilarities in gut microbiota composition according to *Bifidobacterium* relative abundance (High or Low) represented by nonmetric multidimensional scaling (NMDS) of zOTU relative abundances for infants (B) 7 days and (D) 1 month of age. The centroids of each group are in blue (Low) and yellow (High). The Top contributing categorical variables are displayed as arrows, with a length proportional to the correlation between the variable and the NMDS ordination. (E) Multivariable association between factors and taxonomy in both clusters while accounting for potentially confounding covariates using MaAsLin,



(Figure II-4)

4.4 *Bifidobacterium* relative abundance modulates antibiotic resistant gene load

Antibiotic resistance load and presence diverged depending on *Bifidobacterium* relative abundance (**Supplementary Table II-7**). The multivariate redundant discriminant analysis (RDA) (**Figure II-5**) showed that, for 7 days infant gut samples, *tetO*, *ermB*, and *tetM* were the ARGs associated with the Low-*Bifidobacterium* cluster, and further analysis suggest that their sources are likely *Escherichia/Shigella*, *Phocaeicola*, *Blautia*, *Veillonella*, *Raoutella*, and *Staphylococcus*, whereas *Streptococcus*, *Enterococcus*, and *Klebsiella* were associated to the high-*Bifidobacterium* cluster.

In 1-month-old infants, the Low cluster was positively associated with *Blautia*, *Enterococcus*, *Veillonella*, *Phocaelcola*, *Streptococcus*, *Escherichia/Shigella*, *Klebsiella*, and *Bacteroides*, and the last three genera were highly correlated with copies of *tetO*, *tetM*, *blaTEM*, *blaSHV* ($p = .016$), and *ermB* resistant genes. On the contrary, *tetW* is negatively correlated with the other ARGs and taxa and is associated with infants from the high-*Bifidobacterium* cluster. This gene (*tetW*) is more prevalent ($p = .02$) in 1-month-old infants in the high-*Bifidobacterium* (73.08% of presence) group compared to the low-*Bifidobacterium* (42.50%).

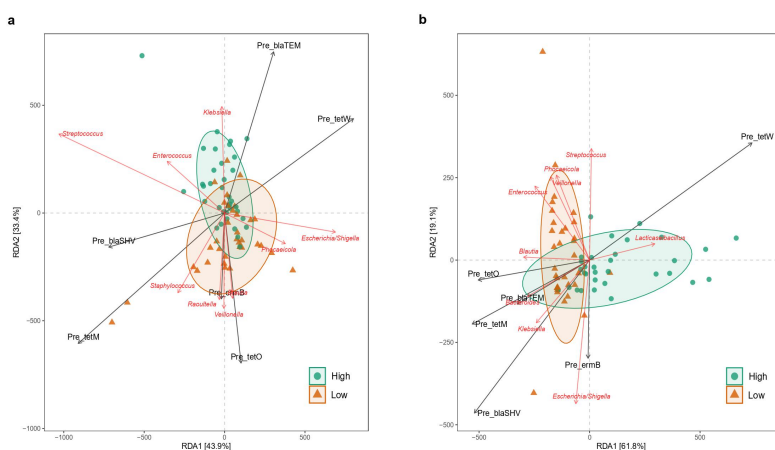


Figure II-5. Redundancy Discriminant Analysis (RDA) biplot depicting the relationship between the bacterial communities and copies of ARGs at (A) 7 days and (B) 1 month of

age. Coloured arrows represent differential bacteria and copies ARGs, coloured dots represent samples from different groups. Angles between the arrows represent correlations; acute angles represent positive correlations and obtuse angles represent negative correlations. The black arrows are the ARGs that best explain the differences between the sample groups. The orange arrows are the taxa that best explain the differences between the sample groups (n=9 taxa).

Spearman correlations between microbiota at the genus level and copies of ARGs were performed (**Figure II-6**). In 7-day-old infants with higher abundance of *Bifidobacterium*, a high abundance of the *Lachnospira* genus was associated with a higher abundance of *blaTEM*, and *tetW* gene ($p < .05$), while in the low-*Bifidobacterium* infants, a higher abundance of *blaTEM* gene was associated to higher abundance of *Escherichia/Shigella* ($p < .05$). In addition, in the low-*Bifidobacterium* cluster, *tetM*, and *ermB* genes appear negatively correlated with *Blautia*, *Clostridium*, *Klebsiella*, and *Pseudomonas* ($p < .01$).

On the other hand, in the High cluster of 1-month-old infants, there is a positive correlation between *blaSHV*, and *tetO* genes and *Enterococcus* ($p < .01$). For the Low cluster, *blaSHV* is negatively correlated with *Clostridium* ($p < .05$), *Lactobacillus* ($p < .001$), and *Pseudomonas* ($p < .001$), and positively correlated with *Escherichia/Shigella* ($p < .001$).

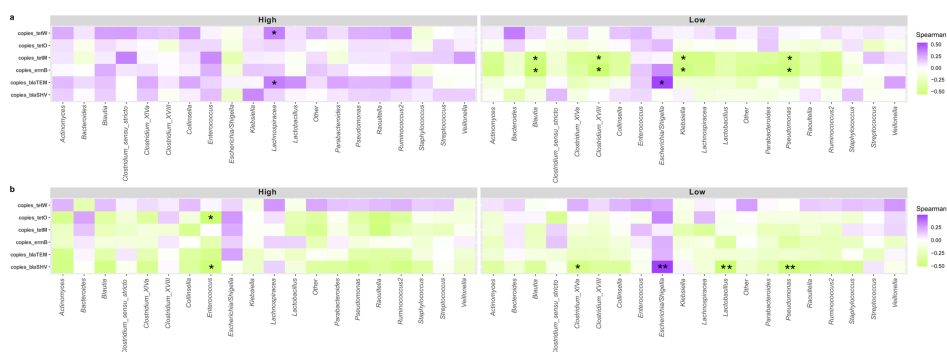


Figure II-6. Spearman correlations between genera and copies of ARGs. Heat map to show Spearman correlations between copies of ARGs and microbiota composition at the genus level, divided by *Bifidobacterium* relative abundance clusters (High and Low) for infants of 7 days of age (A) and 1 month of age (B). Statistical differences are marked as follows: * $p < .05$, ** $p < .01$.

5. Discussion

We demonstrated the association of *Bifidobacterium* spp. with the modulation of early-life microbiota and the antibiotic resistance gene load, and determined which perinatal factors are involved in these complex interactions.

Our primary findings showed that the presence of ARGs in infant's gut correlated with a low- *Bifidobacterium* abundance, so we focused on how the abundance of *Bifidobacterium* could shape the infant resistome in the early stages of life. Results from this resistance epidemiology study align with previous studies⁶: the microbiota of low- *Bifidobacterium* dominated infants is associated with a higher abundance of antibiotic resistance genes, due to a differential microbial composition responsible for carrying these ARGs, as well as the external factors to which the infant is exposed, such as mode of delivery, antibiotic exposure, and breastfeeding. Overall, our results provide insight into the very early acquisition and development of ARGs in a general population cohort of healthy infants.

***Bifidobacterium* relative abundance is associated with a specific gut microbiota**

The infant gut resistome changes primarily in relation to the microbiome as ARGs are carried by the microorganisms in the gut and transferred between them through horizontal gene transfer. As far as we know, this is the first study encompassing the influence of *Bifidobacterium* on antibiotic resistance load in infants of 7 days and 1 month of age. These two time points are of high interest as the gut microbiota at these ages is very diverse and still in the early stages of development. Thus, it is still largely shaped by the initial colonizers that the baby is exposed to during delivery and by feeding mode. This is the reason why the specific composition of the gut microbiota can vary widely between individuals.

To achieve our objective, we first studied the microbial profile of our study population. The application of k-means clustering algorithms elucidated that the infants in the study clustered into two different groups according to *Bifidobacterium* spp. abundance. *Bacteroidota* appeared more abundant in those samples with low

abundance of *Bifidobacterium*, enhancing a potential interaction between *Bacteroides* spp. and *Bifidobacterium* spp. Both are generalist glycan degraders capable of using a wide range of substrates: *Bacteroides* spp. are specialized as primary degraders in the metabolism of complex carbohydrates, whereas *Bifidobacterium* spp. more commonly metabolizes smaller glycans, such as oligosaccharides, sometimes through syntrophic interactions with *Bacteroides* spp., in which they act as secondary degraders^{38,39}. Some bifidobacteria can produce exopolysaccharides (EPS), which can be metabolized by *Bacteroides*⁴⁰. It has been described that cocultures of *Bifidobacterium* and *Bacteroides* behave differently against fermentable carbohydrates as a function of the specific characteristics of the strains from each species⁴¹.

It is also known that *Bifidobacterium* strains have antibacterial activity against various pathogens, including *Escherichia coli*, *Streptococcus* spp., *Salmonella* spp., *Listeria* spp. and *Cutibacterium* spp.⁴², as well as against multidrug-resistant pathogens⁴³, by nutrient competition⁴⁴, biofilm removal or inhibition^{45,46}, production of antimicrobial substances^{47,48}, acidification, and enhancement of mucus barrier function⁴⁹. In addition, *Bifidobacterium* can influence the overall composition and diversity of the gut microbiota, which helps to maintain a diverse and stable microbial community in the gut⁵⁰. In our study we have observed that *Escherichia/Shigella* and *Streptococcus* are more abundant than *Bifidobacterium* in the low-*Bifidobacterium* cluster of infants at 7 days of life, which reverts at 1 month of life. This may be explained by the impact of breastfeeding on the infant microbiome, as it is well-known that breastfeeding enriches the *Bifidobacterium* dominance^{51,52}, and most of our study population is breastfed. Moreover, at both time points, low-*Bifidobacterium* infants had higher abundances of *Staphylococcus*, *Klebsiella*, and *Clostridium* than high-*Bifidobacterium* infants. These genera have been previously found to carry ARGs², therefore, suggesting that the correlation between *Bifidobacterium* and antibiotic resistance load can be a result of the genera that predominate when *Bifidobacterium* are less dominant. All these results highlight the importance of

considering specific species and strain interactions in the relationship among intestinal microbial populations and its dynamics over time.

The application of Bayesian LCA-based method in our microbial population showed a distinct *Bifidobacterium* species profile in the two groups. Specifically, 1-month-old infants with low- *Bifidobacterium* abundance were enriched with *Bifidobacterium dentium*, which is found primarily in the oral cavity and possesses various enzymes that enable it to break down complex carbohydrates and degrade protective glycans on mucin proteins^{53,54}. Moreover, *B. dentium* is capable of assimilating type 1 and type 2 HMOs^{55,56}. By fermenting these carbohydrates, *B. dentium* produces SCFAs and other metabolites that provide energy to both itself and other gut bacteria⁵⁷. This metabolic activity may contribute to the overall functioning and health of the gut ecosystem, enhancing the potential beneficial role of this species in the gut of low- *Bifidobacterium* infants. A non-classified *Bifidobacterium* spp. was enriched in the high- *Bifidobacterium* group of infants, highlighting the importance of species-specific identification in microbial studies to understand the role of each species in the early gut establishment and maturation.

Antibiotic resistance genes in the infant gut

Antibiotic-resistant bacteria carry a set of antibiotic resistance genes, and this set in a population of bacteria is known as the resistome. Most predominant resistance genes encode for proteins that confer resistance to beta-lactams, tetracyclines, macrolides, aminoglycosides, and quinolones¹⁷. Beta-lactamases have been prescribed for many decades, and are commonly used during pregnancy and labour, so it is understandable that resistance appears in infant fecal samples². Although tetracycline antibiotics are no longer used in pregnancy, *tet* genes have been reported to be the most abundant among infant fecal samples and the most representative resistance gene in the infant resistome^{58,59}. Klassert et. Al² determined that genes conferring resistance to macrolides (*mefA*, *ermB* and *ermC*) showed the highest dissemination in a sample set of mother-infant pairs and also present the highest likelihood of vertical transmission⁶⁰.

Our primary results showed a lower number of *tetM* copies in the guts of those infants clustered in the high- *Bifidobacterium* group, although, as mentioned, these antibiotics are not used during pregnancy. The prevalence of tetracycline resistance genes in various bacteria species, coupled with the common utilization of tetracycline to treat infections, may explain the acquisition of *tet* genes by women. Although this antibiotic is not used during pregnancy and early life due to potential dental staining effects, it is still extensively used in animals and, therefore, the environment and the diet may be sources of *tet* genes without direct exposure to the antibiotic⁵⁹. Hence, the vertical transfer of antibiotic resistances from the mother to their offspring, together with the high prevalence of this resistance in the environment, may be the principal mechanism of spread of tetracycline resistance in early life.

To complete the study, other ARGs conferring resistance to antibiotics that are used during pregnancy and labour (beta-lactams and macrolides) were measured and the relationship with specific bacterial communities was assessed. We quantified of *blaTEM*, *blaSHV*, and *ermB* genes in infant fecal samples, and similarly to *tet* genes, we showed that their presence has been associated with a low abundance of *Bifidobacterium*.

Antibiotic resistance load is affected by the specific microbiota associated with Bifidobacterium relative abundance

Some studies have shown that *Bifidobacterium* can help to reduce the risk of antibiotic resistance by competing with pathogenic bacteria for nutrients and space in the gut and by producing antimicrobial compounds that inhibit the growth of other bacteria^{61,62}. A study conducted in 2019 described a change in the gut microbiome and a reduction in ARG abundance in infants supplemented with *Bifidobacterium infantis* EVC001⁶³. In some studies, the negative correlation between *Bifidobacterium* relative abundance and ARG load has been described in infants from 6 weeks of age⁶. Moreover, it has been found that *Bifidobacterium* themselves are less likely to possess ARGs than other taxa such as *Enterococcus*,

Streptococcus, *Staphylococcus*, and *Bacteroides*^{6,7}. Pseudomonadota, more specifically, Gammaproteobacteria, have been demonstrated to be the major carriers for ARGs in studies either employing culture-based or metagenomics methods⁶⁴. Members of the family Enterobacteriaceae, such as *Escherichia*, *Klebsiella*, *Citrobacter*, and *Enterobacter*, which are responsible for a large range of infections, contribute the most abundant and unique ARGs in the infant's gut².

Comparably, our results showed that at 7 days of age, *tetO*, *ermB*, and *tetM* are positively correlated with the bacterial species predominate in the Low cluster of infants, namely *Escherichia/Shigella*, *Phocaeicola*, *Blautia*, *Veillonella*, *Raoutella*, and *Staphylococcus*. *Streptococcus*, *Enterococcus*, and *Klebsiella* were associated with the high- *Bifidobacterium* cluster at 7 days old gut microbiota and with the low- *Bifidobacterium* cluster at 1 month old. This result shows the gain of stability of gut microbiota at 1 month of age compared to 7 days, as it has been previously described⁶⁵.

Moreover, at 1 month of age, low- *Bifidobacterium* infants are associated with *Blautia*, *Veillonella*, *Phocaelcola*, *Escherichia/Shigella*, *Klebsiella*, and *Bacteroides*, the last three of which highly correlated with copies of *tetO*, *tetM*, *blaTEM*, *blaSHV* and *ermB* resistant genes.

It is important to recall that *tetW* was negatively correlated with the other ARGs and associated with infants from the high- *Bifidobacterium* cluster at this time point, which matches with previous findings that indicate that *tetW* gene is an ARG from the *Bifidobacterium* genus^{66,67}. Spearman correlations also highlighted the influence of *Escherichia/Shigella* on antibiotic resistance load: in low- *Bifidobacterium* infants, this genus was positively correlated with the *blaTEM* resistance gene.

Overall, these findings suggest that low levels of *Bifidobacterium* may lead specific ARG-carrying genera to dominate the infant's gut, thus increasing the antibiotic resistance load. But how does this *Bifidobacterium* variation occur? Resistome composition is strongly influenced by the phylogenetic profile of the

bacterial population. Considering that there are still numerous bacteria possessing unidentified ARGs, the known ARGs may represent only a small part of the true resistome. In this vein, it would be interesting to include genes conferring resistance to other classes of antibiotics, such as aminoglycosides or quinolones, that, together with a metagenomic approach, would provide a wider view of the infant resistome. Aminoglycosides are widely used for neonatal sepsis and are usually combined with beta-lactams⁶⁸, and resistance to quinolones also emerged with clinical use and has become common in some bacterial pathogens. Although quinolones are limited in the paediatric population, the presence of ciprofloxacin-resistant *E. coli* in the infant's fecal microbiota has been described⁶⁹.

Factors influencing infant's gut microbiota

High *Bifidobacterium* dominance in infants' guts has been directly related to the mode of delivery: vaginal or C-section. C-section-born infants have a decreased abundance of *Bifidobacterium* compared to vaginally born infants^{70,71}. For this reason, we assessed whether our microbial clusters may be a result of the procedure of delivery, but we again obtained two different clusters according to *Bifidobacterium* relative abundance, independent of the mode of delivery. All infants born vaginally had a significantly different microbiota depending on if they had high or low *Bifidobacterium* relative abundance, at both 7 days and 1 month of age, so we could discard that mode of delivery was shaping our microbiota clusters. Indeed, k-means hierarchical clustering was the variable with the highest contribution to the separation of the microbial composition of our population, as expected. However, we observed role of delivery in the modulation of gut microbiota, as it was associated with genera such as *Enterococcus*, *Klebsiella*, and *Clostridium*, which have been previously related to the microbiota of C-section born infants⁷².

In addition, it has been seen that early-life antibiotic treatment disrupts the proper and natural development of gut microbiota with a potential negative influence on later health⁷³. It has also been previously described that intrapartum

antibiotic prophylaxis (IAP) has a negative effect on the later bifidobacterial establishment in the neonatal gut^{7,74}. To assess this effect, antibiotic exposure of our cohort was recorded during pregnancy and also during the first month of life of the infants. During a C-section procedure, antibiotics are commonly administered as a preventive measure to reduce the risk of infection (e.g., cefazolin, vancomycin, clindamycin and erythromycin)⁷⁵. On the other hand, there is the possibility that vaginal-born infants have been exposed to antibiotics at birth too, and this has been recorded and considered in our study. Interestingly, the antibiotic exposure at delivery and how childbirth occurred had a similar contribution to the microbial composition of infants of 7 days of age, while this interaction dissociated at 1 month of age, with the antibiotic exposure losing importance, indicating a possible mitigation of its effects over time. Nevertheless, in both time points, C-section procedures and exposure to antibiotics contributed to a gut microbiota with low *Bifidobacterium* spp. Therefore, our results confirm previous knowledge on the effect of antibiotics on the early microbial composition but highlight the importance of *Bifidobacterium* abundance in the infant's gut⁷⁶.

The feeding mode showed a significant contribution to 1-month-old infants' microbiota but not to that at 7 days old, maybe because of the short period infants consumed milk or because a reduced number of infants that were mixed or formula-fed. Formula-feeding contributed to the low- *Bifidobacterium* microbiota profile, while mixed and exclusive breastfeeding contributed to the high-*Bifidobacterium* infants, highlighting the widely known benefits of breastfeeding practises for infant health⁷⁷⁻⁷⁹. Nevertheless, breastmilk plays a crucial role on the acquisition of antibiotic resistance in early life⁸⁰. Human milk has been found to contain antibiotic-resistant bacteria, such as *Staphylococcus*, *Streptococcus*, *Acinetobacter*, *Enterococcus*, and *Corynebacterium*, resistant to antibiotics and with multidrug-resistant profiles⁸¹. Moreover, metagenomic analyses have highlighted the high levels of ARGs and MGEs in breastmilk and the similarity of the breastmilk and infant gut resistomes³. Overall, more understanding on mother's gut and breastmilk resistomes may shed light to decipher how neonates acquire

antibiotic resistance genes in early life and the role that environmental factors may play, so new approaches and decisions can be made to reduce antibiotic resistance. The interplay between early microbial colonization, environmental factors, and dietary changes might have lasting effects that extend beyond infancy. Understanding the persistence of these early exposures and their potential link to ARGs could offer valuable insights into strategies for promoting healthy gut microbiota development and combating antibiotic resistance.

Our results support the notion that microbiota plays a crucial role in early-life antibiotic resistance gene acquisition, focusing on infants of 7 days and 1 month of age. *Bifidobacterium* dominance has been found to be associated with the load of ARGs: low- *Bifidobacterium* abundance is associated with a higher presence of ARGs in infant's gut, which is also associated with a specific microbial profile. Moreover, we assessed that antibiotic exposure and mode of delivery are two of the key factors influencing microbial composition, and thus, antibiotic resistance load, and we suggest the potential impact of new or current antibiotic prescription practices during pregnancy, at birth, or during the first month of life on the infant gut microbiome and resistome. Our findings highlight the public health concern of antibiotic resistance and elucidate that more studies are needed to gain further insights into how prevalent antibiotic resistance can be in the population and to develop new strategies to ameliorate the ARG load.

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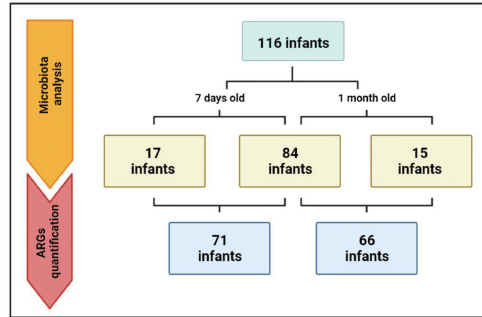
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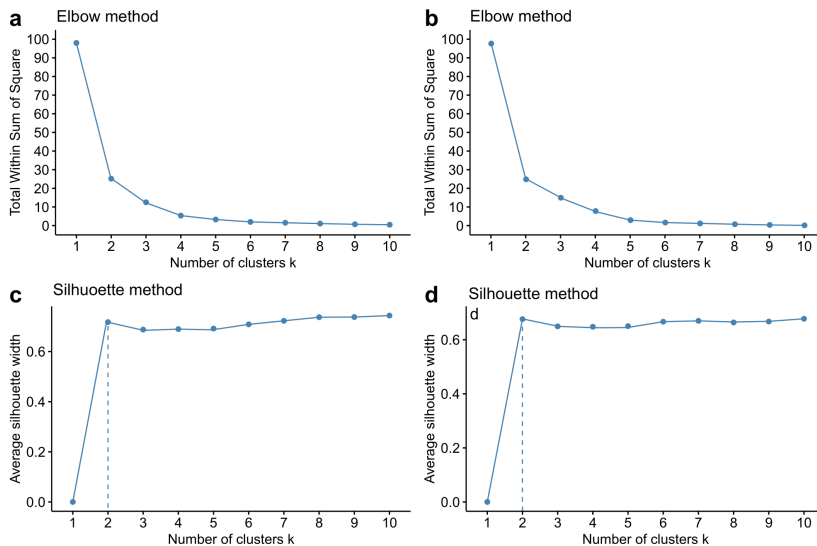
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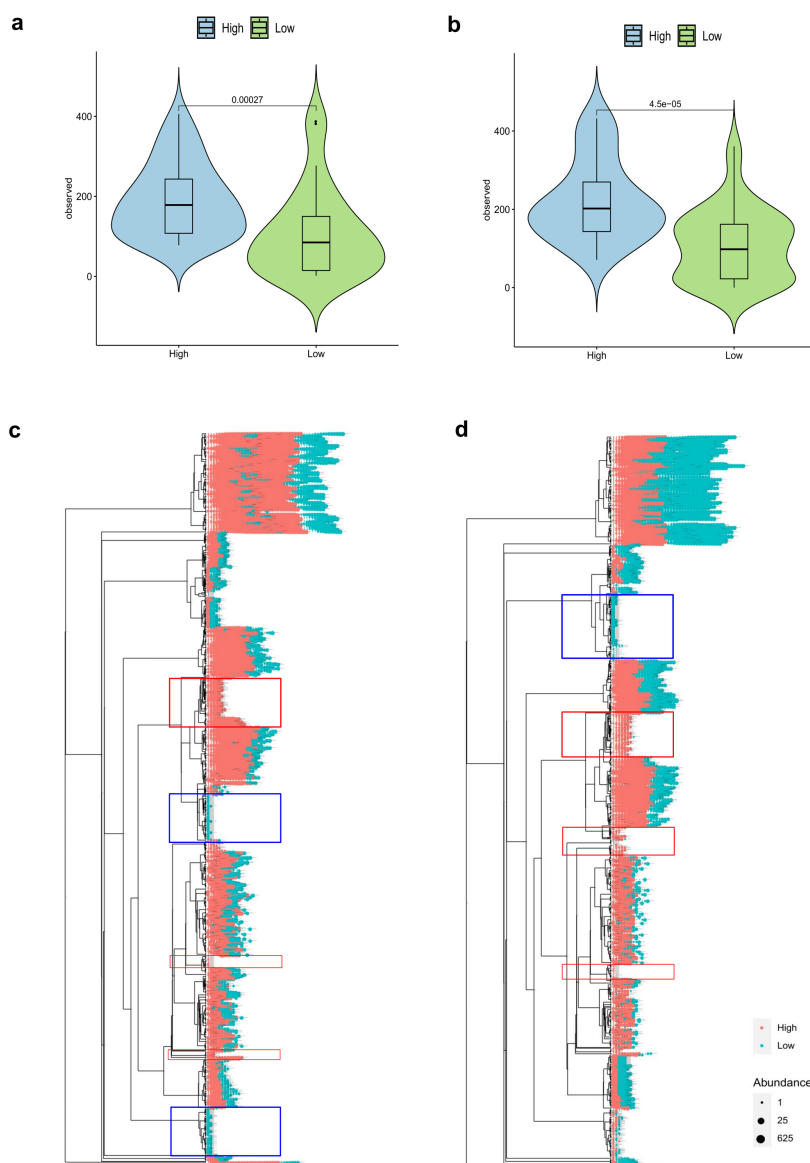
7. Supplementary Material



Supplementary Figure II-1. Flowchart of the study participants. A total number of 116 infant fecal samples of 7 days and 1 month of age (N=84 paired, 17 and 15 unpaired, respectively for each timepoint) were included in the study. A subset of samples from each time point (n=71 of 7 days, n=66 of 1 month) was used to assess and the presence and abundances of each ARG.



Supplementary Figure II-2. The optimal number of clusters (K=2) for infants of 7 days of age (A and C) and 1 month (B and D) of age is represented according to elbow and average silhouette methods of the K-means partitioning clustering algorithm. The elbow method is a graphical representation for finding the optimal 'K' in a K-means clustering. It works by finding WCSS (Within-Cluster Sum of Square). The average silhouette method computes the average silhouette of observations for different values of k. The optimal number of clusters k is the one that maximizes the average silhouette over a range of possible values for k. Both elbow and average silhouette methods suggest that the solution is 2 clusters, so it is possible to define k=2 as the optimal number of clusters in the data.



Supplementary Figure II-3. *Bifidobacterium* spp. alpha diversity and phylogenetic trees.

Observed alpha diversity and phylogenetic trees based on *Bifidobacterium* genus for each timepoint: (a) and (c) for 7 days, (b) and (d) for 1-month infants. The phylogenetic trees show the unique zOTUs present in each timepoint depending on *Bifidobacterium* abundance clusters. Red squares remark the zOTUs that cluster in the tree for each time and show the single zOTUs in the High group. On the other hand, the blue square marks zOTUs that cluster only in the Low group for each time.

Supplementary Table II-1. Primers used in the study.

Primer	Forward	Reverse	T (°C)	Class
<i>tetM</i>	GGAGCGATTACAGAATTAGG AAGC	TCCATATGTCCTGGCGTGCT	62	Tetracycline
<i>tetW</i>	ATGAACATTCCCACCGTTATC TTT	ATATCGCGCGAGAGCTTAT CC	60	Tetracycline
<i>tetO</i>	ACGGARAGTTTATTGTATACC	TGGCGTATCTATAATGTTGA C	62	Tetracycline
<i>blaTEM</i>	CGCCGCATACACTATTCTCA G	GCTTCATTCAGCTCCGGTTC	66	Beta Lactam
<i>blaSHV</i>	CGTAGGCATGATAGAAATGG ATC	CGCAGAGCACTACTTTAAA GG	65	Beta Lactam
<i>ermB</i>	GAACACTAGGGTTGTTCTTG CA	CTGGAACATCTGTGGTATG GC	62	MLS _B
<i>Bif.</i> <i>spp.</i>	GATTCTGGCTCAGGATGAAC GC	CTGATAGGACGCGACCCC AT	60	16S rRNA

Supplementary Table II-2. Summary of ADONIS pairwise model statistics using Bray-curtis dissimilarities at 7 days and 1 month of age by mode of delivery.

Variables	Bray-Curtis		p-value
	R ²	F value	
7 days			
Vaginal_High vs Vaginal_Low	0.0431	3.247	0.006*
C-section_High vs C-section_Low	0.062	1.532	0.486
Vaginal_High vs C-section_High	0.041	1.726	0.300
Vaginal_Low vs C-section_Low	0.043	2.476	0.030*
Vaginal_High vs C-section_Low	0.098	6.246	0.006*
Vaginal_Low vs C-section_High	0.031	1.224	1.000
1 month			
Vaginal_High vs Vaginal_Low	0.048	3.751	0.006*
C-section_High vs C-section Low	0.077	1.934	0.060
Vaginal_High vs C-section_High	0.038	1.633	0.438
Vaginal_Low vs C-section_Low	0.037	2.166	0.012*
Vaginal_High vs C-section_Low	0.085	4.671	0.006*
Vaginal_Low vs C-section_High	0.037	1.852	0.036*

Vaginal_High: infants born vaginally and clustered in the high-*Bifidobacterium* group; Vaginal_Low: infants born vaginally and clustered in the low-*Bifidobacterium* group; C-section_High: infants born by C-section and clustered in the high-*Bifidobacterium* group; C-section_Low: infants born by C-section and clustered in the low-*Bifidobacterium* group. Asterisks (*) indicate a significant difference ($p < .05$) between groups.

Supplementary Table II-3. Summary of ADONIS pairwise model statistics using Bray-curtis dissimilarities at 7 days and 1 month of age by antibiotic consumption

Variables	Bray-Curtis		p-value
	R ²	F value	
7 days			
High_no vs Low_no	0.038	2.573	0.012*
High _yes vs Low_ yes	0.060	1.919	0.150
High yes vs Low_no	0.025	0.958	1.000
High_yes vs High_no	0.013	0.536	1.000
Low_ yes vs High_no	0.093	5.950	0.006*
Low_Yes vs Low_no	0.043	2.477	0.012*
1 month			
High_no vs Low_no	0.046	4.081	0.006*
High _yes vs Low_ yes	0.080	1.222	0.990
High yes vs Low_no	0.023	1.277	0.792
High_yes vs High_no	0.017	0.736	1.000
Low_ yes vs High_no	0.060	2.749	0.006*
Low_Yes vs Low_no	0.019	1.104	1.000

High_no: infants with no antibiotic exposure clustered in the high-*Bifidobacterium* group; High_yes: infants exposed to antibiotics and clustered in the high-*Bifidobacterium* group Asterisks; Low_no: infants with no antibiotic exposure clustered in the low-*Bifidobacterium* group; Low_yes: infants exposed to antibiotics and clustered in the low-*Bifidobacterium* group. Asterisks (*) indicate a significant difference ($p < .05$) between groups.

Supplementary Table II-4. Alpha diversity indices and statistical analyses.

7-days old infants (N=99)					1-month old infants (N=101)			
	High (N=42)	Low (N=57)	χ^2	p-value	High (N=43)	Low (N=58)	χ^2	p-value
Richness								
Observed	1127 ±514.75	1291 ±513	98	0.368	1218 ±528	1677.5 ±769	97.97	0.510
Chao	1594.32 ±724	1639.66 ±825.62	98	0.481	1561.05 ±652.84	2083.55 ±949.94	100	0.481
ACE	1573.03 ±767.83	1567.97 ±806.22	97	0.480	1613.96 ±616.61	2035.89 ±892.80	100	0.481
Evenness								
Shannon	5.75 ±0.59	5.86 ±0.74	98	0.481	5.63 ±0.67	6.133 ±0.72	100	0.481

Simpson	0.99 ±0.002	0.99 ±0.004	98	0.481	0.99 ±0.004	0.99 ±0.004	100	0.481
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Alpha diversity richness (Observed, Chao and ACE) and evenness (Shannon and Simpson) indices according to *Bifidobacterium* relative abundance groups. Shapiro-Wilk test was used to check normality and non-parametric indices were evaluated for statistical significant differences by Kruskal Wallis test, and expressed as mean $\pm$ SD

Supplementary Table II-5. Genus relative abundance (%) according to *Bifidobacterium* relative abundance groups (High and Low) of 7-day old infants.

Genus	High (%)	Low (%)	W	p-value
<i>Bacteroides</i> spp.	3.682	5.772	1198.0	0.997
<i>Bifidobacterium</i> spp.	54.388	11.263	2161.0	***
<i>Bilophila</i> spp.	0.016	0.002	1200.0	0.975
<i>Blautia</i> spp.	0.040	1.695	910.5	0.036
<i>Clostridium_IV</i> spp.	0.003	0.013	1176.0	0.759
<i>Clostridium_sensu_stricto</i> spp.	0.740	5.298	1240.5	0.591
<i>Clostridium_XI</i> spp.	0.092	0.140	1049.0	0.183
<i>Clostridium_XIVa</i> spp.	0.007	0.049	1179.0	0.793
<i>Clostridium_XVIII</i> spp.	0.241	0.622	722.5	***
<i>Collinsella</i> spp.	1.006	0.869	1143.5	0.694
<i>Corynebacterium</i> spp.	0.042	0.043	1384.0	0.165
<i>Eggerthella</i> spp.	0.156	0.105	1312.5	0.322
<i>Enterobacter</i> spp.	0.173	1.014	963.0	0.062
<i>Enterococcus</i> spp.	2.657	9.108	869.5	**
<i>Erysipelotrichaceae</i> spp.	0.164	0.014	1243.5	0.602
<i>Escherichia/Shigella</i> spp.	11.788	18.552	1167.5	0.837
<i>Finegoldia</i> spp.	0.093	0.487	1366.0	0.173
<i>Flavonifractor</i> spp.	0.001	0.002	1190.5	0.911
<i>Gemella</i> spp.	0.062	0.083	1217.0	0.890
<i>Gemmiger</i> spp.	0.000	0.027	1222.0	0.714
<i>Haemophilus</i> spp.	0.011	0.057	1014.0	0.162
<i>Intestinibacter</i> spp.	0.001	0.001	1166.5	0.608
<i>Klebsiella</i> spp.	1.611	8.423	835.5	**
<i>Lachnospira</i> spp.	0.179	1.408	1154.0	0.754
<i>Lactobacillus</i> spp.	0.298	0.152	1389.5	0.137
<i>Lysinibacillus</i> spp.	0.001	0.028	1220.5	0.694
<i>Parabacteroides</i> spp.	0.942	0.850	1221.5	0.856
<i>Peptoniphilus</i> spp.	0.005	0.020	1142.0	0.524
<i>Phascolarctobacterium</i> spp.	0.097	0.025	1240.0	0.565
<i>Prevotella</i> spp.	0.000	0.019	1194.0	0.968
<i>Cutibacterium</i> spp.	0.002	0.002	1175.0	0.747
<i>Rothia</i> spp.	0.070	0.266	989.5	0.134
<i>Ruminococcus 2</i> spp.	0.030	0.001	1170.0	0.650
<i>Staphylococcus</i> spp.	7.039	9.622	913.0	*
<i>Streptococcus</i> spp.	11.176	18.140	1036.0	0.256
<i>Veillonella</i> spp.	1.815	4.888	958.5	0.091

Mann-Whitney U test values and adjusted p values for the differential log-transformed abundances are also shown. Asterisks (*) denotes the significant covariates at each time point: * p < .05, ** p < .01, *** p < .001.

Supplementary Table II-6. Genus relative abundance (%) according to *Bifidobacterium* relative abundance groups (High and Low) of 1-month old infants.

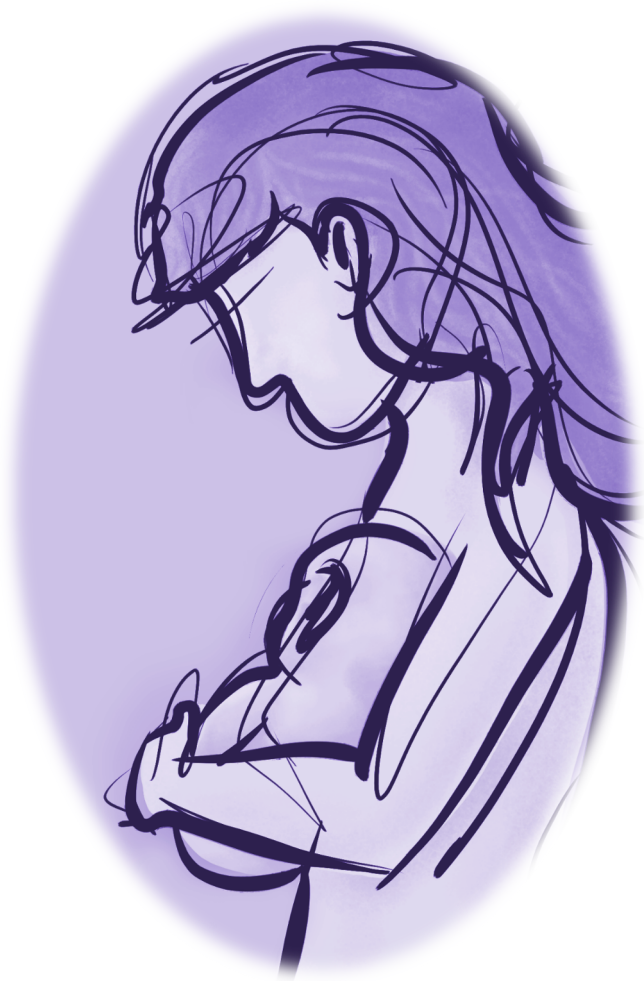
Genus	High (%)	Low (%)	W	p-value
<i>Actinomyces</i> spp.	0,062	0,0226	1470	*
<i>ATop obium</i> spp.	0,0023	0,0012	1325	0,36
<i>Bacteroides</i> spp.	1,5324	10,5543	759	***
<i>Bifidobacterium</i> spp.	61,7243	17,0834	2375	***
<i>Bilophila</i> spp.	0,0006	0,0204	1109	0,105
<i>Blautia</i> spp.	0,1084	1,0702	1095	0,232
<i>Burkholderia</i> spp.	0,0028	0,0015	1285	0,619
<i>Citrobacter</i> spp.	0,0076	0,0315	1101	0,116
<i>Clostridium_IV</i> spp.	0,049	0,1018	1266	0,855
<i>Clostridium_sensu_stricto</i>	1,1958	6,744	1288	0,683
<i>Clostridium_XI</i> spp.	0,0874	0,0727	1198	0,719
<i>Clostridium_XIVa</i> spp.	0,0074	0,6133	1053	0,056
<i>Clostridium_XVIII</i> spp.	1,0294	3,0869	737	***
<i>Collinsella</i> spp.	2,1647	1,3189	1263,5	0,908
<i>Corynebacterium</i> spp.	0,0047	0,0036	1354	0,375
<i>Dorea</i> spp.	0,0006	0,0083	1217	0,621
<i>Eggerthella</i> spp.	0,1999	0,1655	1197	0,704
<i>Enterobacter</i> spp.	0,1082	0,7765	1015	0,089
<i>Enterococcus</i> spp.	1,8789	3,0878	1383	0,352
<i>Erysipelotrichaceae</i> spp.	0,5482	0,2124	1276	0,786
<i>Escherichia/Shigella</i> spp.	6,7657	14,5084	966	0,054
<i>Finegoldia</i> spp.	0,0603	0,5	1532	*
<i>Flavonifractor</i> spp.	0,0037	0,0309	1117	0,126
<i>Gemella</i> spp.	0,029	0,3253	1358	0,441
<i>Haemophilus</i> spp.	0,1016	0,2492	1098	0,251
<i>Klebsiella</i> spp.	2,9703	6,7493	931	*
<i>Lachnospira</i> spp.	1,6533	5,479	1277	0,835
<i>Lactobacillus</i> spp.	1,359	1,2754	1458	0,13
<i>Negativicoccus</i> spp.	0,0126	0,0543	1174	0,421
<i>Parabacteroides</i> spp.	0,0929	1,0055	991,5	*
<i>Peptoniphilus</i> spp.	0,0167	0,0053	1210	0,693
<i>Phascolarctobacterium</i> spp.	0,0066	0,1004	1091	0,057
<i>Cutibacterium</i> spp.	0,0123	0,0016	1226,5	0,799
<i>Pseudomonas</i> spp.	0,0005	0,0135	1320	0,288
<i>Rothia</i> spp.	0,3879	0,7414	1428	0,208
<i>Staphylococcus</i> spp.	1,7968	3,2151	1109	0,345
<i>Streptococcus</i> spp.	9,9055	15,1917	1126	0,408
<i>Veillonella</i> spp.	3,5594	4,5997	1081	0,256

Mann-Whitney U test values and adjusted p values for the differential log-transformed abundances are also shown. Asterisks (*) denotes the significant covariates at each time point: * p < .05, ** p < .01, *** p < .001.

Supplementary Table II-7. Antibiotic resistance genes presence (%) and load (log n° copies/μL) in the study population.

		7-days-old infants (N=71)			1-month-old infants (N=66)		
		High (N=44)	Low (N=27)	p value	High (N=26)	Low (N=40)	p value
<i>tetM</i>							
	Presence (%)	83.36	70.37	0.181	76.92	85.00	0.3613
	Median (IQR)	5.530 (1.763)	5.988 (1.947)	0.392	4.730 (1.505)	4.920 (2.158)	0.964
<i>tetO</i>							
	Presence (%)	56.82	66.67	0.565	34.62	52.50	0.4
	Median (IQR)	3.825 (2.028)	2.571 (2.552)	0.175	3.772 (1.240)	3.648 (3.086)	0.4399
<i>tetW</i>							
	Presence (%)	70.45	59.26	0.477	73.08	42.50	0.02*
	Median (IQR)	3.926 (2.425)	4.022 (1.711)	0.486	3.447 (2.411)	3.946 (1.555)	0.645
<i>blaTEM</i>							
	Presence (%)	54.55	33.33	0.135	26.92	35.00	0.676
	Median (IQR)	4.333 (3.600)	4.019 (1.919)	0.824	3.706 (2.241)	4.399 (2.772)	0.794
<i>blaSHV</i>							
	Presence (%)	40.91	40.74	1	7.57	21.24	0.133
	Median (IQR)	1.423 (1.201)	1.212 (1.055)	0.981	2.812 (0.307)	3.106 (1.808)	0.1977
<i>ermB</i>							
	Presence (%)	59.09	51.85	0.725	42.31	57.50	0.339
	Median (IQR)	2.824 (0.973)	2.904 (1.543)	0.853	2.631 (1.323)	2.839 (0.910)	0.7966

Presence of ARGs is expressed as percentage (%) of individuals Pearson's-Chi-square test were used for calculating statistical significance. Load of ARGs by log copies/μL of DNA of each ARG is expressed as median (IQR) and only positive cases are included. Kruskal Wallis test was used for calculating statistical significance. Asterisks (*) indicate a significant difference (p<.05) between two groups.



CHAPTER III

CHAPTER III

Breastfeeding and Early *Bifidobacterium*-driven Microbial Colonization Shape the Early Infant Gut Resistome

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My contribution to this research article was:

Conceptualization. Perform the experiments: DNA extraction, quantification, and purification. Data analysis and curation, statistical analysis and interpretation. Writing-original draft preparation.

1. Abstract

The assembly of the gut microbiome in early life is key to infant health, and specific perinatal factors such as cesarean section (C-section), antibiotic exposure and lack of breastfeeding practice are detrimental to proper microbial development and increase the antimicrobial resistance genes (ARGs). Using 265 gut longitudinal metagenomes from 66 mother-infant pairs, we investigated how perinatal factors influence the acquisition and dynamics of ARGs during the first year of life. Our findings reveal that *Bifidobacterium* plays a crucial role in modulating the infant resistome, with its high relative abundance being associated with a lower ARG load. Exclusive breastfeeding during the first month of life accelerates the reduction of ARGs and ensures a lower resistome burden at six months. Moreover, early breastfeeding cessation correlates with a higher ARG load, underscoring its long-term influence on microbial resilience. Importantly, we identify exclusive breastfeeding as a key strategy to mitigate the impact of C-section delivery on the infant gut resistome, counteracting the early-life antibiotic exposure associated with this procedure and the resulting resistance acquisition. By promoting a microbiome enriched in *Bifidobacterium*, breastfeeding may help suppress ARG-carrying taxa, reducing the risk of resistance dissemination. Our findings underscore the importance of breastfeeding as a natural intervention to shape the infant microbiome and resistome. Supporting breastfeeding through public health policies could help limit the spread of antimicrobial resistance in early life.

2. Introduction

The establishment of the infant gut microbiota begins immediately after birth, closely linked with the development of the host's immune system, metabolism, and intestinal homeostasis. The process of colonization occurs from multiple origins¹ and varies according to the infant's age². Previous studies have reported that the infant gut microbiota development during the first year of life is critical for later risk of diseases and host health³. At birth, prenatal conditions, host genetics and mode of delivery influence the development of the gut microbiome^{4,5}. Later, antibiotic treatment, mode of feeding, the start of weaning, geographical location and other environmental exposures, will be associated with the maturation of the gut microbiota^{6,7}.

In parallel with the development of the early-life microbiota, the infant gut assembles its own resistome, which encompasses all the antibiotic resistance genes (ARGs) within a microbial community⁸. Thus, the development of the infant resistome is linked to the dynamics of the microbial composition, contributing to the long-term dynamics and stabilization of the resistome into adulthood. In the early stages of life, antibiotic acquired resistance can result from the vertical transmission from the mother at birth or during lactation, and/or maternal, intra-partum, and infant antibiotic treatment⁹. Moreover, mobile genetic elements (MGEs), such as most plasmids, can transfer ARGs between members of the gut microbial community in a process known as horizontal gene transfer. The same factors that influence the gut microbiome composition can drive the spread and selection of resistance genes within the developing gut microbiota¹⁰. The types of ARGs that dominate in infancy typically confer resistance to common antibiotics such as beta-lactams, tetracyclines, macrolides, aminoglycosides, and quinolones¹¹, as well as to drugs which infants are not exposed¹². Previously studies have indicated that infants have a higher relative abundance of these genes compared to older children^{13–15}.

As microbes evolve resistance mechanisms, previously effective treatments become obsolete, increasing clinical complications, mortality, and healthcare costs¹⁶. The problem is especially concerning during infancy, a time when early exposure to antibiotic resistance can have lasting health effects. With approximately 214,000 neonatal deaths annually linked to sepsis from ARG-carrying pathogens¹⁷, the importance of understanding how the resistome develops with age is clear. Yet, how various factors impact the resistome's development during this crucial period of time is less explored.

Our research aims to delineate the assembly of the infant gut resistome during the first year of life and to identify the factors that shape its dynamics through the analysis of metagenomic data from infants and their mothers. By mapping out the resistome landscape within the infant gut, and the dynamics within their microbial hosts, our work seeks to provide future strategies to ameliorate the spread of antimicrobial resistance in early life.

3. Materials and methods

3.1. Study design and volunteers

A total of 66 of healthy, full-term mother-infant pairs from the MAMI cohort¹⁸ were selected for inclusion based on sample availability and completeness of clinical and anthropometric records. Maternal-infant data including gestational age, neonate gender, birth weight, place and mode of delivery, type of lactation, and antibiotic exposure of the infant were collected. Infant length and weight were registered at birth, 1, 6, and 12 months and the data were used to calculate BMI Z-scores. These anthropometric measures that are standardised by age and sex were electronically computed using WHO Anthro software (<https://www.who.int/childgrowth/software/en/>).

Among the longitudinal sampling performed in the MAMI cohort, infant fecal samples from 7 days, 1, 6 and 12 months were included in this analysis (N=265). One fecal sample was collected 1-month postpartum from each mother.

Ethical approval for the study was obtained from the Ethics Committee for Clinical Research of the healthcare centres involved in the recruitment including the Hospital La Fe, Hospital Clínico Universitario, Parc de Salut MAR, and CSIC (Consejo Superior de Investigaciones Científicas) [ClinicalTrials.gov NCT03552939]. The clinical characteristics of the study participants can be found in **Supplementary Table III-1**.

3.2. Biosamples, DNA extraction and sequencing

A total of 265 mother-infant faecal samples were collected at home by their parents who were previously trained by clinical personnel in the health care centres where they were enrolled. Briefly, faecal samples were deposited in provided sterile containers and immediately kept at -20°C, before the final storage at -80°C until further analysis. DNA was isolated from the faecal samples (50-100 mg) using the Master-Pure DNA extraction kit (Epicentre, Madison, WI, USA) following the recommended protocol with some additional steps as described in Selma-Royo *et al*¹⁹. Briefly, a pre-treatment with lysozyme and mutanolysin was included as well as a cell disruption step with 3-µm diameter glass beads in a FastPrep 24-5G Homogenizer (MP Biomedicals). After extraction, DNA Purification Kit (Macherey-Nagel, Duren, Germany) was used for DNA purification.

Shotgun sequencing libraries were constructed using the NexteraXT DNA Library Preparation Kit (Illumina) and sequenced on the Illumina HiSeq2500 platform with a target depth of 5Gb/sample (**Supplementary Table III-2**).

3.3. Bioinformatic analysis of the metagenomic sequencing data and resistome and mobilome investigation

The quality of the raw sequencing data was firstly evaluated by using FastQC v0.11.9 (<https://www.bioinformatics.babraham.ac.uk/projects/fastqc/>) and MultiQC v1.11²⁰ and further trimmed by using FastP v0.23.2²¹, excluding reads with a length shorter than 75 bp and a mean Phred score quality below 25. Residual adapters and barcodes were also excluded by using FastP. Residual PhiX and human reads were discarded by using Bowtie2 v2.4.2²². The efficiency of the quality

control (QC) over the dataset was again evaluated with FastQC and MultiQC, and the reads overcoming the process were used for downstream analysis by following two main approaches: assembly-free and assembly-based.

In the assembly-free approach, the QC reads were subjected to MetaPhlAn 4²³ in order to perform a microbial profiling of the different samples by using the CHOCOPhlan_202403 database. The resistome profiling per sample was assessed by aligning the QC reads against the ResFinder database²⁴ (downloaded on 18th June 2024) as follows: i) the ARGs contained in the ResFinder database were clustered at 90% identity and coverage by using CD-HIT²⁵ in order to prevent spurious alignments of the short sequencing reads to conserved regions from multiple closely-related ARGs; ii) alignment of the QC reads against the clustered ResFinder database by using Bowtie2 v2.4.2 and enabling the “--end-to-end” and “--very-sensitive” flags; iii) alignments were polished with featureCounts²⁶ considering only as positive hits those reads that aligned just once against the clustered ResFinder database iv) the obtained counts matrix was processed to calculate the counts per million reads (CPMs) adding a “bacterial marker” modification according to the formula:

$$CPM = \frac{(ARG \times \text{Ratio of bacterial assigned reads})}{\text{Total number of reads}} \times 10^6$$

where “CPM” is the total counts per million reads value for each ARG and “Ratio of bacterial assigned reads” represent the ratio of reads per sample to be considered to belong to the kingdom “Bacteria” according to MetaPhlAn 4, in order to normalise the dataset to consider only those reads derived from bacteria.

In the assembly-based approach, the QCed metagenomes were assembled by using SPAdes v3.15.5²⁷ by enabling the --meta option, 96 threads and 1024 GB RAM per assembly using the k-mer lengths 21, 33, 55, 77, 99 and 121. The resulting FASTA files containing the metagenomic contigs were imported into TORMES v1.3.1²⁸ with ²⁸me modifications to automate the downstream bioinformatic analysis as follows: first, contigs shorter than 500bp were excluded, and quality control of the assemblies was performed by using QUAST v5.0.2²⁹ and aligning the QC reads of each sample against its own assembly by using Bowtie2, in

order to assess the proportion of reads that were finally included in the assembly. The taxonomy of each contig was assessed by using kraken2 v2.0.8³⁰ the --use-names option and the PlusPFP database (downloaded on 12th January 2024). Taxonomic nomenclature was refined by using Taxonkit v0.16.0³¹ (taxdump NCBI files downloaded on 6th June 2024). Bacterial and archaeal coding sequences (CDS) were predicted and amino acid sequences were translated using Prodigal v2.6.3³² and by enabling the --only_gene_prediction flag in TORMES. Contigs with less than 3 CDS were excluded for downstream analysis. Resistome profiling was performed by aligning the assemblies against the ResFinder database (downloaded on 18th June 2024) by using BLASTN v2.14.1+³³. Alignments below 80% sequence similarity and coverage were excluded. Plasmid occurrence in the metagenome was evaluated by using geNomad v1.8.1³⁴ and by aligning the assemblies against the PlasmidFinder database³⁵ (downloaded on 27th March 2024) by using BLASTN and excluding alignments below 95% sequence similarity and coverage ARGs occurring in contigs that were classified as “plasmid” with geNomad and/or that harbored a plasmidic replicon according to PlasmidFinder were considered to be plasmidic.

3.4. Statistical analyses and data visualization

All statistical analyses were performed under R environment v. 3.6.0, and visualization was performed by using *ggplot2*³⁶. Normality of the data was evaluated with Shapiro – Wilk tests. Categorical variables are expressed as positive cases-prevalence and (percentage, %). Normally distributed variables are presented as mean $\pm$ standard deviation (SD), while non-normal distributed variables are presented as as median and interquartile range [IQR]. Pearson’s-Chi-square test was used for categorical variables, and Mann–Whitney U test or Fisher’s exact test were used for continuous variables, as appropriate, for calculating statistical significance. All p-values calculated in the manuscript were adjusted using the BH method.

We used the *vegan* package³⁷ diversity analyses (α - and β -diversity). The Shannon diversity of ARGs or bacterial species was used as a measure of within-individual diversity (α -diversity). The β -diversity was conducted using the function

‘vegdist’ of the *vegan* package with Principal Coordinate Analysis (PCoA), and Bray–Curtis dissimilarity index was used for microbiota and Jaccard for resistome data. Permutational multivariate analysis of variance (PERMANOVA) was performed using the function ‘adonis’ of the *vegan* package with 9999 permutations to determine the statistical significance of composition differences. Differentially abundant features between time points were identified using the *DESeq2*³⁸ with the p-values attained by the Wald test were corrected for multiple testing using the Benjamini Hochberg method.

Differences in the abundance of a given ARG or taxa were compared using the Wilcoxon test (for two groups) or Kruskal-Wallis test (for three or more groups). Pairwise comparisons were carried out by pairwise Wilcoxon tests. Differences in the relative abundance of taxa between two clusters were evaluated using the Mann Whitney U test (Wilcoxon rank-sum test for 2 levels). In all of these tests, the p-value was adjusted for multiple comparisons by the FDR/Bonferroni correction.

The main separation of the microbiota in groups or clusters at each time point was performed with an unsupervised non-linear algorithm of k-means partitioning, and confirmed with the k-medoids clustering method Partitioning Around Medoids (PAM). Both methods require specifying the number of clusters *k* to be generated and we used the elbow and average silhouette method from *factoextra* R package³⁹ to estimate the optimal *k* value. Next, clustering of the microbial data was performed with the *stats* R core package.

To further explore patterns in community composition and identify potential associations with environmental factors, we employed an unconstrained ordination approach. Unconstrained ordination is particularly valuable in this context as it allows for the identification of intrinsic patterns in ARG distributions without being restricted to pre-specified explanatory variables. Subsequently, we used the *envfit* function from *vegan* R package to fit environmental variables onto the unconstrained ordination space.

MaAsLin⁴⁰ was used to build general linear models to efficiently determine the multivariate association between relevant factors (high R² in *envfit* analyses) and resistome while accounting for potentially confounding covariates. In this case, the analysis with MaAsLin2 was a longitudinal analysis, so the first added variable was time (7 days, 1, 6 and 12 months, considering 7 days as reference). The following variables were added depending on the strength of each variable seen in the other analyses and reported before, so we added the *Bifidobacterium* abundance based k-means clustering (High vs Low), the mode of delivery (vaginal vs C-section), exclusive breastfeeding (yes vs. no), antibiotic consumption (yes vs. no), and the presence of domestic pets in the household (yes vs. no). No more variable groups or other variables were added. The command runs a multivariable regression model to test for the association between ARGs abundance (or groups of resistances) versus the above-mentioned variables as fixed effects. Finally, the input data were transformed to logarithmic format.

To assess the statistical impact of clinical variables on infant resistome, a generalised linear model (GLM) was estimated using the *stats* R core package. The best model was selected using the Akaike Information Criterion (AIC), following a forward selection approach and choosing the one with the lowest AIC value. Additionally, collinearity among explanatory variables was reviewed using the Variance Inflation Factor (VIF) with *car* R package⁴¹. The influence of fixed effects was calculated via Nagelkerke's R^2 . The final model was as follows, and the response *YResistance* represented the observed value of CPMs of total ARGs:

$$YResistance = \beta_1(Infant\ age) + \beta_2(Bifidobacterium\ level)$$

For the prediction of the variables infant age, lactation type and *Bifidobacterium* cluster from the abundance in CPMs and the different variables present, the Random Forest (RF) machine learning algorithm was applied using *randomforest* R⁴² package. We split the infant-based data in a 70:30 ratio (training set:test set). The number of predictors to be used in the RF model was determined by 10-fold cross-validation over 100 iterations using *caret* R package⁴³. The number of trees, variables for each split (mtry) and the total number of variables (node size)

to grow the forest were set at 500, 18 and 18, respectively for infant age, 500, 8 and 8 for *Bifidobacterium* cluster and 500, 7 and 7 for lactation type. In addition, to evaluate models in detail according to their performance by comparing model predictions with the actual values of the test set *caret* R package was used to obtain the confusion matrices, and to see in more detail the relationship between the sensitivity and specificity of the RF models obtained, a pROC curve study was performed on the categorical variables of *Bifidobacterium* cluster (high, low) and lactation type (maternal, artificial) using *pROC* R package⁴⁴.

4. Results

4.1. Dynamics of the gut resistome during the first year of life

Overall, 265 samples were subjected to metagenomic sequencing and further resistome screening by using assembly-free and assembly-based approaches (**Supplementary Table III-2**). The assembly-free approach allowed us to quantify (based on CPM) the occurrence and abundance of ARG in the different samples. ARGs were found in all samples collected. In total, 208 different ARGs were identified in infants and 134 in the mothers, and 122 ARGs (35.67% of the total ARGs) were shared among them. Infants shared more ARGs with their own mothers than with unrelated mothers at 1 month postpartum (**Supplementary Figure III-1**). Mother-infant dyads shared ARGs conferring resistance to lincosamides, macrolides, streptogramin B, tetracycline, aminoglycosides, beta-lactams and amphenicol (**Figure III-1A**), but unique ARGs were found in mothers and infants. The proportion of shared ARGs is lower than the proportion of unique ARG in mothers and infants (**Figure III-1A**). Tetracycline ARGs were the most abundant in infant fecal samples, with *tet(S/M)* being the most abundant and prevalent (93.4% of samples), followed by *tetB*, *tetA*, *tetW* and *tetO* (over 75% of samples). Macrolide ARGs were the second most abundant overall, with *msrD* and *mefA* present in more than 95% of the samples. *ermB*, categorized as a Macrolide-lincosamide-streptogramin B (MLS_B) resistance gene, was prevalent in 80.6% of the infant samples, followed by *ermX* and *ermT* (over 60% of the infant samples). The

aminoglycoside resistance gene *aph(3')-IIIa* was found in 61.7% of the infant fecal samples, and *blaTEM* was identified as the most abundant beta-lactam resistance gene (55.1% of the samples) (**Supplementary Table III-3**).

The total load of ARGs in the infant gut decreased significantly during the first year of life. The amount of ARG in 7-days-old infants was significantly higher than later time points and also compared to the mothers' load ($p < 0.01$). However, infants at 6 and 12 months of age appeared to have lower ARG load than mothers ($p < 0.025$) (**Figure III-1B**). The resistome diversity increases over time and tends to resemble their mothers ($p < 0.01$) (**Figure III-1C**). The infant resistome composition also changed significantly over time (for all timepoints $p < 0.05$) (**Figure III-1D**) towards resembling the mothers' resistome (**Figure III-1E**). Antibiotic resistances differing significantly with child age were calculated using the DESeq 2 approach (**Supplementary Figure III-2**). Briefly, ARGs conferring resistance to aminoglycosides started to increase from 1 month of age, while beta-lactam, macrolide and MLSB ARGs decreased through time, Tetracycline resistance genes increased during the first month of life, but then diminished towards 12 months of life.

We subsequently utilized a RF model to identify time-specific indicators in the resistome (**Supplementary Figure III-3**). This decision was based on the GLM analysis, which revealed a negative relationship between time and ARG load ($p < 0.001$), indicating that as the infant's age increased, the ARG load decreased. The developing gut resistome was linearly related to infant age. RF, with a ten-fold cross-validation analysis ($R^2 = 65.24\%$), established that ten ARGs and five variables (type of diet, exclusive breastfeeding, type of breastfeeding, duration of exclusive breastfeeding and duration of lactation in days) are the most predictive of age. A tetracycline resistance gene, *tet(40)* (present in 34.5% of the samples) was ranked as the most important variable within the ARGs differentiating infant age, followed by other genes of the same antibiotic type *tet(32)*, *tet(W)*, *tet(O)*, *tet(A)* and

tet(S), as well as the *blaZ*, *ant(6)*, *lsa(A)*, and *erm(B)* genes, which confer resistance to beta-lactamases, aminoglycosides, lincosamides and macrolides, respectively.

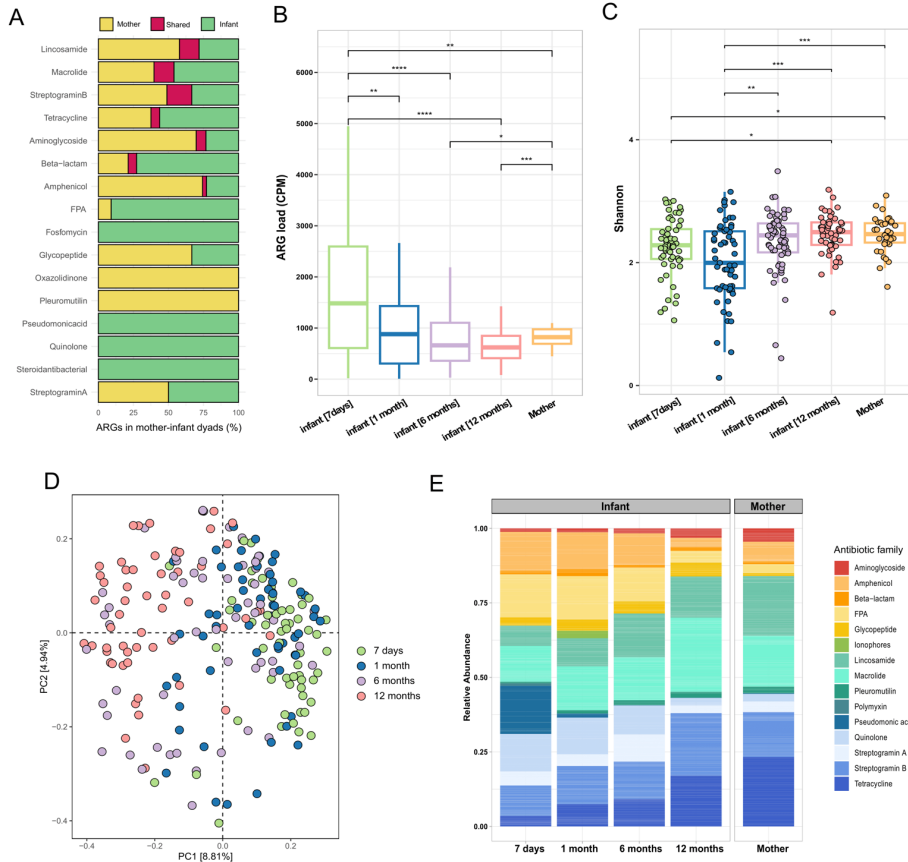


Figure III-1. Mother-infant's gut resistome during the first year of life. (A) Abundance of assembled ARGs in mother-infant pairs at 1 month postpartum (N=39 complete dyads), classified as shared, unique in mothers and unique in infants, and grouped by antibiotic class. **(B)** Antibiotic resistance load in the gut of infants during the first year of life and their mother's, expressed as total CPM of ARGs per infant in each timepoint. ARG load was the summed abundance of all ARGs in a given sample. Significance values for ARG load were calculated using the pairwise rank-sum Wilcoxon test (Mann-Whitney U), two-sided. **(C)** Alpha diversity of the mother-infant gut resistomes based on two-sided Kruskal-Wallis test on Shannon index. **(D)** Composition of the infant resistome through time colored by infant's age. Two-sided PERMANOVA on Jaccard distances was applied to assess differences between timepoints ($p < 0.05$ for all comparisons). **(E)** Composition of the resistome at antibiotic class level of mother-infant pairs. ARGs have been grouped according to the antibiotic(s) they confer resistance. All samples represent independent biological replicates, derived from

individual participants. Sample sizes per group are as follows: day 7 infants (N=58), 1-month-old infants (N=58), 6-month-old infants (N=56), 12-month-old infants (N=55), and mothers (N=39). Box plots show the median (center line), the first and third quartiles (bounds of box). Whiskers are chosen to show the 1.5 of the IQR. Asterisks indicate statistical significance: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$. FPA: Folate Pathway Antagonist.

4.2. Influence of the microbiome on the resistome acquisition

The investigation of the microbial composition by using assembly-free approaches showed that the microbiome diversity of the infants increased significantly over time even though it remained lower than the mothers' ($p < 0.05$) (Figure III-2A), which was in concordance with the results observed for the resistome. The microbial composition in infants of 7 days of age differed significantly to older ages ($p = 0.05$) (Figure III-2B and Supplementary Figure III-4).

Moreover, an assembly-based approach was also conducted in order to infer the microbial taxa associated with the ARGs identified. Overall, Bacillota was the main phyla carrying ARG, and *Staphylococcus*, *Streptococcus*, *Enterococcus* and *Klebsiella* were the main ARG-carrying genera, particularly at 7 days of life. *Staphylococcus aureus*, *S. epidermidis*, and *K. pneumoniae* abundances decreased during the first year of life, while *Streptococcus salivarius*, *S. parasanguinis*, *S. lactarius*, *E. faecium* and *E. fecalis* maintained constant over time (Figure III-2C). *Escherichia coli*, *Clostridium perfringens* and *Bacteroides fragilis* were some of the species with the greater impact over the infants' resistome and appeared to remain stable during the first year of life. The species of *Bifidobacterium* that carry resistances increased over time, being *B. longum* and *B. breve* the most representative (Figure III-2C).

Tetracyclines and macrolides ARGs were harbored by many different genera, but *Streptococcus* were the main hosts of ARGs conferring resistance to those antibiotics (accounting for 28% and 42% of all tetracycline and macrolide ARGs, respectively). Similarly, aminoglycoside ARGs were mainly carried by *Staphylococcus* (23.39% of the total number of aminoglycosides ARGs), *Escherichia* (35.5%) and *Enterococcus* spp. (19.02%) (Figure III-2D and Supplementary Table III-4). Many of these genes were also associated with

plasmids, as *aph(3'')*-*lb* and *blaTEM* were mainly associated with plasmids of *Escherichia* spp., and *blaZ* was mainly associated with plasmids in *Staphylococcus* spp (Figure III-2E). Compared to the other genera, *Klebsiella* is the one responsible for carrying resistances against folate pathway antagonist (FPA, 66.66%), amphenicol (75.32%) and quinolone (95.08) antibiotics, and copies of *fosA* and *oxqA* genes in *Klebsiella* were identified in plasmids (Figure III-2E). Moreover, fosfomycin resistance was only associated with *Klebsiella* and *Staphylococcus*, and these two genera were also the main carriers of beta-lactam resistances (24.42% and 35.48%, respectively) (Figure III-2D and Supplementary Table III-4). Macrolide ARGs were mainly carried by *Streptococcus*, being *mefA* the most representative non-mobile gene, but also by *Staphylococcus*, *Enterococcus* and some *Bifidobacterium* (Figure III-2D, E). In addition, *Bifidobacterium*, *Enterococcus* and *Bacteroides* species were the main responsible for carrying tetracycline, macrolide, streptogramin B and lincosamide related resistances (Figure III-2D and Supplementary Table III-4).

► **Figure III-2. Infant's gut microbiome during the first year of life and bacterial hosts of resistances.** (A) Alpha diversity of the mother-infant gut resistomes based on Kruskal-Wallis test on Shannon index. Box plots show the median (centre line), the first and third quartiles (bounds of box). Whiskers are chosen to show the 1.5 of the IQR. All samples represent independent biological replicates, derived from individual participants. Sample sizes per group are as follows: day 7 infants (N=58), 1-month-old infants (N=58), 6-month-old infants (N=56), 12-month-old infants (N=55), and mothers (N=39). Asterisks indicate statistical significance: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$. (B) Microbial composition at species level of infants gut over the first year of life. Samples are colored according to infants' age. Two-sided PERMANOVA on Bray-Curtis distances was applied to assess differences between timepoints. $p=0.05$ for 7 days compared to older timepoints. (C) Microbial hosts of resistance during the first year of life. ARG-carrying contigs were detected within the bins. (D) Antibiotic resistances and their microbial hosts during the first year of life. (E) Relative abundance (%) of genus carrying ARGs and number of copies of these genes associated with a plasmid. FPA: Folate Pathway Antagonist; MLSB: Macrolide-Lincosamide-StreptograminB.

4.3. High *Bifidobacterium* abundance is associated with a lower resistome load and diversity during the first year of life

The microbial composition of the infants' gut throughout time was clustered into two distinct groups according to hierarchical clusterization based on k-means (**Supplementary Figure III-4**) and further confirmed with PAM clustering (**Supplementary Figure III-5**). The strength of association between the PAM and the k-means clustering was 0.77 (Cramer's V correlation). The overall microbiome composition was statistically different between the two groups during the first year of life ($p < 0.001$) (**Supplementary Figure III-6A**). Strikingly, *Bifidobacterium* abundance appeared to differ greatly between both groups in all the investigated timepoints, an observation that was further confirmed statistically ($p < 0.0001$) (**Figure III-3A**). Moreover, the microbial diversity was statistically higher in the group of infants with a lower abundance of *Bifidobacterium* ($p < 0.05$ in all timepoints) (**Figure III-3A**). Hence, we named the groups according to the relative abundance of *Bifidobacterium* as "High" ($> 70\%$) or "Low" ($< 30\%$), and further investigated the role of species belonging to this genus in shaping the infants' gut resistome. During the first year of life, 42.25% of infants remained in the same *Bifidobacterium* abundance-based group. Stability within the groups was 65.96% from 7 days to 1 month, 67.39% from 1 to 6 months, and 58.33% from 6 months to 1 year (**Figure III-3B**).

Among the species of *Bifidobacterium*, *B. longum* and *B. bifidum* were statistically more abundant in High-*Bifidobacterium* infants during the entire first year of life ($p < 0.05$) (**Figure III-3C**). Similarly, *B. breve* was also more abundant in High-*Bifidobacterium* infants at 7 days, 1 month, and 1 year of age ($p < 0.01$). Indeed, the presence of *B. breve* in Low-*Bifidobacterium* infants seems to first appear at 6 months of age. On the other hand, Low-*Bifidobacterium* infants seem to be highly colonized by other genera, including *E. coli* during the first year of life ($p < 0.05$ in all timepoints), *Clostridium perfringens* and *Klebsiella pneumoniae* during the first 6 months of life, *S. aureus* during the first month of life and including *E. fecalis* at 7 days of age (**Supplementary Figure III-3E**).

Based on the premise that vaginal deliveries and exclusive breastfeeding have been associated with *Bifidobacterium* spp. dominance in the infant gut⁴⁵, we assessed the potential influence of these two factors to drive the clusterization of the samples in the High- or Low-*Bifidobacterium* groups. Our analysis confirmed that the groups of samples based on *Bifidobacterium* abundance were still significantly different and independently to the mode of delivery and lactation ($p < 0.05$ in all timepoints) (**Supplementary Figure III-6B and C**).

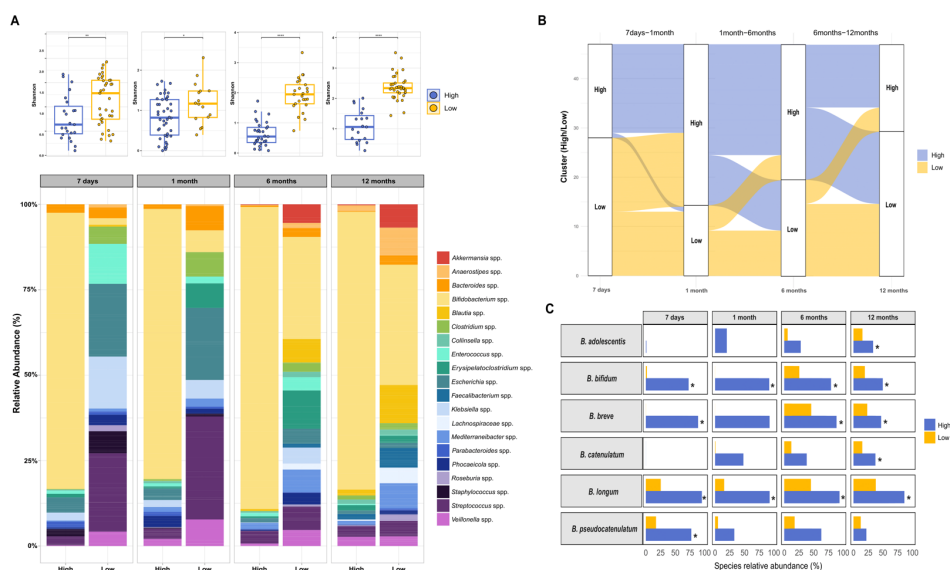


Figure III-3. *Bifidobacterium* abundance drives microbial composition. (A) Genus relative abundance over time depending on the *Bifidobacterium* abundance-based clusters. Two distinct clusters (optimal number clusters $k = 2$) were identified via k-means clustering. Mann Whitney U test revealed that statistically significant differences in relative abundance were found in *Bifidobacterium* genus, in all timepoints, so clusters were named as High- and Low-*Bifidobacterium*. Upper boxplots represent alpha diversity of the mother-infant gut resistomes based on two-sided Kruskal-Wallis test on Shannon index. Box plots show the median (centre line), the first and third quartiles (bounds of box). Whiskers are chosen to show the 1.5 of the IQR. Sample sizes per group and timepoint were as follows: day 7: High-*Bifidobacterium* $N = 23$, Low-*Bifidobacterium* $N = 35$; 1 month: High $N = 42$, Low $N = 16$; 6 months: High $N = 31$, Low $N = 25$; 12 months: High $N = 19$, Low $N = 36$. Asterisks denote statistical significance: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$. **(B)** Stability of the hierarchical clustering based on *Bifidobacterium* spp. abundance over the first year of life. **(C)** Relative abundance of *Bifidobacterium* species in each cluster during the first year of life. Only species with statistically different abundances in any timepoint are shown (Mann Whitney U test, * $p < 0.05$).

Considering the effect on the microbiome, we assessed if *Bifidobacterium* abundance could have an association with the resistome of the infants. Low-*Bifidobacterium* infants have a significantly higher ARG load than High-*Bifidobacterium* ones (for all timepoints $p < 0.001$) (**Figure III-4A**), and the composition of the resistome is also significantly different (for all timepoints $p = 0.001$). Overall, most of the classes of antibiotic resistances are more abundant in Low-*Bifidobacterium* infants than High-*Bifidobacterium* infants during the first month of life (**Figure III-4B**). Moreover, the mobilome was also influenced by the *Bifidobacterium* abundance in the infants' gut. Low-*Bifidobacterium* infants had more copies of ARGs associated to plasmids than High-*Bifidobacterium* infants, including genes conferring resistance to tetracycline, beta-lactam, amphenicol, and MLSB resistances (**Figure III-4C**).

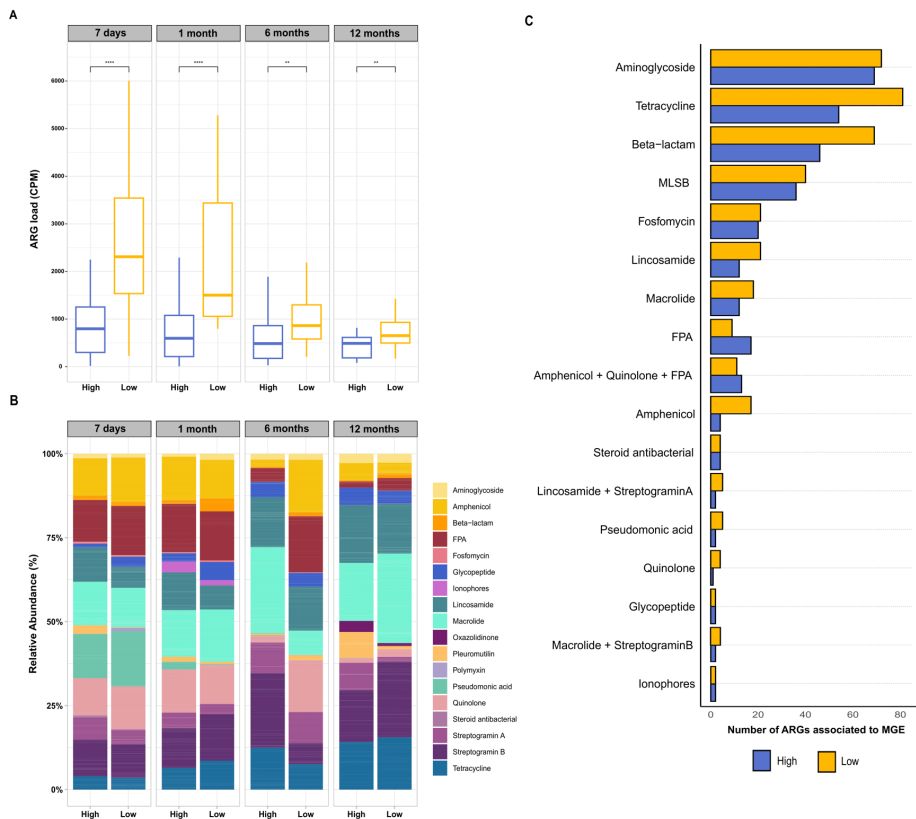


Figure III-4. *Bifidobacterium* abundance drives resistance load and composition. (A) Antibiotic resistance load in the gut of infants during the first year of life depending on their *Bifidobacterium* abundance (cluster High or Low), expressed as total CPM of ARGs per infant in each timepoint. ARG load was the summed abundance of all ARGs in a given sample.

Significance values for ARG load were calculated using the pairwise rank-sum Wilcoxon test (Mann Whitney U), two-sided. Box plots show the median (center line), the first and third quartiles (bounds of box). Whiskers are chosen to show the 1.5 of the IQR. All samples represent independent biological replicates, derived from individual participants. Sample sizes per group and timepoint were as follows: day 7: High-*Bifidobacterium* N = 23, Low-*Bifidobacterium* N = 35; 1 month: High N = 42, Low N = 16; 6 months: High N = 31, Low N = 25; 12 months: High N = 19, Low N = 36. Asterisks denote statistical significance: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$. **(B)** Antibiotic resistance profiles in each group. ARGs have been grouped according to the resistance they confer. **(C)** Number of copies of ARGs associated with a plasmid in High- and Low-*Bifidobacterium* grouped infants. FPA: Folate Pathway Antagonist; MLSB: Macrolide-Lincosamide-StreptograminB. Source data are provided as a Source Data file.

Another RF model was performed to identify those *Bifidobacterium* cluster indicators in the resistome (**Supplementary Figure III-8**). The GLM analysis showed a significant negative association between high *Bifidobacterium* cluster infants and ARG load, compared to the low cluster group ($p < 0.001$). RF, classified infants into high and low *Bifidobacterium* clusters with an accuracy of 82.35%, while the pROC curve showed an AUC value of 0.90 using only resistance genes as predictive variables. The macrolides resistance gene *erm(B)* and the tetracycline resistance gene *tet(S)*, which were also present in the infant-age model, were ranked as the most important variables. Other genes present a high importance in the model: *tet(32)*, *erm(X)*, *tet(B)*, *OqxB* or *mef(A)*.

4.4. Association of the resistome with perinatal factors

Our next goal was to assess the ways in which the distribution of ARGs in the infant gut was shaped by various environmental exposures. At 7 days of age, mode of delivery and *Bifidobacterium* abundance-based clustering (referred as “kmeans_bifis”) are key factors driving the antibiotic resistance composition, although their association is not significant. At 1 month of age, exclusive breastfeeding significantly influenced infants’ resistome ($R^2=0.119$, $p=0.001$), and the type of diet (exclusive breastfeeding, mixed or formula feeding) appeared to also have a slight influence ($R^2=0.143$, $p=0.08$). “kmeans_bifis” is the following factor with high influence. At 6 months of age, the consumption of antibiotics has a higher impact on infant’s resistome, but is not statistically significant, and exclusive

breastfeeding loses its importance as most of the infant's start weaning. Finally, the presence of domestic pets in the same household of 1-year-old infants seemed to have a great impact on their resistome as well ($p=0.05$) (**Figure III-5A**).

Further analysis of the effect of these factors on the resistome diversity, composition and abundance were done. As reported above, *Bifidobacterium* abundance influences resistome load, diversity and composition during the first year of life. Regarding mode of delivery, C-section born infants had higher ARG load at 7-days of age than those born vaginally (**Figure III-5B**). The mode of delivery showed a direct effect on the resistome composition of 7-days and 1-month-old infants ($p<0.05$) (**Figure III-5C**).

Our data suggests that any contact with human milk during the first 6 months of life reduces the ARG load on the infant's resistome (**Supplementary Figure III-9**). At 7 days of age, breastfed infants have less abundance of ARG than formula-fed infants. At 1 month of age, some infants received a combination of both formula and breastfeeding (mixed), and we observed that in those cases, their ARG load was higher compared to those infants that kept exclusively breastfeeding ($p<0.01$). Formula fed infants at 1 month of age showed higher ARG abundances than those receiving breastfeeding, although the differences were not significant. At 6 months of age, most of the infant's stopped receiving breastfeeding and started weaning. In those cases, their ARG load is higher than those infants that are still breastfed ($p<0.05$). At 12 months of age, none of the infants were receiving exclusive breastfeeding (**Figure III-5D**). In addition, we have observed that exclusive breastfeeding directly influences the resistome during the first month of life, as infants that are not exclusively fed with human milk, and receive either only formula feeding or combination of formula and breastfeeding, have a higher ARG load than those exclusively breastfed ($p=0.003$) (**Figure III-5E**). The ARG composition is also different at 1 month of age ($p=0.012$). Exclusive breastfeeding during the first month of life accelerates the decrease of antibiotic resistance in the infant gut during that time and ensures a lower abundance of ARGs at 6 months (**Figure III-5F**). Early termination of breastfeeding (before 6 months according to WHO

recommendations⁴⁶) was associated with an enrichment of several ARGs in infants ($p < 0.05$) (**Figure III-5G**). The enriched genes included genes conferring resistance to beta-lactams, amphenicol, quinolones, FPA, macrolides, and tetracyclines, among others.

The mode of delivery clearly influenced the ARG load of 7-days old infants, as C-section-born infants have more resistances than vaginal-born infants ($p < 0.01$). However, exclusive breastfeeding until 1 month of age reduces the ARG load in infants born by C-section (**Figure III-5H**). From vaginal delivered infant's, those exclusively breastfeeding showed less ARG than those not exclusively breastfed ($p < 0.05$). Hence, we observe that the effects that a C-section procedure has on ARG load during the first month of life, can be erased if the infant is exclusively breastfed.

C-section not exclusively breastfeed infants correlate positively with genes conferring resistance to lincosamide and streptogramin A, glycopeptides and amphenicol+quinolone+FPA (**Figure III-5I**). C-section procedures correlate positively with fosfomycin, lincosamide, quinolones and aminoglycoside resistances, while vaginal deliveries are positively correlated with tetracycline, macrolide and lincosamide resistances. The lack of exclusive breastfeeding is associated with many resistances too, such as macrolides, lincosamides and streptogramins. Fosfomycin, pseudomonic acid and steroid antibacterial resistances are negatively correlated with infant age, while lincosamides, tetracyclines, and quinolones increase over time. However, genes conferring resistance to macrolides, lincosamides and beta-lactams are more associated with 7 days infants. Finally, a low abundance of *Bifidobacterium* is associated with lincosamides, tetracyclines, amphenicol, beta-lactam and quinolone resistances (**Figure III-5I**). Additionally, RF with a cross-validation analysis (accuracy =66.18%, AUC=0.79), established that seven ARGs are the most predictive for type of lactation (**Supplementary Figure III-10**). *bla(Z)* beta-lactam resistance gene was ranked as the most important variable within the ARGs differentiating between maternal or artificial lactation, followed by other genes *mph(C)* and *msr(A)*, *tet(O)*, *tet(32)* and *tet(W)*.

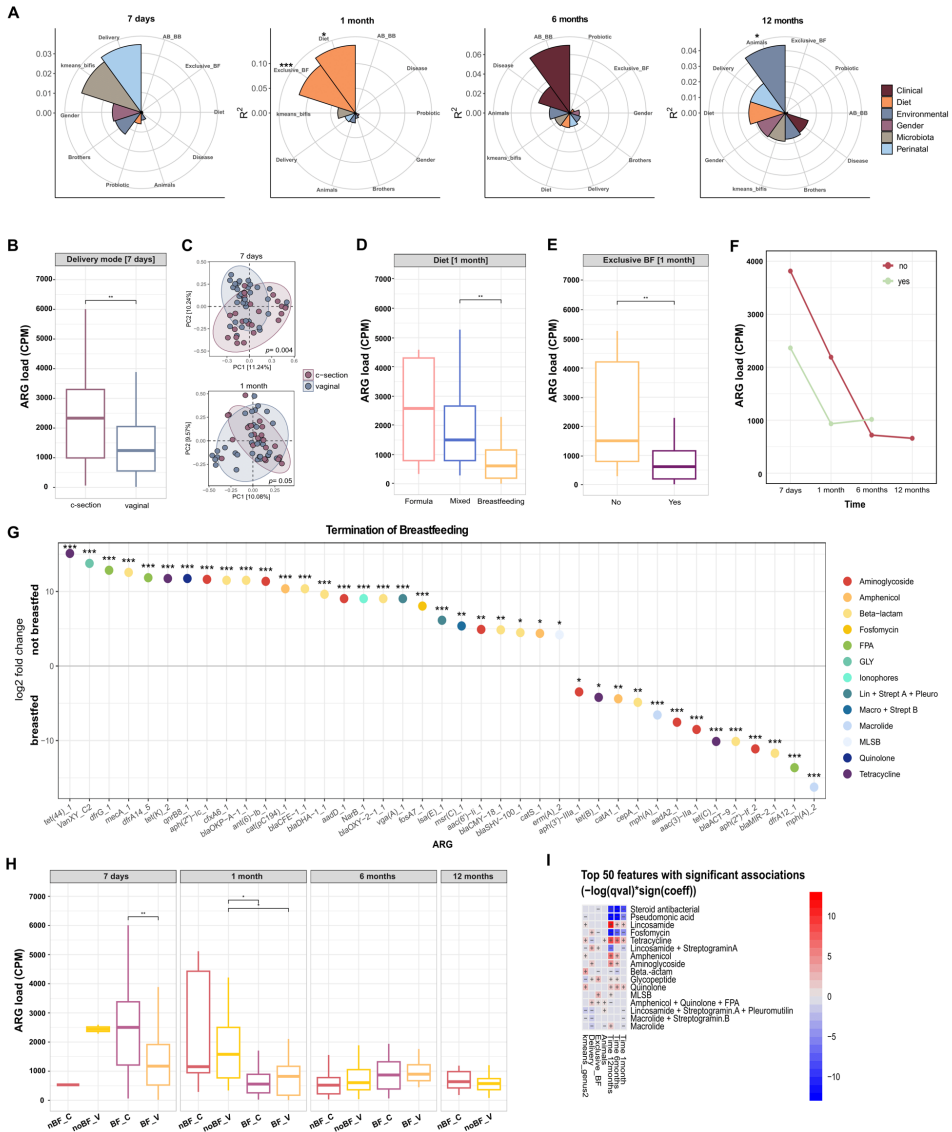


Figure III-5. The influence of environmental factors on shaping infant's resistome during first year of life. (A) Environmental factors driving infant's resistome, ranked by effect size ('envfit' R2, false discovery rate (FDR)-corrected two-sided test, $p < 0.05$). **(B)** Antibiotic resistance load in the gut of infants of 7 days of age, depending on their mode of delivery ($N = 40$ vaginal, $N = 26$ C-section), expressed as total CPM of ARGs per infant in each timepoint. **(C)** Principal Coordinate Analysis (PCoA) of the composition of the resistome depending on the mode of delivery (vaginal or C-section) during the first 7 days and 1 month of life, based on Jaccard distances. Two-sided PERMANOVA on Jaccard distances was applied to assess

differences between groups. **(D)** Antibiotic resistance load in the gut of infants of 1 month of age depending on their diet (N= 40 breastfeeding, N= 4 formula feeding, N= 14 mixed), at each timepoint during the first year of life, expressed as total CPM of ARGs per infant in each timepoint. **(E)** Antibiotic resistance load in the gut of infants during the first month of life depending on exclusive breastfeeding (N= 40 yes and N= 18 no), at each timepoint during the first year of life, expressed as total CPM of ARGs per infant in each timepoint. **(F)** Tendency of antibiotic resistance during the first year of life according to breastfeeding during the first month of life. The trajectory of the total load of ARGs was studied considering if the infant received exclusive breastfeeding during the first month of life. **(G)** ARGs differentially abundant due to breastfeeding in 6-month-old infants compared to non-breastfed infants, calculated using *DESeq2* with the p-values attained by the Wald test corrected for multiple testing using the Benjamini Hochberg method. **(H)** Antibiotic resistance load in the gut of infants during the first year of life depending on the mode of delivery and if they were exclusively breastfed, at each timepoint during the first year of life, expressed as total CPM of ARGs per infant in each timepoint. **(I)** Multivariable association between factors and resistome composition using *MaAsLin*. Antibiotic resistance load was the summed abundance of all ARGs in a given sample. Box plots show the median (centre line), the first and third quartiles (bounds of box). Whiskers are chosen to show the 1.5 of the IQR. Significance values for ARG load were calculated using the pairwise rank-sum Wilcoxon test (Mann Whitney U), two-sided. Asterisks denote statistical significance: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$. kmeans_bifis: *Bifidobacterium* abundance-based clustering; AB_BB: infant's antibiotic consumption; BF: breastfeeding; nBF_C: not exclusively breastfed infants born by C-section; nBF_V: not exclusively breastfed infants born vaginally; BF_C: exclusively breastfed infants born by C-section; BF_V: exclusively breastfed infants born vaginally.

5. Discussion

Our study highlights the crucial role of *Bifidobacterium* and breastfeeding in shaping the infant gut resistome. We demonstrate that a high abundance of *Bifidobacterium* is associated with a more beneficial microbial composition and a lower ARG load throughout the first year of life, and this high abundance is independent of mode of delivery or feeding, highlighting an alternative way for a lower resistome burden. While the impact of C-section delivery on the resistome is evident at 7 days of life, exclusive breastfeeding becomes a key determinant by one month of age, significantly reducing ARG burden. Moreover, early breastfeeding cessation correlates with a higher ARG load, underscoring its long-term influence on microbial resilience. Notably, this is the first study to identify exclusive breastfeeding as a strategy to counteract the resistome-altering effects of C-section birth, reinforcing its protective role. Our findings position breastfeeding as a critical,

modifiable factor in fostering a healthier microbiome and mitigating antibiotic resistance in early life.

In this study we utilized over 265 fecal metagenomes from 66 mother-infant pairs to explore the characteristics and dynamics of the intestinal resistome during the first year of life, and how the microbiome and external factors influence the infant resistome and its dynamics. We identified ARG in all the samples investigated, even though the type of resistance, load and associated taxa varied greatly during the different timepoints.

The developing gut resistome was linearly associated with infant age, suggesting a dynamic trajectory of resistance over time as described in other studies such as that of Xu, X., *et al.*⁴⁷. Over time, an infant's resistome tends to become more similar to that of its mother. While we have observed that mother-infant dyads at one month postpartum share common ARGs and exhibit similar ARG loads, but their overall resistome composition remains distinct. Therefore, the extent of maternal gut influence on shaping the infant's resistome is still unclear, and further evidence is needed to better understand this relationship. Tetracycline was the most abundant resistance class across all ages in the childrens' samples, although infants are no longer exposed to this antibiotic⁴⁸. The second most abundant resistance was against macrolides, which include genes encoding multiresistance (such as MLSB), as previously reported^{12,47,49,50}. The use of macrolides, which still remains an important antibiotic in pediatric care⁵¹, has been associated with an increase in resistance to this class of antibiotic, with long-lasting effects on the composition of the infant gut microbiota^{52,53}.

The resistome development during the first year of life occurs in parallel with the gut microbiota development and tends to resemble the mother's -adult's- resistome. ARGs in neonates have been most frequently associated with *Bacteroides*, *Clostridioides*, *Escherichia*, and *Staphylococcus*⁵⁴⁻⁵⁷, being enterobacteria particularly abundant in early life and important ARG carriers^{12,58}. On Top of those, we have also found *Enterococcus*, *Streptococcus*, *Enterococcus* and

Klebsiella to be the main genera responsible for carrying a higher variety of antibiotic resistances in our cohort.

In terms of the potential mobilization of the resistome, we analyzed the presence of plasmids carrying ARGs in our samples. We observed a high presence of ARGs conferring resistance to beta-lactams and aminoglycosides in plasmids, and highly associated with *Escherichia*. Although it has been recently published that *Bacteroides* are the main hosts for the infant gut plasmidome⁵⁹, the overrepresentation of plasmidic genomic sequences from enterobacteria, such as *Escherichia* and *Klebsiella*, might hinder the identification of the exact bacterial host in metagenomic studies. Similarly, most plasmidic contigs were assigned *de novo* without the occurrence of a plasmidic replicon that might share light of the family and characteristic of such plasmids, including their mobilization ability. Despite these limitations, we aimed to infer the ratio of ARGs that occur in potential mobilizable elements and their potential host, due to the key role they pose in AMR spread and dissemination.

Our primary finding was the association of *Bifidobacterium* with the modulation of the early-life microbiota and the resistome abundance and profiles. High *Bifidobacterium* levels have been previously associated with low ARG abundance⁶⁰. In a previous study, we investigated the resistome of the same cohort by using qPCR⁶¹. Here, we confirm and expand our previous results using an in-depth metagenomic sequencing approach on a larger dataset: a high relative abundance of *Bifidobacterium* on the infant's gut drives a specific microbial composition and reduced ARG load during the first year of life.

The study conducted by Taft *et al.*⁶⁰ correlates with our findings, as they also found that High-*Bifidobacterium* infants had lower levels of *Enterobacteriaceae* than the Low-*Bifidobacterium* infants, suggesting that the abundance of *Bifidobacterium* may suppress AMR-carrying taxa. The inhibitory effect of *Bifidobacterium* on the growth of other commensals and pathogens has been described before⁶². We have observed that a low abundance of *Bifidobacterium* is

associated with higher load of ARGs conferring resistance to lincosamides, tetracyclines, amphenicols, beta-lactams and quinolones. However, it is important to highlight that some species of *Bifidobacterium* carry resistances (mainly against tetracyclines)⁶³ and that can increase over time. Indeed, the RF model indicated that seven ARGs, associated with macrolide resistance (*erm(B)*, *erm(X)*, *mef(A)*), tetracycline resistance (*tet(S)*, *tet(32)*, *tet(B)*) and beta-lactam resistance (*blaTEM*), were the most important variables in predicting the *Bifidobacterium* cluster, some of which also had an important influence in the infant-age prediction model.

Shao *et al.*⁶⁴ previously reported that one-month-old infants with high *Bifidobacterium bifidum* colonization carried fewer ARGs than those colonized by *Enterococcus fecalis*, which exhibited elevated extended-spectrum beta-lactamase (ESBL) levels. Likewise, Li *et al.*¹² identified *Escherichia coli* as a key determinant of the microbiome in one-year-old infants, with *Proteobacteria* contributing the most diverse and abundant ARGs. Aligning with these findings, our study found that infants with low *Bifidobacterium* levels were predominantly colonized by *E. coli* and *E. faecium*, both carrying a higher proportion of ARGs throughout the first year. Additionally, our longitudinal analysis, spanning from seven days to one year of age, provided further insights into these microbial patterns.

Historically, the infant microbiome is dominated by *Bifidobacterium* and associated with a variety of beneficial health effects^{65–67}. While we have observed the effect of *Bifidobacterium* abundance on resistome load and composition in all timepoints during the first year of life, the Bangladeshi infants from Taft *et al.*⁶⁰ lose the association between early-life *Bifidobacterium* levels and antimicrobial resistance at 2 years of age. This suggests the important role of *Bifidobacterium* on the gut microbiota development only during the first year of life, as gut microbiota will tend to evolve towards an adult-like microbiota, where *Bifidobacterium* is less abundant. The dominance of the infant gut by *Bifidobacterium* is linked to the consumption of human milk oligosaccharides (HMO), the third most abundant component in human milk⁶⁸. The essential role of breastfeeding in shaping a healthy

infant gut microbiome by supporting the growth of beneficial microbes has been studied before⁴⁵. Interestingly, our study found that the clustering based on *Bifidobacterium* abundance is not influenced by the mode of delivery or breastfeeding practices, with infants from diverse birth and feeding modes converging in the high-*Bifidobacterium* group. This underscores the unique role of *Bifidobacterium* in shaping the infant microbiome and resistome, which we have treated as an independent variable in our analysis. However, the interplay of host genetics and other factors that may drive the early-life abundance of *Bifidobacterium* remains to be explored. While breastfeeding did not show a strong association with *Bifidobacterium* abundance in our cohort overall, previous studies have demonstrated that, when *Bifidobacterium* is present, breastfeeding typically supports its growth^{68–70}. This highlights the complex relationship between feeding practices and microbiota composition, which may vary depending on other individual or environmental factors.

In this vein, we also provide insights on the benefits of breastfeeding on resistome acquisition and maintenance. Exclusive breastfeeding during the first month of life accelerates the decrease of antibiotic resistances in the infant gut during that time, and ensures a lower abundance of ARGs at 6 months. We also provide more evidence that early termination of breastfeeding -recommended for at least 6 months by the WHO⁴⁶- is associated with an enrichment of ARGs. Based on our study and previous work⁷¹, breastfeeding for at least 6 months may decrease the prevalence of Gammaproteobacteria while simultaneously increasing the presence of bifidobacteria, contributing to lowering the load of antibiotic resistance genes in the infant gut. Previous findings suggest that HMO-supported bifidobacteria growth could mediate these associations^{60,61,72}.

Nevertheless, the benefits of breastfeeding on shaping infant's resistome are not observed until the first month of age. Before that time, the mode of delivery exerts the greatest influence in modulating the infant resistome. The WHO suggests that women who undergo a c-section should receive a single dose of penicillin or a first-generation cephalosporin as a preventive measure, instead of other types of

antibiotics⁷³. Consequently, all infants are currently exposed to beta-lactams antepartum via the umbilical cord, as these antibiotics rapidly cross the placenta. In concordance with previous studies^{49,58,74,75}, c-section born infants have a higher abundance of ARGs than vaginal-born infants, and the composition of their resistome is different. This difference is likely driven primarily by the altered colonization patterns typical of C-section births, including reduced transmission of maternal microbes and increased acquisition of hospital-associated taxa such as *Staphylococcus*, *Enterococcus*, and *Klebsiella*, which are commonly associated with higher ARG carriage^{75,76}. Therefore, the enrichment of ARGs in C-section infants may result from a combination of altered microbial succession, environmental exposure, and, potentially, transient antibiotic pressure. Notably, these differences diminish by one month of age, when breastfeeding becomes a dominant factor influencing resistome composition.

Guo *et al.*⁷⁷ reported that breastfeeding restores the gut microbiota of c-section infants. In their study based on 16S rRNA gene amplicon sequencing, they observed that breastfeeding did not compensate for the lack of *Bifidobacterium* spp. in children born by c-section: children born by c-section and receiving breastfeeding had less bifidobacteria than formula fed vaginally-born children. A recent study conducted by Sinha *et al.* reflected that feeding mode is by far the most defining factor for microbial composition and functionality in early life in c-section born infants⁷⁸. In our study, we extend these results to the infant' gut resistome, observing that exclusive breastfeeding till 1 month of age reduces the ARG load of infants born by c-section. The positive contribution of human milk to the gut microbiota development^{79,80} may explain why breastfeeding could restore the delayed microbiome development in c-section, extending to a reduction in antibiotic resistances.

Jokela *et al.*⁷⁵ described how the resistome in early life is primarily shaped by the natural development of the gut microbiota, with *Bifidobacterium* abundance correlating negatively, and *Bacteroides* positively, with ARG load. In our study, we confirm the association between *Bifidobacterium* abundance with a decreased

ARG load, but also focus specifically on feeding practices and their influence on ARG acquisition. The inclusion of earlier sampling timepoints—at 7 days and 1 month—allowed to uncover that exclusive breastfeeding during the first month of life is associated with a rapid decrease in ARG abundance and leads to a significantly lower resistome burden by six months. Pärnänen *et al.*⁸¹, in a cross-sectional study of 1-month-old preterm infants, identified higher ARG loads in formula-fed compared to breastfed infants. Our longitudinal study in full-term infants aligns and builds upon these observations, demonstrating how exclusive breastfeeding during the first month not only lowers ARG burden but also mitigates the impact of cesarean-associated antibiotic exposure.

At 6 months of age, as the infants are more exposed to external microbes, the influence of environmental factors over their microbiome increases. This period typically coincides with the onset of weaning, during which most infants either discontinue breastfeeding or combine it with the introduction of solid foods and other liquids. At this stage, the direct effects of breastfeeding may become less pronounced due to the influence of multiple external factors. Nonetheless, the significance of the so-called "window of opportunity" for shaping the infant resistome cannot be overstated. Any contact with breastfeeding during this period offers substantial benefits to the infant microbiome and resistome, with both immediate and long-term effects. Moreover, lack of breastfeeding feeding has been observed to be the most influential variable in plasmid assemblage in early life, which has a related impact to the resistome dynamics⁸².

The establishment of a well-balanced microbiome during early life is critical for ensuring the controlled development of the resistome. Our evidence suggests that breastfeeding may help to modulate potential microbial dysbiosis associated with C-section births, a procedure that is sometimes unavoidable. These findings underscore the value of breastfeeding as a scalable and cost-effective intervention to address antimicrobial resistance, complementing its well-established health benefits⁸³. In high-income nations, where fewer than half of infants are breastfed at six months of age⁸³, promoting breastfeeding practices has

the potential to decrease early-life antibiotic usage and enhance the gut's ability to resist colonization by AMR pathogens, which are becoming increasingly common in community environments. Establishing evidence that breastfeeding or components of human milk offer protection against colonization or infection with AMR bacteria would provide strong support for intensifying efforts to advocate for breastfeeding through policy initiatives.

6. References

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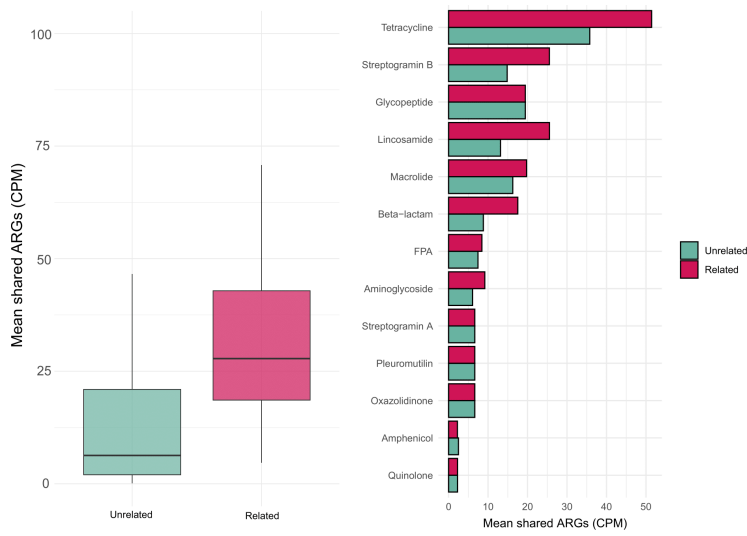
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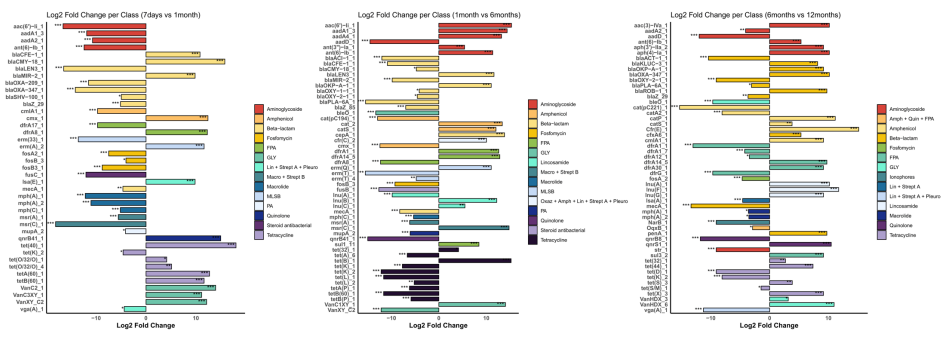
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7. Supplementary materials



Supplementary Figure III-1. Comparison among the shared ARGs between infants and their own mothers (related mother-infant dyads) and unrelated mothers, shown as the total mean of shared ARG (CPM) and grouped by antibiotic resistance class. Box plots show the median (center line), the first and third quartiles (bounds of box). Whiskers are chosen to show the 1.5 of the IQR. Wilcoxon sum test was performed to assess statistical differences between related and unrelated mother-infant dyads.

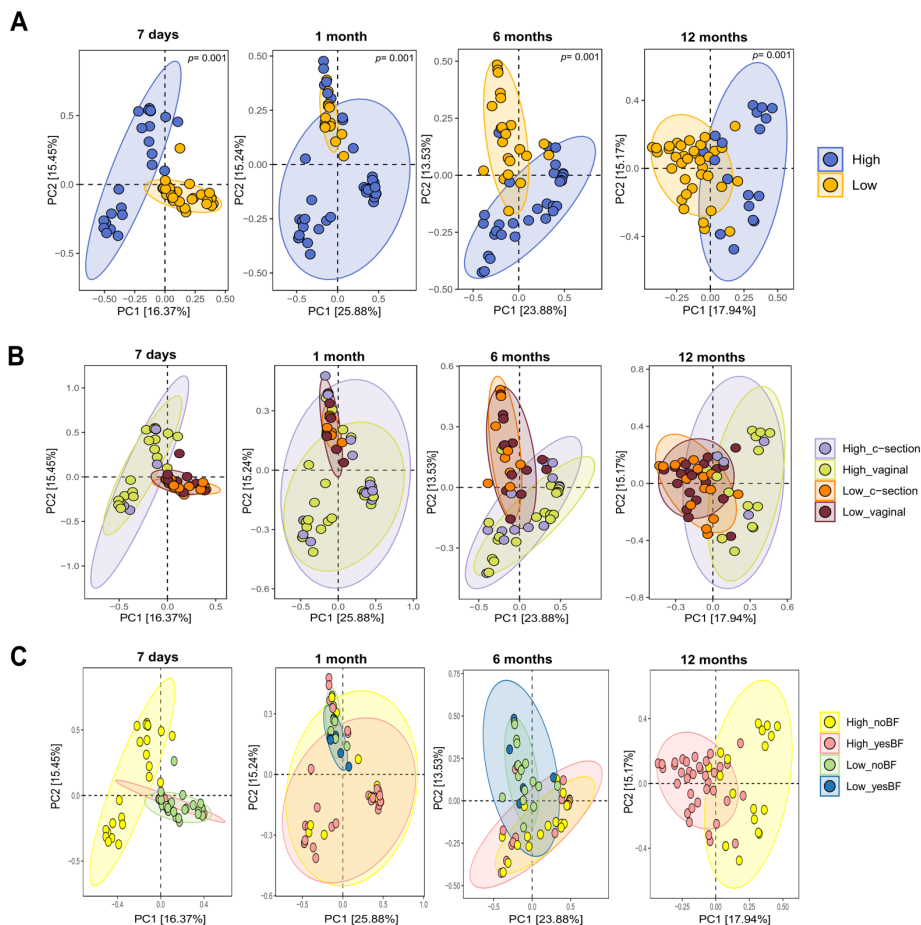


Supplementary Figure III-2. Differential ARG abundance over time using DESeq2. Differences were considered significant with the p-value (adjusted for false discovery rate using Benjamini-Hochberg correction) cut-off of 0.05. Log 2fold changes greater than zero indicated an increase in the relevant genera, whereas log 2fold changes less than zero indicated a decrease in the relevant genera, between the two timepoints compared. Negative binomial GLMs, Wald's test, DESeq 2, adjusted p-value < 0.05)

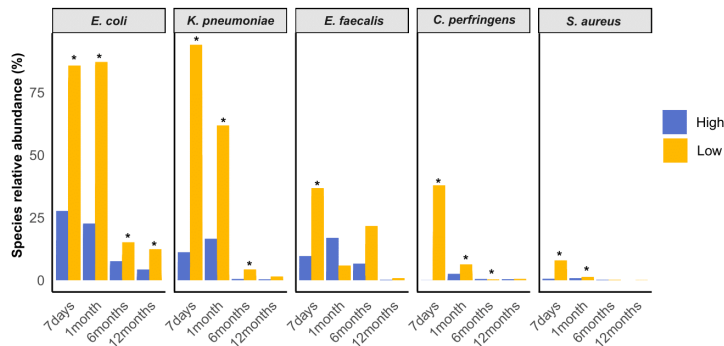
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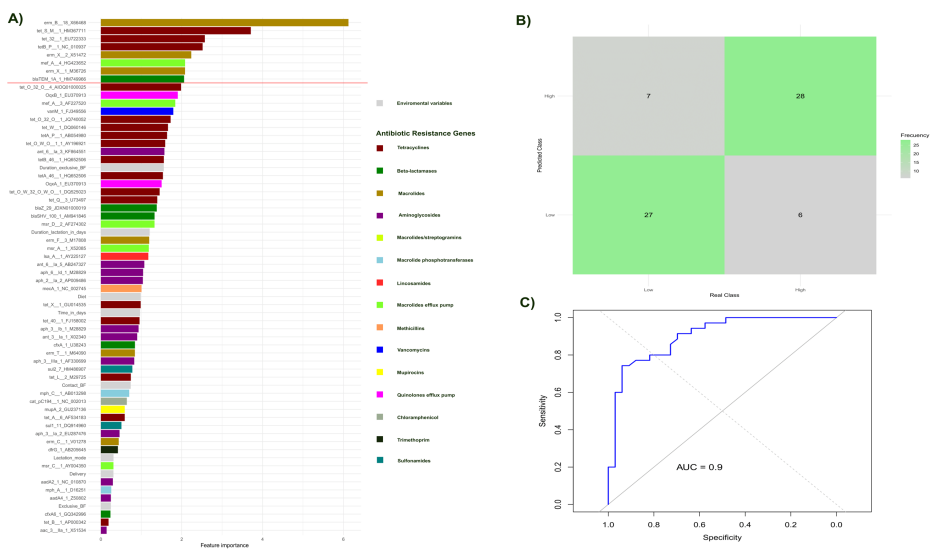
clustering. Mann Whitney U test revealed that statistically significant differences in relative abundance were found in *Bifidobacterium* genus, in all timepoints, so clusters were named as High- and Low-*Bifidobacterium*. **(C)** Prevalence of hierarchical clustering based on *Bifidobacterium* spp. abundance over the first year of life.



Supplementary Figure III-7. Principal coordinates analysis of the (A) hierarchical clustering of *Bifidobacterium* relative abundance of the infants (high or low), (B) mode of delivery (vaginal or C-section) and hierarchical *Bifidobacterium*-based clusters (high or low), and (C) exclusive breastfeeding (yes or no) and hierarchical *Bifidobacterium*-based cluster (high or low), in the four timepoints, based on Bray Curtis distances regarding the microbial community composition at species level (β -diversity, Bray-curtis distance, PERMANOVA; $p<0.001$).

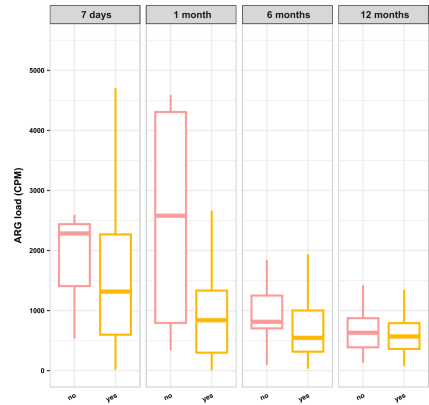


Supplementary Figure III-8. Species relative abundance (%) in each cluster during the first year of life. Only selected species with statistically different abundances in any timepoint are shown.

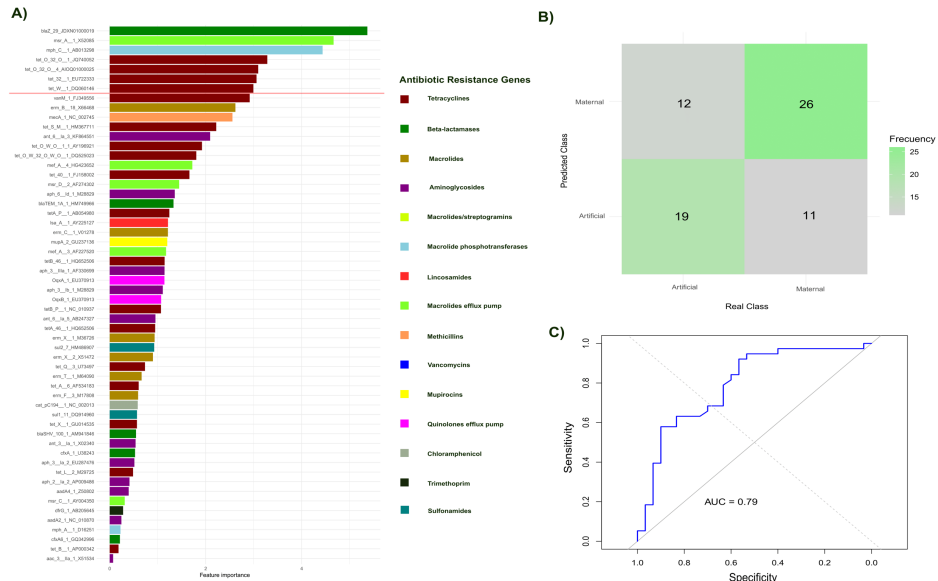


Supplementary Figure III-9. Predictive Modeling of *Bifidobacterium* Cluster (High or Low) Using Random Forest. (A) Ranked lists of the Top environmental variables and ARGs and their feature importance in model. ARGs are coloured according to the type of antibiotic(s) they confer resistance. The red line indicates variables that are sufficient for RF predictions of infant age according to the ten-fold cross-validation. **(B)** Confusion Matrix for Model Evaluation: The matrix shows the performance of the classification model by comparing the predicted labels to the true labels. Each cell represents the count of instances for each combination of predicted and actual values. The diagonal cells (Top-left to bottom-right) represent the correct classifications (True Positives and True Negatives), while the off-diagonal cells represent misclassifications (False Positives and False Negatives). The model has an accuracy of 80.88%. **(C)** ROC Curve for Model Performance. The plot illustrates the Receiver Operating Characteristic (ROC) curve, showing the trade-off between the True Positive Rate (Sensitivity) and the False Positive Rate (1 - Specificity) at various classification

thresholds. The gray diagonal line represents random classification performance. The blue curve represents the model's performance, with a higher curve indicating better discrimination between the 'High' and 'Low' cluster. The Area Under the Curve (AUC) value of 0.9 indicates excellent model performance, where a value closer to 1.0 denotes near-perfect accuracy.



Supplementary Figure III-10. Influence of contact with breastfeeding on the antibiotic resistance gene load during the first year of life, expressed as total CPM of ARGs per infant in each timepoint. ARG load was the summed abundance of all ARGs in a given sample.



Supplementary Figure III-11. Predictive Modeling of type of lactation (Maternal or Artificial) Using Random Forest. (A) Ranked lists of the Top environmental variables and ARGs and their feature importance in model. ARGs are colored according to the type of antibiotic(s) they confer resistance. The red line indicates variables that are sufficient for RF predictions of infant age according to the ten-fold cross-validation. (B) Confusion Matrix for

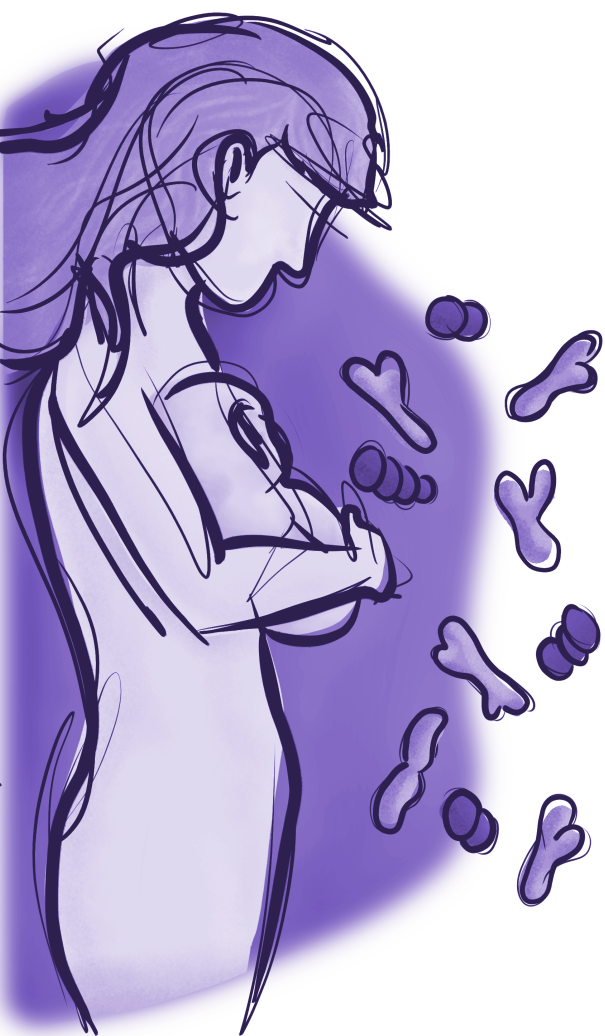
Model Evaluation: The matrix shows the performance of the classification model by comparing the predicted labels to the true labels. Each cell represents the count of instances for each combination of predicted and actual values. The diagonal cells (Top -left to bottom-right) represent the correct classifications (True Positives and True Negatives), while the off-diagonal cells represent misclassifications (False Positives and False Negatives). The model has an accuracy of 66.18%. (C) **ROC Curve for Model Performance.** The plot illustrates the Receiver Operating Characteristic (ROC) curve, showing the trade-off between the True Positive Rate (Sensitivity) and the False Positive Rate (1 - Specificity) at various classification thresholds. The gray diagonal line represents random classification performance. The blue curve represents the model's performance, with a higher curve indicating better discrimination between the 'High' and 'Low' cluster. The Area Under the Curve (AUC) value of 0.79 indicates excellent model performance, where a value closer to 1.0 denotes near-perfect accuracy.

Supplementary Table III-1. Characteristics of study participants.

Infant characteristics		Total (N=66)
Gender: female (%)		30 (54.54%)
Birth mode (%)		
	vaginal birth (%)	40 (60.60%)
	C-section	26 (39.39%)
Exclusive breastfeeding (%)		
	7 days	62 (93.93%)
	15 days	49 (74.24%)
	1 month	47 (71.21%)
	12 months	0
Antibiotic exposure (%)		
	At birth	40 (39.39%)
	7 days	0
	1 month	1 (1.51%)
	6 months	2 (3.03%)
	12 months	5 (7.57%)
Mother characteristics		
Gestational age (years)		39 [39-40]
Weight gain (kg)		13.436 ±4.631
Pre-gestational BMI (kg m ⁻²)		23.104 ±3.094
Antibiotic consumption during pregnancy (%)		15 (22.72%)

Categorical variables are expressed as positive cases-prevalence and (percentage, %). Normally distributed data are presented as mean ± standard deviation (SD) and non-normal data as median and interquartile range [IQR]. BMI: body mass index z-score.

Supplementary Table III- 2 -4. (See Annex Chapter III)



CHAPTER IV

CHAPTER IV

Maternal Secretor Status and Human Milk Oligosaccharides Modulate the Impact of Cesarean Birth on the Infant Gut Resistome

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My contribution to this research article was:

Conceptualization. Perform the experiments: DNA extraction, quantification, and purification. Sample preparation. HPLC analysis. Data analysis and curation, statistical analysis and interpretation. Writing-original draft preparation.

This chapter presents results obtained during a research stay in Dr. Bode laboratory at the University of California San Diego (UCSD).

1. Abstract

The infant gut resistome develops early in life and is shaped by delivery mode and feeding practices. We analyzed fecal samples from 57 one-month-old infants and milk from their respective 50 mothers using shotgun metagenomics and HPLC to investigate how human milk oligosaccharides (HMOs) and maternal secretor status influence the resistome. Despite no direct antibiotic exposure, 208 distinct antibiotic resistance genes (ARGs) were identified, with tetracycline and beta-lactam resistance genes most abundant. Cesarean section (C-section) delivery was linked to greater ARG diversity, while exclusive breastfeeding was associated with reduced ARG abundance and diversity. Infants breastfed by secretor mothers showed resistome profiles independent of delivery mode, likely due to enrichment of beneficial gut microbes. Specific HMOs, such as 2'FL and 6'SL, were negatively associated with certain ARGs. These findings suggest that maternal secretor status and HMO composition play key roles in shaping the early life resistome and may help modulate the impact of C-section delivery on antibiotic resistance risk.

2. Introduction

Antibiotic resistance is one of the most pressing global health challenges with its emergence in early life being particularly concerning due to the on-going development of the immune system¹. Immediately after birth, the early-life microbiota and resistome (i.e., all the antibiotic resistance genes (ARGs) within a biome) begin to establish and develop^{2,3}. Understanding the factors that influence microbiome maturation and the acquisition and composition of the resistome in infancy is crucial, as early establishment of resistances may contribute to a long-term reservoir that persists into later life⁴.

C-section birth and antibiotic exposition play a pivotal role in the initial colonization of the microbiome and resistome⁵⁻⁷. Infants born via C-section (C)-section are characterized by higher microbial diversity and colonized predominantly by environmental and skin-associated microbes⁸. These differences extend to the

resistome, as C-section-born infants harbor higher ARG diversity and abundance, largely driven by opportunistic pathogens such as *Staphylococcus aureus* and *Klebsiella pneumoniae*^{6,9,10}. In contrast, vaginally born infants are primarily colonized by commensal microbes such as *Bifidobacterium* and *Bacteroides*^{6,11}, which are associated with lower microbial diversity and a reduced load of ARGs^{12,13}. While exclusive breastfeeding supports the establishment of beneficial microbiota, there is limited data on its specific influence on the resistome.

Human milk is the main postnatal link between mother and infants and beyond nutritional components, it contains a wide range of bioactives including microbes and human milk oligosaccharides (HMOs)^{14–16}. In human milk, the predominant bacterial genera include *Lactobacillus*, *Bifidobacterium*, *Staphylococcus*, and *Streptococcus*^{17,18}. *Bifidobacterium* are among the microbes that thrive in the gut of breastfed infants due to their capacity to efficiently metabolize HMOs^{19–21}, which are the third most abundant component in human milk²². HMO concentrations fluctuate throughout lactation and vary between individuals, with maternal secretor status, dictated by *FUT2* functionality, playing a key role in shaping HMO composition^{23,24}. These variations significantly influence the infant's gut microbiota, as studies show that infants of secretor mothers are predominantly colonized by *Bifidobacterium*^{25,26}.

The critical role of breastfeeding in shaping microbiome maturation raises the question of whether it exerts a similar influence on the resistome. Previous studies have reported that breastfeeding can help restore the gut microbiota of C-section-born infants and lower the risk of infection in early life²⁷, potentially due to the fucosylated oligosaccharides in secretor milk²⁸.

We therefore hypothesized that exclusive breastfeeding fosters a gut environment that not only promotes the growth of beneficial microbiota such as *Bifidobacterium*, but also modulates the acquisition and abundance of ARGs, thereby supporting a healthier microbiome and potentially limiting the reservoir of resistance genes that could contribute to future antibiotic-resistant infections. By

analyzing stool and milk samples from mother-infant pairs at one-month postpartum using shotgun metagenomic sequencing and HMO glycoprofiling, our study demonstrated that maternal secretor status plays a key role in modulating the impact of delivery mode on the infant resistome, resulting in a more uniform resistome composition in infants breastfed by secretor mothers regardless of birth mode.

3. Materials and methods

3.1. Study design and volunteers

A subset of mother-infant pairs (N=57) from the MAMI cohort²⁹ was selected for analysis based on the sample availability and the homogeneity of the clinical/anthropometric characteristics such as the breastfeeding practices. Maternal-infant clinical records, including gestational age, neonate gender, birth weight, mode of delivery, type of lactation, and antibiotic exposure of the infant were collected. Infant length and weight were registered at birth, 1, 6, and 12 months and the data were used to calculate BMI Z-scores. These anthropometric measures were standardized by age and sex and electronically computed using WHO Anthro software (<https://www.who.int/childgrowth/software/en/>). From the longitudinal sampling performed in the MAMI cohort, infant fecal samples collected at one month of age (N= 57) were included in this analysis. In addition, one human milk sample per mother (N= 50) was collected at one-month postpartum from breastfeeding mothers. The clinical characteristics of the study participants can be found in **Supplementary Table IV-1**.

Ethical approval for the study was obtained from the Ethics Committee for Clinical Research of the healthcare centres involved in the recruitment including the Hospital La Fe, Hospital Clinico Universitario, and CSIC (Consejo Superior de Investigaciones Científicas) [ClinicalTrial.gov NCT03552939].

3.2. Sample collection, DNA extraction, and sequencing

Infant fecal samples were collected at home by their parents who were previously trained by clinical personnel in the health care centres where they were enrolled. Briefly, fecal samples were deposited in provided sterile containers, immediately frozen at -20°C , and subsequently transferred to -80°C for long-term storage until further analysis. Similarly, milk samples were collected by mothers following provided instructions. Breast skin was sanitized with 0.5% chlorhexidine solution and the first drops were discarded. Next, human milk was collected using a sterile pumper in sterile bottles to normalize milk collection.

DNA was isolated from fecal samples (50–100 mg each) and human milk samples as described previously³⁰. DNA extraction was performed using the Master-Pure DNA extraction kit (Epicentre, Madison, WI, USA) following the recommended protocol with additional steps as described in Selma-Royo *et al.* Briefly, a pre-treatment with lysozyme and mutanolysin was included as well as a cell disruption step with 3- μm diameter glass beads in a FastPrep 24-5G Homogenizer (MP Biomedicals). After extraction, DNA samples were purified using a DNA Purification Kit (Macherey-Nagel, Duren, Germany). For human milk samples (2 ml), an additional preliminary step of centrifugation at $4,000 \times g$ for 20 min was carried out to separate fat and cellular components from the whey. DNA extraction was then initiated from the resulting pellet. Controls from the DNA extraction were collected to include in the sequencing process.

Sequencing libraries were constructed using the NexteraXT DNA Library Preparation Kit (Illumina Inc., San Diego, CA, USA) and sequenced on a HiSeq2500 platform (Illumina) with a target depth of 5 Gb/sample (**Supplementary Table IV-2**).

Even though the DNA concentration of milk samples was enough for sequencing, three samples were removed from the final analysis due to the low number of bacterial-associated reads after quality control (**Supplementary Table IV-2**). Thus, 47 milk were retained for further analysis.

3.3. Human milk oligosaccharide profiling

The profile of HMOs was obtained as previously described³¹. Briefly, human milk samples (20 μ L) were first spiked with maltose (20 mg/mL) as an internal standard to correct for sample losses during sample processing and allow for absolute oligosaccharide quantification. Oligosaccharides were fluorescently labeled with 2-aminobenzamide (2AB; Sigma-Aldrich, St. Louis, MO, USA) at 65 °C for 2 h. Labeled oligosaccharides were analyzed by high-performance liquid chromatography (HPLC; Dionex Ultimate 3000, Dionex, now Thermo Fisher Scientific, Waltham, Massachusetts, USA) on an amide-80 column (15 cm length, 2 mm inner diameter, 3 μ m particle size; Tosoh Bioscience, Tokyo, Japan) with a 50-mmol L⁻¹ ammonium formate–acetonitrile buffer system. Separation was performed at 25 °C and monitored with a fluorescence detector at 360 nm excitation and 425 nm emissions. HMO peaks were annotated based on standard retention times and quantified by reference to the internal standard.

The identified HMOs in the samples were: 2'-fucosyllactose (2'FL), 3-fucosyllactose (3FL), 3-sialyllactose (3SL), 6-sialyllactose (6'SL), difucosyllactose (DFLac), difucosyl-lacto-N-hexaose (DFLNH), difucosyl-lacto-N-tetraose (DFLNT), disialyl-lacto-N-hexaose (DSLNH), disialyl-lacto-N-tetraose (DSLNT), fucosyl-disialyl-lacto-N-hexaose (FDSLNH), fucosyl-lacto-N-hexaose (FLNH), lacto-N-fucopentaose (LNFP)I, LNFPII, LNFPIII, lactose-N-hexaose (LNH), lacto-N-neotetraose (LNnT), lacto-N-tetraose (LNT), sialyl-lacto-N-tetraose b (LSTb), and sialyl-lacto-N-tetraose c (LSTc). HMO Simpson diversity and evenness indexes were also calculated based on relative HMO abundances.

3.4. Maternal secretor status determination

Maternal phenotypic secretor status was determined by the presence or near absence of 2'FL. For women with a functional FUT2 enzyme (Se+), 2'FL is the most abundant HMO in their milk, whereas for women with a nonfunctional FUT2 enzyme (Se-), 2'FL has been shown to be in lower concentrations^{32,33}. Moreover, the

secretor/non-secretor phenotype assignment was validated by genotyping the *FUT2* gene from total DNA extracted from human milk by polymerase chain reaction (PCR-random fragment length polymorphisms (RFLPs) as described previously³⁴.

3.5. Bioinformatic workflow for microbiome and resistome profiling

The quality of the raw sequencing data was first evaluated using FastQC v0.11.9 (<https://www.bioinformatics.babraham.ac.uk/projects/fastqc/>) and MultiQC v1.11.³⁵ Sequences were then trimmed using FastP v0.23.2³⁶, with reads shorter than 75 bp and/or a mean Phred quality score below 25. Residual adapters and barcodes were also removed. Additionally, potential residual PhiX and human reads were discarded using Bowtie2 v2.4.2³⁷ by aligning the reads against their reference genomes. The quality-controlled human-free (QC) reads were re-evaluated using FastQC and MultiQC to inspect the effectiveness of the quality control process and further subjected to the downstream analysis towards microbiome and resistome profiling by following two main approaches: i) assembly-free for microbiome profiling and semi-quantitative resistome investigation and ii) assembly-based in order to evaluate the microbial taxa that carried the ARGs (**Supplementary Table IV-2**).

Microbiome profiling was performed over the QC-reads (human-free) by using MetaPhlAn4³⁸ and the CHOCOPhIAn_202403 database. The resistome profiling was performed as follows: i) the ARGs contained in the ResFinder database³⁹ (downloaded on 18th June 2024) were clustered at 90% identity and coverage by using CD-HIT⁴⁰ in order to prevent spurious alignments of the short sequencing reads to conserved regions from multiple closely-related ARGs; ii) alignment of the QC reads against the clustered ResFinder database by using Bowtie2 v2.4.2 and enabling the “--end-to-end” and “--very-sensitive” flags; iii) alignments were polished using featureCounts⁴¹, considering as positive hits only those reads that mapped uniquely to the clustered ResFinder database iv) ARGs with fewer than 10 total aligned reads were excluded from the analysis and v) the resulting count matrix was used to calculate the counts per million reads (CPMs), incorporating a “bacterial marker” modification according to the formula:

$$CPM = \frac{(ARG \times \text{Ratio of bacterial assigned reads})}{\text{Total number of reads}} \times 10^6$$

where “CPM” is the total counts per million reads value for each ARG and “Ratio of bacterial assigned reads” represent the ratio of reads per sample to be considered to belong to the kingdom “Bacteria” according to MetaPhlAn4, in order to normalize the dataset to consider only those reads derived from bacteria.

To infer the taxonomic provenance of the ARGs we assembled the QCed reads by using SPAdes v3.15.5⁴² and enabling the --meta option, 96 threads and 1,024 GB RAM per assembly using the k-mer lengths 21, 33, 55, 77, 99 and 121. The resulting FASTA files containing the metagenomic contigs were imported into TORMES v1.3.1⁴³ with some modifications to automate the downstream bioinformatic analysis, which consisted of the following steps: i) contigs shorter than 500 bp were excluded; ii) quality control of the assemblies was performed using QUAST v5.0.2⁴⁴ and by aligning the quality-controlled reads of each sample against its own assembly using Bowtie2 in order to assess the proportion of reads that were finally included in the assembly; iii) the taxonomy of each contig was assessed by using Kraken2 v2.0.8⁴⁵ enabling the --use-names option and the PlusPFP database (downloaded on 12th January 2024), and the taxonomic nomenclature was further refined using Taxonkit v0.16.0⁴⁶ (taxdump NCBI files downloaded on 6th June 2024); iv) bacterial and archaeal coding sequences (CDS) were predicted and amino acid sequences were translated using Prodigal v2.6.3 and by enabling the --only_gene_prediction flag in TORMES; v) Contigs with less than 3 CDS were excluded for downstream analysis; vi) resistome investigation was performed by aligning the assemblies against the ResFinder database (downloaded on 18th June 2024) using BLASTN v2.14.1⁴⁷ and by excluding alignments below 80% sequence similarity and coverage.

3.6. Statistical analyses and data visualization

All statistical analyses were performed using R version 3.6.0 and figures were created using the ggplot2 R package⁴⁸. For all methods, p-values were adjusted for multiple comparisons using the Benjamini–Hochberg (BH) method.

Normality of the data was evaluated with Shapiro–Wilk tests. Categorical variables are expressed as positive cases-prevalence and percentage (%). Normally distributed data are presented as mean $\pm$ standard deviation (SD) and non-normal data as median and interquartile range [IQR]. Pearson’s Chi-square test was used for categorical variables, and Mann–Whitney U test or Fisher’s exact test were used for continuous variables, as appropriate, for calculating statistical significance. The *vegan* package⁴⁹ was used to calculate the alpha and beta diversity metrics. The Shannon diversity of ARGs or bacterial species was used as a measure of within-individual diversity (alpha diversity). The beta diversity was assessed using the function ‘vegdist’ and visualized as Principal Coordinate Analysis (PCoA), employing Bray–Curtis distances for microbiome data and Jaccard distances for resistome profiling. Permutational multivariate analysis of variance (PERMANOVA) was performed using the function ‘adonis’ with 999 permutations to determine the statistical significance of composition differences.

To further explore patterns in community composition and identify potential associations with environmental factors, we employed an unconstrained ordination approach. Unconstrained ordination is particularly valuable in this context as it allows for the identification of intrinsic patterns in ARG distributions without being restricted to pre-specified explanatory variables. Subsequently, we used the ‘envfit’ function from *vegan* R package to fit environmental variables onto the unconstrained ordination space.

Differences in the abundance of a given ARG or taxa were evaluated using Wilcoxon tests for comparisons between two groups, and Kruskal–Wallis tests or comparisons involving three or more groups. Pairwise comparisons were carried out by pairwise Wilcoxon tests. Differences in the relative abundance of taxa between two clusters were evaluated using the Mann–Whitney U test (Wilcoxon rank-sum test for 2 levels). All p-values were adjusted for multiple comparisons by the FDR - Bonferroni correction.

To assess the statistical impact of different HMO structures on the abundance of tetracycline, aminoglycoside, fosfomycin, and lincosamide ARGs in the infant resistome, a generalized linear model (GLM) was estimated using *stats* R package. The best model was selected using the Akaike Information Criterion (AIC), following a forward selection approach and choosing the one with the lowest AIC value. Additionally, collinearity among explanatory variables was reviewed using the Variance Inflation Factor (VIF) with the *car* R package. The influence of fixed effects was calculated via Nagelkerke's R^2 . The antibiotics included in this analysis were selected based on the presence of at least one statistically significant predictor in each corresponding model.

4. Results

4.1. Cesarean birth and lack of breastfeeding shape the early resistome of one-month old infants

A total of 57 stool samples obtained from one-month-old infants in the MAMI cohort²⁹ underwent metagenomic sequencing. Although none of the infants had been directly exposed to antibiotics, assembly-free approaches identified 208 ARGs in this population. Overall, these ARGs were linked to resistance against 16 different antibiotic classes.

ARGs associated with resistance to tetracycline and beta-lactams were the most abundant, followed by those associated with macrolides, streptogramin B, aminoglycosides, lincosamides and folate pathway antagonists (FPA) resistance (**Figure IV-1A**). *tet(S/M)* and *tetA* were the most abundant and prevalent tetracycline ARGs (present in 84.2% and 71.9% of samples, respectively), followed by *tetW*, *tetB*, *tet(O/W/O)* and *tet(O/32/O)* (all present in more than 65% of the samples). The macrolide and streptogramin B resistance gene *msr(d)* was present in more than 90% of the samples, followed by *erm(B)* and *erm(X)* from the MLSB resistance phenotype. Finally, *blaZ* and *blaTEM* were identified as the most abundant beta-lactam resistance genes (61.4% and 50.8% of the samples, respectively) (**Table S3**). The main mechanism of resistance encoded by ARGs was enzymatic inactivation,

followed by enzymatic modification and target protection (**Supplementary Table IV-3**). The main mechanism of resistance encoded by ARGs was enzymatic inactivation, followed by enzymatic modification and target protection (**Figure IV-1B**).

To identify the taxa carrying ARGs in the infant fecal samples, we performed an assembly-based bioinformatic approach. The majority of ARGs in the infant gut originated from Bacillota (**Figure IV-1C**). *Staphylococcus aureus* was the most significant contributor, carrying 33 ARGs (15.8% of the total) across 11 distinct ARG variants. The second-largest contribution came from Pseudomonadota, particularly *Klebsiella pneumoniae* and *Escherichia coli*, which harbored 30 and 26 ARGs, respectively, spanning 9 and 12 different variants. Additionally, certain commensal members of the human microbiome were identified as ARG carriers, including *Bifidobacterium longum* (10 ARGs from 4 distinct variants) and *Bifidobacterium breve* (4 ARGs from 2 variants). A detailed distribution of the bacterial ARGs is shown in **Supplementary Table IV-4**.

Next, we evaluated the influence of different environmental exposures on the distribution of ARGs in the infant gut. Forty infants in this study (70.2%) were exclusively breastfed during the first month of life, 13 (22.8%) received a combination of breastfeeding and formula (“mixed feeding”), and only 4 (7.0%) were exclusively formula-fed. Seventeen (29.8%) infants continued breastfeeding until 6 months of age. Thirty-five (61.4%) infants were born vaginally, 27 (47.4%) had siblings and 21 (36.8%) lived with pets in the same household. A balanced representation of infant genders was maintained in this study (**Supplementary Table IV-1**).

At one month of age, exclusive breastfeeding ($R^2=0.1474$, $p=0.001$) explained the greatest variance in resistome profiles, followed by animals, delivery, and siblings (**Figure IV-1D**). Among these factors, only exclusive breastfeeding and mode of delivery had a direct significant impact on the resistome composition. Exclusively breastfed infants contained a lower ARG load and diversity

(**Supplementary Figure IV-1**), and their resistome composition significantly differed from that of non-exclusively breastfed infants ($p=0.01$) (**Figure IV-1E**). Similarly, while vaginally-born infants had a higher total ARG load in their gut compared to those born via C-section, resistome diversity was greater in C-section-born infants (**Supplementary Figure IV-1**). The resistome composition also differed significantly between vaginally and C-section-born infants ($p=0.031$) (**Figure IV-1F**). Notably, vaginally born infants who were not exclusively breastfed had higher ARG levels than those who were exclusively breastfed, regardless of delivery mode (**Figure IV-1G**).

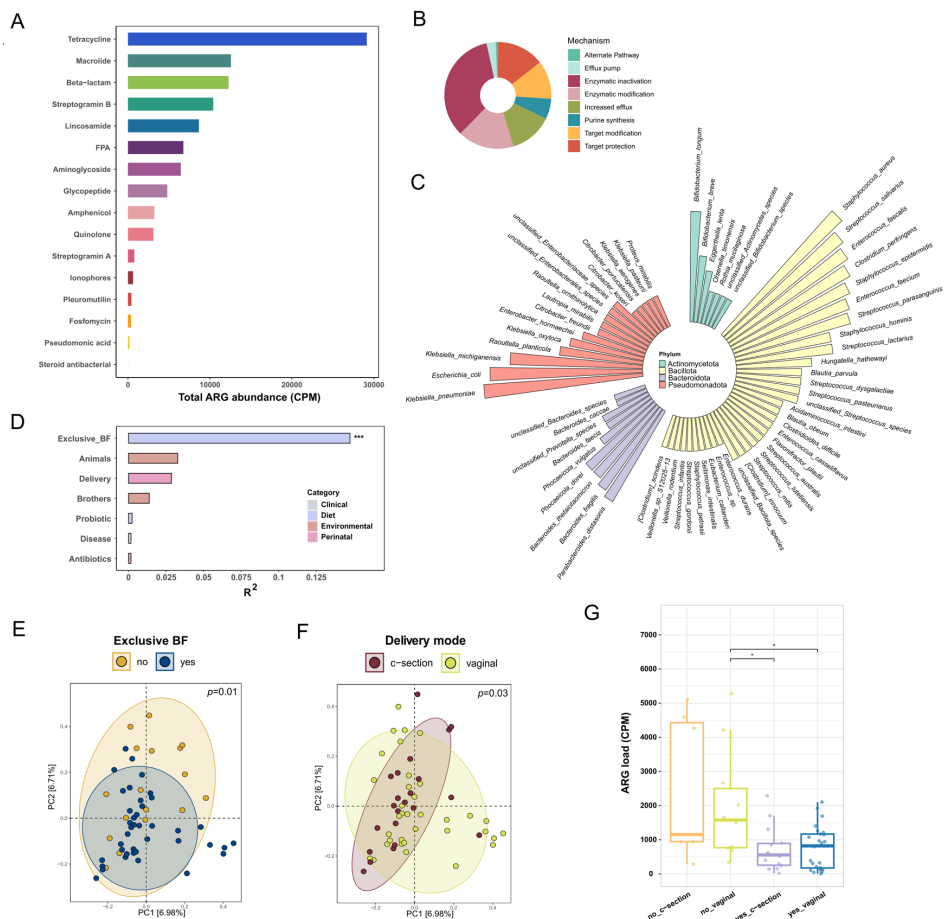


Figure IV-1. Resistome profile of the gut microbiota in one-month-old infants and the influence of perinatal factors. (A) The abundance of ARGs associated with different drug classes. **(B)** Mechanism of resistance of each ARG, depicted as a proportion of all ARGs detected in the cohort. **(C)** Distribution among bacterial species carrying ARGs. Bars

represent the total ARG among bacterial species and phyla. To facilitate viewing, only those species with at least two types of ARGs (a total of 52 species and 4 phyla) are shown. **(D)** Environmental factors driving infant's resistome ranked by effect size (*vegan* R package 'envfit' R2, false discovery rate (FDR)-corrected two-sided test, * $p < 0.05$). **(E-F)** Principal Coordinates Analysis based on Jaccard distances regarding the resistome composition according to exclusive breastfeeding **(E)** and mode of delivery **(F)**. **(G)** Antibiotic resistance load in the gut of infants during the first month of life in relation to mode of delivery and exclusive breastfeeding, expressed as total CPM of ARGs per infant in each timepoint.

4.2. Human milk harbors both a microbiome and a resistome

Considering the significant impact of breastfeeding on the infant's resistome, we next sought to identify specific milk components driving this influence. Milk samples (N=50) were collected from mothers of the infants included in this study and subjected to metagenomic sequencing (N=47) and/or HMO glycoprofilng (N=40).

The most abundant genera in the milk samples were *Streptococcus* and *Staphylococcus*, while *Streptococcus mitis* and *S. epidermidis* were found to be the most predominant species, followed by *Rothia mucilaginosa* and *Acinetobacter johnsonii* (**Supplementary Figure IV-2**). ARGs associated with resistance to macrolides, streptogramin B, pseudomonic acid, and beta-lactams were found to be highly abundant in human milk, followed by tetracyclines, lincosamides and glycopeptides (**Figure IV-2A**) *msr(D)*, *mef(A)*, *msr(A)* and *mph(C)* were the macrolide resistance genes most prevalent in the human milk samples (present in more than 48% of the samples). Beta-lactam resistance gene *blaTEM* was the next highly prevalent ARG (44.68% of the samples), followed by *tet(S/M)* (42.55% of the samples) (**Supplementary Table IV-5**).

The resistome of non-breastfed infants was markedly different from that found in human milk, with no shared ARGs. Similarly, the gut resistome of breastfed infants, regardless of whether their mothers were secretors, also differed from the resistome of milk (**Figure IV-2B-C**). Compared to the breastfed infants' resistome, human milk harbored more unique aminoglycoside and beta-lactam ARGs, but fewer unique ARGs conferring resistance to lincosamide, macrolide and

streptogramin B. On the other hand, amphenicol, FPA and quinolone ARGs were shared between the sample types (**Figure IV-2D**).

We observed that HMO composition of maternal milk varied based on secretor status. Specifically, 2'FL, 3SL, 6'SL, DFLac, LNFP I, LSTc, DFLNT, DFLNH, DSLNH were more abundant in the milk of secretors, while 3FL, LNT, LNFP II and FDSLNH were predominate in non-secretor milk (**Figure IV-2E**). Overall, milk from secretor mothers contained a significantly higher total HMO concentration than that of non-secretors ($p < 0.0001$). The median total HMO concentration was 12.19 mg/mL (IQR=11.93-12.34) and 8.24 mg/mL (IQR=8.06-8.308) for secretors and non-secretor, respectively.

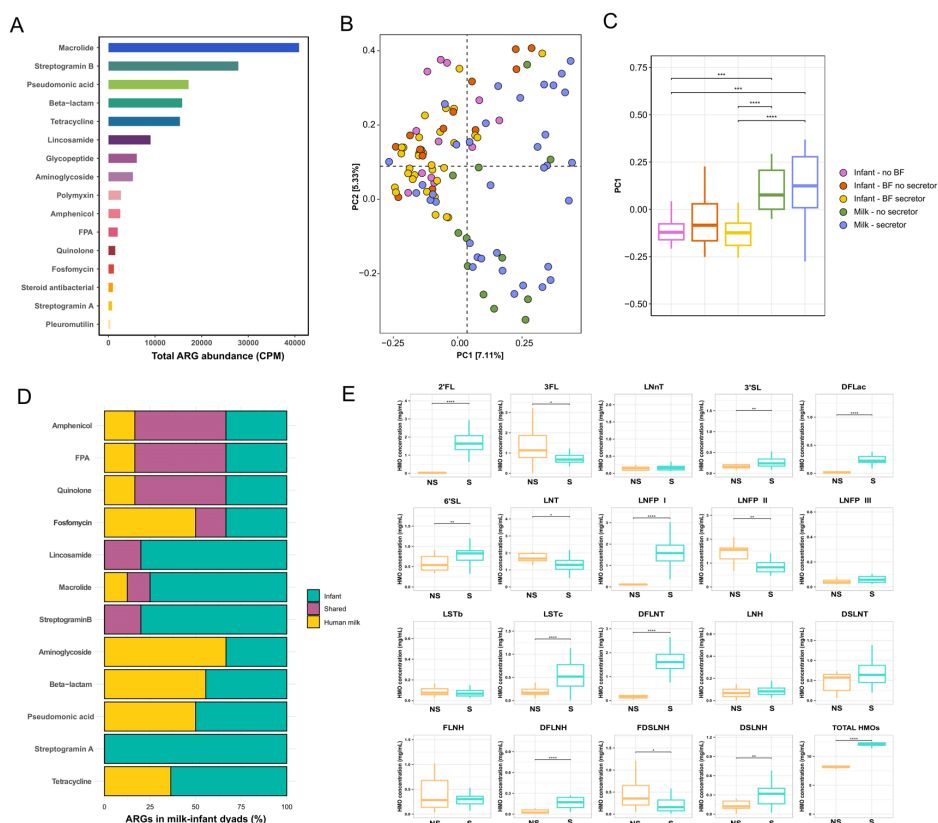


Figure IV-2. The human milk resistome and oligosaccharide composition. (A) The human milk resistome of samples collected at one month postpartum (N=47). **(B)** Principal Coordinates Analysis (PCoA) based on Jaccard distances regarding the resistome

composition of infant fecal and human milk samples, according to maternal secretor status. **(C)** Boxplot showing the differences along the first axis of the PCoA, assessed using multiple Wilcoxon tests. **(D)** Shared ARGs between human milk and breastfeeding infants' gut resistome. Abundance of assembled ARGs in mother-infant pairs at one month postpartum, classified as shared, unique in milk or unique in infants, and grouped by antibiotic class. **(E)** Concentration of individual HMOs (mg/mL) in maternal milk stratified by secretor status. BF: breastfeeding; NS: non-secretor; S: secretor.

4.3. Maternal secretor status influences milk composition and infant gut resistome

The milk resistome composition is different according to secretor status, as milk from secretors had a lower abundance of beta-lactam ($p=0.009$) and pseudomonic acid ($p=0.03$) ARGs (**Supplementary Figure IV-3A**). Similarly, milk from non-secretor donors showed a higher ARG diversity and load than that of secretor donors (**Figure IV-3**). No differences were found according to donor secretor status on the milk microbiome composition.

The overall gut microbiome composition of infants did not significantly differ based on their mothers' secretor status. However, infants that were breastfed by non-secretor mothers exhibited higher abundances of *S. salivarius*, *S. vestibularis*, *Phoeicola dorei*, and *K. pneumoniae*, while *S. vestibularis*, *Veillonella dispar*, and *L. reuteri* were highly abundant in infants fed by secretors (**Supplementary Figure IV-4**). Despite these microbiome similarities, resistome analysis revealed significant differences based on maternal secretor status ($p=0.007$). Infants receiving human milk from non-secretors harbored a greater abundance and diversity of ARGs than those receiving milk from secretors (**Figure IV-3**), particularly ARGs associated with macrolides and streptogramin B resistance (**Supplementary Figure IV-3B**).

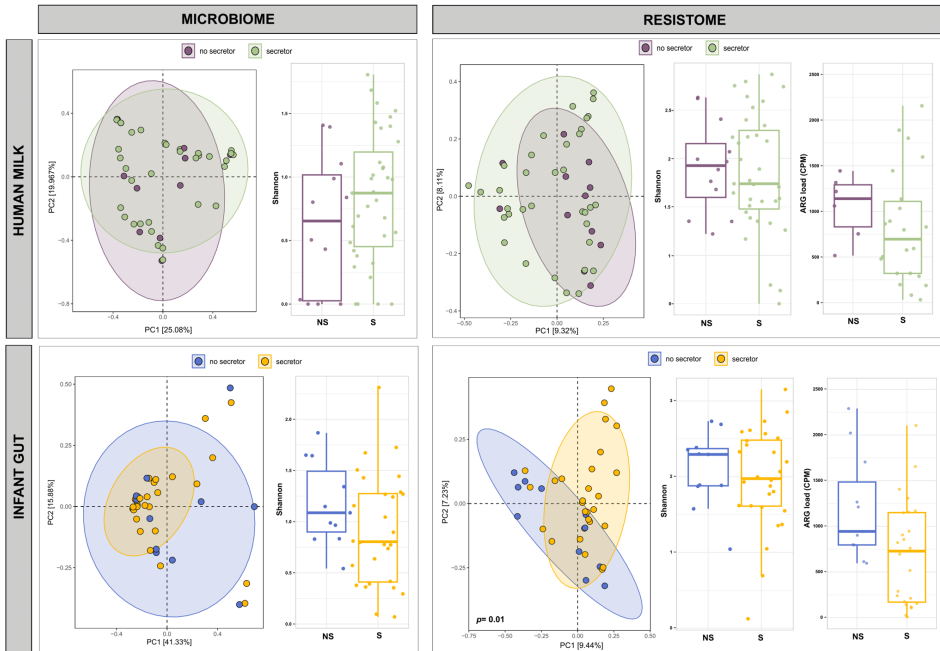


Figure IV-3. Maternal secretor status influences the maternal milk and infant gut microbiome and resistome. Principal Coordinate Analysis (PCoA) on the composition of the human milk and infant's gut microbiome and resistome based on Bray-curtis distances, stratified by maternal secretor status (secretor vs. non-secretor). Alpha diversity of the microbiomes and resistomes based on Shannon diversity index, and the antibiotic resistance load in the gut of infants and maternal milk, depending on maternal secretor status, expressed as total CPM of ARGs.

4.4. Maternal secretor status mitigates the impact of cesarean delivery on the resistome of breastfed infants

Given that breastfeeding and delivery mode had the greatest impact on the one-month-old infant resistome and considering the direct effect of mothers' secretor status on the breastfed infant resistome, the effect of delivery mode could be mitigated by the composition of mother's milk. Infants were categorized as either VS (vaginal-born infants breastfed by secretors), VNS (vaginal-born infants breastfed by non-secretors), CS (C-section-born infants breastfed by secretors) or CNS (C-section-born infants breastfed by non-secretors). As non-breastfed infants (nBF) were all born vaginally, only comparisons within that mode of delivery could be performed.

Delivery mode did not affect the resistome of infants breastfed by secretor mothers but had a significant impact on those fed by non-secretors ($p=0.021$) (**Figure IV-4A**). This suggests that in secretor-fed infants, milk composition overrides the influence of delivery mode, whereas in non-secretor-fed infants, delivery mode plays a key role. While both factors independently influenced the infant resistome, the impact of delivery mode was diluted when infants were breastfed by secretor mothers. CS harbored significantly more aminoglycoside, FPA and glycopeptide ARGs than VS ($p<0.05$), but less fosfomycin and macrolide ARGs than CNS ($p=0.03$ and $p=0.002$ respectively). Moreover, CS had fewer ARGs conferring resistance to amphenicol, quinolone and FPA compared to CNS ($p<0.05$). Indeed, CS infants contained a similar gut resistome profile to vaginal-born infants. The abundance of beta-lactams was driven by mode of delivery in infant's breastfed by non-secretors, as VNS had higher abundances of beta-lactams than CNS ($p=0.01$). This pattern was not observed in infant's breastfed by secretors. Indeed, VS had statistically similar beta-lactam abundance than CS, but higher than VNS ($p=0.04$). Lincosamide resistances were less abundant in non-secretor infants regardless of mode of delivery. Compared with exclusively formula-fed infants, VS had fewer amphenicol and FPA resistances but more resistance to macrolide and streptogramin B ($p<0.05$) (**Figure IV-4B**).

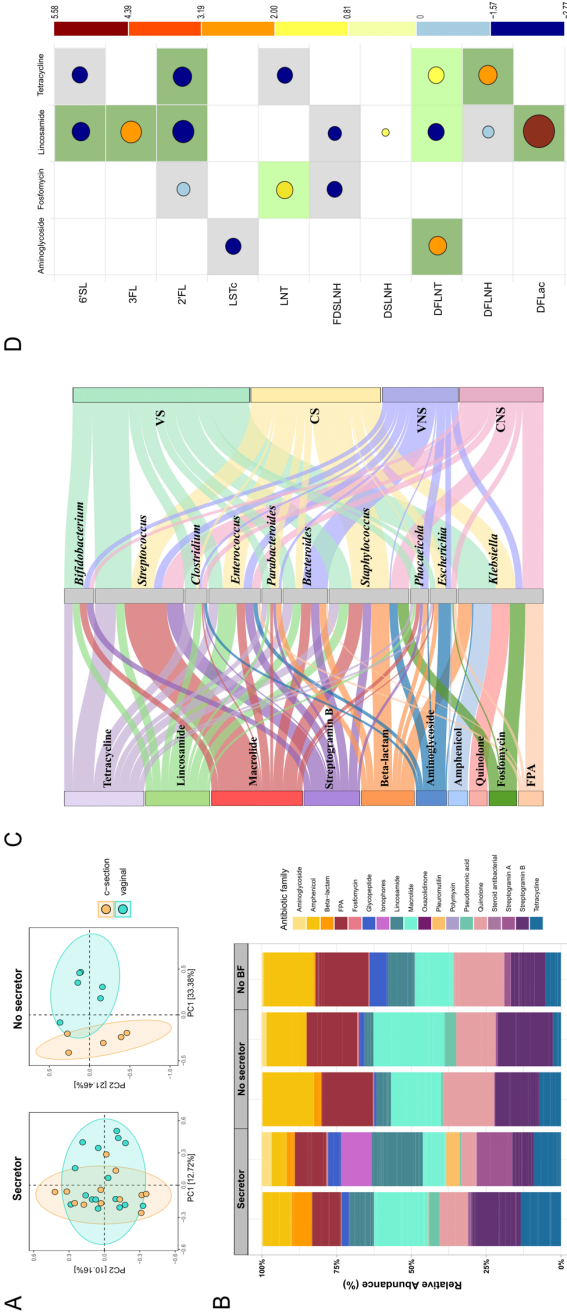
Considering that the infant gut microbiome and resistome composition varied with mode of delivery and maternal secretor status, we next sought to understand the specific microbial antibiotic resistance contributions within each group. CS contained more *Bifidobacterium* spp. than those born vaginally, while *B. bifidum* was highly abundant in VS compared to VNS and CNS ($p<0.05$) (**Supplementary Figure IV-5**). Compared to breastfed infants, non-breastfed infants had higher abundances of *Klebsiella* spp. In particular, *K. pneumoniae* was highly abundant in infants breastfed by non-secretor infants, regardless of delivery mode compared to infants breastfed by secretors ($p<0.05$). CNS contained higher abundances of *E. coli*, followed by VNS. CS had similar abundances of *E. coli* to VNS but significantly more than VS ($p<0.01$) (**Supplementary Figure IV-5**).

Streptococcus salivarius was significantly more abundant in CNS ($p < 0.05$ compared to CS, VS and VNS). For instance, tetracycline, lincosamide, macrolides, streptogramin B and betalactams were the most predominant resistances carried by bacteria. *Streptococcus* spp. are the major hosts of macrolide resistances, but also carry tetracyclines, lincosamide and streptogramin B resistances. *Staphylococcus* spp., associated with CS, hosted a diverse range of antibiotic resistances, including macrolides, betalactams, aminoglycosides and fosfomycin. *Escherichia* spp. carried aminoglycoside resistance genes and were present in all groups of infants. *Klebsiella* spp. were responsible for the majority of ARGs related to amphenicol, quinolone, fosfomycin, FPA and beta-lactam resistances, mainly to VS. *Bifidobacterium* and *Bacteroides* emerged as hosts of tetracycline, macrolide, lincosamide and streptogramin B resistances. While *Bacteroides* was mainly linked with VSN, *Bifidobacterium* was associated with VS (**Figure IV-4C**).

The associations between CPMs of ARGs for each antibiotic class and HMO concentrations are shown in **Figure IV-4D**. Among the HMOs negatively associated with ARGs, 2'FL exhibited a negative association with tetracycline and lincosamide, while 6'SL and DFNLT were negatively correlated with lincosamide. In contrast, positively associated HMOs included 3FL with lincosamide, LNT with fosfomycin, DFNLT with aminoglycosides and tetracyclines, DFNLH with tetracyclines, and DFLac with lincosamide.

► **Figure IV-4. Maternal secretor status modulates the impact of C-section delivery on the resistome of breastfed infants.** (A) Beta diversity of infants' resistome based on mode of delivery, stratified by mother's secretor status. (B) Antibiotic resistance profiles in each group. ARGs have been grouped according to the resistance(s) they confer. Statistical differences were determined using Mann–Whitney U tests to compare the abundance of each group of ARG. (C) Alluvial plot depicting the antibiotic resistance contributions of the 10 most abundant genera in the gut of breastfed infants, stratified by mode of delivery and maternal secretor status. Assignment of a microbial taxon to the resistance gene-containing contigs was performed for these analyses. ARGs have been grouped according to the drug they confer resistance to. VS: vaginal-born infants breastfed by secretors; VNS: vaginal-born infants breastfed by non-secretors; CS: C-section-born infants breastfed by secretors; CNS: C-section-born infants breastfed by non-secretors; no_BF: non-breastfed. (D) General linear models showing significant relationships between individual HMOs and ARGs in the gut microbiota of one-month-old infants. ARGs were grouped according to the type of antibiotic to which they confer resistance, including aminoglycosides, fosfomycin, lincosamide, and tetracycline. The colors on the right-side bar represent the range of statistical t-values, with

warmer colors indicating positive association, while cooler colors represent negative association. The magnitude of the t-value reflects the strength of the effect of HMOs on ARGs. Circle size reflects the magnitude of the test statistic value (absolute t-value). The highlighted boxes behind the circles indicate statistical significance based on the p-value: dark green = $p < 0.05$, light green = $p < 0.075$, and gray = $p < 0.1$.



(Figure IV-4)

5. Discussion

To the best of our knowledge, this is the first study to report an association between breastfeeding, specific bioactive compounds in human milk, particularly specific HMOs, and variation in the resistome of breastfed infants, potentially mitigating differences linked to C-section delivery. Our findings highlight the crucial role of HMO composition, determined largely but not exclusively by secretor status, in shaping the infant gut resistome. The observed association between breastfeeding and resistome composition is unlikely to be primarily driven by the direct transfer of bacteria and ARGs through milk, but may instead be linked to other components, especially HMOs. Once in the infant gut, HMOs can influence microbiota composition and, in turn, shape the resistome, potentially helping to counteract the effects of cesarean delivery.

In this study, we comprehensively analyzed ARG profiles in stool samples from 57 healthy one-month-old infants, in order to evaluate their relationship with the gut microbiome and key environmental exposures, including breastfeeding and mode of delivery. Mapping ARGs to specific taxa enabled us to identify genera carrying resistance genes, while statistical analyses confirmed that delivery mode and infant diet are major factors associated with resistome composition.

Notably, ARGs conferring resistance to antibiotics classified as highly and critically important by the World Health Organization (WHO)⁵⁰ were detected, despite the absence of direct antibiotic exposure. This suggests a potential role for vertical transmission or early environmental exposure⁴. In line with previous reports, members of the Bacillota and Pseudomonadota phyla, particularly *S. aureus*, *K. pneumoniae*, and *E. coli*, were among the most frequent ARG carriers⁵¹.

The developing infant gut resistome appears highly sensitive to environmental influences, with breastfeeding and the mode of delivery showing the strongest determinants of its composition. It has been widely reported that cesarean delivery disrupts gut microbiome development and increases ARG abundance^{6,7,10,52}. In contrast, breastfeeding is globally promoted for its health

benefits, with the WHO recommending exclusive breastfeeding for the first six months of life⁵³. Our findings build on previous work showing that maternal secretor status is associated with differences in HMO composition and infant gut microbiota^{54,55}, extending this association to include the resistome.

Building on this knowledge, we identified a direct association between maternal secretor status and the infant resistome. We observed that maternal secretor status was linked to differences in both milk HMO content and the infant resistome. While both delivery mode and secretor status were independently associated with resistome variation, the effect of delivery mode was less evident in infants breastfed by secretor mothers. This suggests that milk composition, particularly HMO content, may moderate the influence of delivery mode on the resistome. Breastfed cesarean-born infants of secretor mothers exhibited resistome profiles more similar to those of vaginally born infants, a pattern not observed in infants of non-secretor mothers.

Differences in the gut microbiota of infants also aligned with secretor status. Infants fed by secretor mothers showed higher abundances of *Bifidobacterium* species, particularly *B. bifidum*, which is known to degrade HMOs efficiently^{56,57}. In contrast, infants fed by non-secretor mothers had higher abundances of *Klebsiella* and *Escherichia*, regardless of delivery mode. These genera, which lack HMO-degrading capacity⁵⁸, may take advantage of ecological niches in the absence of dominant HMO-utilizing bacteria. Notably, *Bifidobacterium* was most abundant in vaginally delivered infants fed by secretors, while enterobacteria were more prevalent in infants of non-secretors, suggesting that secretor status may play a stronger role than delivery mode in shaping resistome-related microbial communities.

To further investigate this, we performed metagenomic analysis on human milk to characterize its microbial and resistome profiles. However, the technical challenges, such as the high proportion of human DNA in milk, hindered the retrieval of high-quality metagenomes, as observed in previous studies³⁰. Despite these limitations,

we successfully obtained high-quality metagenomes for 25 human milk samples, which allowed us to provide a description of their microbiome and resistome. Consistent with previous reports^{17,59}, *Staphylococcus* spp. and *Streptococcus* spp. were the most predominant genera in our human milk samples, reflecting the enrichment of oral and skin bacteria in milk⁶⁰. Although we identified ARGs in milk, particularly those conferring resistance to macrolides and lincosamides, the overall resistome composition of milk differed from that of infant stool. Furthermore, secretor status appeared to influence the milk resistome but not its microbial composition. These findings support the idea that breastfeeding may shape the infant resistome through non-bacterial milk components, such as HMOs, rather than direct microbial transfer. While human milk could be a potential source of ARGs for infants, our study did not focus on this aspect, as addressing it would require strain-level metagenomic analysis and larger cohorts. Future research using high-resolution techniques could provide further insights, building on existing evidence.⁶¹.

Our HMO glycorofiling confirmed that milk from secretor mothers had higher total HMO concentrations and distinct oligosaccharide profiles compared to non-secretors. Specifically, secretor milk contained higher levels of 2'FL and 6'SL, which were negatively associated with ARGs conferring resistance to tetracyclines and lincosamides. Conversely, 3FL and LNT, more abundant in non-secretor milk²², were positively associated with resistance to lincosamides and fosfomycin. These associations reinforce the link between milk composition and resistome variation and provide further evidence that differences in HMO profiles may influence resistome dynamics in early life.

While our study was not designed to determine causality, these findings contribute to a growing body of evidence suggesting that human milk composition, which is influenced by maternal genetics⁶², is associated with the early-life resistome. Screening for maternal *FUT2* status or milk HMO profiles could help identify infants more likely to harbor elevated ARG loads, particularly among those born by C-section. These infants may benefit from targeted interventions such as HMO

supplementation aimed at promoting microbiome maturation and potentially limiting ARG acquisition.

Despite providing novel insights and the application of high-resolution metagenomic and glycomic techniques, this study has limitations. The relatively small sample size and cross-sectional design may limit generalizability and preclude causal inference. Moreover, our analysis was restricted to one-month-old infants from a single geographic region, and the cohort included few exclusively formula-fed infants. Larger, longitudinal studies including diverse populations and additional feeding types are needed to validate these results and explore the long-term impact of early-life exposures on resistome development. Nevertheless, our study provides a foundation for future research into the role of breastfeeding and HMOs in modulating the infant resistome and offers potential avenues for intervention in infants at greater risk of developing antibiotic-resistant microbiota.

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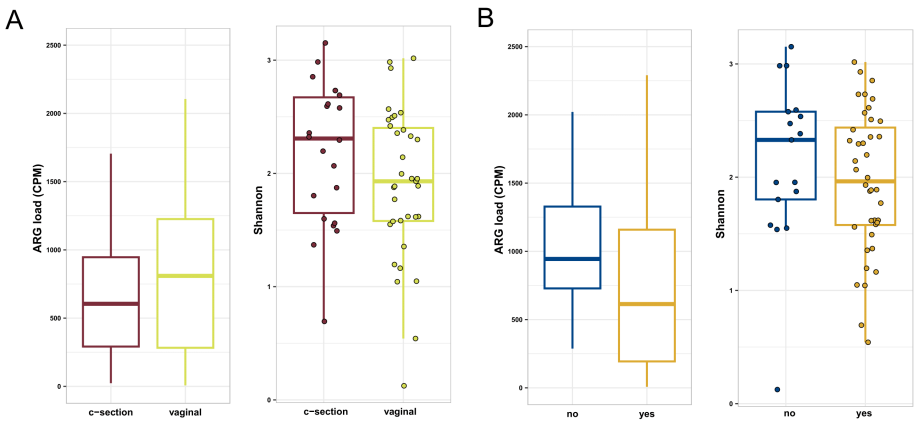
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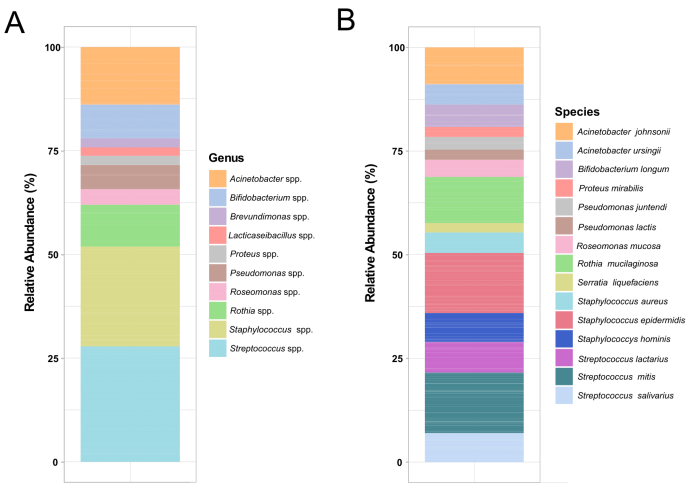
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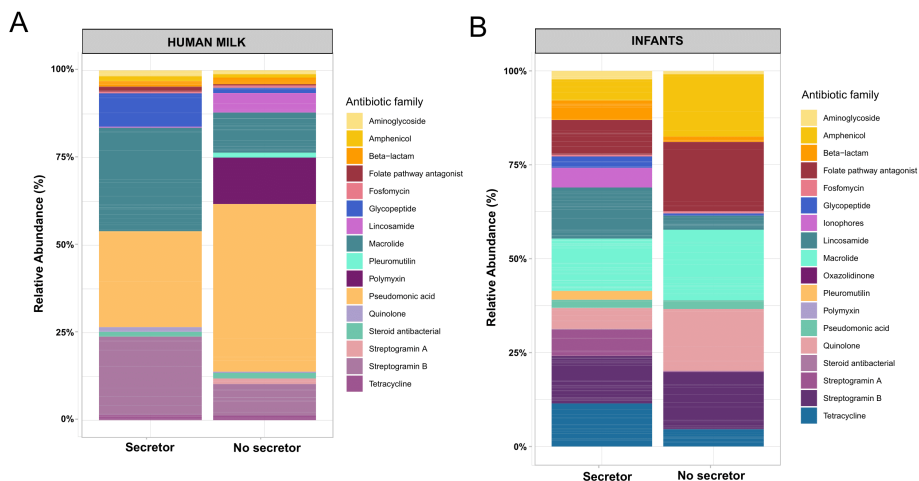
7. Supplementary materials



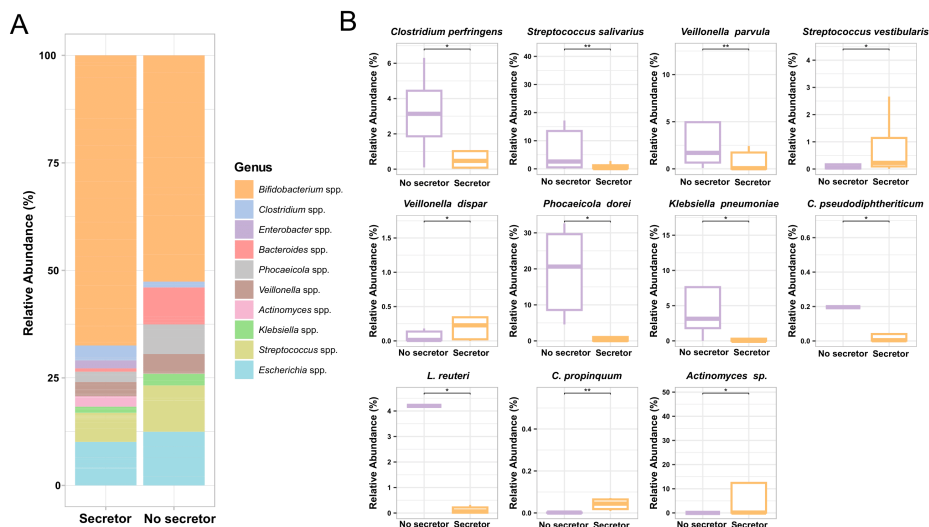
Supplementary Figure IV-1. Antibiotic resistance gene load, expressed as total CPM of ARGs, and alpha diversity of the infant gut resistome based on Shannon index, depending on mode of delivery (A) and exclusive breastfeeding (B).



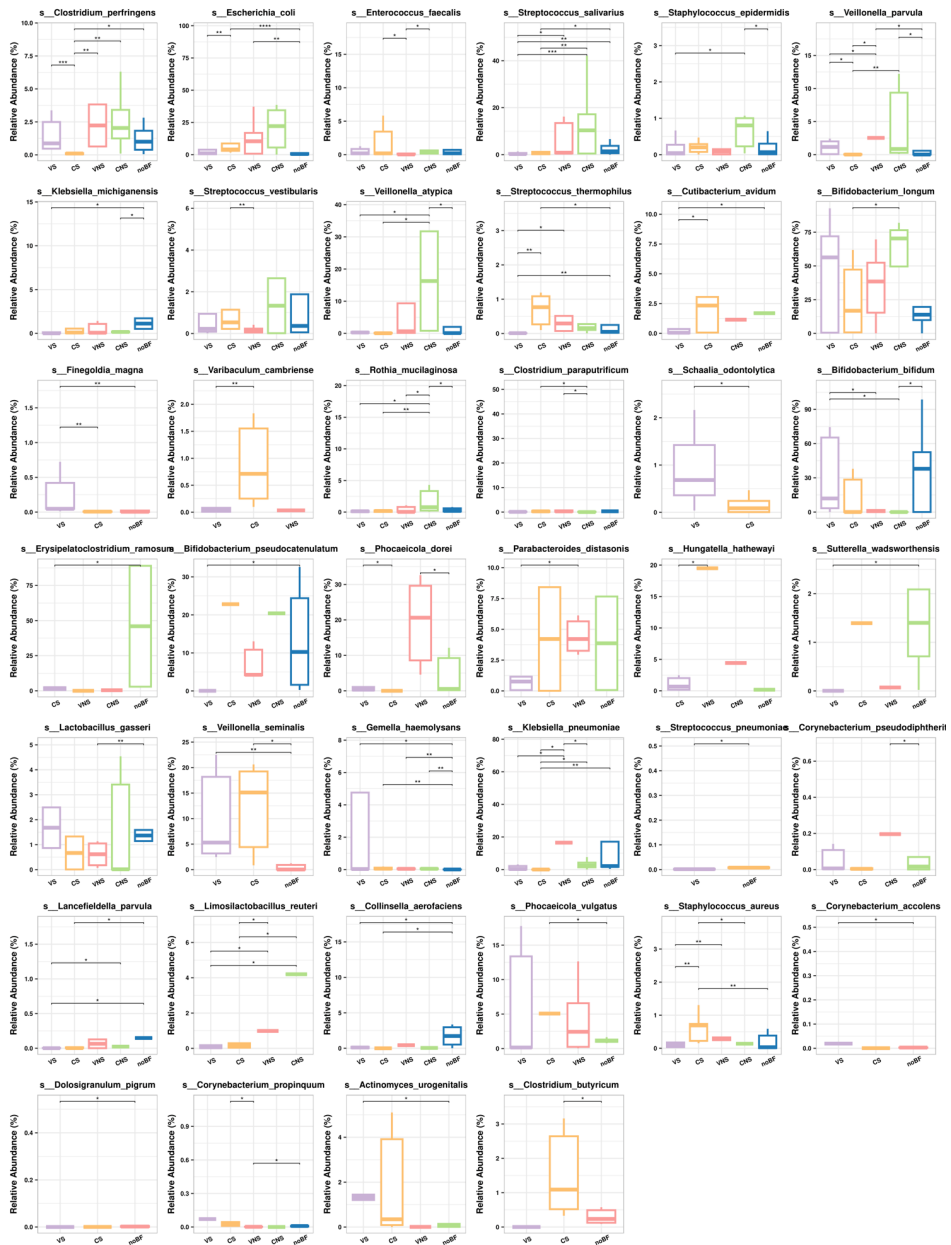
Supplementary Figure IV-2. Human milk microbiome profile at genus (A) and species (B) level.



Supplementary Figure IV-3. Antibiotic resistance profiles in human milk (A) and infant's gut (B), depending on maternal secretor status. ARGs have been grouped according to the resistance they confer.



Supplementary Figure IV-4. Infant's gut microbiome composition depending on maternal secretor status at genus level (A) and species level (B). Only significant species ($p > 0.05$, Wilcoxon rank sum test) are shown.



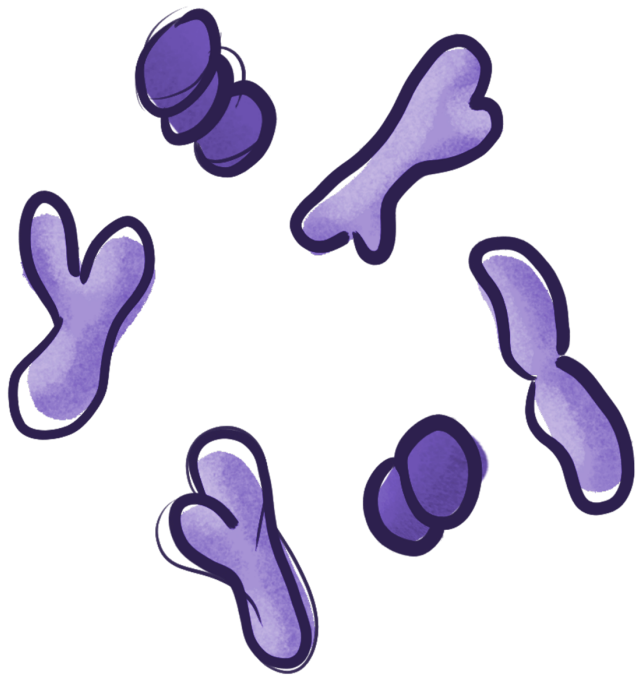
Supplementary Figure IV-5. Infant's gut microbiome composition at species level depending on secretor status and mode of delivery. VS: vaginal-born infants breastfed by secretors; VNS: vaginal-born infants breastfed by non-secretors; CS: C-section-born infants breastfed by secretors; CNS: C-section-born infants breastfed by non-secretors.

Supplementary Table IV-1. Characteristics of study participants.

Infant characteristics		Total (N=57)
Gender: female		31 (54.38%)
Birth mode		
	vaginal birth	35 (61.40%)
	c-section	22 (38.59%)
Diet		
	Breastfeeding	40 (70.17%)
	Mixed	13 (22.80%)
	Formula-feeding	4 (7.01%)
Antibiotic exposure		23 (40.35%)
Probiotic		5 (8.77%)
Siblings		27 (47.36%)
Pets		21 (36.84%)
Mother characteristics		
Gestational age (years)		39 [38-40]
Weight gain (kg)		13.436 ± 4.631
Pre-gestational BMI (kg m ⁻²)		23.104 ± 3.094
Antibiotic consumption during pregnancy (%)		16 (28.07%)

Categorical variables are expressed as positive cases-prevalence and (percentage, %). Normally distributed data are presented as mean ± standard deviation (SD) and non-normal data as median and interquartile range [IQR]. BMI: body mass index z-score.

Supplementary Table IV-2--5. (See Annex Chapter IV)



CHAPTER V

CHAPTER V

Human Milk Oligosaccharide Metabolism and Antibiotic Resistance in Early Gut Colonizers: Insights from Bifidobacteria and Lactobacilli in the Maternal-Infant Microbiome

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Samarra A, Renwick S, Arzamasov A., et al. Human milk oligosaccharide metabolism and antibiotic resistance in early gut colonizers: insights from bifidobacteria and lactobacilli in the maternal-infant microbiome. *Gut Microbes*. 2025;17(1):2501192. doi:10.1080/19490976.2025.2501192

My contribution to this research article was:

Conceptualization. Perform the experiments: bacterial isolation, DNA extraction, quantification, and purification. Growth kinetics and HPLC analysis. Data curation, statistical analysis and interpretation. Writing-original draft preparation.

This chapter presents results obtained during a research stay in Dr. Bode laboratory at the University of California San Diego (UCSD).

1. Abstract

Human milk, rich in human milk oligosaccharides (HMOs), supports the early-life colonization of beneficial bacteria such as bifidobacteria and lactobacilli, potentially reducing early-life antibiotic resistance. However, antibiotic treatment may interfere with the beneficial functions of HMO-degrading bacteria. This study investigated the metabolism of HMOs by bifidobacteria and lactobacilli isolated from human milk and mother-infant paired fecal samples, along with their antibiotic resistance profiles. Understanding these species- and sample-type-specific interactions will provide valuable insights into how bioactive components in human milk may shape the infant resistome during early life.

A total of 39 *Bifidobacterium* and 14 *Lactobacillaceae* strains were isolated from paired mother-infant fecal and human milk samples. Whole genome sequencing (WGS) allowed functional predictions on the HMO metabolism abilities and the resistance genotype of each strain. In vitro HMO utilization was assessed using growth kinetics assays combined with HMO glycoprofiling in culture supernatant. The minimum inhibitory concentration (MIC) was also determined for each strain.

HMO metabolism by the bifidobacteria was species-specific. *Bifidobacterium bifidum* (*B. bifidum*) and *Bifidobacterium longum* subsp. *infantis* (*B. infantis*) exhibited the highest capacity for HMO degradation, consistent with genomic predictions. In contrast, lactobacilli were unable to degrade HMOs in vitro but were predicted to metabolize the by-products of HMO degradation. Phenotypic analysis revealed that *B. bifidum* strains had the lowest levels of antibiotic resistance, while *Bifidobacterium animalis* subsp. *lactis* (*B. lactis*) strains were resistant to most tested antibiotics. Overall, *B. bifidum* demonstrated the strongest HMO-degrading ability while remaining the most antibiotic-susceptible species.

Early-life colonizing bifidobacterial species possess the essential machinery required to degrade HMOs and are highly susceptible to antibiotics. A better understanding of these dynamics could inform clinical strategies to protect

and restore the infant gut microbiome, particularly in neonates exposed to antibiotics.

2. Introduction

Human gut microbial colonization begins at birth when the neonate is exposed to microorganisms of the environment, primarily from maternal sources, which serve as the main microbial inoculum^{1,2}. The establishment and development of the microbiota occurs in parallel with other maturation processes and is modulated by external factors to which the infant is exposed during the early stages of life³. Among these, mode of birth is a widely known determinant of the infant gut microbiota.⁴⁻⁶ Additionally, antibiotic use during pregnancy, delivery, or following a Cesarean (C)-section can further disrupt maternal microbial transmission⁷. Antibiotic therapy in early life can alter microbiome diversity and disrupt microbiota-host crosstalk, resulting in microbial imbalances that can lead to short- and long-term health consequences⁸. Furthermore, the abundance of microorganisms carrying antibiotic resistance genes (ARGs) increases in the infant's gut following exposure to antibiotics, which has been linked to an increased risk of early-life infections⁹. The threat of antibiotic-resistant microorganisms is especially severe in newborns, whose underdeveloped gut microbiota and immune systems make them more vulnerable than adults. Globally, antibiotic resistance is responsible for approximately 214,000 neonatal deaths annually due to septic infections, underscoring its grave effects on infant health¹⁰. In early life, antibiotic resistance may emerge through multiple pathways, including horizontal gene transfer among bacteria, vertical transmission from the mother during pregnancy, childbirth, and breastfeeding, or direct exposure to antibiotics in infants¹¹. In this context, external factors to which the infant is exposed not only influence their microbiome, but also affect their resistome, which is defined as the set of ARGs carried by a population of bacteria³.

Breastfeeding plays a crucial role in shaping the infant's microbiome and resistome¹². Human milk provides personalized nutrition during the first months of

a baby's life through a wide range of bioactive compounds that promote infant health and mold the developing infant's microbiome¹³. In addition, human milk also serves as a source of commensal and potentially probiotic bacteria for the newborn's gut, with *Staphylococcus* and *Streptococcus* being the most dominant genera found in human milk, followed by members of the families *Lactobacillaceae* and *Bifidobacteriaceae*^{14,15}. These latter families are highly prevalent in the gut of breastfed infants due to their ability to utilize the prebiotic substrates found in human milk, such as human milk oligosaccharides (HMOs)^{16,17}. HMOs are a diverse group of structurally complex glycans that constitute the third most abundant solid component of human milk¹⁸. The concentration of HMOs varies among mothers and changes over time, depending on the stage of lactation¹⁹. The capacity to metabolize HMOs by bacteria is highly species-specific, with certain species demonstrating superior proficiency in metabolizing these complex carbohydrates due to the presence of a large array of carbohydrate-active enzymes (CAZymes) and glycan transporters encoded in their genomes^{20–23}.

With the rising concern over the early life acquisition of antibiotic resistance, early interventions like breastfeeding could protect children against the intestinal colonization of antibiotic-resistant microorganisms (ARM)^{24,25}. The species-specific capacity to utilize HMOs may play a crucial role in shaping the composition and functional dynamics of the infant gut microbiome and resistome. However, antibiotic treatment may disrupt the positive effect of HMO-degrading bacteria. The aim of this study was to explore the metabolism of HMOs by bifidobacteria and lactobacilli isolated from human milk and mother-infant paired fecal samples, as well as their antibiotic resistance profiles. Understanding these species-specific and sample-type-specific interactions will provide more evidence on the potential role of human milk in shaping the early-life resistome.

3. Materials and methods

3.1. Sample collection and ethics statement

A total of 39 maternal-infant fecal and mature human milk samples collected during the first month of life from the MAMI birth cohort ²⁶ were included in the study. Maternal-infant clinical records, including gestational age, neonate gender, birth weight, mode of delivery, type of lactation, and antibiotic exposure of the infant were collected (**Supplementary Table V-1**). All infants were born term and were exclusively breastfed at the time of sample collection. All participants received oral and written information about the study, and written consent was obtained. The study was approved by the Ethical Committee of Hospital Clínico Universitario de Valencia and CSIC. The trial was registered on the ClinicalTrial.gov (number NCT03552939).

3.2. Bacterial isolate collection

Bacterial isolations were performed in agar media including Difco™ Lactobacilli MRS Broth (Fisher Scientific, Hampton, NH, USA) supplemented with 0.05% cysteine and TOS Propionate Agar Base (Condalab, Madrid, Spain) supplemented with 0.005% mupirocin. For identification, morphologically unique colonies (1-7 maximum) were chosen from the agar media, re-streaked for isolation, grown in broth and viewed under a phase contrast microscope. Glycerol stocks were made from isolates and stored at -80°C for subsequent analyses. Colonies from the isolation step were further identified by sequencing of the 16S rRNA gene using the 27F-924R primer pair (27F 5'-GAGTTTGATCMTGGCTCAG-3' and 924R 5'-CTTGTGCGGGYCCCGTCAA-3'). The obtained sequence was searched on the BLAST database for the genus level taxonomic identification.

3.3. Human milk oligosaccharide utilization assay of bacterial strains

Bacterial growth on a pool of human milk oligosaccharides

The ability of the isolated bacterial strains to utilize a pool of HMOs was assessed as previously described²⁷. Briefly, pooled HMOs (pHMOs) were extracted

and purified following the method outlined by Jantscher-Krenn *et al.*²⁸. Human milk samples, donated by healthy volunteers to a milk bank, were pooled and centrifuged to remove the lipid layer. Cold ethanol was added to precipitate proteins, which were then removed by centrifugation. Ethanol was eliminated via rotary evaporation. Lactose (Lac) and salts were removed using size exclusion and fast protein liquid chromatography. The resulting oligosaccharide mixture was lyophilized and stored at -20°C until use.

Strains were cultured overnight in modified peptone-yeast-glucose broth (mPYG, **Supplementary Note 1**) under anaerobic conditions (5% H_2 , 10% CO_2 , balanced with N_2) in a chamber (Whitley A 45, Don Whitley Scientific, West Yorkshire, UK) to obtain the seed cultures for the assays. Assays were performed in diluted complex media, rather than minimal media, so that both positive and negative growth effects could be compared to a baseline level of growth. For anaerobic conditions, media was degassed prior to inoculation.

Strains were grown under 4 different conditions: 1) mPYG dextrose-free medium, as the negative control (mPYG); 2) mPYG medium (mPYG_{gluc}); 3) mPYG dextrose-free medium supplemented with 15 g/L of pHMOs (pHMOs); 4) mPYG medium supplemented with 15 g/L of pHMOs (pHMOs_{gluc}). Each medium condition was inoculated with 5% (v/v) seed culture in biological triplicate in 96-well plates and overlaid with 50 μL sterile mineral oil to prevent evaporation. Plates were transferred into a BioTek Epoch 2 microplate spectrophotometer (Agilent, Santa Clara, CA, USA) for overnight growth measurements, which was placed under anaerobic conditions (5% H_2 , 10% CO_2 , balanced with N_2). Plates were incubated for 24–48 h at 37°C while optical density (OD_{600}) readings were recorded every 30 min with 10s double orbital shaking immediately prior to each reading.

Individual HMOs and by-products utilization assays of bacterial strains

The growth of selected strains on individual HMOs or their degradation by-products was established as previously described^{29,30}, using the mPYG medium. For *Lactobacillaceae* strains, N-acetyllactosamine (LacNAc; Elicityl, Crolles,

France) and lacto-N-neotetraose (LNnT; Glycom, Esbjerg, Denmark) were added individually to the mPYG dextrose-free medium at a concentration of 4 mM. For *Bifidobacterium* strains, lacto-N-tetraose (LNT; Glycom, Esbjerg, Denmark) and LNnT were added to the mPYG dextrose-free medium at a concentration of 2 mM. Glucose (Sigma-Aldrich, St. Louis, MO, USA), Lac (PanReac AppliChem, Barcelona, Spain) and galactose (PanReac AppliChem, Barcelona, Spain) were used as positive controls of growth, and mPYG dextrose-free medium was used as a negative control. Briefly, the strains were cultured overnight in mPYG medium and diluted to an OD₆₀₀ of 0.1 in 200 µl of mPYG dextrose-free medium supplemented with each carbohydrate in 96-well plates. Cultures were grown at 37 °C under the anaerobic conditions described above. Bacterial growth was determined by measuring the OD₆₀₀ with Cerillo Stratus™ Microplate Reader (Cerillo, Charlottesville, VI, USA) every 30 min with a constant shaking of 80 rpm for 48 h. At least two independent biological replicates and three technical replicates were performed for each growth assay.

HMO utilization by each bacterial strain

The utilization of HMOs was estimated by measuring the HMO profile of the bacterial growth supernatants. Bacterial cultures were centrifuged 10 min at 5,000 rpm and supernatants were collected and stored at –80 °C. Each supernatant (20 µL) was spiked with maltose (20 mg/mL) as an internal standard at the beginning of sample preparation to correct for sample losses during sample processing and allow for absolute oligosaccharide quantification. Oligosaccharides were fluorescently labeled with 2-aminobenzamide (2AB, Sigma) at 65 °C for 2 h. Labeled oligosaccharides were analyzed by high performance liquid chromatography (Dionex Ultimate 3000, Dionex, now Thermo Fisher) on an amide-80 column (15 cm length, 2 mm inner diameter, 3 µm particle size; Tosoh Bioscience) with a 50-mmol L⁻¹ ammonium formate–acetonitrile buffer system. Separation was performed at 25 °C and monitored with a fluorescence detector at 360 nm excitation and 425 nm emissions. HMO peaks were annotated based on standard retention times and quantified in reference to the internal standard.

The measured HMO structures included 2'-fucosyllactose (2'FL), 3-fucosyllactose (3FL), 3'-sialyllactose (3'SL), 6'-sialyllactose (6'SL), difucosyllactose (DFLac), LNT, LNnT, lacto-N-fucopentaose (LNFP) I, LNFP II, LNFP III, sialyl-lacto-N-tetraose b (LSTb), sialyl-lacto-N-tetraose c (LSTc), difucosyllacto-N-tetraose (DFLNT), disialyllacto-N-tetraose (DSLNT), lacto-N-hexaose (LNH), fucosyllacto-N-hexaose (FLNH), difucosyllacto-N-hexaose (DFLNH), fucodisialyllacto-N-hexaose (FDSLNH) and disialyllacto-N-hexaose (DSLNH).

3.4. Antibiotic susceptibility testing on bacterial isolates

The resistance or susceptibility to antibiotics and the minimum inhibitory concentration (MIC) of bifidobacteria and lactobacilli isolates were determined following European Food Safety Authority (EFSA) guidelines³¹ and protocols described elsewhere³². Following the definition used by EFSA, a bacterium was deemed susceptible when its growth was inhibited at a concentration of a specific antimicrobial equal to or lower than the established cut-off value ($S \leq x$ mg/l); and was deemed resistant when it was able to grow at a concentration of a specific antimicrobial compound higher than the established cut-off value ($R > x$ mg/l). Hence, we performed the broth microdilution methodology to pre-screen which antibiotic resistances were exhibited by the isolates.

First, we prepared a battery of double-strength dilutions of the cut-off values of each antibiotic determined by the EFSA and distributed 50 μ L in 96-well plates. Bacteria were grown in Difco™ Latcobacilli MRS broth (Fisher Scientific, Hampton, NH, USA) supplemented with 0.05% cysteine, and 50 μ L were inoculated into each well from a standardized suspension in the same broth medium for a final concentration of 3×10^4 CFU/well. Plates were incubated for 48h and the final OD₆₀₀ was measured. Isolates showing resistance to any of the tested antibiotics were subject to obtaining MIC. For this, the same procedure as mentioned above was followed but included a series of two-fold double-strength dilutions representing the final concentration range of dilutions established by EFSA.

3.5. Whole genomic sequencing and data pre-processing

Bacterial isolates were grown overnight in a liquid culture and centrifuged for 6 min at 10,000 rpm to obtain the pellet for genomic DNA extraction. Briefly, each pellet was treated with lysozyme (20 ng/mL), liticase (500 U/mL) and mutanolysin (10 U/mL) for 40 min at 37 °C and cells were disrupted by bead-beating for 1 min at 6 m/s using 3-µm diameter glass and FastPrep 24-5G Homogenizer (MP Biomedicals). Proteinase K (12 ng/mL) was added following an incubation of 20 min at 65 °C and 15 min at room temperature. Next, MPC (Promega, Madison, WI, USA) was added to precipitate lipids and proteins, and the supernatant was taken into two consecutive centrifugations (15 min, 13000 rpm at 4 °C) with 70% and 96% ethanol, respectively. RNA was removed by treating each washed pellet with RNase A (5 mg/mL) for 1 h at 37 °C. Finally, 3M sodium acetate was added to precipitate the DNA, and another two consecutive centrifugations with 96% ethanol were performed. Finally, DNA was resuspended with 50 µL of MilliQ water for further purification and quantification. Purification of the DNA was performed using a DNA Purification Kit (Macherey-Nagel, Duren, Germany) according to manufacturer's instructions. DNA concentration was measured using Qubit 2.0 Fluorometer (Life Technology, Carlsbad, CA, USA). Genomic DNA isolated from liquid culture was used for library preparation with Illumina DNA Prep and Tagmentation Kit (Illumina, San Diego, CA, USA), and was subjected to a cleaning step (0.6× Agencourt AMPure XP beads). Whole genome sequencing was performed on Illumina Sequencing Platforms (Novaseq 6000 and Nextseq 2000) at the Department of Cellular, Computational and Integrative Biology, University of Trento (Trento, Italy) and at the Quadram Institute Bioscience (Norwich, UK).

Genome annotation and phylogenetic analysis

Quality of the reads was assessed using FastQC (version 0.11.5)³³. Specifically, reads were filtered for length, while those with a quality score less than Q 30 were discarded using Trimmomatic (version 0.39)³⁴. De-novo contigs were generated using SPAdes v 4.0.0 (parameters: --careful -k 21,33,55,77,99,127)³⁵. Assembly statistics computed using QUAST v 5.1.0rc 1³⁶. Genome completeness

and contamination were estimated with CheckM v 1.1.2³⁷. Mean coverage was computed using CMseq v 1.0.4 (<https://github.com/SegataLab/cmseq>). Genomes with completeness >95% and contamination <1.5% were included in the downstream analyses. Taxonomic assignment was performed using GTDB-Tk v.2.4.0³⁸ against bacterial and archaeal genomes based on the Genome Database Taxonomy (GTDB; Release 09-RS 220 (24th April 2024)). Unless specified, all the computational tools used in this work were applied with default parameters. Average Nucleotide Identity (ANI) was calculated using FastANI (with default settings (K-mer size = 16, threads count for parallel execution = 1, fragment length default = 3,000)³⁹. We analyzed multiple genomes using a genome list with command options of “—ql” and “--al.” For visualization, we used the “-matrix” command. For subsequent analysis, genomes with over 80% similarity were selected using FastANI. The assemblies and plasmid were annotated using Prokka (v.1.14.5)⁴⁰. A phylogenetic tree was constructed using all species from *Bifidobacterium* and *Lactobacillaceae*, following the methodology with GToTree (v 1.8.2)⁴¹, utilizing the protein sequences of 138 curated single-copy genes specific to *Actinomycetota* (i.e., *Bifidobacterium*), and 119 curated single-copy genes for *Bacillota* (i.e., *Lactobacillaceae*). Homologous sequences were aligned and concatenated using MUSCLE v 3.8.1551⁴². The resulting phylogenetic tree was constructed with FastTree v 2.1.10⁴³. The tree was rooted using *Bifidobacterium animalis* subsp. *lactis* (*B. lactis*) for *Bifidobacterium* and *Lactobacillus paragasseri* for *Lactobacillaceae*. The iTOL web tool version 6⁴⁴ was employed for the tree visualization and annotation.

Genomics-based reconstruction of HMO metabolism

We used a subsystems-based approach implemented in the SEED platform⁴⁵ for the reconstruction of HMO utilization pathways across the set of 39 *Bifidobacterium* and 14 *Lactobacillaceae* genomes. The genomes were annotated via RAST⁴⁶ and uploaded to mcSEED (microbial community SEED), a clone of the SEED annotation environment. Homology and genomic context-based methods in mcSEED were used to analyze the representation of groups of orthologous genes

implementing a curated set of 67 and 57 functional roles in bifidobacteria and lactobacilli, respectively, that contribute to HMO metabolism. These functional roles included components of glycan transporters, extracellular and cytoplasmic glycoside hydrolases (GHs), monosaccharide catabolic enzymes, and transcriptional regulators^{23,47}. The genomic distribution of functional roles was used to reconstruct glycan utilization pathways in two metabolic subsystems containing the genomes of bifidobacteria and lactobacilli isolates, respectively. For bifidobacteria, we reconstructed pathways for Lac, lacto-N-biose (LNB), LNT, LNnT, 2'FL, 3FL, DFLac, LNFP I, 3'SL, 6'SL, and lcHMO catabolism (**Supplementary Figure V-2** and **Supplementary Table V-2**). The lcHMOs are HMOs with a degree of polymerization ≥ 5 (e.g., LNFP II/III, LNDFH I/II) for which separate utilization pathways could not be unequivocally predicted due to the insufficient information on the substrate specificity of corresponding HMO transporters. For lactobacilli, pathways for individual HMO (LNT and LNnT), and the products of HMO degradation (Lac, LacNAc, LNB, and lacto-N-triose [LNTriose], L-fucose [Fuc], N-acetylneuraminic acid [Neu 5Ac], N-acetyl-D-glucosamine [GlcNAc], D-galactose [Gal], and D-glucose [Glc]) were reconstructed (**Figure V-4b** and **Supplementary Table V-3**).

The reconstructed pathways contained alternative biochemical routes implemented by different subsets of catabolic enzymes and diverse glycan transporters corresponding to different ecological strategies of HMO metabolism (extracellular vs. intracellular). To enable comparison across different strains, the detailed pathway variants were simplified to binary utilization phenotypes (1 and 0) corresponding to the predicted ability to metabolize a specific HMO species or its degradation by-products.

Identification of antibiotic resistance genes, virulence factors and plasmids

The ARGs were detected from the assembly output of Resistance Gene Identifier (RGI) v 6.0.3 with the CARD database v 3.3.0⁴⁸, and Strict significance based on CARD curated bitscore cut-offs. The virulence factors were identified by

ABRicate v 1.0.1 (<https://github.com/tseemann/abricate>) using the Virulence Factor Database (VFDB) ⁴⁹ with a cut-off coverage of 80 and 70% identification. Plasmids were detected by the Platon v 1.7 ⁵⁰, which is based on a taxon-independent approach to extract both plasmid and genomic sequences. The pathogenicity towards humans was predicted by PathogenFinder v 1.1 (<https://cge.food.dtu.dk/services/PathogenFinder/>).

3.6. Statistical analysis

All statistical analyses were performed using R version 3.6.0, and Figures were created with the *ggplot2* R package ⁵¹. For all methods, p-values were adjusted for multiple comparisons using the Benjamini-Hochberg (BH) method ⁵². Normality of the data was evaluated using Shapiro-Wilk tests. Categorical variables are expressed as positive cases-prevalence and (percentage, %). Normally distributed data are presented as mean $\pm$ standard deviation (SD) and non-normal data as median and interquartile range [IQR]. Pearson's Chi-square test or Fisher's exact test was used for categorical variables, and the Mann-Whitney U test was applied to continuous variables, to assess statistical significance.

Growth curve analysis

Growth curves were analyzed using the R package *gcplyr* ⁵³ which carries out model-free and non-parametric analyses to directly quantify attributes of growth dynamics. The underlying OD₆₀₀ of the base medium was subtracted from each condition at each timepoint (blank subtraction). The area under the curve (AUC), the maximum growth rate (r) and carrying capacity (k) were calculated to study the impact of HMOs on the growth properties of the strains.

Log fold changes in growth curve metrics were calculated by dividing the values of the growth curve metric from condition 1 by condition 2 and applying logarithmic transformation. Growth curve metrics between the different conditions of each strain were compared by Mann-Whitney U tests with p-values adjusted using the BH method.

HMO utilization analysis

HMO utilization at 24–48 h was calculated in reference to the initial HMO concentrations in the medium at the beginning of each experiment (t=0) and represented as the percentage of HMOs remaining in the medium. The *ComplexHeatmap* R package⁵⁴ was used to draw the HMO utilization profile of each group of species.

Resistance and HMO consumption profiles association analyses

Bray-Curtis distance-based Multivariate Redundancy Analysis (db-RDA) was conducted to test the correlation between HMO consumption capacity and antibiotic resistance profiles, by terms of MIC values. Both datasets were log transformed and the mean of overall HMO consumption and MIC values of each species were used for db-RDA. Antibiotic susceptibility was indicated as a MIC value of 0. The 'adonis 2' function from *vegan* R package⁵⁵ was used to perform permutations to evaluate overall differences in HMO consumption between species and to assess the association with the resistance profile (taken as the variables). Mann-Whitney U tests with p-values corrected using the BH method were performed to study the differences between the log transformed mean HMO consumption values and the log transformed mean MIC values of each species. The significance value was determined based on 999 permutations.

4. Results

4.1. Targeted isolation and genome sequencing of *Bifidobacteriaceae* and *Lactobacillaceae* strains

Isolates from maternal-infant paired fecal and human milk samples identified as *Bifidobacterium* (N=39) or *Lactobacillaceae* (N=14) with a mean genome coverage of 95.59% were included in this study (**Figure V-1**). The median genome size was 2.32 Mb with an average of 44.24 contigs per genome and the GC content of the genomes varied in the range between 34.74% and 62.90%, with a median of 59.63% (**Supplementary Table V-4**).

In summary, 22 (39.28%) unique isolates were obtained from infant fecal samples (N=15), 30 (53.71%) from maternal fecal samples (N=23), and 4 (7.14%) from human milk (N=3) (**Supplementary Table V-1**). The 39 *Bifidobacterium* strains belonged to 6 species: 3 *B. adolescentis*, 8 *B. lactis*, 8 *B. bifidum*, 7 *B. breve*, 2 *B. pseudocatenulatum*, 2 *B. infantis* and 9 *B. longum* subsp. *longum* (*B. longum*). The 14 *Lactobacillaceae* strains represented 7 species: 4 *Lacticaseibacillus paracasei*, 2 *Lacticaseibacillus rhamnosus*, 2 *Lactobacillus gasseri*, 4 *Lactobacillus paragasseri*, 1 *Lactilactobacillus sakei*, 1 *Limosilactobacillus mucosae*, and 1 *Ligilactobacillus ruminis*.

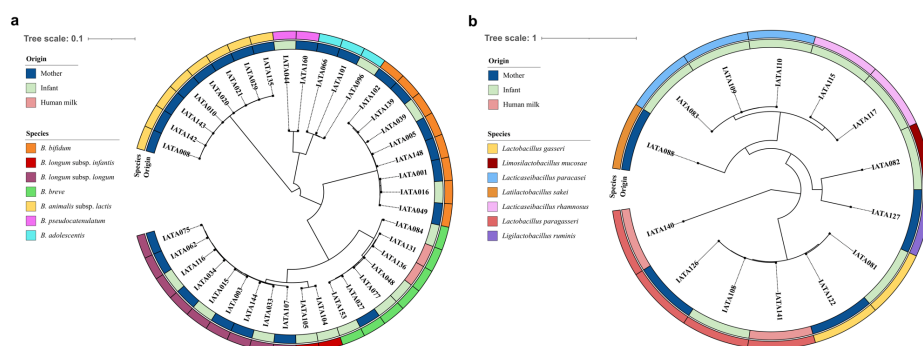


Figure V-1. Phylogenetic trees of *Bifidobacteriaceae* (a) and *Lactobacillaceae* (b) isolates from mother-infant pairs.

4.2. Bacterial growth on pHMOs

The growth of all *Bifidobacterium* isolates in the presence of glucose and/or pHMOs was assessed (**Figure V-2a**). All *B. bifidum* strains were able to grow with pHMOs as the sole carbon source, as indicated by a positive log fold change of pHMOs/mPYG. For most of the strains, pHMOs were a more favorable energy source as strains reached a slightly higher AUC and *r* compared to pHMOs_{gluc} treatment (**Figure V-2b**). Most isolates of *B. breve* and *B. infantis* displayed similar growth properties as the *B. bifidum* isolates. In addition, the growth of the two *B. pseudocatenulatum* strains tested was enhanced in the presence of pHMOs.

B. longum isolates showed strain-level heterogeneity in their growth properties under the various conditions tested. For *B. longum* strains IATA 015, IATA

033, IATA 062 and IATA 075, pHMOs resulted in a higher k compared to mPYG_{gluc} (Figure V-2a,b). In contrast, *B. longum* IATA 144 did not grow in the presence of only pHMOs (Figure V-2b). The remaining *B. longum* strains grew better in the presence of glucose compared to pHMOs despite an ability to utilize pHMOs as a sole carbon source. Interestingly, all *B. adolescentis* strains and most *B. lactis* strains, including IATA 135, IATA 143, IATA 021, and IATA 008, tested were incapable of using pHMOs as the sole carbon source but displayed enhanced growth in the presence of both pHMOs and glucose compared to glucose only. On the other hand, the growth of *B. lactis* IATA 020, IATA 029 and IATA 142 was not affected by pHMOs. Only *B. lactis* IATA 010 displayed an ability to metabolize pHMOs as the sole carbon source. Overall, *B. lactis* displayed the lowest capacity to grow on pHMOs alone, as strains displayed a lower log fold change of the AUC, r and k compared to other species tested (Kruskal-Wallis test, $p < 0.05$) (Figure V-2c). The ability of *Bifidobacterium* strains to metabolize HMOs was influenced by their isolation source. Strains isolated from infant stool produced a higher k (log fold change 5.18 ± 2.87) than those species isolated from mother's stool (log fold change: infants $= 5.18 \pm 2.87$, mothers 2.17 ± 1.71 , Wilcoxon test, $p = 0.04$).

Among the *Lactobacillaceae* strains tested, growth with pHMOs as the sole carbon source was poor ($OD_{600} < 0.3$). However, the strains displayed enhanced growth when both pHMOs and glucose were added to the medium, even higher than when only glucose was present (Supplementary Figure V-1). There were no significant differences in the activities of the *Lactobacillaceae* strains, nor did their behavior vary statistically based on their isolation source.

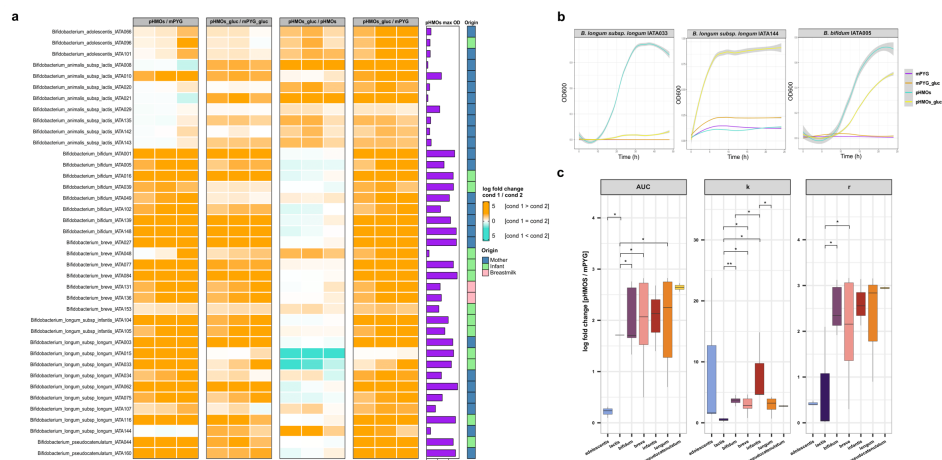


Figure V-2. Growth of *Bifidobacterium* isolates on human milk oligosaccharides. a) Growth of the 39 *Bifidobacterium* strains isolated from human milk and mother-infant fecal samples in diluted media supplemented with 4 carbon sources: pHMOs (pHMOs), pHMOs and glucose (pHMOs_gluc), glucose (mPYG_gluc), and no carbon source (mPYG). Log fold changes of area under the curve (AUC), carrying capacity (k), and growth rate (r) between the two conditions tested (condition 1/condition 2) are represented. A gradient-coloured in orange represents a higher growth curve metric of condition 1 in respect to condition 2, whereas a gradient-coloured in blue represents a higher metric of condition 2 in respect to condition 1. Horizontal bars in purple represent the highest OD reached in the condition of pHMOs as the sole carbon source (pHMOs). The origin of each isolate is indicated as a column annotation (from mother feces in blue, from infant's feces in green, and from human milk in pink). **Supplementary Table V-5** shows significant comparisons for each growth metric between treatments for each *Bifidobacterium* strain. **b)** Growth curves *B. longum* IATA 033, IATA 144, and *B. bifidum* IATA 005 under the four growth conditions. **c)** Mean log fold change between the pHMOs and mPYG conditions of the *Bifidobacterium* strains grouped by species. * $p < 0.05$, ** $p < 0.01$.

4.3. Genotype-to-phenotype predictions of HMO utilization

We analyzed the representation of reconstructed HMO utilization pathways encoded in the genomes of *Bifidobacterium* strains, and associated them with their HMO utilization profiles, expressed as the percentage of remaining HMOs in the culture supernatant after 48 h of growth (**Figure V-3** and **Supplementary Figure V-2**). A detailed description of genotype-phenotype comparisons can be found in **Supplementary Note 2**. Overall, the *in silico* predictions were in good agreement

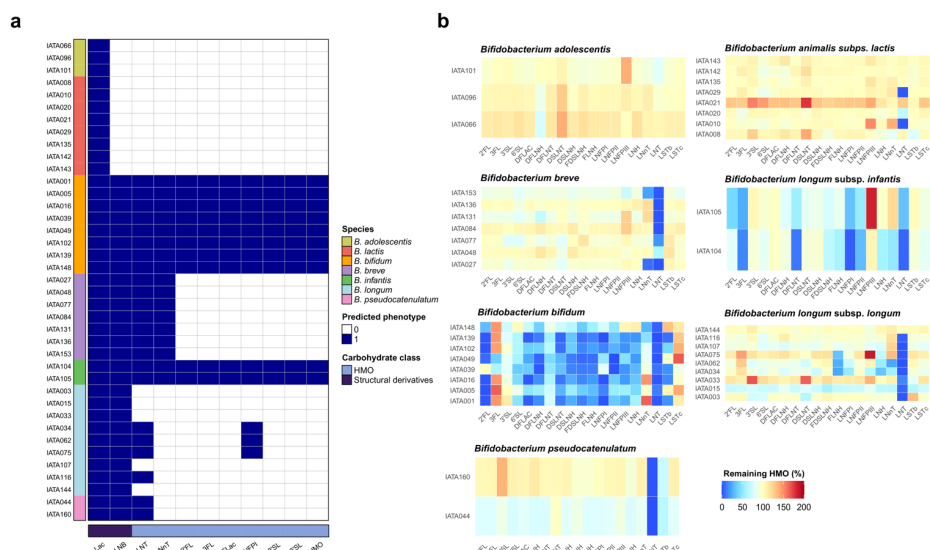
with *in vitro* HMO utilization profiles for most strains; however, several discrepancies were observed.

B. bifidum strains degraded most measured HMO structures consistent with the presence of multiple HMO-acting extracellular GHs encoded in the genomes of these strains (**Supplementary Table V-2**). However, some strain-level variability of HMO utilization was noted. For example, *B. bifidum* IATA 039 could not degrade 2'FL, likely due to a 5'-end truncation of the gene encoding the alpha-1,2-fucosidase (BbAfcA; GH 95) required for 2'FL metabolism⁵⁶ (**Supplementary Figure V-3**). We also observed an interplay between concentrations of 3FL and DFLac in the supernatants of all strains except *B. bifidum* IATA 049 and IATA 039 (**Figure V-3b**). The increased 3FL concentration could be explained by the unequal (separated in time) removal of fucose residues from DFLac by the alpha-fucosidases of *B. bifidum*. The concentration of LNnT in the supernatants of *B. bifidum* strains varied considerably, decreasing in some samples (IATA 049, IATA 102, IATA 139, IATA 148) and increasing in others (IATA 001, IATA 016). Since LNnT is an intermediate product of the degradation of multiple more complex HMOs (e.g., LNFP III), the observed results may reflect strain-level variability in the kinetics of long-chain HMO degradation. *B. bifidum* strains IATA 102, IATA 005, IATA 001, and IATA 049 were able to grow in the medium supplemented with LNnT as a sole carbon source, confirming their capacity to utilize this HMO (**Supplementary Figure V-4a**).

B. infantis strains efficiently utilized multiple HMO species, including 2'FL, 3FL, LNFP I, LNT, LNFP II, and DFLNT, consistent with the *in silico* predictions (**Figure V-3a**). *B. infantis* IATA 105, however, did not utilize LNnT, potentially due to mutations in genes encoding the respective utilization pathway. *B. longum* strains displayed varying capacities to utilize HMOs. *B. longum* strains IATA 034, IATA 062, and IATA 075 degraded LNT, LNFP I, LNH, and FLNH via a mechanism involving an extracellular lacto-N-biosidase (LnbX; GH 136)⁵⁷ (**Supplementary Figure V-2**). Other *B. longum* strains utilized only LNT, likely via an intracellular mechanism, although the variant of the LNT transporter (GltABC) found in most of these strains was previously shown to have low affinity to LNT⁵⁸.

All *B. pseudocatenulatum* strains were predicted to utilize LNT, which was confirmed by their ability to consume over 95% of the LNT present in the pHMO mixture (**Figure V-3a,b**). In agreement with *in silico* predictions, *B. breve* IATA 027 and IATA 153 efficiently consumed both LNT (>90%) and LNnT (>75%) (**Figure V-3b**). The remaining *B. breve* strains depleted only LNT. Consistent with glycoprofiling data, *B. breve* IATA 048 and IATA 131 grew in a medium supplemented with LNT but not LNnT (**Figure V-3c**). The discrepancy between genomic predictions and experimental observations of LNnT utilization could be attributed to mutations in the genes involved in the metabolism of this oligosaccharide. For example, in *B. breve* IATA 131, the *nahS* gene was disrupted by a premature sTop codon, suggesting that the encoded substrate-binding component of the LNnT-specific ABC transporter might be nonfunctional (**Supplementary Figure V-3**).

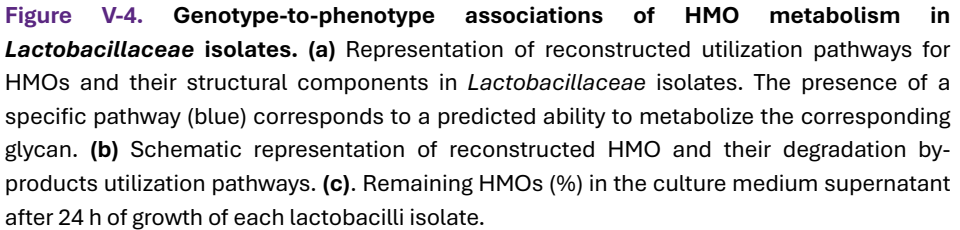
B. adolescentis and *B. lactis* strains were predicted to lack the capability to utilize any HMOs due to the absence of required transport systems and GHs (**Figure V-3a** and **Supplementary Table V-2**). Glycoprofiling results supported this prediction for most strains. Unexpectedly, *B. lactis* strains IATA 010, IATA 020, and IATA 029 depleted LNT from the pHMO mixture and grew in a medium where LNT served as the sole carbon source (**Supplementary Figure V-4a**).



◀ **Figure V-3. Genotype-to-phenotype associations of HMO metabolism in *Bifidobacterium* isolates.** (a) Representation of reconstructed utilization pathways for HMOs and their structural components in bifidobacterial strains. The presence of a specific pathway (blue) corresponds to a predicted ability to metabolize the corresponding glycan. (b) Remaining HMO (%) in the culture medium supernatant after 48 h of growth of each *Bifidobacterium* isolate.

We also analyzed the representation of HMO utilization pathways in 14 *Lactobacillaceae* and compared it with HMO glycoprofiling data (**Figure V-4** and **Supplementary Table V-3**). Unlike the bifidobacteria tested, most lactobacilli except *L. mucosae* IATA 082, *L. rhamnosus* IATA 115, and *L. sakei* IATA 088 were predicted not to utilize any HMO structures. Despite lacking complete HMO utilization pathways, all analyzed strains were predicted to metabolize mono- and disaccharide products of HMO degradation, namely, lactose, N-acetylglucosamine, galactose, and glucose, and some strains were predicted to also utilize N-acetyllactosamine, lacto-N-biose, and lacto-N-triose II (**Figure V-4a,b**).

L. mucosae IATA 082, *L. rhamnosus* IATA 115, and *L. sakei* IATA 088 were predicted to partially degrade LNnT via a mechanism involving an extracellular beta-galactosidase (**Supplementary Table V-3**). Consistent with this prediction, *L. mucosae* IATA 082 and *L. sakei* IATA 088 depleted LNnT from the pHMO mixture and grew in the medium with LNnT as the sole carbon source (**Figure V-4c** and **Supplementary Figure V-4b**). Notably, these were the only strains lacking the N-acetyllactosamine utilization pathway and failed to grow when N-acetyllactosamine was the only carbon source in the medium (**Supplementary Figure V-4b**). *L. rhamnosus* IATA 115, however, did not degrade LNnT present in the pHMO mixture. Other discrepancies between genomic predictions and experimental data include the unexpected partial degradation of LNFP III by *L. paracasei* strains IATA 083 and IATA 109 (**Figure V-4c**). Additionally, *L. mucosae* IATA 082 depleted LNT, LNH as well as fucosylated HMOs (LNFP III, FLNH, DFLNH) despite lacking genes encoding alpha-fucosidases (**Figure V-4c** and **Supplementary Table V-3**).



RGI analysis revealed 26 ARGs in bifidobacteria and 8 ARGs in the lactobacilli isolates, with perfect and strict hits (**Supplementary Figure V-5**). These genes were predicted to have > 80% sequence identity to well-characterized ARGs in the CARD database. The antibiotic resistance phenotypes (**Supplementary Table V-s 7,8** and **Supplementary Note 3**) and genotypes (**Figure V-5a**) of the *Bifidobacterium* and *Lactobacillaceae* strains were compared.

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genes were only identified in *B. breve* and *B. longum* strains. Although no beta-lactam resistances were identified at the genome level, 89.7% of the bifidobacteria were resistant to ampicillin.

The *tetW* gene, which confers resistance to tetracycline, was identified in the genomes of strains of *B. lactis*, *B. longum* and *B. breve*, all of which phenotypically exhibited resistance to tetracycline. In contrast, all *B. bifidum* and *B. infantis* strains, as well as 77.7% of *B. longum* strains, were susceptible to tetracycline. Interestingly, all *B. infantis* strains carried a *tetO* resistance gene despite displaying a susceptibility to tetracycline.

Resistance to aminoglycosides was tested experimentally with gentamicin and streptomycin. Only three *B. breve* strains carried two aminoglycoside resistance genes in their genomes (*aad* 6 and *aph*(3')-IIIa). However, only *B. breve* IATA 153 exhibited resistance to both gentamicin and streptomycin, while *B. breve* IATA 048 and IATA 077 were susceptible to gentamicin. Although no aminoglycoside resistance genes were found in their genomes, *B. bifidum*, *B. infantis*, and *B. longum* strains were generally resistant to gentamicin but susceptible to streptomycin.

All *Lactobacillaceae* strains were susceptible to chloramphenicol, while only *L. sakei*, *L. mucosae*, and *L. ruminis* strains were resistant to tetracyclines. Indeed, the *tetM* gene was identified in the genomes of *L. ruminis* isolates. *L. rhamnosus* strains showed lower levels of resistance. Namely, *L. rhamnosus* IATA 117 was susceptible to all antibiotics tested and *L. rhamnosus* IATA 116 was resistant to only erythromycin and ampicillin.

Although not tested *in vitro*, other resistances were detected in the genomes of the *Bifidobacterium* and *Lactobacillaceae* strains. These included resistance to lincosamides, mainly present in *B. breve*, *B. longum*, *L. rhamnosus* and *L. mucosae* strains. All *Bifidobacterium* strains contained *rpoB* mutants that confer resistance to rifampicin. Mupirocin-like resistance was identified in 9 bifidobacterial strains. Overall, these results emphasize the importance of

combining *in silico* and *in vitro* analyses to confirm that the detected ARGs are phenotypically expressed.

Genomic analyses also allowed the detection of lateral gene transfer (LGT) events in each strain, as well as the presence of plasmids (**Supplementary Table V-9**). All *B. infantis* strains and *B. longum* IATA 116 possessed the potential ability to exchange genes with species of *Clostridium*, *Eubacterium*, *Blautia* or *Coprococcus*. Similarly, *B. bifidum* IATA 005 and IATA 049 could potentially transfer genes to members of *Propionibacterium* and *Coprococcus*. On the other hand, the potential for LGT was detected in six of the 14 *Lactobacillaceae* isolates included in this study. *L. mucosae* IATA 082 displayed the potential to transfer genes to *L. fermentum* species. Moreover, the other lactobacilli strains displaying LGT capacity could both acquire genes from or transfer genes to *Enterococcus* spp. Finally, the plasmids detected in the bacterial isolates were primarily mobilization or replication plasmids, although some strains carried conjugation plasmids.

4.5. Associations between antibiotic resistance and HMO metabolism

We performed multivariate distance-based Redundancy Analysis (db-RDA) to examine the potential association between HMO metabolism capabilities and the antibiotic resistance profiles of the bacterial isolates (**Figure V-5b**). The analysis demonstrated that bifidobacterial strains of the same species clustered together according to their HMO metabolism capacity. *B. bifidum* strains formed a distinct cluster in the db-RDA ordination, supported by significant separation from *B. lactis* ($R^2 = 0.602$, $p = 0.021$), *B. breve* ($R^2 = 0.561$, $p = 0.042$), and *B. longum* ($R^2 = 0.539$, $p = 0.021$), as determined by PERMANOVA. Moreover, resistance to ampicillin ($R^2 = 0.196$, $p = 0.001$) and tetracycline ($R^2 = 0.883$, $p = 0.016$) were not associated with the *B. bifidum* cluster.

In fact, the contrast between the high capacity to consume HMOs and the low antibiotic resistance phenotype of *B. bifidum* was highly significant (Mann-Whitney U, $p = 0.003$) (**Figure V-5c**). A similar trend was observed in *B. longum* and *B. infantis*, though the differences were not statistically significant. In contrast,

other *Bifidobacterium* species, such as *B. lactis*, had a lower capacity for HMO utilization but a higher resistance to antibiotics (Wilcoxon test, $p=0.0003$).

The RDA analysis for *Lactobacillaceae* isolates did not yield conclusive results, primarily due to their limited ability to utilize HMOs (**Supplementary Figure V-6**). *L. mucosae* IATA 082 was the only strain capable of degrading HMOs and, accordingly, clustered separately from the other *Lactobacillaceae* strains, although this separation was not statistically significant. Overall, the *Lactobacillaceae* strains lacked both the ability to metabolize HMOs and resistance to the tested antibiotics, and no association between these two independent traits was observed.

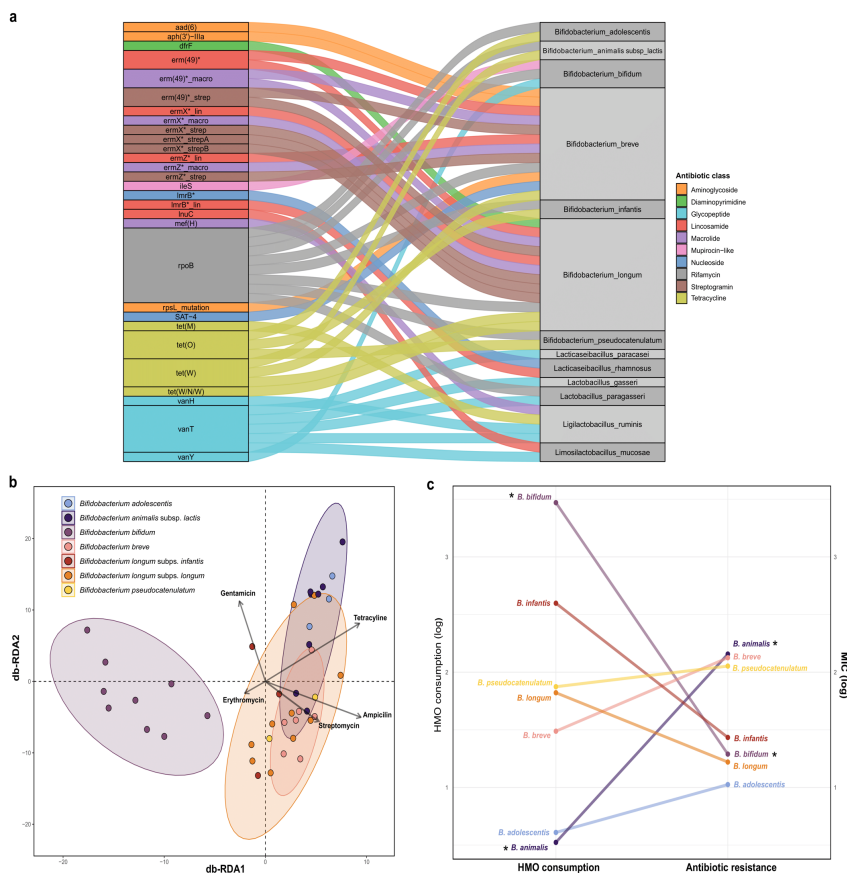


Figure V-5. Antibiotic resistance genes present in the genome of the *Bifidobacteriaceae* and *Lactobacillaceae* isolates and association with HMO metabolism. (a) Summary of the ARGs detected in each of the bacterial genomes, coloured by class of antibiotic

resistance. An asterisk (*) next to the gene name indicates that it encodes for multiple resistance factors. Bacterial genomes are grouped by species. **(b)** Distance-based Redundant Discriminant Analysis (db-RDA) biplot depicting the relationship between the HMO degradation profile and antibiotic resistance of each strain, coloured by species of *Bifidobacterium*. In db-RDA, angles between the arrows represent correlations; acute angles represent positive correlations and obtuse angles represent negative correlations. The adonis-pairwise was performed to evaluate overall differences in HMO consumption between species and to assess the association with the resistance profile (taken as the variables). Mann-Whitney U test with p-values corrected using the BH method was performed to study the differences between the log transformed mean HMO consumption values and the log transformed mean MIC values of each species. **(c)** Differences in HMO consumption (log of remaining HMOs) and antibiotic resistances (log MIC values) of *Bifidobacterium* species. Susceptibility to an antibiotic was considered as a MIC value of 0. An asterisk (*) indicates differences that were significant ($p < 0.05$).

5. Discussion

With growing concern over the early-life acquisition of antibiotic resistance, interventions such as breastfeeding may offer protection by limiting intestinal colonization with antibiotic-resistant microorganisms¹². One proposed mechanism involves HMOs, which selectively nourish beneficial bacteria in the infant gut. Species-specific HMO utilization may significantly influence the composition and function of the infant gut microbiome and indirectly affect early-life acquisition of antibiotic resistance. However, external factors like antibiotic treatment can disrupt this process by hindering the establishment of HMO-degrading bacteria, thereby reducing their positive effects. This disruption may favour the growth of pathogenic and antibiotic-resistant microorganisms⁵⁹. Understanding the strain-level capabilities for HMO metabolism and the impact of antibiotics on early-life colonizers is therefore critical for evaluating long-term consequences for the infant resistome.

This study aimed to enhance our understanding of strain-specific HMO metabolism among early-life gut colonizers and to investigate how antibiotic exposure may influence these microbes. We found that HMO utilization by bifidobacteria is highly species-specific. *B. bifidum* and *B. infantis* exhibiting the most extensive capacity to degrade HMOs, consistent with their genomic profiles, and are known early colonizers of the breastfed infant gut. In contrast, *B. longum*

and *B. breve* showed more limited HMO-degrading capabilities, although some *B. longum* strains possessed genes encoding extracellular lacto-N-biosidase, supporting a stronger metabolic potential. These findings are consistent with previous studies that have documented interspecies variation in HMO utilization and validated corresponding metabolic pathways at the genomic level^{17,23,60,61}. On the other hand, *B. pseudocatenulatum*, *B. adolescentis* and *B. lactis*, species commonly associated with the adult gut, did not efficiently degrade HMOs and lacked the necessary genetic machinery for their metabolism, in concordance with previous literature⁶².

Interestingly, some *Bifidobacterium* strains exhibited enhanced growth and more efficient HMO consumption in the presence of HMOs compared to glucose or HMOs and glucose combined. This highlights their specific and unique capacity to metabolize HMOs efficiently and suggests a potential role for glucose metabolism in regulating HMO-degrading pathways. However, further experiments are needed to validate these observations.

Regarding antibiotic resistance, *B. bifidum* strains showed the lowest resistance levels, whereas *B. lactis* displayed resistance to multiple antibiotics. Notably, *B. bifidum* emerged as the species with the highest HMO degradation capacity and the lowest antibiotic resistance, emphasizing its potential significance in early-life gut colonization. Together, these findings underscore the pivotal role of breastfeeding in shaping the infant gut microbiome by promoting the growth of HMO-degrading bacteria, particularly bifidobacteria. At the same time, our results highlight the potential disruptive effects of antibiotic exposure in early life, which could interfere with the establishment of key microbial species critical for infant health.

In the case of *Lactobacillaceae*, it has been previously reported that their ability to utilize HMOs is not as widespread as *Bifidobacterium* members^{17,20,21}. Consistent with this, we identified fewer HMO-active GHs in the genomes of the *Lactobacillaceae* strains compared to the *Bifidobacterium* strains. Many

Lactobacillaceae members are capable of metabolizing HMO-derived components, such as glucose, Lac, LacNAc, LNB and GlcNAc, to support their growth, classifying them as “secondary degraders”^{63,64}. Consistent with these reports, we identified the relevant metabolic pathways in *L. rhamnosus* and *L. paracasei*. Furthermore, in both our *in vitro* and *in silico* analyses, *L. mucosae* emerged as one of the most versatile carbohydrate-degrading *Lactobacillaceae* species, followed by *L. sakei*, with no previous literature documenting these findings.

As observed in this study, HMO degradation profiles vary significantly among species and their coexistence in the infant gut microbiota may reflect complex metabolic interactions^{65–67}. Certain species possess enzymes that cleave more complex HMOs to produce by-products that can be degraded by other taxa, thus acting as a driver of community structure through the provision of substrates for others to consume⁶⁸. For example, *Lactobacillaceae* members may depend on the presence of bifidobacteria or other gut microbes to liberate di- and monosaccharide components from HMOs to support their growth⁶⁹. Although the present study focused on HMO metabolism in monocultures, future studies involving co-cultures or complex fecal microbial communities could provide deeper insights into these interspecies metabolic interactions and their role in early-life gut microbiome development.

We found that both bifidobacteria and lactobacilli strains harbored ARGs and exhibited resistance *in vitro*, despite reports that they carry fewer ARGs than other genera and are less prone to horizontally transfer of ARGs^{70–72}. In some cases, the resistance may be the result of punctual mutations in specific genes^{32,73}. *B. bifidum* and *B. infantis* were more susceptible to antibiotics than *B. lactis* and *B. adolescentis*. *L. sakei*, *L. mucosae* and *L. ruminis* strains were resistant to tetracyclines, though the majority of the identified genotypic resistances were related to glycopeptides. Given the widespread acceptance of lactic acid bacteria as probiotics with established benefits for infant gut health, the ability of some strains to survive antibiotic exposure could potentially be advantageous. For

example, *B. pseudocatenulatum* strains have previously been noted for their antibiotic survivability in this context⁷⁴. While some bifidobacterial strains exhibited intrinsic resistance to antibiotics, generally considered low-risk for horizontal transfer⁷³, our analysis of LGT and plasmid content revealed the potential cross-species exchange of ARGs between bifidobacteria, lactobacilli, and other gut microbes.

Discrepancies between the *in silico* and *in vitro* antibiotic resistance profiles also emerged in our findings. Discrepancies may arise from regulatory mechanisms, mutations that disrupt the potential function, or new and/or evolving resistance mechanisms not captured in ARG reference databases. Moreover, a strain may be resistant to an antibiotic although no related ARGs are identified. For example, we found that *B. lactis* strains were the most resistant to antibiotics despite their genomes containing only two known ARGs. This highlights the critical need to consistently corroborate genomic predictions with phenotypic results.

Our findings reveal an inverse relationship between HMO degradation capabilities and antibiotic resistance. Strains such as *B. bifidum*, *B. infantis*, and *B. longum*, frequently isolated from infant sources, efficiently degrade a variety of HMOs yet remain highly susceptible to antibiotics. Conversely, *B. lactis* and *B. adolescentis* exhibit greater antibiotic resistance but a reduced ability to utilize HMOs. Only one previous study has linked the development of the infant resistome to microbial carbohydrate metabolism during early life²⁵. That study, using metagenomic functional profiling and metagenome-assembled genome analyses, demonstrated that shifts in microbial carbohydrate metabolism correspond to the temporal development of the gut resistome. Our findings align with these previous reports and are further supported by our *in vitro* observations. These results underscore the crucial role of breastfeeding in shaping the infant gut microbiome by fostering the growth of HMO-degrading bacteria, particularly bifidobacteria. However, early-life antibiotic exposure may disrupt this process by depleting these beneficial bacteria, thereby favoring antibiotic-resistant strains. Since early-life colonizers are highly susceptible to antibiotics, their suppression could impair

microbial cross-talk, such as interactions between bifidobacteria and lactobacilli, ultimately compromising the protective effects of breastfeeding and HMOs in mitigating antibiotic resistance. Given their ability to selectively promote beneficial bacteria, HMOs hold promise as a potential therapeutic to support microbiome resilience and counteract the adverse effects of early-life antibiotic exposure.

In conclusion, our findings demonstrate that temporal variation in *Bifidobacterium* and *Lactobacillaceae* species in the infant gut is influenced not only by their specific ability to utilize HMOs but also by their antibiotic resistance profiles. We have highlighted that early-life antibiotic exposure can disrupt colonization by beneficial HMO-degrading bacteria, impeding their role in supporting a balanced gut microbiome, while, in turn, promoting the growth of antibiotic-resistant bacteria. Future research should explore the transmission dynamics of these species and ARG reservoir in complex environments to elucidate the trajectory of an infant's resistome. This includes investigating the role of breastfeeding, human milk composition, and the impact of antibiotic treatment, while ultimately aid in the proper monitoring and management of antibiotic prescriptions.

Strengths and Limitations

This study employed a combination of methods to model the prediction of antibiotic resistance and the degradation of HMOs, focusing on bifidobacteria and lactobacilli within the maternal-infant microbiotas. By identifying ARGs, the study assessed the impact of antibiotic use on neonatal gut colonization, laying groundwork for strategies to maintain beneficial microbiota and curb early-life antibiotic resistance spread. Culturing bacteria from mother-infant pairs and examining glucose's effect on HMO degradation provided insights into microbial metabolism. However, the study faced limitations including a small sample size, reducing the generalizability of the findings. Its cross-sectional, region-specific design restricted exploration into microevolution and comparisons across different geographic populations. Additionally, differences in diet between breastfed infants and adults complicated comparisons of HMO degradation and antibiotic resistance

among strains. Future research should focus on expanding the collection and analysis of beneficial strains, incorporating broader genomic data, and conducting longitudinal studies to better understand microbial adaptation and the persistence of beneficial strains in the gut over time.

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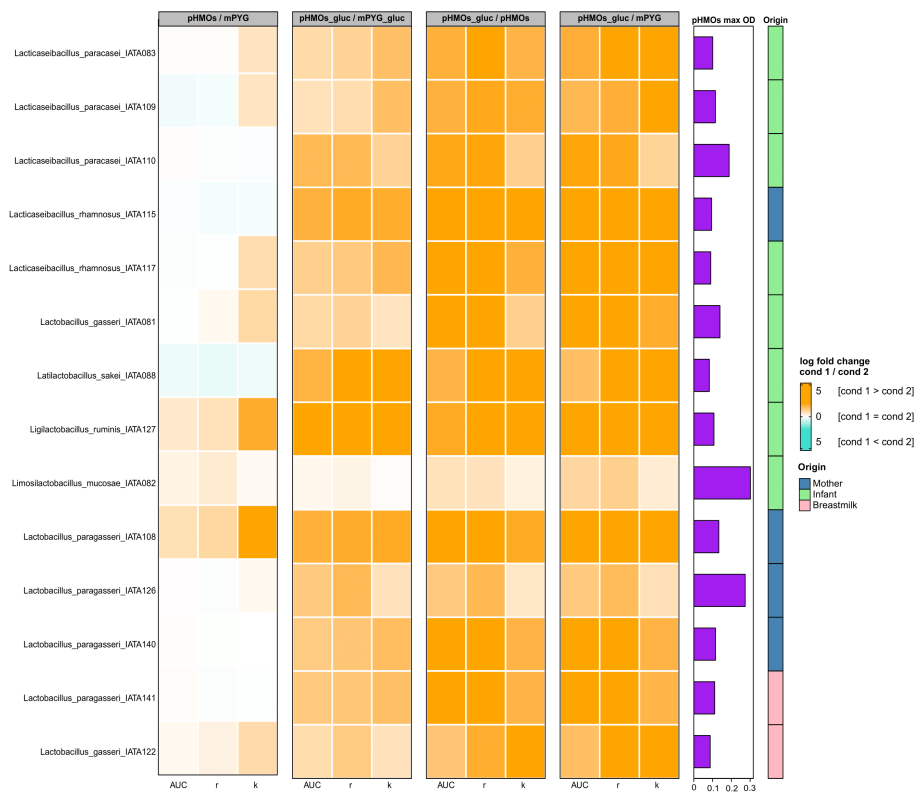
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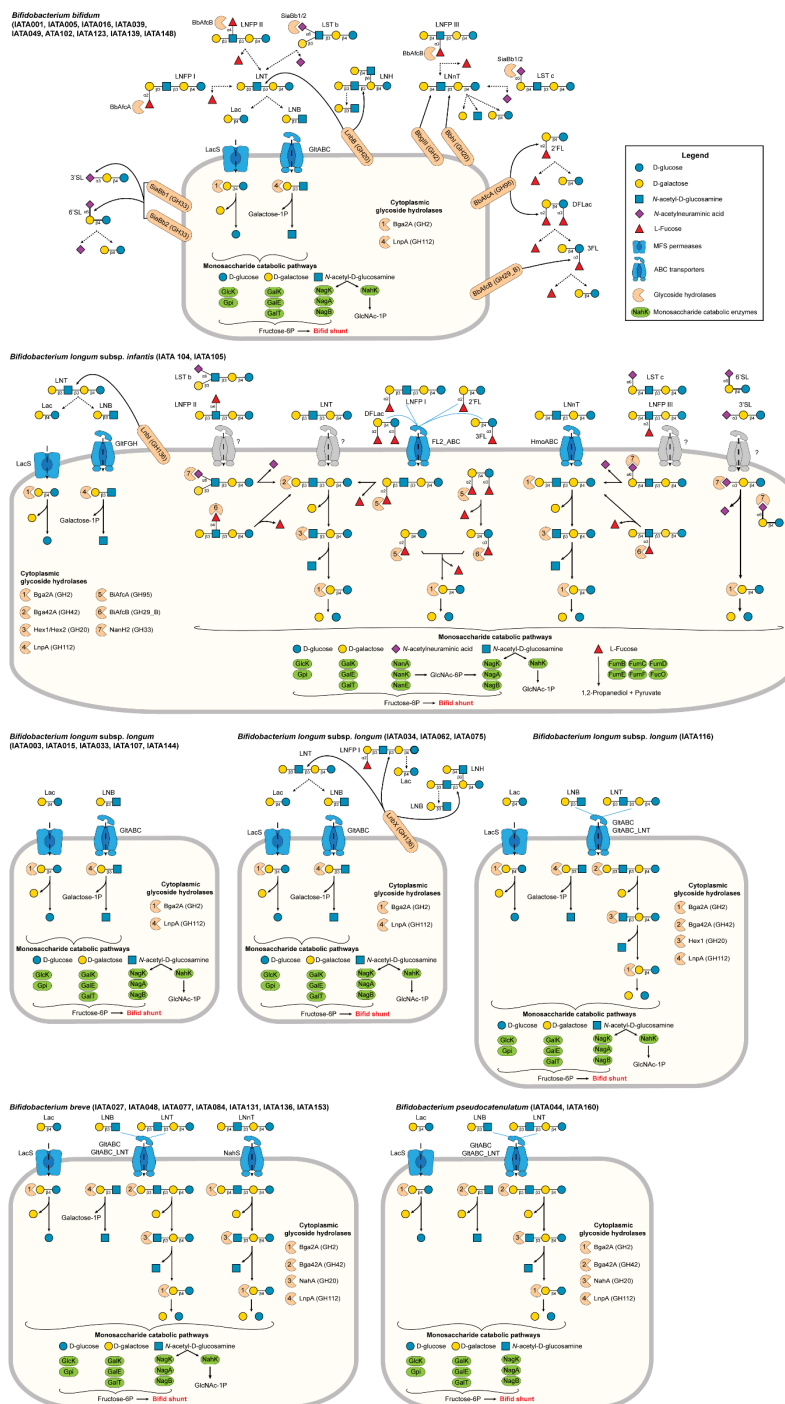
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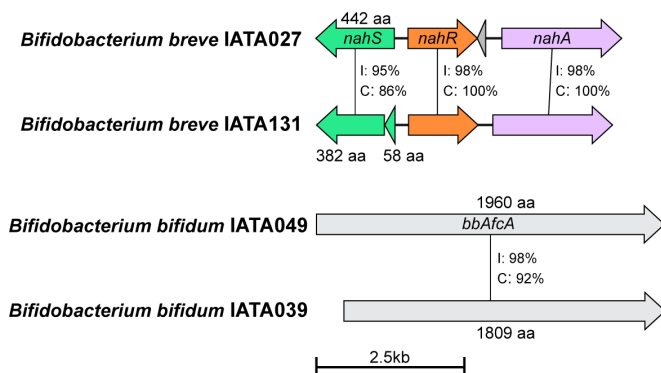
7. Supplementary material



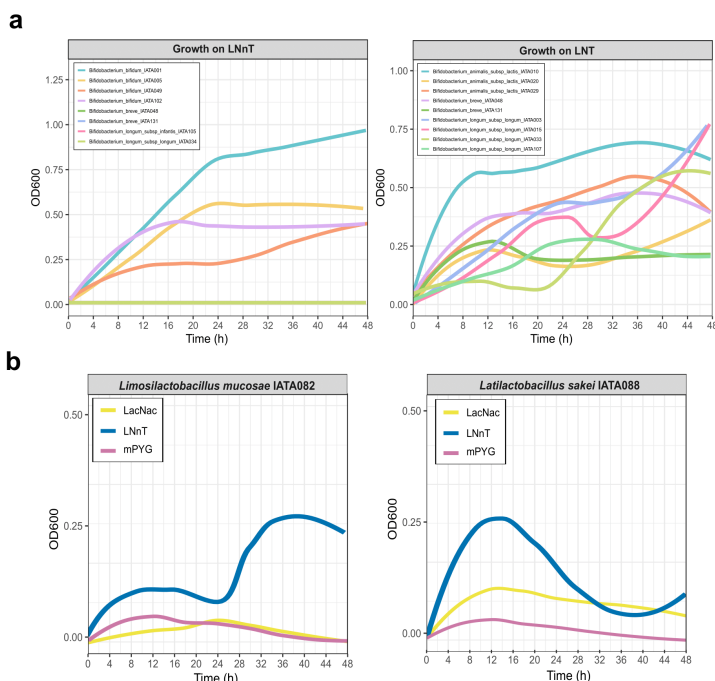
Supplementary Figure V-1. *Lactobacillaceae* growth on human milk oligosaccharides. Growth of 14 *Lactobacillaceae* strains isolated from human milk and mother-infant fecal samples under the 4 growth conditions. Representation follows the same patterns as in Figure V-2a. **Supplementary Table V-6** shows significant comparisons for each growth metric between treatments for each strain.



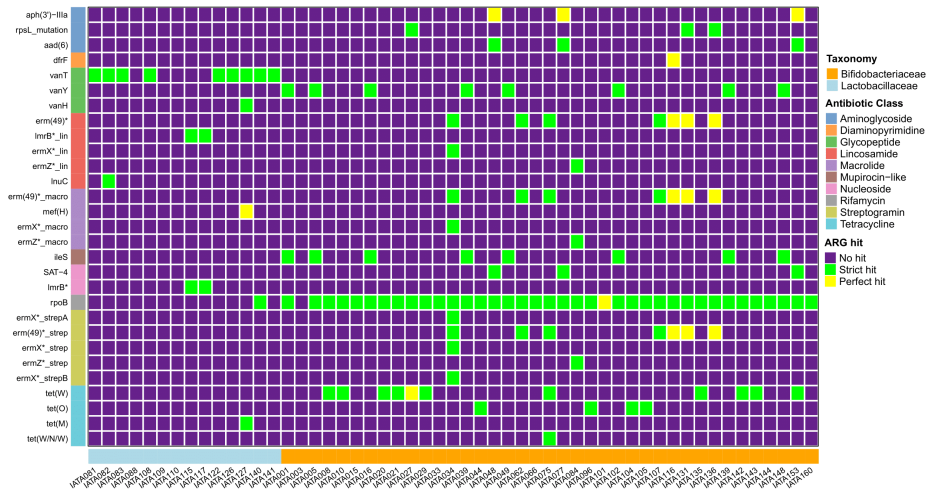
Supplementary Figure V-2. Schematic representation of reconstructed HMO utilization pathways in bifidobacteria.



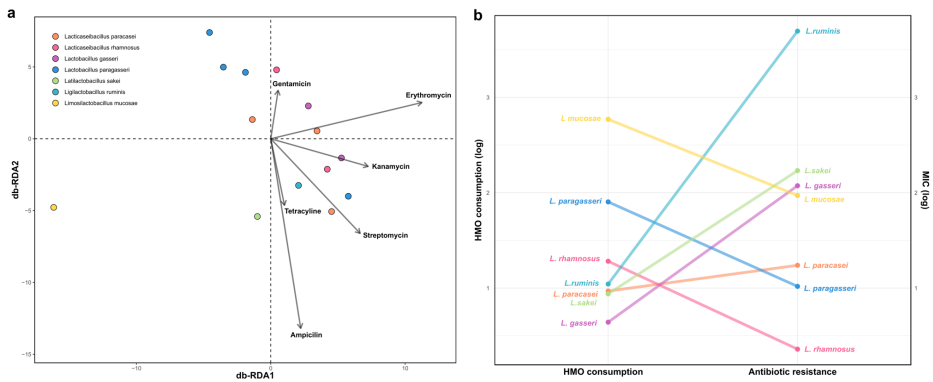
Supplementary Figure V-3. Gene clusters depicting the 5'-end truncation of the gene encoding the alpha-1,2-fucosidase (BbAfcA; GH 95), required for 2'FL metabolism in *B. bifidum* IATA 039, and the disruption of *nahS* gene in *B. breve* IATA 131, due to a premature sTop codon.



Supplementary Figure V-4. Bacterial growth on individual HMOs and by-products. (a) Growth of specific strains in the medium supplemented with LNT or LNT as the sole carbon source. **(b)** Growth of specific strains in the medium supplemented with LNT or LNT as the sole carbon source.



Supplementary Figure V-5. Heatmap showing ARGs detected in each genome. In the heatmap, purple represents no hit, yellow represents perfect hit, and green represents strict hit.



Supplementary Figure V-6. Association between HMO metabolism and antibiotic resistance profiles. (a) RDA biplot depicting the relationship between the HMO degradation profile and antibiotic resistance of each strain, colored by species of *Lactobacillus*. In RDA, angles between the arrows represent correlations; acute angles represent positive correlations and obtuse angles represent negative correlations. (b) Differences in HMO consumption (log-transformed of consumed HMOs) and antibiotic resistances (log-transformed MIC values) *Lactobacillus* spp. Susceptibility to an antibiotic was considered as a MIC value of 0. Asterisk (*) indicates significant differences ($p < 0.05$).

Supplementary Table V-1. Sample information.

Supplementary Table V-2. Predictions of bifidobacteria HMO metabolism.

Supplementary Table V-3. Predictions of lactobacilli HMO metabolism.

Supplementary Table V-4. Genome quality.

Supplementary Table V-5. Results of the statistical analysis of the comparison of each growth metric between the different treatments bifidobacteria were exposed.

Supplementary Table V-6. Results of the statistical analyses of the comparison of each growth metric between the different treatments lactobacilli were exposed.

Supplementary Table V-9. Lateral gene transfer and plasmids of each genome.

Given their considerable size, these tables are available in the supplementary materials hosted in the *Gut Microbes* journal repository, which are accessible under the following doi: 10.1080/19490976.2025.2501192.

Supplementary Table V-7. *Bifidobacterium* spp, phenotypic antibiotic resistance and MIC (µg/mL).

	GEN	STREP	TET	ERY	CHLORA	AMP	VAN
<i>B. adolescentis</i>							
IATA 066	256	S	S	16	S	8	S
IATA 096	S	512	64	16	S	8	S
IATA 101	R	S	S	R	S	S	S
<i>B. animalis subsp. lactis</i>							
IATA 008	256	256	64	16	S	16	S
IATA 010	256	S	32	16	S	32	S
IATA 020	256	S	32	16	S	32	S
IATA 021	256	256	64	16	S	S	S
IATA 029	256	S	0.5	16	S	S	S
IATA 135	512	512	64	16	S	32	S
IATA 142	512	256	64	16	S	8	S
IATA 143	512	256	64	16	S	8	S
<i>B. bifidum</i>							
IATA 001	512	S	1	16	S	4	S
IATA 005	512	S	S	16	S	S	S
IATA 016	256	S	S	16	S	4	S
IATA 039	256	64	0.5	16	R	4	S
IATA 049	512	S	S	16	S	S	S
IATA 102	256	S	S	16	S	2	S
IATA 139	S	S	S	16	S	2	S

IATA 148	256	512	32	16	R	16	S
<i>B. breve</i>							
IATA 027							
IATA 048	S	2048	64	16	S	8	S
IATA 077	S	2048	64	16	S	4	S
IATA 084	256	S	S	16	S	32	S
IATA 131	256	2048	S	16	S	32	S
IATA 136	256	2048	S	16	S	32	S
IATA 153	256	1024	64	16	S	8	S
<i>B. longum subsp. infantis</i>							
IATA 104	32	S	32	16	S	8	S
IATA 105	32	S	32	16	S	4	S
<i>B. longum subsp. longum</i>							
IATA 003	S	S	S	16	S	16	S
IATA 015	R	S	S	16	S	16	S
IATA 033	128	64	0.5	16	R	32	S
IATA 034	S	S	S	16	S	32	S
IATA 062	128	S	S	16	S	32	S
IATA 075	256	S	S	16	S	32	S
IATA 107	128	S	S	16	S	32	S
IATA 116	256	S	S	16	S	32	S
IATA 144	128	S	2	16	S	32	S
<i>B. pseudocatenulatum</i>							
IATA 044	256	128	64	8	R	32	S
IATA 160	32	64	1	16	R	32	S

GEN: gentamycin; STREP: streptomycin; TET: tetracycline; ERY: erythromycin; CHLORA: chloramphenicol; AMP: ampicillin; VAN: vancomycin.

Supplementary Table V-8. Lactobacilli phenotypic antibiotic resistance and MIC ($\mu\text{g/mL}$).

	GEN	KAN	STREP	TET	ERY	CHLORA	AMP
<i>L. paragasseri</i>							
IATA 108	S	S	1024	S	16	S	32
IATA 126	512	S	S	S	16	S	2
IATA 140	S	S	S	S	16	S	16
IATA 141	S	S	S	S	16	S	16
<i>L. gasseri</i>							
IATA 081	128	1024	S	S	16	S	8
IATA 122	128	1024	S	S	16	S	16
<i>L. paracasei</i>							
IATA 109	S	S	S	S	16	S	32
IATA 083	S	S	S	S	S	S	S
IATA 110	256	1024	256	S	16	S	32
<i>L. rhamnosus</i>							
IATA 115	S	S	S	S	16	S	32
IATA 117	S	S	S	S	S	S	S
<i>L. sakei</i>							
IATA 088	32	128	128	8	S	S	32

<i>L. mucosae</i>							
IATA 082	16	128	64	4	S	S	32
<i>L. ruminis</i>							
IATA 127	512	1024	512	64	16	S	32

GEN: gentamycin, KAN: kanamycin; STREP: streptomycin; TET: tetracycline; ERY: erythromycin; CHLORA: chloramphenicol; AMP: ampicillin

Supplementary Note 1. Modified peptone-yeast-glucose (mPYG) medium reagents and preparation.

Solution 1: combine the following and stir at room temperature:

Reagent	Supplier	Cat. No.	Concentration
Tryptone	Teknova	T 9011	5 g/L
BBL Biosate Peptone	BD	211862	5 g/L
Yeast Extract		Y 1003-1001	10 g/L
Beef Extract Powder	Sigma-Aldrich	B 4888-50G	5 g/L
Dextrose (anhydrous)	Millipore	DX 0145-1	5 g/L
K ₂ HPO ₄	Sigma-Aldrich	P 3786-500G	2 g/L
Cysteine-HCl	Sigma-Aldrich	C 1276-10G	0.5 g/L

Solution 2 (salt solution): Combine the following and stir until all reagents are dissolved. Filter sterilize and store at room temperature:

Reagent	Supplier	Cat. No.	Quantity
CaCl ₂ x 2 H ₂ O	Sigma-Aldrich	C 5080-500G	125 mg/L
MgSO ₄ x 7 H ₂ O	Sigma-Aldrich	M 5921-500G	250 mg/L
K ₂ HPO ₄	Sigma-Aldrich	P 3786-500G	0.50 g/L
KH ₂ PO ₄	Sigma-Aldrich	P 0662-500G	0.50 g/L
NaHCO ₃	Sigma-Aldrich	S 8875-500G	5.00 g/L
NaCl	Fisher Scientific	BP 358-212	1.00 g/L

Solution 3 (haemin solution): Combine the following in a 15 mL conical and vortex to dissolve. Filter sterilize into a new 15 mL conical tube and store at 4°C wrapped in foil.

Reagent	Supplier	Cat. No.	Quantity
Haemin chloride	Sigma-Aldrich	3741	50 mg/L
1 N NaOH	Sigma-Aldrich	S 5881-500G	0.1%

Solution 4 (vitamin k solution): Combine the following in a 15 mL conical and vortex to dissolve. Filter sterilize into a new 15 mL conical tube and store at 4°C wrapped in foil.

Reagent	Supplier	Cat. No.	Quantity
Vitamin K 1	Thermo Scientific	L 10575.06	100 uL
95% EtOH	Koptec	V 1101	20 mL

Add the following volumes of Solutions 2-4 to Solution 1. Filter sterilize combined medium in biosafety cabinet. Store bottle(s) wrapped in foil at 4°C.

Solution	Volume
Solution 2 (salt solution)	120 mL
Solution 3 (haemin solution)	1 mL
Solution 4 (Vitamin K solution)	200 μ L

Supplementary Note 2. Genotype-to-phenotype predictions of bacterial HMO utilization.

All *B. adolescentis* strains were predicted to utilize lactose (Lac) but not any of the HMO structures due to the absence of HMO transporters and most glycoside hydrolases (GHs) required for HMO metabolism (Figure V-3a, Supplementary Table V-2). In agreement with *in silico* predictions, *B. adolescentis* IATA 066, IATA 096, and IATA 101 did not utilize any HMOs (Figure V-3b).

Similarly, all *B. lactis* strains were predicted to utilize Lac but not any HMO species (Figure V-3a, Supplementary Table V-2). In agreement with *in silico* predictions, strains IATA 008, IATA 021, IATA 135, IATA 142, and IATA 143 did not utilize any HMOs (Figure V-3b). However, there was a discrepancy for strains IATA 010, IATA 020, and IATA 029, which depleted lacto-*N*-tetraose (LNT) from the pHMO mixture. These strains also grew in a medium supplemented with LNT as the sole carbon source (Supplementary Figure V-4a). Given the very high genomic similarity of tested *B. lactis* strains, additional studies are required to explain the observed discrepancy.

B. pseudocatenulatum strains were predicted to utilize Lac, lacto-*N*-biose (LNB), and LNT intracellularly (Figure V-3a, Supplementary Figure V-2). In agreement with *in silico* predictions, all strains consumed over 95% of LNT from the pHMO mixture (Figure V-3b).

All *B. breve* strains were predicted to utilize Lac, LNB, LNT, and lacto-*N*-neotetraose (LNnT) via intracellular catabolic pathways (Figure V-3a, Supplementary Figure V-2). In agreement with *in silico* predictions, *B. breve* strains IATA 027 and IATA 153 efficiently consumed both LNT (>90%) and LNnT (>75%) (Figure V-3b). The remaining *B. breve* strains depleted only LNT. Consistent with glycoprofiling data, *B. breve* IATA 048 and IATA 131 grew in a medium supplemented with LNT but not LNnT (Supplementary Figure V-4a). The discrepancy between genomic predictions and experimental data about LNnT utilization could be potentially linked to insufficient induction or mutations in genes involved in the metabolism of this oligosaccharide. For example, the *nahS* gene in *B. breve* IATA 131 was disrupted due to a premature sTop codon, suggesting that the encoded substrate-binding component of the LNnT-specific ABC transporter¹ might be nonfunctional (Supplementary Figure V-3).

All *B. bifidum* strains possessed extracellular GHs with diverse specificities, potentially enabling them to completely or partially degrade all tested HMOs and utilize released Lac and LNB (Figure V-3a, Supplementary Figure V-2). In agreement with this notion, all *B. bifidum* strains depleted most HMO species from the pHMO mixture (Figure V-3b). The depletion of 2'-fucosyllactose (2'FL) by all strains, except IATA 039, was consistent with the presence of an extracellular α -fucosidase (BbAfcA; GH 95) that releases terminal α -1,2-linked fucosyl residues from fucosylated HMOs² (Supplementary Table V-2). The *bbAfcA* gene in *B. bifidum* IATA 039 was truncated at the 5'-end, resulting in a 151 amino acid shorter protein, which was likely nonfunctional due to the removal of the N-terminal signal peptide (Supplementary Figure V-3). Thus, the *bbAfcA* gene truncation could explain the strain's

inability to utilize 2'FL as well as the decreased degradation of lacto-*N*-fucopentaose I (LNFP I).

We observed an interplay between concentrations of 3-fucosyllactose (3FL) and difucosyllactose (DFLac) in the supernatants of all strains except IATA 049 and IATA 039 (**Figure V-3b**). The increased 3FL concentration could be explained by the unequal (separated in time) removal of fucosyl residues from DFLac by the α -fucosidases of *B. bifidum*. Specifically, the terminal α -1,2-fucosyl residues would be removed first by BbAfcA, generating 3FL and thus temporally increasing its concentration. A similar interplay likely occurred between concentrations of sialyl-lacto-*N*-tetraose c (LSTc) and disialylated HMO species, given the increase of LSTc concentration in supernatants of all *B. bifidum* strains. For example, extracellular α -sialidases SiaBB 1 and SiaBB 2 would remove a single terminal *N*-acetylneuraminic acid residue from disialylated HMOs generating LSTc.

In agreement with *in silico* predictions, all *B. bifidum* strains efficiently (>90%) depleted the LNT in the pHMO mixture (**Figure V-3a,b**). In contrast, the concentration of LNNt in the supernatants of *B. bifidum* strains varied considerably. It decreased in the case of IATA 139 and IATA 148, remained relatively unchanged in the supernatants of five strains (IATA 005, IATA 039, IATA 049, IATA 102, IATA 123), and increased in supernatants of IATA 001 and IATA 016 strains. Since LNNt is an intermediate product of the degradation of multiple more complex HMOs, the observed results may reflect strain-level variability in the kinetics of long-chain HMO utilization. *B. bifidum* strains IATA 001, IATA 005, IATA 049, and IATA 102 were able to grow on LNNt as a sole carbon source, confirming their capacity to utilize this oligosaccharide (**Supplementary Figure V-4a**).

All *B. bifidum* strains were predicted to degrade 3'- and 6'-sialyllactoses (3'SL and 6'SL) by extracellular α -sialidases SiaBB 1 and SiaBB 2 (GH 33)^{3,4} (**Figure V-3a, Supplementary Figure V-2**). However, only 6'SL was partially degraded for all strains except IATA 148, whereas the depletion of 3'SL was strain-specific (**Figure V-3b**). In agreement with predictions, all *B. bifidum* strains degraded various lchMOs, e.g., lacto-*N*-fucopentaoses II/III (LNFP II/III), disialyllacto-*N*-tetraose (DSLNT), fucosyllacto-*N*-hexaose (FLNH), difucosyllacto-*N*-hexaose (DFLNH), and disialyllacto-*N*-hexaose (DSLNH).

B. longum subsp. *infantis* (*B. infantis*) strains were predicted to utilize Lac, LNB, LNT, LNNt, 2'FL, 3'FL, DFLac, LNFP I, 3'SL, 6'SL as well as lchMOs intracellularly (**Figure V-3a, Supplementary Figure V-2**). In agreement with *in silico* predictions, all strains utilized 2'FL, 3'FL, LNT, and LNFP I, and certain lchMOs (LNFP II and DFLNT). In contrast, *B. infantis* IATA 104 and IATA 105 did not utilize DFLac and 3'SL/6'SL, and the IATA 105 strain did not utilize LNNt. *B. infantis* IATA 105 also did not grow in a medium supplement with LNNt (**Supplementary Figure V-4a**). Low DFLac and 3'SL/6'SL consumption could be related to the preferential utilization of HMO species from the pHMO mixture. The absence of LNNt utilization by *B. infantis* IATA 105 was potentially due to mutations in genes constituting the respective catabolic pathway.

All *B. longum* subsp. *longum* (*B. longum*) strains were predicted to utilize Lac and LNB (**Figure V-3a**). Strains IATA 034, IATA 062, and IATA 075 possessed an extracellular lacto-*N*-biosidase (LnbX; GH 136; **Supplementary Table V-2**). This enzyme has a broad substrate specificity and can cleave the internal -GlcNAc(b 1-3)Gal- glycosidic bond in various HMOs, including LNT, LNFP I, and lacto-*N*-hexaose (LNH)⁵. Thus, these three *B. longum* strains were predicted to extracellularly degrade LNT (to LNB and Lac) and LNFP I (to H 1 trisaccharide and

Lac; **Supplementary Figure V-2**). In agreement with *in silico* predictions, all three strains completely (>95%) depleted LNT from the pHMO mixture, and two strains (IATA 062 and IATA 034) degraded 30-50% of LNFP I (**Figure V-3b**). The partial hydrolysis by LnbX might also explain the depletion of LNH and FLNH in the supernatants of *B. longum* IATA 034, IATA 062, and IATA 075.

B. longum IATA 116 was predicted to utilize LNT intracellularly and, in agreement with the predictions, consumed over 95% of this HMO species (**Figure V-3a,b**). The remaining *B. longum* strains (IATA 003, IATA 015, IATA 144, IATA 033, IATA 107) were predicted not to utilize LNT because their variant of the substrate-binding component (GltA) of the LNB/GNB/LNT transporter (GltABC) was previously shown to have low affinity for LNT⁶. However, only *B. longum* IATA 144 did not consume LNT, whereas the remaining three strains efficiently depleted it. Consistent with glycoprofiling data, *B. longum* IATA 003, IATA 015, IATA 033, and IATA 107 grew in a medium supplemented on LNT as the sole carbon source (**Supplementary Figure V-4a**). Additional studies on the substrate-specificity of different GltA orthologs are required to explain the observed discrepancies.

The decrease of LNNt and LNFP III concentration in supernatants of certain strains (e.g., IATA 034) was likely not due to the intracellular utilization of these HMOs but rather partial extracellular degradation by a β -1,4-galactosidase which would cleave the terminal galactosyl residue from LNNt (leaving lacto-*N*-triose II) and LNFP III (leaving fucosyllacto-*N*-triose II). Consistent with this hypothesis, *B. longum* IATA 034 did not grow on LNNt as the sole carbon source (**Supplementary Figure V-4a**).

Reconstruction of 11 carbohydrate utilization pathways suggested that all lactobacilli could utilize specific structural components of HMOs, namely Lac, *N*-acetylglucosamine (GlcNAc), galactose, and glucose (**Figure V-4a, Supplementary Table V-3**). However, the predicted abilities to metabolize LNNt, lacto-*N*-triose II (LNTriose), *N*-acetyllactosamine (LacNAc), LNB, fucose, and *N*-acetylneuraminic acid were species- and strain-specific.

Only *Limosilactobacillus mucosae* IATA 082, *Lactocaseibacillus rhamnosus* IATA 115, and *Latilactobacillus sakei* IATA 088 genomes encode the two-subunit extracytoplasmic β -galactosidase LacLM that cleaves the terminal non-reducing galactosyl residue from LNNt, releasing LNTriose⁷ (**Figure V-4b**). However, these genomes lacked the *bnxG* gene encoding a secreted β -*N*-acetylglucosaminidase that releases a GlcNAc residue from LNTriose⁸. Therefore, the proposed LNNt utilization mechanism for these strains involved the partial extracellular degradation of LNNt and the uptake of released galactose. Consistent with this prediction, *L. mucosae* IATA 082 and *L. sakei* IATA 088 depleted LNNt from the pHMO mixture and grew in the medium supplemented with LNNt as the sole carbon source (**Figure V-4c, Supplementary Figure V-4b**).

None of the 14 lactobacilli strains were predicted to metabolize LNT, which was in agreement with glycoprofiling data for all strains except *L. mucosae* IATA 082. In addition to LNT, the latter strain partially depleted LNFP I/III, LNH, DFLNH, and FNLH from the culture medium. Given that the genome of *L. mucosae* IATA 082 lacks genes encoding GH 29 and GH 95 α -fucosidases (**Supplementary Table V-3**), the decrease in concentrations of these HMOs could be potentially linked to partial degradation via the removal of terminal non-reducing galactosyl residues by β -galactosidases. However, further studies are required to test this hypothesis.

References of Supplementary Note 2

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Supplementary Note 3. Antibiotic resistance phenotype of bifidobacteria and lactobacilli.

All *Bifidobacterium* strains exhibited resistance to erythromycin but were susceptible to chloramphenicol and vancomycin. Among the *Bifidobacterium bifidum* strains, strain IATA 139 is the only one susceptible to gentamicin, whereas IATA 001 and IATA 005 exhibited the highest resistance to gentamicin, with MIC values reaching 512 μ g/mL. For ampicillin, IATA 148 exhibited the highest MIC (16 μ g/mL), IATA 005 was the only strain susceptible, and the remaining strains showed moderate resistance (MIC =2-4 μ g/mL). IATA 039 and IATA 148 were the only strains resistant to streptomycin (64 and 512 μ g/mL, respectively) and tetracycline (0.5 and 32 μ g/mL, respectively).

In the *Bifidobacterium longum* *subsp. longum* group, strain IATA 033 was susceptible to most antibiotics. Other strains, such as IATA 015, IATA 062, IATA 075, IATA 107, IATA 116, and IATA 137, were resistant to gentamicin and ampicillin. Interestingly, resistance to tetracycline was observed in IATA 033 and IATA 144, but not in IATA 075.

Bifidobacterium longum subsp. infantis strains had a similar resistance profile: they showed resistance to gentamicin (MIC =32 µg/mL) and ampicillin (MIC =4-8 µg/mL). Strain IATA 045 was the only one resistant to streptomycin.

Bifidobacterium animalis strains exhibited high resistance to gentamicin (MIC =256-512 µg/mL). Strain IATA 029, exhibited lower resistance levels (32 µg/mL for tetracycline) compared to other strains in this subspecies, indicating potential variations in resistance mechanisms or gene expression levels. IATA 021 and IATA 029 were the only strains susceptible to ampicillin, while IATA 142 and IATA 143 had MICs of 8 µg/mL, IATA 010 and IATA 020 had MICs of 32 µg/mL, and IATA 008 had an MIC of 16 µg/mL. All strains, except IATA 010, IATA 020, and IATA 029, showed high resistance to streptomycin (MIC =256-512 µg/mL).

Bifidobacterium breve strains showed significant variability in their resistance profiles. For instance, IATA 048 and IATA 077 exhibited extremely high resistance to streptomycin (MIC =2048 µg/mL). In contrast, IATA 084 remained susceptible to streptomycin and tetracycline but was resistant to gentamicin (MIC =256 µg/mL). IATA 131, IATA 136, and IATA 153 showed resistance to gentamicin (MIC =256 µg/mL) and streptomycin (MIC =1024-2048 µg/mL), with IATA 153 also resistant to tetracycline.

Bifidobacterium adolescentis strains generally showed low resistance levels, with strains like IATA 066 showing resistance to gentamicin (MIC =256 µg/mL) and ampicillin (MIC =8-16 µg/mL). However, IATA 101 stood out with resistance to streptomycin, tetracycline, and ampicillin, in addition to the already mentioned erythromycin.

All *Lactobacillus* strains showed susceptibility to the antibiotic chloramphenicol. Regarding *Lactobacillus paragasseri*, all strains were resistant to erythromycin (MIC =16 µg/mL) and ampicillin, with IATA 126 being the least resistant to ampicillin (MIC =2 µg/mL). However, this strain was the only one resistant to gentamicin (MIC =512 µg/mL) among the *L. paragasseri* strains. Additionally, IATA 108 exhibited resistance to streptomycin (MIC =1024 µg/mL).

For *L. gasseri*, both strains (IATA 081 and IATA 122) displayed the same resistance profile: they were resistant to gentamicin (MIC =128 µg/mL), kanamycin (MIC =1024 µg/mL), erythromycin (MIC =16 µg/mL), and ampicillin (MIC =8-16 µg/mL), as well as resistant to streptomycin, tetracycline, and chloramphenicol.

Lactocaseibacillus paracasei IATA 110 was resistant to most of the antibiotics tested, while IATA 109 was only resistant to erythromycin (MIC =16 µg/mL) and ampicillin (MIC =32 µg/mL). The strain IATA 083 was the only one susceptible to all antibiotics, and no antibiotic resistance genes were found in its genome.

Similarly, *L. rhamnosus* IATA 117 was susceptible to all antibiotics as well. IATA 116 was resistant to erythromycin (MIC =16 µg/mL) and ampicillin (MIC =32 µg/mL) but susceptible to tetracycline, despite the presence of the tetM gene in its genome.

The unique strain of *L. sakei* (IATA 088) showed resistance to all antibiotics except erythromycin and chloramphenicol. The same phenotype was observed in *L. mucosae* IATA

081, which carries the lincosamide resistance gene *lncC*. Finally, *Ligilactobacillus ruminis* IATA 127 exhibited high resistance to gentamicin (MIC =512 µg/mL), kanamycin (MIC =1024 µg/mL), streptomycin (MIC =512 µg/mL), tetracycline (MIC =64 µg/mL), erythromycin (MIC =16 µg/mL), and ampicillin (MIC =32 µg/mL), but was susceptible to chloramphenicol, as were all other *Lactobacillus* strains.

DISCUSSION



GLOBAL OVERVIEW OF THE MAIN FINDINGS

In this thesis, a deep, comprehensive investigation to understand infant gut resistome development during the first year of life is presented. To achieve this, maternal and neonatal samples were collected longitudinally, and a combination of different microbiological and biocomputational approaches were applied. This study examines how both prenatal and postnatal factors -such as mode of delivery and feeding practices- influence resistome acquisition and dynamics. Furthermore, it explores the underlying mechanisms linking the evolving gut microbiota with antibiotic resistance during the infant's breastfeeding period (**Figure 8**).

The first approach employed focused on characterization of infant resistome assembly and evolution during the first year of life (**Chapter I-III**), focusing on the microbial and external factors shaping resistome dynamics. We revealed that the infant gut resistome follows a complex and dynamic evolutionary trajectory, progressively resembling the maternal resistome over time. This transition is characterized by a reduction in ARG load and an increase in diversity. We identified that the abundance of *Bifidobacterium* is a key factor influencing this process, as infants with a microbiota dominated by this genus harbored a lower abundance of ARGs, regardless of breastfeeding or delivery mode (**Chapter II-III**). However, the key perinatal factors that most significantly influence the development of the infant resistome remain poorly understood. Our study reported that mode of delivery (C-section) and breastfeeding practices play a crucial role, with breastfeeding not only fostering the growth of beneficial microbiota like *Bifidobacterium* but also modulating the acquisition and abundance of ARGs, thereby influencing overall health and resistance to infections. Cessation of breastfeeding before 6 months of age was associated with a higher ARG load. Moreover, we hypothesized that breastfeeding can modulate the impact of C-section birth on the resistome. Indeed, our findings suggest that breastfeeding helps restore the microbiome disrupted by C-section and antibiotic exposure, leading to reduced ARGs, with HMOs playing a role in this process (**Chapter III- IV**). As the HMO profile is determined by the maternal *FUT2* gene, we observed that maternal

secretor or non-secretor status has a distinct impact on the infant resistome. Interestingly, infants breastfed by secretor mothers had fewer C-section effects on their resistome profile (**Chapter IV**). To understand how antibiotic resistance and specific microbes interact in early life, we investigated how HMO metabolism and antibiotic susceptibility of bifidobacteria and lactobacilli shape the temporal development of the infant resistome (**Chapter V**). Our findings reveal that breastfeeding supports the growth of beneficial HMO-metabolizing bacteria, a process that can be disrupted by antibiotic exposure during infancy. Altogether, these findings underscore the intricate interplay between maternal transmission, microbial composition, perinatal factors, and human milk components in shaping the early life resistome.

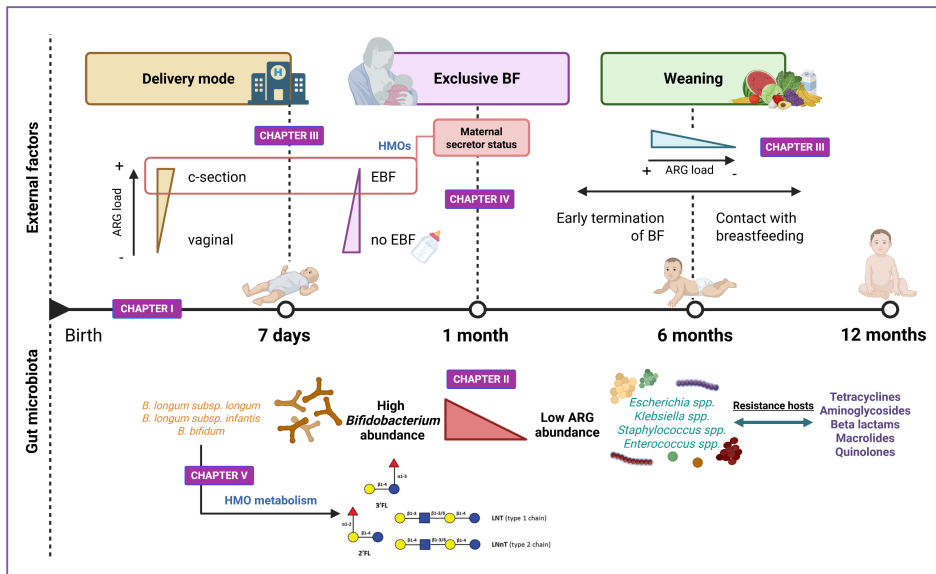


Figure 8. Schematic overview of the main findings of the thesis. The timeline highlights key external factors—delivery mode, exclusive breastfeeding (BF), maternal secretor status, and weaning—and their influence on the infant gut resistome. **Chapter I** focused on resistome assembly from birth, influenced by delivery mode. **Chapter II** showed that high *Bifidobacterium* abundance is linked to lower ARG load. **Chapter III** explored the impact of BF and weaning on resistome dynamics during the first year of life, showing that BF reduces ARGs, especially after C-section. **Chapter IV** investigated how maternal secretor status (FUT2 gene) modulates HMO composition and its protective effect on the infant resistome. **Chapter V** examined HMO metabolism by beneficial bacteria and how antibiotic exposure disrupts this process. Created in BioRender (<https://BioRender.com/54hsrtd>).

GENERAL DISCUSSION

BIFIDOBACTERIUM AS A KEY MODULATOR OF THE INFANT GUT RESISTOME

It is well-known that the abundance of *Bifidobacterium* spp. in the infant gut plays a crucial role in determining microbial composition. One of the key findings presented in this thesis highlights the significant influence of *Bifidobacterium* spp. on shaping the resistome profile during early life (**Chapter II-III**). The application of the k-means clustering algorithm to 16S rRNA sequencing (**Chapter II**) and shotgun metagenomic (**Chapter III**) data revealed that the microbial profiles of infants clustered into two distinct groups: 1) One group was characterized by a high relative abundance of *Bifidobacterium* (>50%), while 2) the other exhibited a low abundance of *Bifidobacterium* (<20%). Moreover, an infant gut that is predominantly colonized by *Bifidobacterium* tends to exhibit a reduced load of ARGs, suggesting a protective role of these bacteria against antimicrobial resistance. This finding underscores the importance of *Bifidobacterium* in driving the early-life microbiota and its potential in reducing the burden of ARGs and thereby limiting the spread of antibiotic resistance.

High-Bifidobacterium abundance is associated with a low ARG load

In this thesis, we hypothesized that as *Bifidobacterium* abundance shapes and drives the infant microbiota, it may also influence the development of the early-life resistome. We first used targeted qPCR to quantify six specific ARGs (*tetM*, *tetO*, *tetW*, *ermB*, *blaSHV* and *blaTEM*) in fecal samples of 7-day and 1-month-old infants (**Chapter II**). These genes were selected based on their high abundance in infant fecal samples as we previously revealed with HT-qPCR of >300 ARGs (**Chapter I**) and include genes conferring resistance to antibiotics commonly used during pregnancy (beta-lactams and macrolides), and those not perscribed during pregnancy and infancy (tetracyclines). We observed that the presence of copies of these ARGs was associated with a low abundance of *Bifidobacterium*. In a second exploration, we used longitudinal shotgun metagenomic data (**Chapter III**) to validate these findings. Metagenomic data allowed us to examine the entire gut

resistome from mothers and infants, as well as that of human milk samples, enabling us to consider additional types of resistances and the microbes responsible for hosting them. A low abundance of *Bifidobacterium* was associated with higher load of ARGs conferring resistance to lincosamides, tetracyclines, amphenicols, beta-lactams, and quinolones.

In agreement with our findings, Shao *et al.*²³⁸ previously reported that one-month-old infants highly colonized with *B. bifidum* exhibited a lower carriage of ARGs compared to those colonized by *E. faecalis*, which was associated with higher levels of ESBLs. Similarly, a study by Li *et al.*⁶³ identified *E. coli* as the key driver shaping the microbiome composition of one-year-old infants, with Pseudomonadota contributing the most abundant and distinct ARGs in the infant gut. Consistent with these observations, our study also found that infants with a low abundance of *Bifidobacterium* were highly colonized by *E. coli* and *E. faecium*, two species that carried a significantly higher proportion of ARGs than other taxa during the first year of life. We expanded on previous knowledge, as our analyses included rich longitudinal sampling with fecal samples from 7 days of life until 1 year of age.

While our study demonstrates that *Bifidobacterium* abundance influences resistome load and composition throughout the first year of life, Taft *et al.*²³⁹ reported that Bangladeshi infants lost this association by the age of two. This suggests that the role of *Bifidobacterium* in shaping the gut microbiota is most critical during the first year of life, likely because the microbiota gradually transitions towards an adult-like composition, where *Bifidobacterium* becomes less dominant. To the best of our knowledge, this is the first study to investigate the influence of *Bifidobacterium* on ARG load in infants as young as 7 days and 1 month of age, using a combination of 16S rRNA sequencing and qPCR of selected ARGs, and later confirmed in infants from 7 days to 1 year of age through shotgun metagenomics.

What drives *Bifidobacterium* abundance over time?

Traditionally, breastfed infants have been characterized by a microbiota dominated by *Bifidobacterium* spp⁴⁵. The dominance of *Bifidobacterium* in the

infant gut has been closely linked to the consumption of HMOs, the third most abundant component of human milk^{66,238}. In fact, we found that *B. breve*, *B. longum*, and *B. bifidum*—species known for their ability to degrade HMOs¹⁶⁸ and further studied in **Chapter V**—were characteristic of infants highly colonized by *Bifidobacterium* species. However, our study revealed that infant microbiome clustering based on *Bifidobacterium* abundance (high vs low) was not influenced by delivery mode or breastfeeding practices. Regardless of birth or feeding methods, infants from diverse backgrounds converged into the high-*Bifidobacterium* group. Shao *et al.*²³⁸ also found that the strong colonization of breastfed infants by *B. bifidum* occurred independently of clinical factors, including delivery mode. These findings support the hypothesis that maternal influences—such as genetic factors affecting human milk composition (further studied in **Chapter IV**), previous pregnancies, cohabitation with other children, and cultural differences in infant care—may play a crucial role in the vertical transmission of the maternal microbiota. Moreover, these findings highlight the unique role of *Bifidobacterium* in shaping both the infant microbiome and resistome, which led us to consider it as an independent variable in our analyses. Further research is still needed to explore the complex interactions between host genetics and other factors that may drive early-life *Bifidobacterium* abundance.

THE IMPORTANCE OF THE GUT MICROBIOTA IN ANTIBIOTIC RESISTANCE ACQUISITION

Given that the gut microbiota harbors both the main carriers of ARGs and the agents of their horizontal transfer, it is plausible that the development of the microbiome and resistome occurs in tandem and are influenced by shared determinants. As infants grow, their microbiota diversify, acquiring a more complex phylogenetic structure, which eventually stabilizes and resembles the maternal microbiota⁴⁰. The increased microbial diversity is accompanied by an increase in the diversity of the resistome, as observed in our study using both 16S rRNA sequencing and shotgun metagenomics (**Chapter I-III**) and in previous research^{64,118}.

Historically, the infant microbiome is dominated by *Bifidobacterium* spp., a characteristic associated with a variety of health benefits, including the production of SCFAs, improved gut barrier function, protection against pathogens, and the modulation of the immune system^{240–242}. It is well established that the genus *Bifidobacterium* plays a key role in shaping the overall gut microbiota composition, contributing to the maintenance of a diverse and stable microbial community²³⁹. Using 16S rRNA gene sequencing (**Chapter II**), we observed that infants with a low abundance of *Bifidobacterium* exhibited higher abundances of the genera *Escherichia*, *Klebsiella*, *Clostridium*, and *Streptococcus*. Additionally, in the metagenomic-based study (**Chapter III**), we identified specific species, including *E. coli*, *C. perfringens*, *K. pneumoniae*, and *S. aureus*, that colonized the microbiome of low-*Bifidobacterium* infants. In agreement with previous research⁶³, our findings confirm that these genera are major carriers of ARGs in the infant gut microbiome.

Although low-*Bifidobacterium* infants had a higher overall diversity of bacterial species, high-*Bifidobacterium* infants had a more diverse range of *Bifidobacterium* species, including *B. longum*, *B. bifidum* and *B. breve*. This distinction underscores that overall microbial diversity does not necessarily reflect functional or ecological complexity within key taxa, and the need to examine specific species and strain interactions to better understand the relationships between intestinal microbial populations and their dynamics over time. A study by Taft *et al.*²³⁹ aligns with our findings, as they also observed that infants with a high abundance of *Bifidobacterium* had lower levels of *Enterobacteriaceae* compared to those with a low abundance, supporting the idea that *Bifidobacterium* may play a role in suppressing ARG-carrying taxa. Similarly, a study conducted by Shao *et al.*²³⁸ showed that the microbial community profile of infants could be separated into three community states according to three dominant species: *B. breve*, *B. longum* and *E. faecalis*.

In line with previous studies⁶², we identified that members of the Bacillota phylum are important carriers of ARGs, with *Staphylococcus*, *Streptococcus*, and *Enterococcus* members being the main carriers, as well as *Escherichia*, *Klebsiella*,

and *Clostridium*. Most plasmids were also attributed to these genera, underscoring the potential risks associated with the establishment of these species in early life (**Chapter III**). Indeed, we observed a high presence of ARGs conferring resistance to beta-lactams and aminoglycosides in plasmids and highly associated with *Escherichia* genus. Accordingly, ESBL-E *E.coli* and *K. pneumoniae* are a public health concern as they can potentially contribute to the neonatal mortality, and they are ranked as a priority among pathogens in need of new treatment by the WHO⁷¹. Tetracycline and macrolide ARGs were found across various genera, with *Streptococcus* being the primary host for ARGs conferring resistance to these antibiotics. Likewise, aminoglycoside ARGs were predominantly carried by *Staphylococcus*, *Escherichia*, and *Enterococcus* spp., in line with previous research⁶³.

Despite their beneficial effects on the proper establishment of the infant microbiota, bifidobacteria can also serve as a reservoir of resistance. The presence of *tet* genes is common in different species of *Bifidobacterium*, including those more abundant in early stages of life, such as *B. longum*, *B. infantis*, *B. breve*, *B. lactis* and *B. bifidum*^{243–245}. Specially, *tetW* is integrated in the chromosome of bifidobacteria²⁴⁶. Nonetheless, bifidobacteria are less likely to possess ARGs and to conduct HGT than genera such as *Enterococcus*, *Streptococcus*, *Staphylococcus*, *Klebsiella*, or *Escherichia*^{64,239}.

The assembly of the gut resistome is thought to occur in parallel with the development of the early-life microbiota, as the microbial community is responsible for carrying and transferring the ARGs that comprise the resistome. The presence of ARGs in certain species aligns with our observation that infants with lower *Bifidobacterium* abundance often exhibit an overrepresentation of ARG-carrying species, such as those mentioned above, potentially contributing to the shaping of the infant resistome. Nevertheless, it remains unclear whether the maturation of the microbiome and resistome are truly synchronized processes, whether they are shaped by the same environmental and host-related factors, or whether the resistome follows an independent developmental trajectory.

THE DYNAMICS OF THE INFANT GUT RESISTOME

Our studies in **Chapter I** and **III** highlight the significant presence of tetracycline resistance genes across all age, as observed in previous studies^{59,63,247}. This is profound because infants have not been exposed to this antibiotic since the early 1980s due to potential staining of the teeth and oral cavity²⁴⁸. Genes conferring resistance to tetracycline (*tet* genes) have been previously reported to be the most abundant genes in the infant resistome and highly prevalent in species of *Bacillota* and *Bacteroidota*²⁴⁷. Moreover, and in agreement with our findings, *tet* genes are one of the most frequently shared ARGs between mothers and infants, suggesting that the maternal gut can be a potential source of tetracycline resistances for infants^{63,75,249}, even though tetracyclines are not used during pregnancy. Hence, the extensive use of tetracyclines in agriculture, the environment, and the food chain may account for the presence of *tet* genes in mothers and infants, even in the absence of direct antibiotic exposure^{76,250}.

Macrolide resistance, including genes encoding multiresistance (such as MLSB), was the second most abundant resistance found using the metagenomic approach (**Chapter III**), in line with previous research^{70,94,102}. The MLSB phenotype represents a notable form of multidrug resistance, commonly associated with MRSA. In neonates, MRSA infections are associated with high morbidity and mortality rates, particularly among preterm infants, who are especially vulnerable to conditions such as necrotizing enterocolitis (NEC) and sepsis^{251,252}. In agreement with previous studies²⁵³, we found the macrolide resistance gene *ermB* to be one of the most prevalent (>80%) in infant fecal samples. *ermB* was mainly carried by *Enterococcus* spp., *Streptococcus* spp., and *Klebsiella* spp., with some pathogenic species harboring the gene on plasmids.

In addition, beta-lactamases, which have been prescribed for many decades and are commonly used during pregnancy and labor, have been previously detected as the second most abundant resistance profile in the infant gut²⁵⁴. Thus it was predictable that this resistance was also identified in our infant fecal samples. Our metagenomic approach (**Chapter III**) showed that the overall beta-lactam

resistance profile of infants was stable during the first year of life, but our quantitative approach (**Chapter I**) on samples collected over the first 4 months of life highlighted that beta-lactam resistance genes exhibit complex patterns, reflecting the dynamics of ARGs in response to external factors. *blaZ* and *blaTEM* were the most abundant ARGs in the infants of our cohort, mainly carried by *Staphylococcus* and *Escherichia* spp., and usually associated to plasmids. Aminoglycosides, often administered alongside beta-lactams, are commonly used to treat neonatal sepsis. In concordance with our findings, resistance to these antibiotics in the infant gut microbiota has been linked to genes encoding enzymes responsible for acetylation, phosphorylation, and adenylation⁸⁰.

The prescription of quinolones to mothers to treat serious gram-negative infections, and the administration of vancomycin to infants to treat colitis, diarrhea induced by *C. difficile*, and gram-positive bacterial infections are common in the medical community²⁵⁵. Although the percentage of infants in our study that were exposed to antibiotics was very low, and none of them suffered from sepsis or colitis, we found quantitative and qualitative evidence of ARGs conferring resistance to quinolones and vancomycin (**Chapter I and III**). Collectively, these findings reinforce the hypothesis that both direct and indirect antibiotic exposure can shape the infant resistome, and that the emergence of antibiotic resistance may occur even in the absence of prior antibiotic exposure⁸².

The role of the mobilome in resistome acquisition

Interestingly, we identified higher amounts of MGE (one transposase and two insertion sequences) in infant fecal samples than in those of mothers (**Chapter I**). Furthermore, and these MGEs were highest in 7-day-old infants and decreased over time, in concordance with previous research²⁵⁶. These findings highlighted the emergence of MGEs as contributors to ARGs in the infant gut. However, we recognize that the amount and type of MGEs measured with HT-qPCR are not representative of the entire infant mobilome. Accordingly, when studying the dynamics of the infant resistome in **Chapter III**, we aimed to provide a wider overview of the role of the mobilome in infant resistome evolution. There is evidence

that an increased presence of MGEs in infant feces may facilitate the transmission of ARGs among and between microbial species, and that MGEs containing ARGs are shared between family members⁶⁴. One challenge when studying the sharing and potential transmission of MGEs between mother-infant dyads is distinguishing between nucleotide level variants of the genetic elements due to the technical challenge of short read assembly of low abundance genes in varying genetic contexts, such as ARGs from diverse environments. For this reason, we analyzed the presence of plasmids carrying ARGs, as they are the most representative group of MGEs. We observed a high presence of ARGs conferring resistance to beta-lactams and aminoglycosides in plasmids and highly associated with the *Escherichia* genus (**Chapter III**). Although recent research suggests that *Bacteroides* are the main hosts of the infant gut plasmidome²⁵⁷, databases still contain thousands of plasmids from *Escherichia* and *Klebsiella* species. Consequently, accurately identifying the exact bacterial hosts of MGEs remains challenging.

The maternal influence on the infant resistome

Through the first year of life, there is a dynamic evolution of resistance over time which gradually resemble the mother's resistome (**Chapter III**). Moreover, we detected higher levels of ARGs in the early stages of infant life compared to the maternal resistome, using both quantitative and qualitative approaches (**Chapter I and III**).

The maternal microbiome is considered a principal source for the initial establishment of both the infant gut microbiome and resistome³. Some previous studies have indicated that infant ARGs and MGEs resemble those of their mothers⁶⁴ but that fathers can also be a source of bacterial strains for their offspring^{122,258}. In contrast to microbiome assembly, a recent study found that the gut resistome shows low similarity between fathers and their infants. Unfortunately, given the lack of paternal fecal samples for our cohort, we did not study the similarity of the resistome among family triads, but rather among mother-infant dyads. We observed that mother-infant pairs at one-month postpartum share common ARGs and exhibit similar ARG loads, although their overall resistome composition remained distinct

(**Chapter III**). Mother-infant pairs had more ARGs in common than unrelated mothers and infants, but the proportion of shared ARGs was still lower than the proportion of unique ARGs in mothers and infants. This mirrors previous findings that infants and mother have some shared ARGs¹⁰¹ while infants tend to carry a higher overall ARG load⁶⁰. This suggests that the infant resistome is, to some extent, influenced by the maternal resistome, but other sources and factors also play a significant role in shaping its composition, including horizontal transmission from fathers, pets, caregivers, and siblings¹²². In this regard, the influence of the maternal resistome on the infant resistome is still unclear. Moreover, it would be valuable to investigate maternal-infant resistome similarity at earlier timepoints, such as at birth, to determine which ARGs are transmitted during delivery. Additionally, studying later time points could provide insights into which ARGs shared in the early stages persist over time and which are acquired (and lost) as the infant's resistome gradually converges with that of the mother.

Two approaches, one goal: integrating quantitative and qualitative approaches to study the gut resistome

To delineate the assembly of the infant gut resistome during the first year of life, we used two different methodologies: a quantitative, targeted approach based on HT-qPCR (**Chapter I**), and a qualitative-based approach based on metagenomic sequencing (**Chapter III**). The use of a high-throughput, quantitative approach to measure and quantify ARGs, was a novel aspect of our study that provided a high-resolution view of ARG abundance. HT-qPCR provides relative abundance data for each gene detected, allowing selective analysis of clinically or ecologically relevant ARGs of interest, with unparalleled sensitivity and precision²⁵⁹. We conducted top-down analysis. Specifically, we first conducted a targeted screening of 385 ARGs in using cross-sectional maternal-infant fecal samples to detect the most abundant and prevalent ARGs among the samples. We then customized a targeted HT-qPCR panel to focus on the most predominant genes enabling individual-level, longitudinal analyses for a large sample. This method provides advantages over techniques like amplicon sequencing or shotgun metagenomics by enabling

accurate absolute quantification of specific resistance genes, which is essential for monitoring changes in ARG levels over time and across different conditions. The development of HT-qPCR has been enhanced by advancements in WGS and the increasing discovery of ARGs. This method supports the analysis of hundreds of ARGs simultaneously but requires designing specific primers for each gene.

Complementarily, metagenomic sequencing provides deeper insights into the distribution of ARGs within gut microbes and microbiomes, down to the species or strain level. To analyse the resistome from shotgun sequencing, one either directly maps sequence reads to reference databases or assembles them into contigs for subsequent annotation. Incorporating an assembly step into the metagenomic workflow—known as assembly-based metagenomics—allows for a more comprehensive range of analyses.

Here, the two methodologies complemented one another in advancing our understanding of infant resistome dynamics (**Figure 9**). Initially employing HT-qPCR (**Chapter I**) provided a clear view of the dynamics within the infant's resistome, and later metagenomic sequencing (**Chapter III**) enhanced our understanding by delving into strain-level antibiotic resistance, exploring the mobilome, and gaining a broader perspective on the resistome.

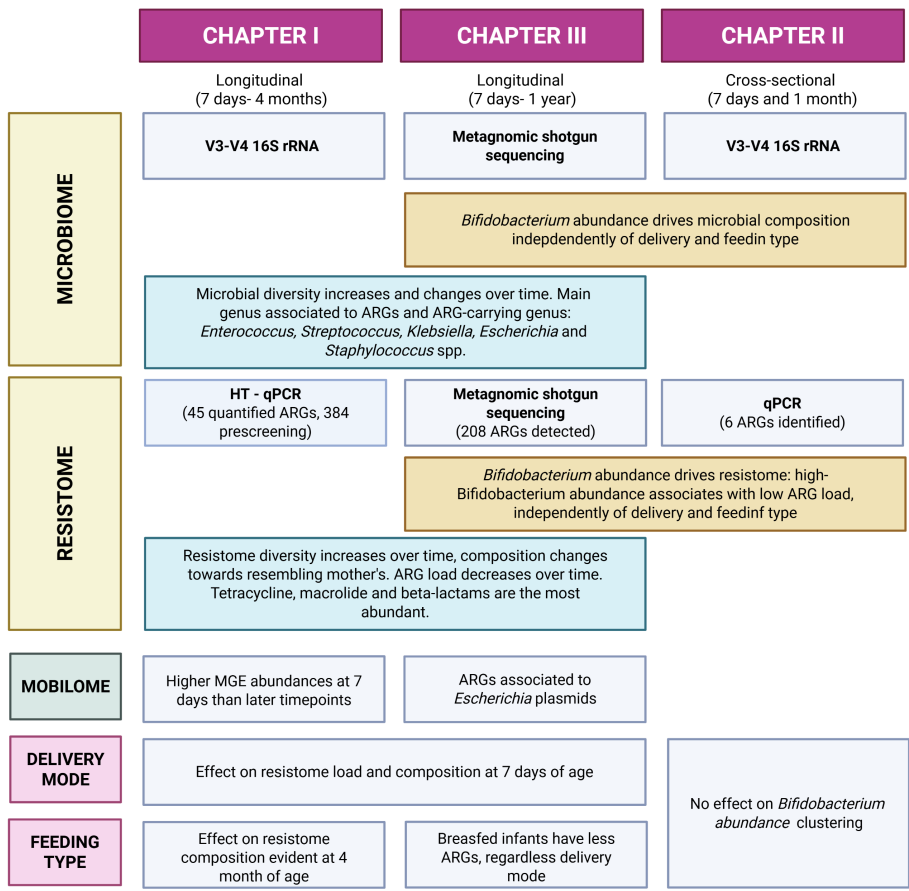


Figure 9. Synthesis of the methodologies and common findings across Chapters I, II and III. Created in BioRender (<https://BioRender.com/55m3k9f>).

THE DEVELOPING RESISTOME: LASTING IMPACTS OF PERINATAL FACTORS

While bacteria serve as the primary reservoirs of ARGs, the dynamics of the microbiota do not necessarily mirror those of the resistome. Notably, we observed that the abundance of *Bifidobacterium* did not vary with delivery mode or breastfeeding practices (**Chapter III**). Interestingly, infants initially classified into the high-*Bifidobacterium* group later shifted to the low abundance group, and vice versa. The underlying driver of these shifts remains unclear, but we hypothesize that external factors play a critical role. For instance, the introduction of solid foods during the weaning period marks a critical moment for the maturation of the gut

microbiome^{260,261}. Consequently, the microbiota transitions from a relatively simple, *Bifidobacterium*-rich community, specialized in metabolising HMOs, to a more complex, diverse ecosystem capable of processing starches and other dietary components⁴⁰.

Given that the microbiome and the resistome can be shaped by similar factors, yet not always in parallel, a fundamental question arises: how strong and consistent is the link between microbial composition and the resistome? Although bacteria are not only the main carriers of ARGs but also key agents of HGT, a shift in microbial diversity and composition does not necessarily imply a corresponding shift in the resistome. Conversely, changes in the resistome may result from multiple HGT events without substantial alterations in the microbiome structure. In this context, external factors can influence both the microbiome and the resistome, potentially leading to compositional imbalances either in a coordinated manner or independently within a single system. Numerous additional variables -many of which are challenging to control or quantify- also play a role in shaping these dynamics.

Delivery mode and antibiotic exposure primarily shape the resistome during the first month of life

During a C-section procedure, antibiotics such as cefazolin, vancomycin, clindamycin, and erythromycin are commonly administered as a preventive measure to lower the risk of infection^{262,263}. However, infants delivered vaginally may also be exposed to antibiotics at birth²⁶⁴, a factor considered in our study.

At 7 days of age, both mode of delivery and antibiotic exposure had a comparable impact on the infant resistome (**Chapters I and III**). By 1 month, however, this association weakened, suggesting either a diminishing influence over time or a growing effect of other environmental or host-related factors. This pattern aligns with longitudinal studies showing that while the impact of early antibiotic exposure on the resistome tends to decline, certain ARGs can persist for over six months following significant antibiotic treatments⁶³.

Mode of delivery was the strongest contributor to resistome composition at 7 days, with infants born by C-section showing a higher abundance of ARGs, including MDR genes (**Chapter I** and **III**). This is consistent with previous findings linking C-section delivery to an increased risk of colonization by opportunistic pathogens such as *Klebsiella*, *Enterococcus*, and *Clostridium*²⁶⁵. Moreover, C-section-delivered infants not only harbored a greater load of ARGs but also presented a distinct resistome profile compared to vaginally delivered infants during the first year of life, in agreement with previous studies^{63,92,94,111}

Data on delivery setting—home or hospital—were available for all infants in our cohort. A recent study from our group found that delivery mode is the primary determinant of initial microbial composition differences, and that the place of birth influences the timing of microbial transmission, which occurs earlier in home births than in C-section deliveries²⁶⁶. In contrast, our findings did not indicate any significant impact of birth location on the infant resistome. This further supports the notion that while the microbiota and resistome develop at the same time, they follow distinct trajectories and are shaped independently of one another.

Breastfeeding is essential for restoring resistome disruption following C-section procedures

As the infant grows, the influence of mode of delivery is overshadowed by other factors, including breastfeeding (**Chapter III**). The crucial role of breastfeeding in fostering a healthy infant gut microbiome by promoting the growth of beneficial microbes has been previously documented²⁶⁶. In this context, our study further highlights the positive impact of breastfeeding on the acquisition and maintenance of the resistome. Prior studies have demonstrated that breastfed infants possess a gut microbiome and resistome profile that differs significantly from that of formula-fed infants^{64,267,268}. Our findings in **Chapters I** and **III** are consistent with these observations; specifically, we noted differences in ARG composition based on feeding practices, particularly from 1 to 6 months of age, and we detected higher ARG abundances in infants receiving formula. Indeed, formula-fed infants had higher prevalence of MDR and vancomycin ARGs. Thanks to the in-depth

longitudinal analysis presented in **Chapter III**, we provide compelling evidence that exclusive breastfeeding during the first month of life accelerates the reduction of antibiotic resistance in the infant gut during this period and results in a lower abundance of antibiotic resistance genes by six months of age.

Moreover, we provide additional evidence that early cessation of breastfeeding—despite WHO recommendations to continue for at least six months²⁶⁹—is linked to an enrichment of ARGs. Based on our findings and previous research⁶⁴, breastfeeding for at least six months may help reduce the prevalence of Gammaproteobacteria while simultaneously promoting bifidobacterial growth, thereby contributing to a lower burden of ARGs in the infant gut. By six months of age, shifts in diet and increased interaction with the external environment begin to play a more dominant role in shaping the infant microbiome. This period typically marks the introduction of solid foods and a shift in feeding practices, as many families either stop breastfeeding or combine it with complementary foods and liquids. At this stage, the direct effects of breastfeeding on the resistome may become less pronounced due to the growing impact of multiple external variables. Nonetheless, the so-called "window of opportunity" for shaping the infant resistome remains crucial. Any level of breastfeeding during this time continues to offer significant benefits to both the microbiome and resistome, with potential long-term effects. Additionally, the absence of breastfeeding has been identified as a key factor influencing early-life plasmid assemblages, which in turn affects resistome dynamics²⁵⁷.

While the benefits of breastfeeding on the resistome are established, this thesis further supports and extends this understanding. To our knowledge, this is the first study to demonstrate that exclusive breastfeeding may serve as a strategy to counteract the impact of C-section delivery on the infant gut resistome during the first month of life (**Chapter III**). We observed that, at 1 month of age, exclusively breastfed, C-section-delivered infants had less ARGs than those not breastfed and similar ARG loads to vaginally-born, breastfed infants. The optimal development of a healthy infant gut microbiota has been observed in full-term, vaginally delivered

infants who are exclusively fed with human milk for the first six months of life²⁷⁰. In these infants, the gut is initially colonized by maternal vaginal, fecal, skin, and oral bacteria, with *Bifidobacterium* and *Lactobacillaceae* species among the dominant colonising genera²⁷¹. This early vertical transmission from mother to infant is further reinforced through breastfeeding, as human milk contains a diverse microbiome that plays a crucial role in shaping the infant's gut health²⁶⁶. The role of breastfeeding in supporting gut microbiota development in infants delivered by C-section has been reported before⁸⁷. However, in that study, which utilized 16S rRNA gene amplicon sequencing, breastfeeding alone did not fully restore *Bifidobacterium* spp. levels. Notably, C-section-born infants who were breastfed still exhibited lower *Bifidobacterium* abundance compared to vaginally delivered, formula-fed infants. Similar to our findings, recent research by Sinha *et al.*¹³⁷ emphasized that, among C-section-born infants, feeding mode is the most influential factor shaping early microbial composition and functionality.

Building on these findings, our study extends this knowledge to the infant gut resistome, demonstrating that exclusive breastfeeding during the first month of life significantly reduces the ARG load in C-section-born infants. Our findings suggest that breastfeeding can help counteract microbial imbalances associated with C-section births, which are increasingly common but sometimes unavoidable. These results reinforce the importance of breastfeeding as an accessible and cost-effective strategy for reducing antimicrobial resistance, complementing its numerous well-established health benefits²⁷². The well-documented benefits of human milk in promoting gut microbiota development may explain its role in mitigating the delayed microbial colonization observed following C-section birth, potentially contributing to a reduction in antibiotic resistance. Prior studies suggest that this association may be mediated by the ability of HMOs to support bifidobacterial colonization²³⁹, a mechanism we have explored in depth throughout this thesis.

MATERNAL SECRETOR STATUS: HMOs AS A MECHANISM TO CONTROL ANTIBIOTIC RESISTANCE

In this thesis we have demonstrated that exclusive breastfeeding fosters a gut environment that not only supports the growth of beneficial microbiota such as *Bifidobacterium* but also modulates the acquisition and abundance of antibiotic resistance genes, thereby influencing the overall health and disease resistance of the infant. In addition, our study shows that maternal secretor status may modulate the impact of delivery mode on the infant resistome (**Chapter IV**).

As mentioned throughout this thesis, human milk serves as the primary postnatal link between a mother her infant, providing not only essential nutrients but also a rich array of bioactive compounds, including microbes and HMOs^{273,274}. *Lactobacillus*, *Bifidobacterium*, *Staphylococcus*, and *Streptococcus* are among the most predominant bacterial genera in human milk^{275,276}. Notably, *Lactobacillus* and *Bifidobacterium* thrive in the gut of breastfed infants due to their ability to metabolize HMOs⁶⁶, which are the third most abundant component in human milk²⁷⁷. The concentration and composition of HMOs vary between mothers and throughout lactation¹⁴⁷, and are influenced by maternal secretor status, which is determined by the activity of the *FUT2* gene²⁷⁸. These variations play a crucial role in shaping the infant gut microbiota, with studies indicating that infants of secretor mothers exhibit higher colonization by *Bifidobacterium*²⁷⁹. Previous research has established that maternal secretor status influences the composition the infant gut microbiome²⁸⁰. In our study, we extend these findings by demonstrating, for the first time, a direct link between maternal secretor status and the infant resistome. Infants breastfed by non-secretor mothers had a greater abundance and diversity of ARGs than those breastfed by secretors. Specifically, we observed higher levels of genes conferring resistance to macrolides and streptogramin B. Indeed, milk from non-secretors also had a higher diversity and ARG load than secretor milk; however, the microbiome composition of both the infant gut and human milk remained unaffected by maternal secretor status. This suggests that the role of breastfeeding in shaping the infant resistome may be driven more by bioactive components in

human milk rather than direct microbial transfer. Beyond bacterial transmission, breastfed infants received other key compounds, such as HMOs, which may actively modulate the gut microbiome and resistome (**Figure 10**).

In this vein, given the impact of both maternal secretor status and mode of delivery on the resistome of breastfed infants, we hypothesized that the effect of delivery mode at 1 month of age could be influenced by the type of human milk an infant receives. Consistent with previous studies²⁸¹, we observed that breastfeeding supports the restoration of gut microbiota in C-section-born infants. Specifically, C-section-born infants breastfed by secretor mothers exhibit *Bifidobacterium* spp. levels comparable to those of vaginally born infants. This aligns with prior observations that infants of non-secretor mothers tend to have lower *Bifidobacterium* abundance²⁸¹. Within our cohort, *B. bifidum* was particularly prevalent in vaginally born infants who received milk from secretor mothers, highlighting the species-specific ability of bifidobacteria to degrade HMOs²⁸². Furthermore, in infants breastfed by non-secretor mothers, we observed a trend toward a higher abundance of enterobacteria²⁸¹. Infants receiving milk from secretors had lower levels of *Klebsiella* and *Escherichia* compared to those breastfed by non-secretors, regardless of delivery mode. Since these species are not known to degrade HMOs¹⁷⁴, they may opportunistically occupy an ecological niche in the infant gut when dominant HMO-degrading bacteria are less abundant.

Notably, the impact of delivery mode on the resistome profile was only evident in infants breastfed by non-secretor mothers, while in those breastfed by secretors, this effect was no longer apparent. This suggests that maternal secretor status exerts a stronger influence on the infant resistome than mode of delivery. Consequently, the effect of C-section birth on the infant resistome appears to be mitigated in infants breastfed by secretor mothers (**Figure 10**).

Through HMO glycoprofiling of human milk samples conducted at the University of California, San Diego during a temporary predoctoral research visit, we measured the concentrations of 20 different HMOs and found that specific HMO

profiles are associated with variations in antibiotic resistance in the infant gut. For example, 2'FL and 6'SL -both highly abundant in secretor milk²⁷⁷- showed a negative correlation with resistance to tetracyclines and lincosamides, whereas 3FL and LNT—more prevalent in non-secretor milk—were positively linked to lincosamide and fosfomycin resistance. These results confirmed that the impact of breastfeeding on the infant resistome is mediated by variations in HMO composition, which in turn depend on maternal secretor status. This insight not only deepens our understanding of how HMOs influence resistome modulation but also opens new avenues for research and potential therapeutic applications. Screening for the *FUT2* gene or analyzing HMO composition in the milk of mothers who delivered by C-section could help identify infants at higher risk for altered gut microbiota and resistome development. These infants might benefit from targeted interventions, such as HMO supplementation, to support microbiome restoration and reduce antibiotic resistance burden.

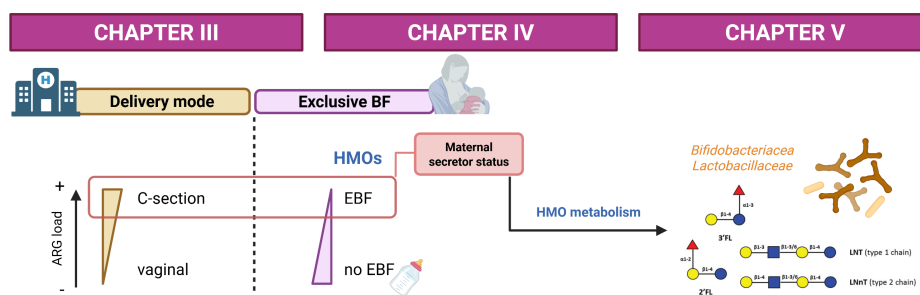


Figure 10. The influence of mode of delivery, breastfeeding, and HMOs on the infant gut resistome. Summary of the main findings in Chapters III, IV and V. III-IV-V. BF: breastfeeding; EBF: exclusive breastfeeding. Created in BioRender (<https://BioRender.com/e2tgq89>).

The human milk resistome: a vehicle for the spread of antibiotic resistance?

To investigate the microbial composition and resistome of human milk, we conducted metagenomic analyses (**Chapter IV**). However, as observed in previous studies²⁶⁶, the high proportion of human DNA in milk samples posed a technical challenge, limiting the recovery of high-quality metagenomes. Despite this limitation, we successfully obtained high-quality metagenomes from 50 human

milk samples, enabling us to characterize their microbiome and resistome. Our findings align with previous research^{139,276}, confirming that *Staphylococcus* spp. and *Streptococcus* spp. are the dominant bacterial genera in human milk, reflecting the enrichment of oral and skin-associated bacteria in these samples²⁸³.

Furthermore, our results suggest that human milk may serve as a reservoir of ARGs during infant development, as we identified shared resistance genes against macrolides and lincosamides in both human milk and infant fecal samples. Various studies have detected resistant strains of *Staphylococcus*, *Streptococcus*, *Acinetobacter*, *Enterococcus*, and *Corynebacterium* in breastmilk, with some of these species exhibiting multidrug-resistant profiles^{64,138,139}. Notably, multidrug-resistant *Streptococcus* and *Staphylococcus* strains have been shown to transfer from mother to infant through breastfeeding^{140,284}.

Although not included in this thesis, the doctoral research has provided the opportunity to isolate *Staphylococcus*, *Streptococcus*, and other bacterial strains from human milk and infant fecal samples, and their resistance profiles are currently being studied. This work aims to characterize ARG-carrying bacteria in human milk and assess the potential transmission of strains through the mother-human milk-infant pathway. Further studies are needed to better understand the extent to which antibiotic resistance is transmitted from mother to infant via the milk. Longitudinal studies analyzing larger cohorts across different geographic regions would provide valuable insights into how maternal factors, diet, antibiotic use, and environmental exposures influence the presence and persistence of resistance genes in breastmilk and their transfer to infants. Investigating the interplay between breastmilk resistomes and the developing infant gut microbiome will be crucial for designing interventions to mitigate the transmission of antibiotic resistance, ultimately supporting a healthier microbiome development in infants.

HMOs AND ARGs IN THE MATERNAL-INFANT MICROBIOME: A FOCUS ON BIFIDOBACTERIA AND LACTOBACILLI

As described, we explored the role of breastfeeding in modulating the infant resistome, with a particular focus on the influence of HMOs (**Chapter IV**). Notably, *Bifidobacterium*, known for their ability to metabolize HMOs, has been identified as a key player in shaping the infant resistome (**Chapter II and III**). In this regard, breastfeeding directly and indirectly promotes the growth of beneficial bacteria, mitigating the early acquisition of antibiotic resistance. For this reason, the final objective of this thesis was to explore the metabolism of HMOs by bifidobacteria and lactobacilli isolated from human milk and fecal samples from mother-infant dyads , as well as their antibiotic resistance profiles, to provide more evidence on the potential role of human milk in early life microbiome and resistome establishment (**Figure 11**). This objective (**Chapter V**) was achieved during the predoctoral research visit at the University of California, San Diego.

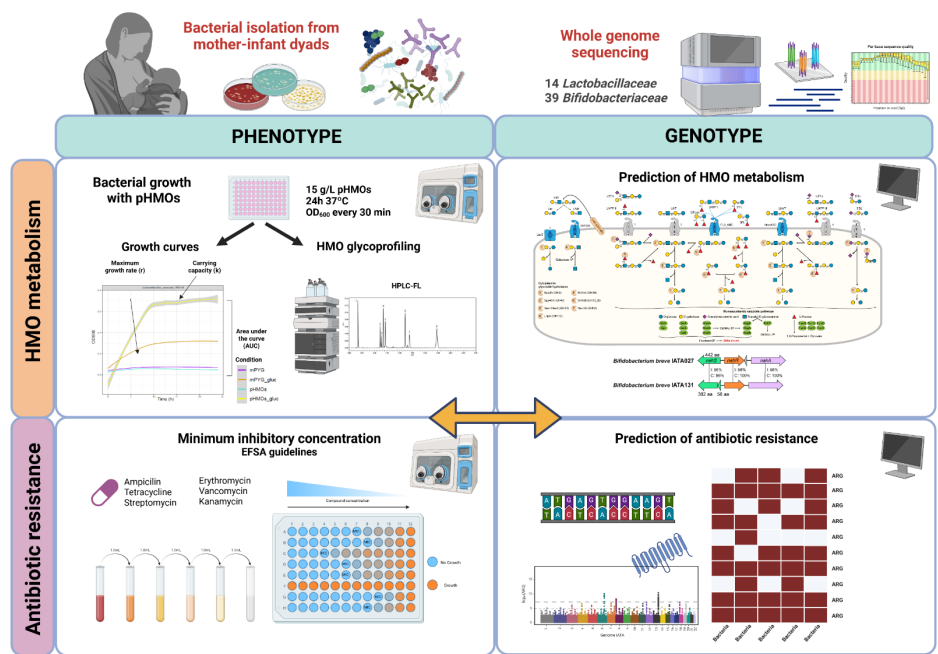


Figure 11. Flowchart of the methodologies used in Chapter V. A total of 39 *Bifidobacterium* and 14 *Lactobacillaceae* strains were isolated from paired mother-infant fecal samples and human milk samples. Whole genome sequencing (WGS) revealed the resistance genotypes

and enabled functional predictions of HMO metabolism abilities for each strain. *In vitro* HMO utilization was assessed using growth kinetics assays combined with HMO glycoprofiling in culture supernatant. The minimum inhibitory concentration (MIC) was also determined for each strain. Created in BioRender (<https://BioRender.com/y60g273>)

Our findings revealed an inverse correlation between HMO degradation capacity and antibiotic resistance. Strains commonly isolated from infant sources, such as *B. bifidum*, *B. infantis*, and *B. longum*, exhibited strong HMO degradation abilities but were highly susceptible to antibiotics. In contrast, *B. lactis* and *B. adolescentis* demonstrated greater antibiotic resistance yet had a more limited capacity for HMO utilization. To date, only one study has explored the link between microbial carbohydrate metabolism and the development of the infant resistome¹⁰². That research utilized metagenomic functional profiling and metagenome-assembled genome analyses and showed that shifts in microbial carbohydrate metabolism during infancy are associated with gut resistome development. Our results are consistent with these findings and are further reinforced by our *in vitro* experiments. Since breastfeeding promotes the colonization of these beneficial microbes and fosters cross-feeding interactions between *Bifidobacterium* and *Lactobacillaceae*, antibiotic exposure may undermine these advantages. Given that early colonizers are highly sensitive to antibiotics, their depletion could create an environment that favours the proliferation of antibiotic-resistant bacteria. This shift may ultimately shape the infant resistome and diminish the protective effects of breastfeeding and HMOs in mitigating antibiotic resistance.

We demonstrated that HMO metabolism in *Bifidobacterium* is highly species-specific. Strains from the species *B. bifidum* and *B. infantis* could degrade a broad spectrum of HMOs, highlighting their significance as key colonizers of the breastfed infant gut. Conversely, while *B. longum* and *B. breve* are also prevalent during early life, their capacity to utilize HMOs appeared more restricted. These differences in HMO utilization among *Bifidobacterium* species have been previously reported^{285,286}, and their associated metabolic pathways have been reconstructed at the genomic level^{168,287}. Furthermore, consistent with prior research²⁸⁸, species commonly associated with the adult gut microbiota, such as *B.*

pseudocatenulatum, *B. adolescentis*, and *B. lactis*, were found to lack the genetic components necessary for efficient HMO metabolism.

Regarding *Lactobacillaceae*, previous studies have indicated that their capacity to utilize HMOs is not as extensive as that of *Bifidobacterium*^{165,166,286}. In line with these findings, our genomic analysis revealed a lower abundance of HMO-degrading GHs in *Lactobacillaceae* compared to *Bifidobacterium*. Many *Lactobacillaceae* species, such as *L. rhamnosus* and *L. plantarum*, can metabolize HMO-derived components such as glucose, lactose, LNB, GlcNAc, and LacNAc, and are therefore considered "secondary degraders"^{289,290}.

Although previous studies suggest that *Bifidobacterium* and *Lactobacillaceae* possess relatively few ARGs and have a lower likelihood of HGT^{239,291}, our findings indicated that both genera carry ARGs and exhibit antibiotic resistance *in vitro*. In some instances, this resistance may result from point mutations in specific genes²⁹². Additionally, we observed discrepancies between *in silico* predictions and *in vitro* antibiotic resistance profiles. These inconsistencies may be attributed to regulatory mechanisms, mutations that alter gene function, or emerging resistance mechanisms that are not yet documented in ARG reference databases. Furthermore, some strains exhibited phenotypic resistance despite lacking identifiable ARGs. For example, *B. lactis* strains exhibited some of the the highest levels of antibiotic resistance, despite harbouring only two recognized ARGs in their genomes. This finding highlights the importance of validating genomic predictions through phenotypic assays to ensure accurate assessments of antibiotic resistance. The *in vitro* genotype-to-phenotype validation approach not only enhances the reliability of resistance profiling but also informs risk assessments in clinical, probiotic, and microbiome-related contexts, where undetected resistance could have significant health implications.

WHAT WE KNOW THAT WE DO NOT KNOW

As with all scientific discoveries, not all gaps are filled. In recent years, growing concerns over antibiotic resistance have driven increased research efforts

to understand this phenomenon, particularly in early life. However, many questions remain unanswered, and greater efforts are required to combat this silent pandemic.

In this thesis, we have revealed that while the development of the resistome occurs alongside the microbiome, alterations in the microbiome do not necessarily have a direct impact on the resistome. Nonetheless, the technical protocols used to study both biomes share fundamental methodological principles and face similar limitations. Many microbiome studies have reported significant variability in their findings, occasionally even yielding contradictory results. This inconsistency may be partly attributed to differences in sample collection and storage procedures, as well as variations in DNA extraction techniques. Additionally, sequencing methodologies can also contribute to discrepancies in results. The rapid advancement of NGS technologies has significantly advanced human microbiome research and decreasing costs have made them accessible to a broad range of laboratories worldwide. Nevertheless, to minimize variability among studies, a standardized workflow for data generation and analysis is essential. Advancing and refining bioinformatics tools that enable a more in-depth analysis of microbial taxa interactions within communities and their antibiotic resistance is crucial for the continued progress of microbiome research. This is particularly vital in the maternal–neonatal microbiome field, as certain target niches are characterized by low-biomass environments. In such cases, distinguishing between contamination and the genetic material of interest is essential for drawing meaningful and reliable conclusions.

Beyond technical considerations, the research community continues to advocate for a shift in microbiome studies—from simply identifying associations to establishing causation. In recent years, numerous studies have linked microbial alterations, environmental factors, and genetic background to an increased risk of various diseases. However, a significant gap remains in understanding the molecular mechanisms that drive these relationships. Ideally, research should begin with large human cohorts and extensive datasets, which serve as the

foundation for formulating a working hypothesis. This hypothesis should then be tested through host-focused proof-of-concept studies to validate initial findings. The next step should involve using targeted models to investigate the specific mechanisms underlying the microbiome's causative role in shaping host phenotypes and health outcomes. Once a key mechanism is identified, an intervention can be designed and evaluated preclinically to determine its ability to modify the affected pathway. If the intervention demonstrates both safety and efficacy, clinical trials should follow²⁹³.

The relatively small sample size in some chapters of this thesis may introduce bias, and the focus on infants from a single geographic region limits its generalizability. Future research should prioritize larger, longitudinal studies, include a higher proportion of exclusively formula-fed infants, and extend research by incorporating experimental approaches to evaluate whether prebiotic or probiotic supplementation could support microbiome maturation and positively influence resistome development in early life. Furthermore, to preserve a healthy infant microbiome after birth and minimize the proliferation of potential pathogens and ARGs, unnecessary antibiotic prescriptions must be avoided. As demonstrated in this study, even in the absence of antibiotic administration, infants harbour a wide range of antibiotic resistances. Ideally, microbial biomarkers or specific gut microbiome patterns associated with pathogen infection could be identified to help determine whether antibiotic treatment is truly necessary.

As discussed in this thesis, various environmental factors can disrupt the infant microbiome during its early developmental phase. One of the most significant factors is the mode of delivery. Medical researchers and healthcare professionals play a critical role in educating expectant parents about the potential consequences of C-section delivery on the infant microbiome and, by extension, on their child's long-term health. Obstetrician-gynecologists acknowledge the rising demand for C-section deliveries based on maternal requests, yet they generally consider the risks to outweigh the benefits. To guide delivery practices, strong evidence regarding the negative impact of C-section on microbiome development,

immune system priming, and long-term health outcomes is essential. Such evidence could support counselling parents toward vaginal delivery when it is safe for both mother and child. However, in cases where C-section delivery is medically necessary due to maternal or infant complications, strategies to restore the infant's microbiome and resistome should be explored. Current research is investigating methods such as vaginal and fecal seeding, yet further studies are required to determine their effectiveness. As highlighted in this thesis, promoting breastfeeding should be the primary strategy for supporting optimal microbiome development, reducing antibiotic resistance, and mitigating the impact of C-section procedures on the infant gut microbiome and resistome.

Additionally, optimizing the dosing and administration of probiotics may be key to achieving healthy long-term microbial colonization. While numerous health benefits have been attributed to probiotics, ongoing research aims to determine their potential for preventing specific diseases or restoring microbial homeostasis. Investigating whether probiotic supplementation can effectively enhance microbiome resilience and mitigate C-section- and antibiotic-induced disruptions would be a valuable avenue for future studies.

Closing the remaining knowledge gaps in maternal-offspring microbiome research will require a multidisciplinary approach, combining expertise from microbiologists, clinicians, bioinformaticians, and other specialists. Collaborative efforts, along with larger and more diverse studies, will be essential to standardizing methodologies, improving data reliability, and uncovering the molecular mechanisms underlying microbiome-related health outcomes. Advancements in sequencing technologies, bioinformatics tools, and experimental models will further enhance our understanding of microbiome and resistome development, antibiotic resistance, and potential therapeutic interventions. By fostering collaboration and investing in rigorous research, we can move closer to translating microbiome science into meaningful clinical applications, ultimately improving early-life health and long-term disease prevention.

CONCLUSIONS

The main conclusions of this thesis are:

- I. The **evolutionary** trajectory of the infant resistome reflects the **intricate dynamics of ARGs** within the **early-life microbiota**, shaped by its **susceptibility to external factors**. Over the first year of life, the infant resistome tends to **resemble** that of its **mother**, with a decrease in ARG abundance, and an increase in both microbial and resistance gene diversity.
- II. The microbiota of **high-*Bifidobacterium*-dominated infants** is associated with a **lower abundance of ARGs**, likely due to a distinct microbial composition that harbors fewer ARGs, along with the **protective effect** of *Bifidobacterium* against antimicrobial resistance.
- III. **Breastfeeding** fosters an infant gut environment that not only supports the growth of **beneficial microbiota**, such as *Bifidobacterium*, but also **modulates** the acquisition and abundance of antibiotic resistance genes, thereby influencing the overall health and disease resistance of the infant.
- IV. The **positive contribution of human milk** to infant gut microbiota development explains why **breastfeeding** can restore the **disrupted** microbiota development associated with **C-section birth**, resulting in a **reduction** of ARGs. These findings highlight exclusive breastfeeding as a **potential strategy** to counteract the resistome-altering effects of C-section birth.
- V. **HMOs** have a direct effect on shaping the infant resistome, as the effect of a **C-section** on the microbiota is **diluted** in infants breastfeeding from **secretor** mothers. **Secretor status modulates the impact of delivery mode on the infant resistome**, homogenizing its gut microbiota composition in infants breastfed by secretor mothers, regardless mode of delivery.
- VI. Temporal variation in bifidobacteria and lactobacilli species in the infant gut is influenced not only by their **specific ability to utilize HMOs** but also by their **antibiotic resistance profiles**. Breastfeeding has an essential role in shaping a healthy infant's gut microbiota by supporting the growth of HMO-utilizing beneficial bacteria, which can be disrupted by the impact of antibiotics during infancy.

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APPENDICES

ORIGINAL PUBLICATIONS

Publications used for the introduction section and general discussion:

1. **Samarra A**, Esteban-Torres M, Cabrera-Rubio R, Bernabeu M, Collado MC. Maternal-infant antibiotic resistance genes transference: what do we know? *Gut Microbes*. 2023;15(1). doi:10.1080/19490976.2023.2194797
2. Bernabeu M, Cabello-Yeves E, Flores E, **Samarra A**, Summers JK, Marina A, Collado MC. Role of vertical and horizontal microbial transmission of antimicrobial resistance genes in early life: insights from maternal-infant dyads. *Curr Opin Microbiol*. 2024;77:102424. doi:10.1016/J.MIB.2023.102424
3. Saturio S, Rey A, **Samarra A**, Collado MC, Suárez M, Matecón L, Solís G, Gueimonde M, Arboleya S. Old Folks, Bad Boon: Antimicrobial Resistance in the Infant Gut Microbiome. *Microorganisms* 2023, Vol 11, Page 1907. 2023;11(8):1907. doi:10.3390/MICROORGANISMS11081907
4. **Samarra A**, Flores E, Bernabeu M, Cabrera-Rubio R, Bäuerl C, Selma-Royo M, Collado MC. Shaping Microbiota During the First 1000 Days of Life. *Adv Exp Med Biol*. 2024;1449:1-28. doi:10.1007/978-3-031-58572-2_1

Original publications included as Chapters of the thesis:

- I. **Samarra A**, Cabrera-Rubio R, Martínez-Costa C, Collado MC. The role of *Bifidobacterium* genus in modulating the neonate microbiota: implications for antibiotic resistance acquisition in early life. *Gut Microbes*. 2024;16(1). doi:10.1080/19490976.2024.2357176
- II. **Samarra A**, Cabrera-Rubio R, Martínez-Costa C, Collado MC. Unravelling the evolutionary dynamics of antibiotic resistance genes in the infant gut microbiota during the first four months of life. *Ann Clin Microbiol Antimicrob*. 2024;23(1):1-16. doi:10.1186/S12941-024-00725-Z/TABLES/4
- III. **Samarra A**, Alcañiz A, Martínez-Costa C, Marina A, Comas I, Segata N, Quijada N, Collado MC. Breastfeeding and Early Bifidobacterium-driven Microbial Colonization Shape the Early Infant Gut Resistome. *Nature Communications*. 2025, *In press*.
- IV. **Samarra A**, Quijada N, Renwick S, Alcañiz A, Spann K, Martínez-Costa C, Quijada N, Bode L, Collado MC. Maternal Secretor Status and Human Milk Oligosaccharides Modulate the Impact of Cesarean Birth on the Infant Gut Resistome. *Manuscript under review*.
- V. **Samarra A**, Renwick S, Arzamasov A., et al. Human milk oligosaccharide metabolism and antibiotic resistance in early gut colonizers: insights from bifidobacteria and lactobacilli in the maternal-infant microbiome. *Gut Microbes*. 2025;17(1):2501192. doi:10.1080/19490976.2025.2501192

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Consent statement to participate

All participants' parent(s) or guardian(s) received oral and written information about the study and written consent was obtained. The study was approved by the Hospital Ethics Committees (HECs) (Hospital Universitario y Politécnico La Fe and Hospital Clínico Universitario de Valencia) in accordance with the Declaration of Helsinki, and the study was registered on the ClinicalTrials.gov platform on the 21-03-2018, with the registration number NCT03552939.

Availability of data and materials

- The 16S sequencing data supporting the conclusions of **Chapter I** and **II** is available in the NCBI's Sequence Read Archive (SRA) repository, under the BioProject ID **PRJNA614975**.
- Raw sequences of shotgun metagenomics samples used in **Chapters III** and **IV** are available on the European Nucleotide archive (ENA) and on SRA under the accession numbers **PRJEB74322** and **PRJNA1227883**.
- Genome sequences of all isolates used in **Chapter V** have been deposited in the National Center for Biotechnology Information (NCBI) under accession **PRJNA1190840**.

Competing Interests

The authors and collaborators declare no competing financial interest.

Declaration on the use of BioRender figures

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ABBREVIATIONS TABLE

2'FL	2'-Fucosyllactose
3'SL	3'-Sialyllactose
3FL	3-Fucosyllactose
6'SL	6'-Sialyllactose
AIC	Akaike Information Criterion
AMP	Ampicillin
AMR	Antimicrobial Resistance
ANI	Average Nucleotide Identity
ANOVA	Analysis of Variance
APS	Antimicrobial Stewardship Programs
ARG	Antibiotic Resistance Gene
ARM	Antibiotic-Resistant Microorganism
AUC	Area Under the Curve
BF	Breastfeeding
BH	Benjamini–Hochberg Procedure
BMIZ	Body Mass Index Z-Score
CARD	Comprehensive Antibiotic Resistance Database
CAZy	Carbohydrate-Active enZymes
CDC	Centers for Disease Control and Prevention
CHLORA	Chloramphenicol
CSIC	Consejo Superior de Investigaciones Científicas
db-RDA	Distance-Based Multivariate Redundancy Analysis
DFLac	Difucosyllactose
DFLNH	Difucosyllacto-N-hexaose
DFLNT	Difucosyllacto-N-tetraose
DNA	Deoxyribonucleic Acid
DSLNH	Disialyllacto-N-hexaose
DSLNT	Disialyllacto-N-tetraose
EBF	Exclusive Breastfeeding
EFSA	European Food Safety Authority
ERY	Erythromycin
ESBL	Extended-Spectrum Beta-Lactamase
FDR	False Discovery Rate
FDSLNH	Fucodisialyllacto-N-hexaose
FLNH	Fucosyllacto-N-hexaose
FMT	Fecal Microbiota Transplant
Fuc	L-Fucose

FUT2	α -1,2-Fucosyltransferase
Gal	D-Galactose
GalNAc	N-Acetyl-D-galactosamine
GBS	Group B Streptococcus
GEN	Gentamicin
GH	Glycoside Hydrolase
GI	Gastrointestinal
GlcNAc	N-Acetylglucosamine
GLM	Generalized Linear Model
Glu	D-Glucose
GNB	Galacto-N-biose
GTDB	Genome Taxonomy Database
HGT	Horizontal Gene Transfer
HMO	Human Milk Oligosaccharide
HPLC	High-Performance Liquid Chromatography
IAP	Intrapartum Antibiotic Prophylaxis
IBD	Inflammatory Bowel Disease
IQR	Interquartile Range
KAN	Kanamycin
LacNAc	N-Acetyl-D-lactosamine
Le ^a	Lewis a
Le ^b	Lewis b
LEfSE	Linear Discriminant Analysis Effect Size
LNB	Lacto-N-biose
LNFP I	Lacto-N-fucopentaose I
LNFP II	Lacto-N-fucopentaose II
LNFP III	Lacto-N-fucopentaose III
LNH	Lacto-N-hexaose
LNnT	Lacto-N-neotetraose
LNT	Lacto-N-tetraose
LNTII	Lacto-N-triose
LST	Sialyllacto-N-tetraose
LSTb	Sialyllacto-N-tetraose b
LSTc	Sialyllacto-N-tetraose c
MAG	Metagenome Assembled Genome
MDR	Multidrug Resistance
MGE	Mobile Genetic Element
MIC	Minimum Inhibitory Concentration
ML	Machine Learning
MLSB	Macrolide–Lincosamide–Streptogramin B

mPYG	Modified Peptone–Yeast–Glucose Medium
MRS	Man, Rogosa and Sharpe Medium
MRSA	Methicillin-Resistant Staphylococcus Aureus
MSS	Metagenome Shotgun Sequencing
NCBI	National Center for Biotechnology Information
NGS	Next-Generation Sequencing
NICU	Neonatal Intensive Care Unit
NMDS	Non-metric Multidimensional Scaling
OD	Optical Density
PAM	Partitioning Around Medoids
PBS	Phosphate-Buffered Saline
PCA	Principal Component Analysis
PCoA	Principal Coordinates Analysis
PERMANOVA	Permutational Multivariate Analysis of Variance
pHMOs	Pooled Human Milk Oligosaccharides
PTS	Phosphotransferase System
qPCR	Quantitative Polymerase Chain Reaction
RF	Random Forest
RFLP	Restriction Fragment Length Polymorphism
RGI	Resistance Gene Identifier
SCFA	Short-Chain Fatty Acid
SD	Standard Deviation
Se	Secretor
sEONS	Suspected Early-Onset Neonatal Sepsis
Sia	Sialic Acid
SRA	Sequence Read Archive
STREP	Streptomycin
TET	Tetracycline
VAN	Vancomycin
VG	Vertical Gene Transfer
VIF	Variance Inflation Factor

ANNEXES

Annex Table 1

Study	Delivery mode	Feeding mode	Antibiotic exposure	Environment	Resistome dynamics
Ojeda et al. 2024	ARG diversity and composition was not influenced by delivery mode. IAP and prematurity have more importance		Post-natal antibiotic exposure increases beta-lactam/tetracycline resistance, lowers diversity		High prevalence of ARGs in preterm infants irrespective of direct antibiotic exposure, which intensified with age in parallel to the developing microbiome.
Sinha et al. 2024		Feeding mode significantly outweighs other factors in shaping the gut microbial	Maternal prophylactic antibiotic use during CS has minimal impact on the early-life composition and functionality of the infant gut microbiome, but subtly impact strain variability and antibiotic resistance genes		
Jokela et al. 2024	C-section infants have less ARGs (but most of them are breastfed). The effect of delivery mode on infants resistome is much stronger than the effect of intrapartum antibiotics and during the first 6 months of life.		The choice of antibiotic mattered in vaginally delivered infants exposed to intrapartum antibiotics.		Yes
Xu et al. 2024		Breastfeeding practices and weaning drive infant's resistome development.			Maturation of gut resistome was driven by infants' changing carbohydrate metabolism, which demonstrated an increasing need for carbohydrate-active enzymes from Bacteroidota and decreasing involvements from Pseudomonadota during infancy.
Yuu et al. 2024			The greatest percentages of ARGs (especially bla genes) were found in the antibiotic-treated infants		

Patan-gia et al. 2024	Most strains carrying antibiotic-resistance genes belonged to phyla Pseudomonadota and Bacillota, with the C-section/antibiotic-exposed group presenting a higher frequency of ARGs.		Most strains carrying antibiotic-resistance genes belonged to phyla Pseudomonadota and Bacillota, with the C-section/antibiotic-exposed group presenting a higher frequency of ARGs.	Yes
Zhao et al. 2024	Mode of delivery influences resistome composition	Breastfeeding and later dietary structure significantly influence the gut microbial composition and abundance of ARGs in early childhood	First exposure to antibiotics led to a dramatic increase in the number of opportunistic pathogens carrying multidrug efflux pump encoding genes.	The abundance of bacteria containing specific ARGs increased after the corresponding antibiotic treatment
Leo et al. 2023	Mode of delivery influences resistome composition	Formula-fed infants had higher loads of ARGs than exclusive breastfed infants.	IAP had little effect on the abundance of ARGs, but it was higher in neonates whose mothers took antibiotics during pregnancy	
Bargh et al. 2023			No association between the use of specific antibiotics and ARG profiles	
Reyman et al. 2022			Decreased abundance of <i>Bifidobacterium</i> spp. and increased abundance of <i>Klebsiella</i> and <i>Enterococcus</i> spp. in infants treated with broad-spectrum antibiotics.	
Pärnär en et al. 2022		Formula-fed neonates had higher relative abundances of opportunistic pathogens and ARGs, and lower abundances of bifidobacteria.		
Lebeaux et al. 2021	Delivery mode was most strongly associated with the diversity and relative abundance of ARGs. Infants born via C-section delivery had a trend towards a higher risk of harboring unique ARGs as well as having a higher ARG relative abundance.	Compared to exclusive breastfeeding at 6 weeks, any formula feeding exposure was associated with an increased risk of having diverse ARGs at 6 months, but a lower risk at 1 year.	Infant antibiotic exposure after delivery influences their resistome.	Yes

Li et al. 2021	Mode of delivery influences resistome composition		Children who had taken antibiotics had significantly higher mean ARG abundance. The use of antibiotics in pregnancy is implicated in shaping ARG profiles in the infant gut.	Slightly higher ARG abundance in childrens without siblings and with dogs in the home.	The importance of antibiotics diminished with time since treatment. Some ARGs persisted more than six months after four major antibiotic treatments.
Sosa-Moreno et al. 2020	No significant impact of delivery mode on the infant resistome at 6 months	Contact with breastfeeding influences resistome composition	No relationship between prenatal antibiotic use and resistome patterns.		
Loo et al. 2020			Offspring of mothers exposed to beta-lactam antibiotics during pregnancy and/or labour had significantly increased detection of the beta-lactam-resistance conferring genes		Yes
Mills et al. 2023				Ruminant and dog fecal contamination house floors, as well as poor water quality were associated with multidrug, tetracycline, macrolide/lincosamide /streptogramin, and beta-lactam resistance	Yes

Annex Chapter I

Supplementary Table I-1. Copy number of the ARGs quantified by HT-qPCR in the pre-screening.

Group	Gene	Assay	Infant gut	Mother gut
Taxonomic	Bacillota	AY477	8.88E+06	3.45E+07
Taxonomic	Bacteroidota	AY474	7.57E+06	1.78E+07
Taxonomic	K. pneumoniae	AY478	1.69E+04	nd
Taxonomic	A. baumannii	AY473	nd	nd
Taxonomic	P. aeruginosa	AY479	nd	nd
Taxonomic	Campylobacter	AY475	nd	nd
Taxonomic	Enterococci	AY476	nd	nd
Taxonomic	Staphylococci	AY480	nd	nd
Integrans	intl1_1	AY293	3.11E+06	1.91E+06
Integrans	intl3	AY500	5.79E+05	1.03E+05
Integrans	intl1_2	AY289	2.79E+05	1.19E+05
Integrans	intl2_2	AY294	2.58E+03	nd
MGE	IS26_1	AY512	3.05E+06	2.10E+05
MGE	tnpA_1	AY299	2.34E+06	2.87E+05
MGE	IS200_2	AY509	1.47E+06	7.64E+04
MGE	ISEcp1	AY520	9.40E+05	nd
MGE	Tp614	AY297	5.28E+04	1.28E+06
MGE	trbC	AY527	5.75E+05	1.78E+04
MGE	IS613	AY298	nd	1.04E+06
MGE	IS1111	AY313	4.08E+05	5.68E+03
MGE	Tn5403	AY524	4.00E+05	1.04E+04
MGE	Tn3	AY523	3.81E+05	5.43E+03
MGE	tnpA_5	AY303	3.58E+05	nd
MGE	IS3	AY513	2.53E+05	nd
MGE	tnpA_6	AY304	2.23E+05	5.58E+04
MGE	orf37-IS26	AY307	2.36E+05	1.48E+04
MGE	IS1247_1	AY506	1.68E+05	1.15E+04
MGE	tnpA_7	AY305	7.59E+04	1.29E+05
MGE	IS6100	AY516	1.01E+05	nd
MGE	IS21-ISAs29	AY510	8.85E+04	3.31E+03
MGE	tnpA_2	AY300	7.09E+04	nd
MGE	IncN_rep	AY316	3.65E+04	nd
MGE	IS1133	AY314	2.69E+04	nd
MGE	IS630	AY517	2.36E+04	4.69E+03
MGE	IncI1_rep1	AY504	1.58E+04	2.47E+03
MGE	IS1247_2	AY310	9.80E+03	2.44E+03
MGE	IS200_1	AY508	5.97E+03	9.25E+03
MGE	IS6/257	AY515	8.59E+03	nd
MGE	IS256	AY511	nd	1.88E+04
MGE	ISCR1	AY519	7.12E+03	nd
MGE	EAE_05855	AY502	6.27E+03	nd
MGE	IncP_oriT	AY318	2.81E+03	nd
MGE	IncQ_oriT	AY319	nd	5.21E+03

MGE	IncW_trwAB	AY320	1.70E+03	nd
MGE	tnpA_4	AY302	nd	2.99E+03
MGE	tnpA_3	AY301	nd	nd
MGE	ISpps	AY309	nd	nd
MGE	ISEfm1	AY312	nd	nd
MGE	cro	AY501	nd	nd
MGE	IncF_FIC	AY521	nd	nd
MGE	trfA	AY306	nd	nd
MGE	ISAb3	AY311	nd	nd
MGE	Tn5	AY315	nd	nd
MGE	IncN_oriT	AY317	nd	nd
MGE	pAMBL	AY321	nd	nd
MGE	pAKD1	AY324	nd	nd
MGE	IncHI2-smr0018	AY503	nd	nd
MGE	IncN_korA	AY505	nd	nd
MGE	IS5/IS1182	AY514	nd	nd
MGE	traN	AY526	nd	nd
Sulfonamide	sul2_2	AY365	4.94E+05	1.91E+04
Sulfonamide	sul1_2	AY245	1.67E+05	1.15E+05
Sulfonamide	folA_1	AY247	5.92E+04	3.48E+03
Sulfonamide	sul3_1	AY244	2.77E+04	3.26E+03
Sulfonamide	folP_2	AY361	1.45E+03	nd
Sulfonamide	sul4	AY241	1.00E+03	nd
Tetracycline	tetW	AY263	2.33E+04	1.33E+06
Tetracycline	tetO_2	AY264	1.79E+05	9.50E+05
Tetracycline	tetPA	AY575	3.52E+05	4.97E+03
Tetracycline	tetM	AY574	2.59E+05	1.12E+05
Tetracycline	tetC_2	AY268	2.07E+05	5.35E+03
Tetracycline	tet32	AY250	1.76E+03	4.85E+05
Tetracycline	tetD	AY571	1.74E+05	2.05E+04
Tetracycline	tetQ	AY259	1.83E+04	3.69E+05
Tetracycline	tetA/B_1	AY255	1.60E+05	2.02E+03
Tetracycline	tetPB_1	AY274	1.46E+05	3.83E+03
Tetracycline	tetR	AY577	1.17E+05	1.10E+04
Tetracycline	tetL_2	AY367	9.83E+04	nd
Tetracycline	tetA_2	AY254	7.09E+04	1.15E+03
Tetracycline	tetX	AY267	nd	1.64E+05
Tetracycline	tetR_1	AY325	5.80E+04	nd
Tetracycline	tet44	AY576	3.35E+04	2.49E+04
Tetracycline	tetG	AY572	1.90E+04	8.76E+03
Tetracycline	tet38	AY570	6.00E+03	nd
Tetracycline	tetS	AY269	8.69E+02	nd
Tetracycline	tet36_1	AY249	nd	nd
Tetracycline	tetK	AY258	nd	nd
Tetracycline	tetE	AY273	nd	nd
Tetracycline	tetH	AY260	nd	nd
Tetracycline	tetT	AY276	nd	nd
Tetracycline	tet39	AY568	nd	nd
Tetracycline	tetJ	AY573	nd	nd

MDR	mdtH	AY486	6.36E+05	7.38E+04
MDR	mdtA	AY485	5.17E+05	7.36E+04
MDR	acrA_1	AY207	4.62E+05	6.34E+04
MDR	mdtG_1	AY212	3.97E+05	7.48E+04
MDR	mdtE	AY211	3.17E+05	8.25E+04
MDR	acrB_1	AY199	2.64E+05	7.06E+04
MDR	cefa_qacelta	AY487	2.03E+05	1.39E+05
MDR	tolC_2	AY353	4.71E+04	9.89E+03
MDR	czcA	AY493	3.85E+04	nd
MDR	emrD_1	AY208	3.34E+04	1.52E+03
MDR	acrF	AY201	nd	6.72E+04
MDR	pcoA	AY495	2.71E+04	nd
MDR	oqxA	AY482	1.79E+04	nd
MDR	arsA	AY490	1.65E+04	nd
MDR	mepA	AY227	1.48E+04	1.61E+03
MDR	mexB	AY240	1.09E+04	5.07E+03
MDR	ttgA	AY226	1.00E+04	nd
MDR	acrR_1	AY355	9.40E+03	nd
MDR	tcrB	AY498	9.30E+03	nd
MDR	pbrT	AY494	8.36E+03	nd
MDR	copA	AY492	7.64E+03	1.37E+03
MDR	terW	AY499	4.77E+03	nd
MDR	adeA	AY202	1.98E+03	nd
MDR	pmrA	AY42	1.93E+03	nd
MDR	mexA	AY215	1.62E+03	nd
MDR	mexE	AY228	1.09E+03	nd
MDR	bexA/norM	AY484	nd	1.97E+03
MDR	cadC	AY491	nd	nd
MDR	marR_3	AY360	nd	nd
MDR	qacA/B	AY488	nd	nd
MDR	qacF/H	AY489	nd	nd
MDR	cmr	AY206	nd	nd
MDR	emrB/qacA_1	AY219	nd	nd
MDR	mtrE	AY222	nd	nd
MDR	oprD	AY224	nd	nd
MDR	cfr	AY234	nd	nd
MDR	mdsA	AY350	nd	nd
MDR	adel	AY483	nd	nd
MDR	sugE	AY497	nd	nd
MLSB	ermX_2	AY546	1.07E+06	2.04E+04
MLSB	ermX_1	AY68	7.03E+05	1.67E+04
MLSB	ermB_3	AY547	3.93E+04	5.12E+05
MLSB	mefA_1	AY65	1.89E+05	1.39E+05
MLSB	ermB_2	AY533	4.22E+04	4.78E+05
MLSB	ermB_1	AY56	nd	5.05E+05
MLSB	ermE	AY544	1.91E+05	1.91E+03
MLSB	lnuC	AY537	4.11E+04	1.89E+05
MLSB	mphA	AY539	1.06E+05	5.78E+03
MLSB	ermF_1	AY46	4.85E+03	2.30E+05

MLSB	mefA	AY538	9.01E+04	nd
MLSB	ermF	AY535	6.69E+03	1.70E+05
MLSB	pncA	AY73	7.44E+04	1.15E+03
MLSB	ereA	AY528	3.46E+04	3.87E+03
MLSB	oleC	AY91	3.26E+04	nd
MLSB	ermO	AY548	1.54E+04	1.05E+03
MLSB	lnuB	AY536	1.21E+04	9.27E+02
MLSB	erm35	AY531	9.87E+03	2.12E+03
MLSB	mefB	AY551	8.89E+03	nd
MLSB	vat(A)	AY554	6.56E+03	nd
MLSB	erm42	AY543	5.18E+03	nd
MLSB	pikR2	AY94	3.98E+03	nd
MLSB	vatE_2	AY77	3.88E+03	nd
MLSB	vgaA_1	AY71	2.42E+03	nd
MLSB	erm36	AY54	1.38E+03	nd
MLSB	lmrA_1	AY53	nd	2.76E+03
MLSB	ermA/ermTR	AY90	9.61E+02	nd
MLSB	ermT_1	AY57	nd	1.10E+03
MLSB	erm34	AY530	nd	nd
MLSB	lsaC	AY550	nd	nd
MLSB	ermA	AY532	nd	nd
MLSB	vat(B)	AY540	nd	nd
MLSB	vga(A)LC_1	AY541	nd	nd
MLSB	msrD	AY552	nd	nd
MLSB	mphB	AY61	nd	nd
MLSB	msrA_1	AY66	nd	nd
MLSB	vgaB_1	AY72	nd	nd
MLSB	ermY	AY83	nd	nd
MLSB	ermD_1	AY43	nd	nd
MLSB	ermD/K	AY44	nd	nd
MLSB	ermD	AY534	nd	nd
MLSB	ermC_2	AY545	nd	nd
MLSB	lnuF	AY549	nd	nd
MLSB	msrE	AY553	nd	nd
MLSB	msrC_1	AY58	nd	nd
MLSB	lnuA_1	AY75	nd	nd
MLSB	carB	AY92	nd	nd
Aminoglycoside	aadA7	AY412	8.45E+05	2.78E+04
Aminoglycoside	strB	AY24	3.68E+05	1.36E+04
Aminoglycoside	apmA	AY426	3.16E+05	1.38E+04
Aminoglycoside	aph4-ib	AY424	2.96E+05	2.16E+04
Aminoglycoside	aadA1_2	AY410	2.02E+05	1.35E+05
Aminoglycoside	aadA2_3	AY331	1.17E+05	1.37E+05
Aminoglycoside	aph3-iii	AY420	5.20E+04	2.04E+05
Aminoglycoside	aac(3)-iid_iiia	AY393	1.34E+05	1.75E+03
Aminoglycoside	spcN	AY390	9.07E+04	3.05E+03
Aminoglycoside	aph3-ib	AY419	7.87E+04	5.72E+03
Aminoglycoside	aphA3_1	AY7	1.37E+04	1.58E+05
Aminoglycoside	ant6-ib	AY416	6.72E+04	2.84E+04

Aminoglycoside	ant6-ia	AY415	3.90E+04	7.93E+04
Aminoglycoside	aadA5_2	AY328	6.13E+04	5.00E+03
Aminoglycoside	aacC2	AY2	6.22E+04	nd
Aminoglycoside	aadA_1	AY10	nd	1.42E+05
Aminoglycoside	aadA16	AY409	5.00E+04	1.45E+03
Aminoglycoside	aadE	AY21	2.48E+04	3.89E+04
Aminoglycoside	aadB	AY413	3.72E+04	4.05E+03
Aminoglycoside	aac3-IVa	AY388	1.82E+04	2.04E+03
Aminoglycoside	aac(6')-Ib_1	AY8	1.76E+04	nd
Aminoglycoside	aac(6)-ir	AY400	1.62E+04	2.46E+03
Aminoglycoside	aac(6)-im	AY399	1.39E+03	3.09E+04
Aminoglycoside	aph(2')-Ib	AY330	8.03E+02	2.96E+04
Aminoglycoside	aadA10	AY408	1.05E+04	nd
Aminoglycoside	rmtB	AY603	1.04E+04	nd
Aminoglycoside	aac(6)-iz	AY404	8.07E+03	nd
Aminoglycoside	aacC4	AY3	6.60E+03	nd
Aminoglycoside	aac6-aph2	AY406	nd	1.28E+04
Aminoglycoside	aadA2_1	AY9	3.13E+03	8.86E+02
Aminoglycoside	aacA/aphD	AY4	nd	8.32E+03
Aminoglycoside	aac(6)-ig	AY396	2.59E+03	nd
Aminoglycoside	aac(6)-iv_ih	AY402	2.14E+03	nd
Aminoglycoside	ant4-ib	AY414	2.00E+03	nd
Aminoglycoside	aac(3)-id_ie	AY392	1.95E+03	nd
Aminoglycoside	aac(6)-iic	AY397	1.49E+03	nd
Aminoglycoside	aph6-ia	AY389	nd	3.42E+03
Aminoglycoside	aadA6	AY411	1.19E+03	nd
Aminoglycoside	aadD	AY13	1.10E+03	nd
Aminoglycoside	aadA9_1	AY15	nd	nd
Aminoglycoside	aacA43	AY407	nd	nd
Aminoglycoside	aphA1/7	AY17	nd	nd
Aminoglycoside	aac(3)-ib	AY391	nd	nd
Aminoglycoside	aph_viii	AY417	nd	nd
Aminoglycoside	armA_1	AY427	nd	nd
Aminoglycoside	str	AY22	nd	nd
Aminoglycoside	strA	AY23	nd	nd
Aminoglycoside	aac(6')-ly	AY385	nd	nd
Aminoglycoside	aac(6')I1	AY386	nd	nd
Aminoglycoside	aac(3)-xa_1	AY394	nd	nd
Aminoglycoside	aac(6)-ij	AY398	nd	nd
Aminoglycoside	aac(6)-is_iu_ix	AY401	nd	nd
Aminoglycoside	aac(6)-iw	AY403	nd	nd
Aminoglycoside	aph(3'')-ia	AY418	nd	nd
Aminoglycoside	aph3-viaa	AY421	nd	nd
Aminoglycoside	aph3-via	AY422	nd	nd
Aminoglycoside	aph4-ia	AY423	nd	nd
Aminoglycoside	aph9-ia	AY428	nd	nd
Aminoglycoside	aac(6')-II	AY6	nd	nd
Aminoglycoside	armA_2	AY602	nd	nd
Other	qacEa ⁺ t1_1	AY218	1.40E+05	9.81E+04

Other	qacEa ⁺ 1_3	AY236	1.18E+05	1.34E+05
Other	crAss64	AY198	nd	4.13E+05
Other	bacA	AY465	1.56E+05	5.10E+03
Other	sat4	AY204	2.09E+04	1.86E+05
Other	crAss56	AY197	nd	2.09E+05
Other	mcr1	AY466	1.67E+04	nd
Other	fabK	AY472	5.84E+03	1.58E+03
Other	fosb	AY468	2.48E+03	nd
Other	arr3	AY470	2.01E+03	nd
Other	arr2	AY471	1.27E+03	nd
Other	ttgB	AY142	nd	nd
Other	nisB_1	AY186	nd	nd
Other	nimE	AY188	nd	nd
Other	merA	AY191	nd	nd
Other	mcr2	AY467	nd	nd
Other	fosX	AY469	nd	nd
Beta Lactam	blaTEM	AY439	1.37E+06	6.87E+03
Beta Lactam	penA	AY138	4.24E+05	4.43E+03
Beta Lactam	blaOXY	AY108	3.02E+05	2.45E+03
Beta Lactam	blaSHV11	AY438	1.83E+05	5.61E+04
Beta Lactam	cfxA	AY114	1.70E+04	2.48E+05
Beta Lactam	blaSFO	AY126	1.06E+05	2.80E+04
Beta Lactam	pbp	AY132	9.13E+04	1.01E+03
Beta Lactam	blaOXY1	AY436	3.74E+04	1.29E+04
Beta Lactam	blaACT	AY444	3.62E+04	3.72E+03
Beta Lactam	blaMIR	AY452	3.16E+04	3.02E+03
Beta Lactam	blaCTX-M	AY432	8.65E+03	nd
Beta Lactam	blaOCH	AY102	8.30E+03	nd
Beta Lactam	blaCMY_2	AY339	6.35E+03	nd
Beta Lactam	blaPER	AY437	5.93E+03	nd
Beta Lactam	cepA	AY115	nd	1.12E+04
Beta Lactam	blaZ	AY128	3.54E+03	nd
Beta Lactam	blaCTX-M_8	AY147	2.78E+03	nd
Beta Lactam	blaACC	AY336	2.46E+03	nd
Beta Lactam	blaFOX	AY433	2.38E+03	nd
Beta Lactam	blaCTX-M_5	AY134	2.18E+03	nd
Beta Lactam	blaCARB	AY446	1.59E+03	nd
Beta Lactam	blaOXA48	AY601	1.34E+03	nd
Beta Lactam	blaHERA	AY448	1.20E+03	nd
Beta Lactam	blaLEN	AY451	1.04E+03	nd
Beta Lactam	blaPAO	AY103	9.95E+02	nd
Beta Lactam	blaROB	AY107	9.88E+02	nd
Beta Lactam	cfiA	AY97	nd	1.06E+03
Beta Lactam	ampC/blaDHA	AY117	nd	nd
Beta Lactam	blaNDM	AY152	nd	nd
Beta Lactam	imiR_2	AY434	nd	nd
Beta Lactam	cphA_1	AY111	nd	nd
Beta Lactam	blaSME	AY431	nd	nd
Beta Lactam	blaOXA51	AY435	nd	nd

Beta Lactam	beta_ccra	AY441	nd	nd
Beta Lactam	blaMOX/blaCMY	AY101	nd	nd
Beta Lactam	blaVEB	AY105	nd	nd
Beta Lactam	blaPSE	AY109	nd	nd
Beta Lactam	bla-L1	AY113	nd	nd
Beta Lactam	blaGES	AY125	nd	nd
Beta Lactam	blaTLA	AY127	nd	nd
Beta Lactam	blaVIM	AY129	nd	nd
Beta Lactam	pbp5	AY131	nd	nd
Beta Lactam	mecA	AY133	nd	nd
Beta Lactam	bla1	AY338	nd	nd
Beta Lactam	ampC_cefa	AY430	nd	nd
Beta Lactam	blaKPC	AY440	nd	nd
Beta Lactam	bl1acc	AY442	nd	nd
Beta Lactam	beta_B2	AY443	nd	nd
Beta Lactam	blaB	AY445	nd	nd
Beta Lactam	blaGOB	AY447	nd	nd
Beta Lactam	blaIMI	AY449	nd	nd
Beta Lactam	blaIND	AY450	nd	nd
Beta Lactam	blaBEL-nonmobile	AY453	nd	nd
Beta Lactam	blaADC-nonmobile	AY454	nd	nd
Quinolone	qepA	AY456	2.56E+05	2.68E+04
Quinolone	qnrB4	AY457	4.53E+04	1.50E+03
Quinolone	qnrB	AY96	1.55E+04	nd
Quinolone	qnrB_2	AY458	5.22E+03	nd
Quinolone	norA	AY455	4.99E+03	nd
Quinolone	qnrS_1	AY460	4.25E+03	nd
Quinolone	qnrS2	AY461	nd	nd
Quinolone	qnrD	AY459	nd	nd
Quinolone	qnrA	AY95	nd	nd
Quinolone	qnrVC1_VC3_VC6	AY462	nd	nd
Quinolone	qnrVC_2	AY463	nd	nd
Phenicol	mdtL	AY34	3.69E+05	9.84E+04
Phenicol	catA1	AY38	8.85E+04	3.63E+03
Phenicol	cmlV	AY563	3.55E+04	1.74E+03
Phenicol	catP	AY561	9.98E+02	1.88E+04
Phenicol	cmlA_2	AY35	6.12E+03	nd
Phenicol	floR_1	AY32	5.63E+03	nd
Phenicol	catA3	AY558	3.37E+03	nd
Phenicol	yidY/mdtL	AY33	2.14E+03	nd
Phenicol	ceoA	AY31	1.59E+03	nd
Phenicol	cmlA_4	AY41	1.12E+03	nd
Phenicol	optrA	AY565	nd	nd
Phenicol	cmxA	AY37	nd	nd
Phenicol	cat	AY555	nd	nd
Phenicol	catB9	AY560	nd	nd
Phenicol	catB3	AY29	nd	nd
Phenicol	catB8	AY30	nd	nd
Phenicol	cat(pC221)	AY556	nd	nd

Phenicol	catA2	AY557	nd	nd
Phenicol	catB2	AY559	nd	nd
Phenicol	catQ	AY562	nd	nd
Phenicol	fexA	AY564	nd	nd
Phenicol	floR	AY566	nd	nd
Vancomycin	vanA	AY595	6.35E+04	3.44E+03
Vancomycin	vanHB	AY163	4.76E+04	2.45E+03
Vancomycin	vanB_1	AY159	1.81E+04	1.21E+03
Vancomycin	vanTC_2	AY381	1.09E+04	1.07E+03
Vancomycin	vanYD_1	AY183	9.18E+03	2.36E+03
Vancomycin	vanC2	AY596	9.05E+03	nd
Vancomycin	vanG	AY597	6.79E+03	nd
Vancomycin	vanYB	AY182	1.39E+03	nd
Vancomycin	vanTG	AY181	nd	1.83E+03
Vancomycin	vanXB	AY170	nd	9.87E+02
Vancomycin	vanD	AY161	nd	nd
Vancomycin	vanWB	AY167	nd	nd
Vancomycin	vanRB	AY174	nd	nd
Vancomycin	vanRC	AY175	nd	nd
Vancomycin	vanC_2	AY156	nd	nd
Vancomycin	vanTE	AY160	nd	nd
Vancomycin	vanHD	AY162	nd	nd
Vancomycin	vanRA_1	AY164	nd	nd
Vancomycin	vanSA	AY165	nd	nd
Vancomycin	vanRC4	AY176	nd	nd
Vancomycin	vanRD	AY177	nd	nd
Vancomycin	vanSC_2	AY380	nd	nd
Vancomycin	vanXA	AY598	nd	nd
Vancomycin	vanSB	AY599	nd	nd
Trimethoprim	dfra21	AY583	6.81E+03	nd
Trimethoprim	dfra22	AY584	2.89E+03	nd
Trimethoprim	dfrc	AY592	2.49E+03	nd
Trimethoprim	dfra27	AY586	1.96E+03	nd
Trimethoprim	dfra17	AY581	1.84E+03	nd
Trimethoprim	dfra12	AY285	1.67E+03	nd
Trimethoprim	dfra1_1	AY284	1.14E+03	nd
Trimethoprim	dfrcB	AY591	1.13E+03	nd
Trimethoprim	dfra25	AY585	nd	nd
Trimethoprim	dfra8	AY589	nd	nd
Trimethoprim	dfra10	AY578	nd	nd
Trimethoprim	dfra15	AY580	nd	nd
Trimethoprim	dfra19	AY582	nd	nd
Trimethoprim	dfra7	AY588	nd	nd
Trimethoprim	dfraB4	AY590	nd	nd
Trimethoprim	dfrcG	AY593	nd	nd
Trimethoprim	dfrcK	AY594	nd	nd
16S rRNA	16S rRNA	AY1	9.97E+06	2.41E+07

nd: not detected

Annex Chapter III

Supplementary Table III-2. Metagenomic sequencing information and quality of the samples included in this study.

Sample	QC reads	Contigs	ARG assembly-	ARG assembly-
MAMI_006B02H	1771406	6562	20	1
MAMI_006B04H	6518960	8948	23	5
MAMI_006B07H	27095504	13633	31	6
MAMI_006B08H	62620042	30325	32	9
MAMI_012B02H	30497672	15991	47	17
MAMI_012B04H	48825300	11288	39	8
MAMI_012B07H	24677450	7765	48	13
MAMI_012B08H	110849260	66583	43	19
MAMI_017B02H	20314776	1771	37	15
MAMI_017B04H	104148	162	2	0
MAMI_017B07H	36919796	6234	25	1
MAMI_017B08H	18489984	33408	79	21
MAMI_01WB02H	69841314	11495	38	8
MAMI_01WB04H	25026696	7044	24	2
MAMI_01WB07H	88861670	23654	43	17
MAMI_01WB08H	96966388	51704	61	11
MAMI_020B02H	63986512	10404	47	15
MAMI_020B04H	94821690	13226	45	13
MAMI_020B07H	57553124	27342	45	11
MAMI_020B08H	83197924	38876	56	10
MAMI_022B02H	39780034	6389	31	13
MAMI_022B04H	67424636	4771	35	14
MAMI_022B07H	55516876	19612	32	4
MAMI_022B08H	219236608	47615	55	18
MAMI_031B02H	23347668	12456	26	5
MAMI_031B04H	267932	3943	0	0
MAMI_031B07H	89831012	48122	42	12
MAMI_032B02H	35980612	6663	29	10
MAMI_032B04H	22187592	6902	26	3
MAMI_032B07H	99154094	18442	45	12
MAMI_032B08H	19599812	28032	34	4
MAMI_045B02H	36868800	2354	46	11
MAMI_046B04H	84626562	8229	27	9
MAMI_046B07H	27357984	11114	28	3
MAMI_07WB02H	95887812	9213	41	17
MAMI_07WB04H	7970466	9269	40	4
MAMI_07WB08H	23763000	17505	33	2
MAMI_09WB02H	82379108	5658	30	8
MAMI_09WB04H	46154186	9148	40	7
MAMI_09WB07H	5141754	12499	32	6
MAMI_09WB08H	25410770	26202	36	4
MAMI_12WB02H	26873354	8231	30	13
MAMI_12WB04H	77293432	5641	31	10

MAMI_12WB07H	39808830	11413	21	3
MAMI_12WB08H	45797516	23268	52	5
MAMI_13WB02H	48386052	12226	43	9
MAMI_13WB04H	35006250	3919	17	1
MAMI_13WB07H	66123674	5381	46	15
MAMI_15WB02H	67979780	15723	27	10
MAMI_15WB04H	135940630	6168	23	8
MAMI_15WB07H	42682872	10127	23	4
MAMI_15WB08H	41067532	16559	43	4
MAMI_17WB02H	49264624	5077	38	10
MAMI_17WB04H	65427040	9128	39	10
MAMI_17WB08H	41019476	37922	59	10
MAMI_18WB02H	97201266	23517	40	18
MAMI_18WB04H	85961516	11971	41	17
MAMI_18WB07H	38553432	2602	25	5
MAMI_18WB08H	100653272	48033	65	12
MAMI_21WB02H	73374212	5130	27	10
MAMI_21WB04H	35189268	3145	31	3
MAMI_21WB07H	26674756	22743	36	10
MAMI_21WB08H	66548110	17319	28	6
MAMI_29WB04H	274286206	5332	32	7
MAMI_29WB07H	119923418	11760	30	2
MAMI_29WB08H	63853396	40995	41	8
MAMI_443B02H	9970146	6975	27	4
MAMI_443B04H	3280510	3585	14	4
MAMI_443B07H	34085552	62920	41	10
MAMI_443B08H	80057156	23945	38	14
MAMI_447B02H	24001984	8016	26	5
MAMI_447B07H	136448382	21010	36	8
MAMI_447B08H	110935772	20295	34	7
MAMI_453B02H	82053156	4107	26	4
MAMI_453B04H	43089466	12281	25	9
MAMI_453B07H	92629074	16538	36	13
MAMI_454B04H	47992500	8373	30	3
MAMI_454B07H	128273316	21801	37	10
MAMI_454B08H	170286148	45219	60	21
MAMI_459B02H	27195626	2659	28	8
MAMI_459B04H	1434652	12587	29	6
MAMI_459B07H	36292116	22280	22	6
MAMI_459B08H	75588956	33098	35	11
MAMI_475B02H	41955148	4452	30	8
MAMI_475B04H	37437230	8801	23	6
MAMI_475B07H	93249048	6821	28	7
MAMI_475B08H	40270084	20990	41	7
MAMI_502B02H	23761432	11726	27	11
MAMI_502B04H	62486846	10205	30	14
MAMI_502B07H	29764854	45380	63	17
MAMI_502B08H	31551972	34203	40	7
MAMI_503B02H	107645322	269	18	3

MAMI_503B04H	26168296	10035	33	13
MAMI_503B07H	52157680	99015	36	13
MAMI_503B08H	40315458	44396	37	12
MAMI_506B02H	41056460	7766	28	7
MAMI_506B04H	25538982	10446	17	6
MAMI_506B07H	53142766	13171	33	4
MAMI_506B08H	298537740	34350	62	20
MAMI_508B02H	33563244	4438	48	18
MAMI_508B04H	103143846	14636	44	1
MAMI_508B07H	66621062	23103	45	15
MAMI_510B02H	108416256	4662	31	15
MAMI_510B07H	31495480	11071	31	2
MAMI_510B08H	26159280	37146	48	11
MAMI_518B02H	59601172	2391	34	11
MAMI_518B04H	34097698	4113	27	7
MAMI_518B07H	99056008	16901	36	14
MAMI_518B08H	64654764	26211	52	9
MAMI_546B02H	98797316	16239	56	25
MAMI_546B04H	115921826	18079	63	13
MAMI_546B07H	48633466	16226	44	11
MAMI_547B02H	36896206	4108	51	26
MAMI_547B04H	53030820	11531	30	7
MAMI_547B07H	28538372	22023	36	8
MAMI_547B08H	65148792	44490	39	11
MAMI_814B02H	71342084	7520	39	14
MAMI_814B04H	100392152	10983	24	7
MAMI_814B07H	46983104	15102	26	2
MAMI_814B08H	103463286	20944	44	9
MAMI_007B07H	50462502	86258	56	15
MAMI_007B08H	44310270	58031	48	17
MAMI_027B02H	3041932	18465	33	7
MAMI_027B07H	54505898	41948	50	15
MAMI_027B08H	48765962	43724	64	23
MAMI_033B04H	47148322	97650	30	17
MAMI_033B07H	61141094	86516	64	21
MAMI_033B08H	65852738	124476	71	22
MAMI_037B04H	48101976	11940	32	11
MAMI_037B08H	55531974	47976	45	13
MAMI_432B02H	43753792	10539	63	31
MAMI_432B04H	47678582	8540	52	25
MAMI_432B08H	49427728	69210	48	19
MAMI_444B07H	48574710	13296	48	17
MAMI_444B08H	46509756	70721	68	29
MAMI_469B02H	47278756	9552	26	13
MAMI_469B06H	53157632	4458	33	11
MAMI_469B08H	51639228	37308	41	17
MAMI_471B02H	46144696	13361	45	12
MAMI_471B07H	68410274	38354	47	15
MAMI_471B08H	48642068	56650	48	19

MAMI_521B02H	58685342	6644	34	18
MAMI_521B08H	49292290	36548	41	12
MAMI_526B03H	45344422	4409	32	10
MAMI_526B08H	62661310	29769	49	18
MAMI_544B03H	54491234	12899	43	24
MAMI_544B08H	47095828	54055	67	23
MAMI_DB5B04H	47844860	7407	38	13
MAMI_DB5B08H	52144264	74198	52	21
MAMI_02WB02H	79194610	10645	33	6
MAMI_02WB04H	91879028	9459	24	3
MAMI_039B02H	51013520	2053	18	10
MAMI_039B04H	62089838	12572	29	7
MAMI_039B07H	51046088	9237	43	14
MAMI_039B08H	53365706	208960	60	26
MAMI_052B02H	53053444	6646	32	15
MAMI_052B07H	50801226	4283	26	12
MAMI_052B08H	62594962	39238	59	20
MAMI_056B02H	63670704	2685	26	17
MAMI_056B04H	56035338	7553	40	16
MAMI_056B08H	55922820	49120	41	11
MAMI_10WB02H	46204222	39239	46	11
MAMI_10WB04H	48381444	10215	39	11
MAMI_10WB08H	56412112	112715	51	16
MAMI_16WB04H	63191388	13560	34	12
MAMI_16WB07H	67026806	17080	49	25
MAMI_16WB08H	63929872	18394	31	10
MAMI_441B04H	45091922	15885	52	16
MAMI_441B07H	54564238	17454	40	7
MAMI_441B08H	54960076	158074	57	22
MAMI_451B02H	53878224	16927	49	23
MAMI_451B04H	66545446	13479	48	19
MAMI_451B08H	48454776	69886	49	16
MAMI_542B02H	81495414	10251	38	12
MAMI_542B04H	75689000	13293	36	14
MAMI_542B08H	47526038	55920	50	18
MAMI_DB2CPB04H	66791238	14366	43	22
MAMI_DB2CPB08H	61732728	69624	57	22
MAMI_DB7CPB02H	47561600	8069	23	11
MAMI_006M04H	40390066	62835	41	10
MAMI_012M04H	30943908	45603	45	4
MAMI_017M04H	46891088	112084	36	18
MAMI_01WM04H	36357488	108113	45	5
MAMI_022M04H	40877734	52166	41	7
MAMI_031M04H	54136880	91231	65	21
MAMI_046M04H	72150046	113241	39	7
MAMI_07WM04H	56948782	136719	41	12
MAMI_09WM04H	26946808	119590	44	11
MAMI_12WM04H	66922302	152533	47	15
MAMI_13WM04H	45594410	54501	37	10

MAMI_15WM04H	79157236	151733	40	16
MAMI_17WM04H	100262236	192938	51	18
MAMI_18WM04H	64690124	154537	44	10
MAMI_21WM04H	6040324	69778	57	5
MAMI_29WM04H	71934382	134704	44	18
MAMI_447M04H	359358380	119549	52	25
MAMI_453M04H	64188760	146152	51	15
MAMI_454M04H	125230958	133027	58	23
MAMI_459M04H	54317582	52866	45	7
MAMI_475M04H	24681130	17413	40	3
MAMI_502M04H	29083630	111249	45	19
MAMI_503M04H	30784376	52072	29	6
MAMI_506M02H	4189392	101989	42	14
MAMI_508M04H	179063618	90775	49	7
MAMI_510M04H	50189404	75376	54	10
MAMI_518M04H	1453464	11058	24	1
MAMI_546M04H	36671696	46978	51	6
MAMI_547M04H	34733262	53362	44	7
MAMI_814M04H	41814920	51428	41	9
TW_ESP_018M02H	31232094	70871	80	17
TW_ESP_019M02H	1021199350	160452	129	38
TW_ESP_030M02H	24217132	129481	88	26
TW_ESP_425M02H	199049908	73836	67	19
TW_ESP_501M04H	86501424	192482	45	19
TW_ESP_018B03B	7757842	2801	19	3
TW_ESP_018B03H	11686260	7258	40	4
TW_ESP_018B04H	43373680	18876	52	11
TW_ESP_018B07H	51478258	19813	41	5
TW_ESP_018B08H	47969156	27614	45	10
TW_ESP_018C03H	755960	3056	9	2
TW_ESP_018C04H	13933298	13657	42	16
TW_ESP_018C07H	50396380	25646	49	23
TW_ESP_018C08H	107487496	35433	55	11
TW_ESP_019B02H	188828052	4343	46	16
TW_ESP_019B04H	46129692	13939	47	14
TW_ESP_019C02H	628412	682	9	2
TW_ESP_019C04H	44674480	15475	47	13
TW_ESP_030B02H	54124104	739	18	1
TW_ESP_030B04H	7059500	2287	10	1
TW_ESP_030B07H	78770100	37411	63	25
TW_ESP_030B08H	112219276	59411	39	13
TW_ESP_030C02H	161761860	16904	50	29
TW_ESP_030C04H	26152900	4125	24	9
TW_ESP_030C07H	46563186	22472	29	11
TW_ESP_030C08H	80845778	57923	43	15
TW_ESP_425B02H	88329154	7840	42	16
TW_ESP_425B04H	127465892	1279	30	5
TW_ESP_425B07H	43616420	18620	33	3
TW_ESP_425B08H	96449382	51756	31	7

TW_ESP_425C02H	14369286	6136	27	11
TW_ESP_425C04H	9364488	10460	13	2
TW_ESP_425C07H	37520306	91105	62	12
TW_ESP_425C08H	85406050	22594	37	6
TW_ESP_501B02H	127666066	20371	87	29
TW_ESP_501B04H	8382360	10739	60	23
TW_ESP_501B07H	24624320	13945	36	5
TW_ESP_501C02H	112611730	34981	40	13
TW_ESP_501C04H	110171238	10143	48	18
TW_ESP_501C07H	151293452	15391	35	16
TW_ESP_501C08H	92248720	23146	40	6
MAMI_442B04H	80243064	10945	41	16
MAMI_442M04H	64938680	152368	50	22
MAMI_522B04H	91645754	15681	51	21
MAMI_522M04H	88572182	181665	54	22
MAMI_523M04H	72923570	224733	61	24
MAMI_819B04H	69444176	14341	50	24
MAMI_819M04H	7218060	69049	36	14
MAMI_037B02H	42826858	5231	33	19
MAMI_037B06H	37051980	25833	45	16
MAMI_432B06H	36339614	23244	60	16
MAMI_521B07H	37544044	19279	45	16
MAMI_523B03H	35307988	2594	26	10
MAMI_523B04H	38378360	7437	19	4
MAMI_523B06H	44014638	6697	34	10
MAMI_526B06H	69587264	21288	32	8

Supplementary Table III-3. Information of the abundance, prevalence, class and mechanism of each ARG detected in the samples included in the study.

ARG	Abundance	Prev. (%)	Class	Mechanism
<i>tet(S/M)</i>	22843.408	93.363	Tetracycline	Target protection
<i>blaTEM-1A</i>	20163.241	54.867	Beta-lactam	Enzymatic inactivation
<i>vanM</i>	19503.253	88.053	Glycopeptide	Target modification
<i>tet(O/32/O)</i>	16297.665	73.451	Tetracycline	Target protection
<i>tet(W)</i>	16197.102	74.779	Tetracycline	Target protection
<i>OqxB</i>	11047.907	63.274	Amph + Quin + FPA	Efflux pump
<i>msr(D)</i>	10719.070	96.903	Macrolide + Strept B	Target protection
<i>tet(A)</i>	10507.949	25.221	Tetracycline	Increased efflux
<i>tet(Q)</i>	9893.634	59.735	Tetracycline	Target protection
<i>sul2</i>	9840.963	40.265	FPA	Purine synthesis

<i>aph(3'')-Ib</i>	8049.771	41.150	Aminoglycoside	Enzymatic modification
<i>erm(B)</i>	7646.500	80.531	MLSB	Target modification
<i>aph(6)-Id</i>	7363.447	46.460	Aminoglycoside	Enzymatic modification
<i>mupA</i>	6202.492	40.265	Pseudomonic acid	Alternate Pathway
<i>tet(L)</i>	5195.650	27.876	Tetracycline	Increased efflux
<i>erm(X)</i>	5094.547	63.274	MLSB	Target modification
<i>mef(A)</i>	4846.881	92.478	Macrolide	Increased efflux
<i>cfxA</i>	4324.786	39.823	Beta-lactam	Enzymatic inactivation
<i>tet(O/32/O)</i>	3913.809	66.814	Tetracycline	Target protection
<i>tetB(P)</i>	3854.877	40.708	Tetracycline	Target protection
<i>msr(A)</i>	3480.778	46.903	Macrolide + Strept B	Target protection
<i>tet(O/W/O)-1</i>	3228.646	74.336	Tetracycline	Target protection
<i>OqxA</i>	3222.503	51.327	Amph + Quin + FPA	Efflux pump
<i>aph(3')-IIIa</i>	3142.884	61.504	Aminoglycoside	Enzymatic modification
<i>tetA(46)</i>	2821.543	76.991	Tetracycline	Increased efflux
<i>erm(X)</i>	2688.562	58.407	MLSB	Target modification
<i>tetA(P)</i>	2675.084	47.345	Tetracycline	Increased efflux
<i>erm(F)</i>	2639.986	41.150	MLSB	Target modification
<i>lsa(A)</i>	2561.877	56.637	Lin + StrepA	Target protection
<i>erm(C)</i>	2343.097	26.549	MLSB	Target modification
<i>tet(O/W/32/O/W/O)</i>	2327.407	73.451	Tetracycline	Target protection
<i>mef(A)</i>	2272.199	95.133	Macrolide	Increased efflux
<i>ant(6)-Ia</i>	2239.836	51.770	Aminoglycoside	Enzymatic modification
<i>tetB(46)</i>	2087.744	77.434	Tetracycline	Increased efflux
<i>tet(X)</i>	2069.522	34.513	Tetracycline	Enzymatic inactivation
<i>sul1</i>	2068.143	26.991	FPA	Purine synthesis
<i>blaSHV-100</i>	2046.496	30.973	Beta-lactam	Enzymatic inactivation
<i>ant(6)-Ia</i>	2040.476	53.097	Aminoglycoside	Enzymatic modification
<i>aac(3)-IIa</i>	1678.178	9.735	Aminoglycoside	Enzymatic modification
<i>tet(40)</i>	1622.857	34.513	Tetracycline	Increased efflux

<i>erm(T)</i>	1602.908	16.372	MLSB	Target modification
<i>tet(32)</i>	1540.300	45.575	Tetracycline	Target protection
<i>mph(C)</i>	1513.047	38.496	Macrolide	Enzymatic inactivation
<i>aadA4</i>	1492.997	13.717	Aminoglycoside	Enzymatic modification
<i>aph(2'')-Ia</i>	1403.929	41.593	Aminoglycoside	Enzymatic modification
<i>msr(C)</i>	1240.107	14.602	Macrolide + Strept B	Target protection
<i>mph(A)</i>	1202.097	11.062	Macrolide	Enzymatic inactivation
<i>mecA</i>	1179.423	34.071	Beta-lactam	Target modification
<i>tet(B)</i>	1168.124	13.274	Tetracycline	Increased efflux
<i>aadA2</i>	1097.414	11.947	Aminoglycoside	Enzymatic modification
<i>cat(pC194)</i>	1065.433	11.062	Amphenicol	Enzymatic inactivation
<i>blaZ</i>	1063.573	45.575	Beta-lactam	Enzymatic inactivation
<i>cfxA6</i>	1006.357	10.619	Beta-lactam	Enzymatic inactivation
<i>ant(3'')-Ia</i>	969.299	32.301	Aminoglycoside	Enzymatic modification
<i>aph(3')-Ia</i>	939.163	24.336	Aminoglycoside	Enzymatic modification
<i>dfrG</i>	885.112	10.619	FPA	Purine synthesis
<i>cmlA1</i>	865.773	3.982	Amphenicol	Increased efflux
<i>mph(A)</i>	854.445	9.735	Macrolide	Enzymatic inactivation
<i>floR</i>	792.532	3.540	Amphenicol	Increased efflux
<i>erm(T)</i>	784.832	58.850	MLSB	Target modification
<i>erm(G)</i>	761.839	26.549	MLSB	Target modification
<i>fosA5</i>	749.571	33.628	Fosfomycin	Enzymatic inactivation
<i>dfrA17</i>	725.523	12.389	FPA	Purine synthesis
<i>blaCTX-M-211</i>	714.669	3.097	Beta-lactam	Enzymatic inactivation
<i>lnu(C)</i>	699.675	36.283	Lincosamide	Enzymatic inactivation
<i>blaOXY-1-1</i>	697.684	25.221	Beta-lactam	Enzymatic inactivation
<i>tet(D)</i>	688.451	4.867	Tetracycline	Increased efflux
<i>lsa(E)</i>	668.347	12.832	Lin + StrepA + Pleuro	Target protection
<i>tet(44)</i>	648.884	26.549	Tetracycline	Target protection
<i>ant(6)-Ia</i>	639.249	46.903	Aminoglycoside	Enzymatic modification

<i>blaZ</i>	632.673	42.920	Beta-lactam	Enzymatic inactivation
<i>tet(K)</i>	614.956	30.531	Tetracycline	Increased efflux
<i>tet(S)</i>	590.055	11.504	Tetracycline	Target protection
<i>aadA1</i>	572.308	13.274	Aminoglycoside	Enzymatic modification
<i>sul3</i>	559.581	3.097	FPA	Purine synthesis
<i>blaACI-1</i>	544.489	10.177	Beta-lactam	Enzymatic inactivation
<i>blaCMY-18</i>	537.868	17.699	Beta-lactam	Enzymatic inactivation
<i>aadD</i>	535.160	28.761	Aminoglycoside	Enzymatic modification
<i>lnu(F)</i>	525.023	3.982	Lincosamide	Enzymatic inactivation
<i>blaOXY-2-1</i>	522.046	7.522	Beta-lactam	Enzymatic inactivation
<i>NarB</i>	440.117	5.752	Ionophores	Efflux pump
<i>cepA</i>	433.112	17.257	Beta-lactam	Enzymatic inactivation
<i>aac(6')-II</i>	425.792	14.159	Aminoglycoside	Enzymatic modification
<i>blaACT-5</i>	409.268	15.929	Beta-lactam	Enzymatic inactivation
<i>blaOKP-A-1</i>	403.922	4.867	Beta-lactam	Enzymatic inactivation
<i>dfrA12</i>	399.540	5.752	FPA	Purine synthesis
<i>vga(A)</i>	397.720	12.832	Lin + StrepA + Pleuro	Target protection
<i>tet(X3)</i>	390.066	29.204	Tetracycline	Enzymatic inactivation
<i>fosB</i>	386.024	34.956	Fosfomycin	Enzymatic inactivation
<i>blaOXA-392</i>	355.650	3.097	Beta-lactam	Enzymatic inactivation
<i>lsa(C)</i>	354.324	27.434	Lin + StrepA + Pleuro	Target protection
<i>lnu(B)</i>	330.050	11.947	Lincosamide	Enzymatic inactivation
<i>mcr-10</i>	314.147	1.327	Polymyxin	Target modification
<i>blaLEN3</i>	307.656	24.779	Beta-lactam	Enzymatic inactivation
<i>dfrA8</i>	302.542	3.540	FPA	Purine synthesis
<i>fosB3</i>	264.425	7.080	Fosfomycin	Enzymatic inactivation
<i>lnu(P)</i>	233.602	31.858	Lincosamide	Enzymatic inactivation
<i>cat</i>	228.988	11.947	Amphenicol	Enzymatic inactivation
<i>tet(K)</i>	224.442	20.796	Tetracycline	Increased efflux
<i>catB3</i>	218.555	1.327	Amphenicol	Enzymatic inactivation

<i>bleO</i>	216.556	23.009	Glycopeptide	Enzymatic modification
<i>blaPLA-6A</i>	215.170	6.637	Beta-lactam	Enzymatic inactivation
<i>NarA</i>	201.889	5.752	Ionophores	Efflux pump
<i>VanC1XY</i>	189.220	13.717	Glycopeptide	Target modification
<i>catS</i>	179.897	19.912	Amphenicol	Enzymatic inactivation
<i>cat(pC221)</i>	167.807	4.425	Amphenicol	Enzymatic inactivation
<i>lnu(A)</i>	162.547	7.522	Lincosamide	Enzymatic inactivation
<i>blaMIR-2</i>	157.297	3.097	Beta-lactam	Enzymatic inactivation
<i>blaOXA-347</i>	147.197	5.310	Beta-lactam	Enzymatic inactivation
<i>fosA2</i>	137.419	13.717	Fosfomycin	Enzymatic inactivation
<i>fusB</i>	129.472	18.584	Steroid antibacterial	Target protection
<i>aac(6')-Ib</i>	126.288	1.327	Aminoglycoside	Enzymatic modification
<i>blaCTX-M-64</i>	115.697	3.097	Beta-lactam	Enzymatic inactivation
<i>ant(6)-Ib</i>	109.222	11.504	Aminoglycoside	Enzymatic modification
<i>erm(A)</i>	105.401	17.699	MLSB	Target modification
<i>fosA</i>	103.793	11.062	Fosfomycin	Enzymatic inactivation
<i>ere(D)</i>	101.837	3.097	Macrolide	Enzymatic inactivation
<i>erm(33)</i>	101.391	7.522	MLSB	Target modification
<i>mef(B)</i>	99.477	1.770	Macrolide	Increased efflux
<i>catA1</i>	88.584	7.080	Amphenicol	Enzymatic inactivation
<i>qnrB41</i>	83.280	9.735	Quinolone	Target protection
<i>dfrA14</i>	66.198	4.425	FPA	Purine synthesis
<i>cfr(C)</i>	64.991	9.292	Oxa + Amph + Lin + StrepA + Pleuro	Target modification
<i>VanC2</i>	64.217	11.947	Glycopeptide	Target modification
<i>aph(2'')-Ib</i>	60.141	8.850	Aminoglycoside	Enzymatic modification
<i>ant(3'')-II-aac(6')-IId</i>	57.823	2.212	Aminoglycoside	Enzymatic modification
<i>aph(2'')-Ic</i>	54.788	1.770	Aminoglycoside	Enzymatic modification
<i>aac(6')-If</i>	53.373	1.327	Aminoglycoside	Enzymatic modification
<i>Cfr(E)</i>	53.150	4.867	Amphenicol	Target modification
<i>VanC3XY</i>	51.207	10.177	Glycopeptide	Target modification

<i>fosD</i>	50.042	3.097	Fosfomycin	Enzymatic inactivation
<i>erm(Q)</i>	47.127	21.681	MLSB	Target modification
<i>tetA(60)</i>	45.069	26.106	Tetracycline	Increased efflux
<i>cml</i>	42.197	2.212	Amphenicol	Increased efflux
<i>blaACT-9</i>	41.841	3.097	Beta-lactam	Enzymatic inactivation
<i>dfrA1</i>	41.682	6.637	FPA	Purine synthesis
<i>blaCFE-1</i>	41.576	10.177	Beta-lactam	Enzymatic inactivation
<i>fusC</i>	39.207	10.619	Steroid antibacterial	Target protection
<i>blaDHA-1</i>	38.390	3.540	Beta-lactam	Enzymatic inactivation
<i>aac(6')-30-aac(6')- lb'</i>	37.982	1.770	Aminoglycoside	Enzymatic modification
<i>dfrA1</i>	37.224	6.195	FPA	Purine synthesis
<i>aac(6')-Im</i>	34.547	7.080	Aminoglycoside	Enzymatic modification
<i>fosA7</i>	30.142	3.540	Fosfomycin	Enzymatic inactivation
<i>VanXY</i>	28.598	8.850	Glycopeptide	Target modification
<i>aac(6')-lb- Hangzhou</i>	27.330	1.327	Aminoglycoside	Enzymatic modification
<i>blaMAL-1</i>	23.316	1.327	Beta-lactam	Enzymatic inactivation
<i>aac(3)-lb-aac(6')-lb</i>	19.349	1.327	Aminoglycoside	Enzymatic modification
<i>cfiA1</i>	19.216	0.885	Beta-lactam	Enzymatic inactivation
<i>dfrA30</i>	16.211	2.655	FPA	Purine synthesis
<i>tet(X)</i>	15.982	3.540	Tetracycline	Enzymatic inactivation
<i>blaOXA-209</i>	14.810	2.212	Beta-lactam	Enzymatic inactivation
<i>penA</i>	14.807	5.310	Beta-lactam	Enzymatic inactivation
<i>VanHDX</i>	14.702	3.982	Glycopeptide	Target modification
<i>VanHDX</i>	14.647	4.425	Glycopeptide	Target modification
<i>qnrS2</i>	13.929	0.885	Quinolone	Target protection
<i>tetB(60)</i>	12.518	16.372	Tetracycline	Increased efflux
<i>qnrB8</i>	11.993	2.655	Quinolone	Target protection
<i>ant(9)-la</i>	11.884	0.442	Aminoglycoside	Enzymatic modification
<i>blaCTX-M-110</i>	10.955	1.327	Beta-lactam	Enzymatic inactivation
<i>lnu(G)</i>	9.763	2.212	Lincosamide	Enzymatic inactivation

<i>catP</i>	9.281	5.310	Amphenicol	Enzymatic inactivation
<i>tet(C)</i>	9.034	6.195	Tetracycline	Increased efflux
<i>tet(J)</i>	8.831	3.540	Tetracycline	Increased efflux
<i>cmx</i>	8.705	7.965	Amphenicol	Increased efflux
<i>blaACT-12</i>	8.036	0.442	Beta-lactam	Enzymatic inactivation
<i>catQ</i>	7.474	4.867	Amphenicol	Enzymatic inactivation
<i>aph(3')-IIa</i>	7.455	1.770	Aminoglycoside	Enzymatic modification
<i>dfrA7</i>	7.040	2.655	FPA	Purine synthesis
<i>blaCARB-1</i>	6.907	0.885	Beta-lactam	Enzymatic inactivation
<i>cat</i>	6.861	4.425	Amphenicol	Enzymatic inactivation
<i>blaHERA-1</i>	6.499	1.327	Beta-lactam	Enzymatic inactivation
<i>tet(L)</i>	6.172	5.752	Tetracycline	Increased efflux
<i>aph(2'')-Id</i>	5.745	0.885	Aminoglycoside	Enzymatic modification
<i>aph(2'')-If</i>	4.466	3.982	Aminoglycoside	Enzymatic modification
<i>aac(6')-Iih</i>	4.404	2.655	Aminoglycoside	Enzymatic modification
<i>ant(9)-Ia</i>	4.342	3.097	Aminoglycoside	Enzymatic modification
<i>ant(2'')-Ia</i>	4.206	1.327	Aminoglycoside	Enzymatic modification
<i>erm(A)</i>	3.871	4.425	MLSB	Target modification
<i>tet(42)</i>	3.862	0.885	Tetracycline	Increased efflux
<i>qnrS1</i>	3.365	3.097	Quinolone	Target protection
<i>blaACT-1</i>	3.364	3.097	Beta-lactam	Enzymatic inactivation
<i>VanHDX</i>	3.118	2.212	Glycopeptide	Target modification
<i>aph(4)-Ia</i>	3.076	2.212	Aminoglycoside	Enzymatic modification
<i>qnrD1</i>	2.746	0.442	Quinolone	Target protection
<i>aph(6)-Ic</i>	2.483	0.442	Aminoglycoside	Enzymatic modification
<i>blaROB-1</i>	2.283	1.770	Beta-lactam	Enzymatic inactivation
<i>dfrD</i>	2.121	0.442	FPA	Purine synthesis
<i>aadA13</i>	2.075	2.655	Aminoglycoside	Enzymatic modification
<i>blaSPU-1</i>	2.032	0.442	Beta-lactam	Enzymatic inactivation
<i>aac(3)-IVa</i>	1.892	1.327	Aminoglycoside	Enzymatic modification

<i>str</i>	1.876	1.770	Aminoglycoside	Enzymatic modification
<i>tet(41)</i>	1.826	0.442	Tetracycline	Increased efflux
<i>aph(3')-VIIa</i>	1.685	0.885	Aminoglycoside	Enzymatic modification
<i>mef(C)</i>	1.523	0.442	Macrolide	Increased efflux
<i>mph(G)</i>	1.523	0.442	Macrolide	Enzymatic inactivation
<i>catA2</i>	1.442	2.655	Amphenicol	Enzymatic inactivation
<i>blaCMY-98</i>	1.384	1.327	Beta-lactam	Enzymatic inactivation
<i>cmlV</i>	1.351	0.885	Amphenicol	Increased efflux
<i>VanHBX</i>	1.256	0.885	Glycopeptide	Target modification
<i>blaOXA-85</i>	1.204	0.442	Beta-lactam	Enzymatic inactivation
<i>cat</i>	0.815	0.885	Amphenicol	Enzymatic inactivation
<i>cat</i>	0.809	0.442	Amphenicol	Enzymatic inactivation
<i>blaKLUC-3</i>	0.774	2.212	Beta-lactam	Enzymatic modification
<i>blaOXA-9</i>	0.692	0.885	Beta-lactam	Enzymatic inactivation
<i>blaSED1</i>	0.679	0.442	Beta-lactam	Enzymatic inactivation
<i>blaPAO</i>	0.527	1.327	Beta-lactam	Enzymatic inactivation
<i>aac(6')-IId</i>	0.400	1.327	Aminoglycoside	Enzymatic modification
<i>mdt(A)</i>	0.279	0.442	Macr + Lin + StreptA + StrepB	Increased efflux

Macr: macrolide; Lin: lincosamide; StrepA: streptogramin A; StrepB: Streptogramin B; Tet: tetracycline; Amph: amphenicol; Quin: quinolone; FPA: folate pathway antagonist; MLSB: macrolide-lincosamide-streptograminB; Pleuro: pleuromutilin; Oxa: Oxazolidinone

Supplementary Table III-4. Proportion of antibiotic resistances carried by bacterial genus.

AMR family	Genus	Count	Percentage
Aminoglycoside	<i>g_Bacteroides</i>	2	1.058
Aminoglycoside	<i>g_Bifidobacterium</i>	10	5.291
Aminoglycoside	<i>g_Clostridium</i>	11	5.82
Aminoglycoside	<i>g_Enterococcus</i>	36	19.048
Aminoglycoside	<i>g_Escherichia</i>	71	37.566
Aminoglycoside	<i>g_Klebsiella</i>	9	4.762
Aminoglycoside	<i>g_Phocaeicola</i>	1	0.529
Aminoglycoside	<i>g_Staphylococcus</i>	48	25.397
Aminoglycoside	<i>g_Streptococcus</i>	1	0.529
Amphenicol	<i>g_Bifidobacterium</i>	1	1.299
Amphenicol	<i>g_Clostridium</i>	2	2.597
Amphenicol	<i>g_Enterococcus</i>	4	5.195

Amphenicol	g_Escherichia	3	3.896
Amphenicol	g_Klebsiella	58	75.325
Amphenicol	g_Streptococcus	9	11.688
Beta-lactam	g_Bacteroides	30	13.825
Beta-lactam	g_Escherichia	26	11.982
Beta-lactam	g_Klebsiella	53	24.424
Beta-lactam	g_Parabacteroides	19	8.756
Beta-lactam	g_Phocaeicola	12	5.53
Beta-lactam	g_Staphylococcus	77	35.484
Folatepathwayantagonist	g_Bacteroides	3	3.333
Folatepathwayantagonist	g_Enterococcus	7	7.778
Folatepathwayantagonist	g_Escherichia	18	20
Folatepathwayantagonist	g_Klebsiella	60	66.667
Folatepathwayantagonist	g_Staphylococcus	2	2.222
Fosfomycin	g_Escherichia	1	0.943
Fosfomycin	g_Klebsiella	56	52.83
Fosfomycin	g_Staphylococcus	49	46.226
Lincosamide	g_Bacteroides	37	14.8
Lincosamide	g_Bifidobacterium	45	18
Lincosamide	g_Clostridium	15	6
Lincosamide	g_Enterococcus	83	33.2
Lincosamide	g_Parabacteroides	4	1.6
Lincosamide	g_Phocaeicola	8	3.2
Lincosamide	g_Staphylococcus	23	9.2
Lincosamide	g_Streptococcus	35	14
Macrolide	g_Bacteroides	42	12.034
Macrolide	g_Bifidobacterium	44	12.607
Macrolide	g_Clostridium	4	1.146
Macrolide	g_Enterococcus	41	11.748
Macrolide	g_Escherichia	9	2.579
Macrolide	g_Parabacteroides	6	1.719
Macrolide	g_Phocaeicola	9	2.579
Macrolide	g_Staphylococcus	46	13.181
Macrolide	g_Streptococcus	148	42.407
Quinolone	g_Escherichia	3	4.918
Quinolone	g_Klebsiella	58	95.082
StreptograminB	g_Bacteroides	37	18.782
StreptograminB	g_Bifidobacterium	44	22.335
StreptograminB	g_Clostridium	4	2.03
StreptograminB	g_Enterococcus	41	20.812
StreptograminB	g_Parabacteroides	4	2.03
StreptograminB	g_Phocaeicola	8	4.061
StreptograminB	g_Staphylococcus	19	9.645
StreptograminB	g_Streptococcus	40	20.305
Tetracycline	g_Bacteroides	31	10.197
Tetracycline	g_Bifidobacterium	36	11.842
Tetracycline	g_Clostridium	37	12.171
Tetracycline	g_Enterococcus	39	12.829
Tetracycline	g_Escherichia	16	5.263

Tetracycline	g_Parabacteroides	12	3.947
Tetracycline	g_Phocaeicola	13	4.276
Tetracycline	g_Staphylococcus	34	11.184
Tetracycline	g_Streptococcus	86	28.289

Annex Chapter IV

Supplementary Table IV-2. Metagenomic sequencing information and quality of the samples included in this study.

Sample	QC reads	Contigs	TotalARG assembly-free	TotalARG assembly-based
MAMI_006B04H	6518960	8948	23	5
MAMI_006M04BM	31564256	11086	29	14
MAMI_012B04H	24304594	11288	39	8
MAMI_015M04BM	95634	151	9	0
MAMI_017B04H	51334	162	2	0
MAMI_017M04BM	472196	4352	4	0
MAMI_01WB04H	12502572	7044	24	2
MAMI_01WM04BM	132844	1664	14	0
MAMI_020B04H	47317072	13226	45	13
MAMI_020M04BM	262070	1148	25	1
MAMI_022B04H	33623496	4771	35	14
MAMI_022M04BM	216418	554	3	0
MAMI_02WB04H	91879028	9459	24	3
MAMI_02WM04BM	4042804	2204	5	0
MAMI_031M04BM	31106220	5456	24	9
MAMI_032B04H	11085480	6902	26	3
MAMI_032M04BM	5707408	14755	37	17
MAMI_033B04H	47148322	97650	30	17
MAMI_033M02BM	4823624	9563	20	1
MAMI_037B04H	48101976	11940	32	11
MAMI_037M02BM	172960	87	3	0
MAMI_039B04H	62089838	12572	29	7
MAMI_039M02BM	4551840	4617	15	0
MAMI_045M04BM	4053884	1945	6	0
MAMI_046B04H	42223300	8229	27	9
MAMI_046M04BM	90522	486	14	0
MAMI_056B04H	56035338	7553	40	16
MAMI_07WB04H	7970466	9269	40	4
MAMI_07WM04BM	128042	2050	18	0
MAMI_09WB04H	22225874	9148	40	7
MAMI_09WM04BM	42512	104	10	1
MAMI_10WB04H	48381444	10215	39	11
MAMI_12WB04H	37403038	5641	31	10
MAMI_12WM04BM	43530	421	4	0
MAMI_13WB04H	16857338	3919	17	1
MAMI_13WM04BM	21272	42	5	0
MAMI_15WB04H	135940630	6168	23	8
MAMI_15WM04BM	2681082	4196	11	1
MAMI_16WB04H	63191388	13560	34	12
MAMI_16WM04BM	1078950	685	7	0
MAMI_17WB04H	31729384	9128	39	10
MAMI_17WM04BM	194670	550	21	1

MAMI_18WB04H	42870274	11971	41	17
MAMI_18WM04BM	55256	481	10	0
MAMI_21WB04H	17521850	3145	31	3
MAMI_21WM04BM	133436	96	17	0
MAMI_29WB04H	137050332	5332	32	7
MAMI_432B04H	47678582	8540	52	25
MAMI_432M04BM	2737954	2787	5	0
MAMI_441B04H	45091922	15885	52	16
MAMI_441M04BM	1271308	3649	6	0
MAMI_442B04H	80243064	10945	41	16
MAMI_442M04BM	28036032	20673	62	21
MAMI_443B04H	1638716	3585	14	4
MAMI_443M03BM	3737782	3887	8	0
MAMI_444M05BM	3792576	3455	9	0
MAMI_447M04BM	3357816	1844	6	0
MAMI_451B04H	66545446	13479	48	19
MAMI_451M04BM	3885098	2875	14	1
MAMI_453B04H	20551806	12281	25	9
MAMI_453M04BM	93638	31	5	0
MAMI_453M04BM	74102	65	5	0
MAMI_454B04H	23978762	8373	30	3
MAMI_454M04BM	3216148	8220	30	5
MAMI_459B04H	457778	12587	29	6
MAMI_471M04BM	14397778	4066	41	17
MAMI_475B04H	18196042	8801	23	6
MAMI_475M04BM	89074	103	10	0
MAMI_501M03BM	1475712	1154	7	1
MAMI_502B04H	30080076	10205	30	14
MAMI_502M03BM	132456	2748	6	0
MAMI_503B04H	12547746	10035	33	13
MAMI_503M03BM	20247118	8399	31	9
MAMI_506B04H	12316080	10446	17	6
MAMI_506M04BM	108526	74	6	0
MAMI_508B04H	51420726	14636	44	1
MAMI_508M04BM	880054	1900	9	2
MAMI_510M04BM	99730	123	5	0
MAMI_518B04H	17039326	4113	27	7
MAMI_522B04H	91645754	15681	51	21
MAMI_522M02BM	730192	237	2	0
MAMI_523B04H	74607178	8694	30	5

Supplementary Table IV-3. Information of the abundance, prevalence, class and mechanism of each ARG detected in the infant fecal samples included in the study.

ARG	Total abund.	Prevalence (%)	Class	Mechanism of resistance
<i>blaTEM-1A</i>	8193.55	50.88	Beta-lactam	Enzymatic inactivation
<i>tet(S/M)</i>	5086.50	84.21	Tetracycline	Target protection
<i>vanM</i>	4655.96	85.96	Glycopeptide	Target modification
<i>tet(O/32/O)</i>	4552.53	66.67	Tetracycline	Target protection
<i>tet(Q)</i>	3404.37	47.37	Tetracycline	Target protection
<i>tet(A)</i>	3314.81	24.56	Tetracycline	Increased efflux
<i>tet(W)</i>	3254.22	66.67	Tetracycline	Target protection
<i>sul2</i>	2912.76	38.60	Folate pathway antagonist	Purine synthesis
<i>msr(D)</i>	2753.07	92.98	Macrolide, Streptogramin B	Target protection
<i>aph(3'')-Ib</i>	2527.29	45.61	Aminoglycoside	Enzymatic modification
<i>OqxB</i>	2370.86	64.91	Amphenicol, Quinolone, Folate pathway antagonist	Efflux pump
<i>aph(6)-Id</i>	2219.87	43.86	Aminoglycoside	Enzymatic modification
<i>erm(X)</i>	2023.50	61.40	Macrolide, Lincosamide, Streptogramin B	Target modification
<i>erm(B)</i>	1972.34	70.18	Macrolide, Lincosamide, Streptogramin B	Target modification
<i>tet(L)</i>	1910.39	24.56	Tetracycline	Increased efflux
<i>cfxA</i>	1441.98	40.35	Beta-lactam	Enzymatic inactivation
<i>tetB(P)</i>	1325.01	36.84	Tetracycline	Target protection
<i>erm(F)</i>	1297.26	33.33	Macrolide, Lincosamide, Streptogramin B	Target modification
<i>tet(X)</i>	1224.84	28.07	Tetracycline	Enzymatic inactivation
<i>mef(A)</i>	1086.12	85.96	Macrolide	Increased efflux
<i>erm(X)</i>	1018.89	59.65	Macrolide, Lincosamide, Streptogramin B	Target modification
<i>tet(O/32/O)</i>	973.57	61.40	Tetracycline	Target protection
<i>tet(O/W/O)-1</i>	875.59	70.18	Tetracycline	Target protection
<i>tetA(P)</i>	775.69	47.37	Tetracycline	Increased efflux
<i>OqxA</i>	725.64	50.88	Amphenicol, Quinolone, Folate pathway antagonist	Efflux pump
<i>tet(40)</i>	603.83	8.77	Tetracycline	Increased efflux

<i>erm(T)</i>	595.25	19.30	Macrolide, Lincosamide, Streptogramin B	Target modification
<i>mef(A)</i>	521.22	89.47	Macrolide	Increased efflux
<i>tet(O/W/32/O/W/O)</i>	480.46	59.65	Tetracycline	Target protection
<i>NarB</i>	429.38	3.51	Ionophores	Efflux pump
<i>sul1</i>	424.57	22.81	Folate pathway antagonist	Purine synthesis
<i>lsa(E)</i>	404.48	7.02	Lincosamide, Streptogramin A, Pleuromutilin	Target protection
<i>cfxA6</i>	383.69	10.53	Beta-lactam	Enzymatic inactivation
<i>blaSHV-100</i>	360.76	29.82	Beta-lactam	Enzymatic inactivation
<i>lsa(A)</i>	350.27	63.16	Lincosamide, Streptogramin A	Target protection
<i>blaACT-5</i>	344.68	17.54	Beta-lactam	Enzymatic inactivation
<i>aadA4</i>	334.70	8.77	Aminoglycoside	Enzymatic modification
<i>tetA(46)</i>	334.25	71.93	Tetracycline	Increased efflux
<i>aph(3')-IIIa</i>	322.71	43.86	Aminoglycoside	Enzymatic modification
<i>erm(T)</i>	281.30	43.86	Macrolide, Lincosamide, Streptogramin B	Target modification
<i>ant(6)-Ia</i>	272.88	33.33	Aminoglycoside	Enzymatic modification
<i>tetB(46)</i>	272.50	66.67	Tetracycline	Increased efflux
<i>ant(6)-Ia</i>	264.20	36.84	Aminoglycoside	Enzymatic modification
<i>blaACI-1</i>	237.56	10.53	Beta-lactam	Enzymatic inactivation
<i>tet(X3)</i>	229.29	26.32	Tetracycline	Enzymatic inactivation
<i>mph(A)</i>	225.43	5.26	Macrolide	Enzymatic inactivation
<i>lnu(B)</i>	197.04	3.51	Lincosamide	Enzymatic inactivation
<i>NarA</i>	196.02	3.51	Ionophores	Efflux pump
<i>fosA5</i>	185.33	33.33	Fosfomycin	Enzymatic inactivation
<i>mupA</i>	173.08	50.88	Pseudomonic acid	Alternate Pathway
<i>msr(A)</i>	167.01	57.89	Macrolide, Streptogramin B	Target protection
<i>blaOXY-1-1</i>	166.75	33.33	Beta-lactam	Enzymatic inactivation
<i>blaCMY-18</i>	163.71	12.28	Beta-lactam	Enzymatic inactivation
<i>mph(A)</i>	160.28	5.26	Macrolide	Enzymatic inactivation
<i>dfrA17</i>	145.63	5.26	Folate pathway antagonist	Purine synthesis
<i>dfrG</i>	141.13	12.28	Folate pathway antagonist	Purine synthesis
<i>blaCTX-M-211</i>	139.68	3.51	Beta-lactam	Enzymatic inactivation
<i>lnu(C)</i>	128.89	15.79	Lincosamide	Enzymatic inactivation

<i>erm(G)</i>	128.83	19.30	Macrolide, Lincosamide, Streptogramin B	Target modification
<i>tet(44)</i>	123.07	14.04	Tetracycline	Target protection
<i>mecA</i>	121.66	45.61	Beta-lactam	Target modification
<i>blaOXA-392</i>	117.25	1.75	Beta-lactam	Enzymatic inactivation
<i>cepA</i>	112.32	12.28	Beta-lactam	Enzymatic inactivation
<i>tet(32)</i>	101.58	19.30	Tetracycline	Target protection
<i>lnu(P)</i>	100.87	21.05	Lincosamide	Enzymatic inactivation
<i>fosA2</i>	95.23	10.53	Fosfomycin	Enzymatic inactivation
<i>ere(D)</i>	90.90	1.75	Macrolide	Enzymatic inactivation
<i>blaOXA-347</i>	88.54	3.51	Beta-lactam	Enzymatic inactivation
<i>aph(2'')-Ia</i>	87.20	36.84	Aminoglycoside	Enzymatic modification
<i>blaOXY-2-1</i>	85.47	7.02	Beta-lactam	Enzymatic inactivation
<i>blaZ</i>	79.02	61.40	Beta-lactam	Enzymatic inactivation
<i>mph(C)</i>	76.59	49.12	Macrolide	Enzymatic inactivation
<i>tet(B)</i>	69.55	10.53	Tetracycline	Increased efflux
<i>ant(6)-Ia</i>	68.40	29.82	Aminoglycoside	Enzymatic modification
<i>catB3</i>	65.65	1.75	Amphenicol	Enzymatic inactivation
<i>erm(C)</i>	64.40	31.58	Macrolide, Lincosamide, Streptogramin B	Target modification
<i>msr(C)</i>	60.57	7.02	Macrolide, Streptogramin B	Target protection
<i>aph(3')-Ia</i>	54.32	26.32	Aminoglycoside	Enzymatic modification
<i>tet(K)</i>	52.26	43.86	Tetracycline	Increased efflux
<i>blaLEN3</i>	51.86	24.56	Beta-lactam	Enzymatic inactivation
<i>tet(D)</i>	51.67	1.75	Tetracycline	Increased efflux
<i>tet(S)</i>	50.36	10.53	Tetracycline	Target protection
<i>aadD</i>	44.72	35.09	Aminoglycoside	Enzymatic modification
<i>blaZ</i>	43.90	54.39	Beta-lactam	Enzymatic inactivation
<i>VanC2</i>	43.01	10.53	Glycopeptide	Target modification
<i>aac(6')-Ib</i>	41.64	1.75	Aminoglycoside	Enzymatic modification
<i>aac(6')-If</i>	39.88	1.75	Aminoglycoside	Enzymatic modification
<i>catA1</i>	34.91	5.26	Amphenicol	Enzymatic inactivation
<i>erm(A)</i>	34.57	21.05	Macrolide, Lincosamide, Streptogramin B	Target modification
<i>VanC3XY</i>	34.24	10.53	Glycopeptide	Target modification
<i>ant(6)-Ib</i>	34.02	8.77	Aminoglycoside	Enzymatic modification

<i>fosB3</i>	32.99	5.26	Fosfomycin	Enzymatic inactivation
<i>ant(3'')-Ia</i>	31.00	21.05	Aminoglycoside	Enzymatic modification
<i>blaDHA-1</i>	30.71	3.51	Beta-lactam	Enzymatic inactivation
<i>lnu(A)</i>	28.87	5.26	Lincosamide	Enzymatic inactivation
<i>tetA(60)</i>	27.99	10.53	Tetracycline	Increased efflux
<i>blaPLA-6A</i>	27.42	7.02	Beta-lactam	Enzymatic inactivation
<i>blaMAL-1</i>	22.96	1.75	Beta-lactam	Enzymatic inactivation
<i>VanC1XY</i>	22.88	1.75	Glycopeptide	Target modification
<i>fosB</i>	22.59	38.60	Fosfomycin	Enzymatic inactivation
<i>aac(6')-Ii</i>	21.44	7.02	Aminoglycoside	Enzymatic modification
<i>blaCFE-1</i>	20.48	8.77	Beta-lactam	Enzymatic inactivation
<i>ant(3'')-Ii-aac(6')-IId</i>	19.22	1.75	Aminoglycoside	Enzymatic modification
<i>bleO</i>	19.11	24.56	Glycopeptide	Enzymatic modification
<i>VanXY</i>	18.29	7.02	Glycopeptide	Target modification
<i>lsa(C)</i>	17.11	21.05	Lincosamide, Streptogramin A, Pleuromutilin	Target protection
<i>fosA7</i>	17.00	5.26	Fosfomycin	Enzymatic inactivation
<i>blaCTX-M-64</i>	15.96	1.75	Beta-lactam	Enzymatic inactivation
<i>aac(3)-IIa</i>	15.24	5.26	Aminoglycoside	Enzymatic modification
<i>tet(K)</i>	14.74	26.32	Tetracycline	Increased efflux
<i>tet(X)</i>	14.17	7.02	Tetracycline	Enzymatic inactivation
<i>qnrB41</i>	14.05	12.28	Quinolone	Target protection
<i>dfrA8</i>	13.92	5.26	Folate pathway antagonist	Purine synthesis
<i>dfrA30</i>	12.68	1.75	Folate pathway antagonist	Purine synthesis
<i>aac(6')-30-aac(6')-Ib'</i>	12.61	1.75	Aminoglycoside	Enzymatic modification
<i>fosA</i>	12.12	10.53	Fosfomycin	Enzymatic inactivation
<i>cmlA1</i>	11.69	3.51	Amphenicol	Increased efflux
<i>blaOXA-209</i>	10.61	3.51	Beta-lactam	Enzymatic inactivation
<i>aac(6')-Ib-Hangzhou</i>	10.00	1.75	Aminoglycoside	Enzymatic modification
<i>fusB</i>	9.45	19.30	Steroid antibacterial	Target protection
<i>fusC</i>	8.64	10.53	Steroid antibacterial	Target protection
<i>lnu(G)</i>	8.04	3.51	Lincosamide	Enzymatic inactivation
<i>penA</i>	8.01	3.51	Beta-lactam	Enzymatic inactivation
<i>aadA2</i>	7.65	5.26	Aminoglycoside	Enzymatic modification
<i>tetB(60)</i>	7.44	8.77	Tetracycline	Increased efflux

<i>sul3</i>	7.11	1.75	Folate pathway antagonist	Purine synthesis
<i>aac(3)-Ib-aac(6')-Ib</i>	6.61	1.75	Aminoglycoside	Enzymatic modification
<i>cmx</i>	6.32	10.53	Amphenicol	Increased efflux
<i>blaHERA-1</i>	5.93	1.75	Beta-lactam	Enzymatic inactivation
<i>tet(J)</i>	5.63	5.26	Tetracycline	Increased efflux
<i>dfrA12</i>	5.48	3.51	Folate pathway antagonist	Purine synthesis
<i>aadA1</i>	4.45	5.26	Aminoglycoside	Enzymatic modification
<i>cat(pC194)</i>	4.40	14.04	Amphenicol	Enzymatic inactivation
<i>cat</i>	3.40	5.26	Amphenicol	Enzymatic inactivation
<i>aph(3')-IIa</i>	3.16	1.75	Aminoglycoside	Enzymatic modification
<i>tet(L)</i>	3.11	10.53	Tetracycline	Increased efflux
<i>vga(A)</i>	2.70	14.04	Lincosamide, Streptogramin A, Pleuromutilin	Target protection
<i>aph(6)-Ic</i>	2.48	1.75	Aminoglycoside	Enzymatic modification
<i>blaMIR-2</i>	2.24	5.26	Beta-lactam	Enzymatic inactivation
<i>mef(B)</i>	2.23	1.75	Macrolide	Increased efflux
<i>blaSPU-1</i>	2.03	1.75	Beta-lactam	Enzymatic inactivation
<i>erm(Q)</i>	1.70	5.26	Macrolide, Lincosamide, Streptogramin B	Target modification
<i>catS</i>	1.36	3.51	Amphenicol	Enzymatic inactivation
<i>dfrA7</i>	1.32	1.75	Folate pathway antagonist	Purine synthesis
<i>blaOXA-85</i>	1.20	1.75	Beta-lactam	Enzymatic inactivation
<i>erm(33)</i>	1.02	7.02	Macrolide, Lincosamide, Streptogramin B	Target modification
<i>mcr-10</i>	0.71	1.75	Polymyxin	Target modification
<i>cml</i>	0.67	1.75	Amphenicol	Increased efflux
<i>tet(C)</i>	0.55	5.26	Tetracycline	Increased efflux
<i>catP</i>	0.42	1.75	Amphenicol	Enzymatic inactivation
<i>ant(9)-Ia</i>	0.41	1.75	Aminoglycoside	Enzymatic modification
<i>erm(A)</i>	0.41	1.75	Macrolide, Lincosamide, Streptogramin B	Target modification
<i>aph(2'')-If</i>	0.37	3.51	Aminoglycoside	Enzymatic modification
<i>dfrA1</i>	0.21	5.26	Folate pathway antagonist	Purine synthesis
<i>cat</i>	0.20	1.75	Amphenicol	Enzymatic inactivation
<i>blaPAO</i>	0.20	1.75	Beta-lactam	Enzymatic inactivation

<i>lnu(F)</i>	0.19	3.51	Lincosamide	Enzymatic inactivation
<i>qnrB8</i>	0.18	1.75	Quinolone	Target protection
<i>blaCMY-98</i>	0.14	1.75	Beta-lactam	Enzymatic inactivation
<i>dfrA1</i>	0.13	1.75	Folate pathway antagonist	Purine synthesis
<i>blaACT-9</i>	0.12	1.75	Beta-lactam	Enzymatic inactivation
<i>catQ</i>	0.12	1.75	Amphenicol	Enzymatic inactivation
<i>catA2</i>	0.11	1.75	Amphenicol	Enzymatic inactivation
<i>aac(6')-lii</i>	0.11	1.75	Aminoglycoside	Enzymatic modification
<i>dfrA14</i>	0.10	1.75	Folate pathway antagonist	Purine synthesis
<i>cfr(C)</i>	0.10	1.75	Oxazolidinone, Amphenicol, Lincosamide, Streptogramin A, Pleuromutilin	Target modification
<i>cat(pC221)</i>	0.09	1.75	Amphenicol	Enzymatic inactivation

Supplementary Table IV-4. Diversity (observed and shannon) and total number of ARGs detected in each bacterial species.

Species	Observed ARG richness	Shannon ARG diversity	Total number ARG
[Clostridium]_innocuum	3	1.099	3
[Clostridium]_scindens	2	0.693	2
Acidaminococcus_intestini	1	0	3
Bacteroides_caccae	2	0.693	2
Bacteroides_faecis	1	0	3
Bacteroides_fragilis	4	1.255	8
Bacteroides_thetaiotaomicron	3	1.004	7
Bifidobacterium_breve	2	0.693	4
Bifidobacterium_longum	4	1.314	10
Blautia_obeum	2	0.637	3
Blautia_parvula	3	1.04	4
Citrobacter_freundii	3	1.099	3
Citrobacter_koseri	2	0.693	2
Citrobacter_portucalensis	2	0.693	2
Clostridioides_difficile	3	1.099	3
Clostridium_perfringens	4	1.136	19
Eggerthella_lenta	1	0	3
Enterobacter_hormaechei	3	1.04	4
Enterococcus_casseliflavus	1	0	3
Enterococcus_durans	2	0.693	2
Enterococcus_faecalis	6	1.388	22
Enterococcus_faecium	8	1.97	14
Enterococcus_sp._FDAARGOS_375	2	0.693	2
Escherichia_coli	12	2.03	26
Eubacterium_callanderi	2	0.693	2

Flavonifractor_plautii	2	0.637	3
Hungatella_hathewayi	3	1.055	5
Klebsiella_aerogenes	2	0.693	2
Klebsiella_michiganensis	4	1.381	17
Klebsiella_oxytoca	3	1.055	5
Klebsiella_pasteurii	2	0.693	2
Klebsiella_pneumoniae	9	1.728	30
Lautropia_mirabilis	3	1.099	3
Olsenella_timonensis	2	0.693	2
Parabacteroides_distasonis	4	1.215	9
Phocaeicola_dorei	5	1.475	7
Phocaeicola_vulgatus	3	1.04	4
Proteus_mirabilis	2	0.693	2
Raoultella_ornithinolytica	3	1.099	3
Raoultella_planticola	3	1.099	6
Rothia_mucilaginosa	1	0	2
Sellimonas_intestinalis	2	0.693	2
Staphylococcus_aureus	11	2.059	33
Staphylococcus_epidermidis	7	1.56	16
Staphylococcus_hominis	4	1.321	8
Staphylococcus_petrassii	1	0	2
Streptococcus_australis	2	0.637	3
Streptococcus_dysgalactiae	2	0.562	4
Streptococcus_gordonii	1	0	2
Streptococcus_infantis	2	0.693	2
Streptococcus_lactarius	2	0.377	8
Streptococcus_lutetiensis	3	1.099	3
Streptococcus_mitis	3	1.099	3
Streptococcus_parasanguinis	3	0.687	13
Streptococcus_pasteurianus	2	0.693	4
Streptococcus_salivarius	2	0.44	25
unclassified_Actinomycetes_species	1	0	2
unclassified_Bacillota_species	3	1.099	3
unclassified_Bacteroides_species	2	0.693	2
unclassified_Bifidobacterium_species	2	0.693	2
unclassified_Enterobacterales_species	2	0.637	3
unclassified_Enterobacteriaceae_species	2	0.637	3
unclassified_Prevotella_species	1	0	3
unclassified_Streptococcus_species	2	0.562	4
Veillonella_rodentium	1	0	2
Veillonella_sp._S12025-13	1	0	2

Supplementary Table IV-5. Information of the abundance, prevalence, class and mechanism of each ARG detected in the human milk samples included in the study.

ARG	Total abund.	Prevalence (%)	Class	Mechanism of resistance
msr(D)	11243.23	65.96	Macrolide, Streptogramin B	Target protection
mef(A)	5374.72	65.96	Macrolide	Increased efflux
msr(A)	8582.57	55.32	Macrolide, Streptogramin B	Target protection
mph(C)	4873.69	48.94	Macrolide	Enzymatic inactivation
blaTEM-1A	3803.07	44.68	Beta-lactam	Enzymatic inactivation
mef(A)	2793.54	44.68	Macrolide	Increased efflux
tet(S/M)	3992.70	42.55	Tetracycline	Target protection
vanM	4966.86	40.43	Glycopeptide	Target modification
blaZ	2646.94	40.43	Beta-lactam	Enzymatic inactivation
aadD	2385.13	40.43	Aminoglycoside	Enzymatic modification
mupA	17191.98	36.17	Pseudomonic acid	Alternate Pathway
tet(C)	2703.79	36.17	Tetracycline	Increased efflux
tet(K)	3263.46	34.04	Tetracycline	Increased efflux
blaZ	1858.03	34.04	Beta-lactam	Enzymatic inactivation
mecA	5049.20	31.91	Beta-lactam	Target modification
tetB(46)	1436.70	31.91	Tetracycline	Increased efflux
fosB	1048.59	31.91	Fosfomycin	Enzymatic inactivation
tetA(46)	710.00	29.79	Tetracycline	Increased efflux
bleO	1110.94	27.66	Glycopeptide	Enzymatic modification
erm(C)	6252.37	25.53	Macrolide, Lincosamide, Streptogramin B	Target modification
fusB	676.02	25.53	Steroid antibacterial	Target protection
blaOXA-280	878.16	23.40	Beta-lactam	Enzymatic inactivation
erm(X)	524.77	23.40	Macrolide, Lincosamide, Streptogramin B	Target modification
erm(B)	685.49	21.28	Macrolide, Lincosamide, Streptogramin B	Target modification
Oqx B	1106.20	19.15	Amphenicol, Quinolone, Folate pathway antagonist	Efflux pump
tet(O/32/O)	590.46	19.15	Tetracycline	Target protection

vga(A)	377.33	19.15	Lincosamide, Streptogramin A, Pleuromutilin	Target protection
aph(3')-Ia	342.27	19.15	Aminoglycoside	Enzymatic modification
erm(X)	324.88	19.15	Macrolide, Lincosamide, Streptogramin B	Target modification
aph(2'')-Ia	1035.94	17.02	Aminoglycoside	Enzymatic modification
tet(K)	897.32	14.89	Tetracycline	Increased efflux
tet(W)	460.97	14.89	Tetracycline	Target protection
aph(3')-IIIa	281.20	14.89	Aminoglycoside	Enzymatic modification
aph(3')-Ib	426.84	12.77	Aminoglycoside	Enzymatic modification
fusC	277.54	12.77	Steroid antibacterial	Target protection
blaSGM-7	153.95	12.77	Beta-lactam	Enzymatic inactivation
lsa(A)	391.89	10.64	Lincosamide, Streptogramin A	Target protection
OqxA	322.05	10.64	Amphenicol, Quinolone, Folate pathway antagonist	Efflux pump
cmx	319.59	10.64	Amphenicol	Increased efflux
tet(L)	226.24	10.64	Tetracycline	Increased efflux
aph(3')-IIc	168.35	10.64	Aminoglycoside	Enzymatic modification
tet(O/W/O)-1	67.12	10.64	Tetracycline	Target protection
tmexD1	36.21	10.64	Tetracycline	Efflux pump
sul2	538.06	8.51	Folate pathway antagonist	Purine synthesis
aph(6)-Id	399.74	8.51	Aminoglycoside	Enzymatic modification
lnu(A)	250.23	8.51	Lincosamide	Enzymatic inactivation
blaOXY-1-1	15.99	8.51	Beta-lactam	Enzymatic inactivation
catB1	13.13	8.51	Amphenicol	Enzymatic inactivation
blaOXA-496	361.10	6.38	Beta-lactam	Enzymatic inactivation
ant(6)-Ia	206.24	6.38	Aminoglycoside	Enzymatic modification
blaL1	173.87	6.38	Beta-lactam	Enzymatic inactivation
penA	171.82	6.38	Beta-lactam	Enzymatic inactivation
blaACT-5	134.72	6.38	Beta-lactam	Enzymatic inactivation
tet(O/32/O)	86.83	6.38	Tetracycline	Target protection
fosA5	84.99	6.38	Fosfomycin	Enzymatic inactivation

erm(T)	50.43	6.38	Macrolide, Lincosamide, Streptogramin B	Target modification
fosA2	25.47	6.38	Fosfomycin	Enzymatic inactivation
sul1	12.75	6.38	Folate pathway antagonist	Purine synthesis
ant(3'')-la	2.77	6.38	Aminoglycoside	Enzymatic modification
cat(pC194)	514.48	4.26	Amphenicol	Enzymatic inactivation
tet(D)	339.76	4.26	Tetracycline	Increased efflux
blaSHV-100	333.48	4.26	Beta-lactam	Enzymatic inactivation
blaLEN3	34.79	4.26	Beta-lactam	Enzymatic inactivation
cfxA	32.13	4.26	Beta-lactam	Enzymatic inactivation
msr(E)	23.88	4.26	Macrolide, Streptogramin B	Target protection
tet(39)	6.05	4.26	Tetracycline	Increased efflux
fosB1	5.44	4.26	Fosfomycin	Enzymatic inactivation
erm(33)	3.59	4.26	Macrolide, Lincosamide, Streptogramin B	Target modification
mcr-4.1	2681.52	2.13	Polymyxin	Target modification
tet(J)	512.57	2.13	Tetracycline	Increased efflux
cat	250.56	2.13	Amphenicol	Enzymatic inactivation
erm(T)	168.79	2.13	Macrolide, Lincosamide, Streptogramin B	Target modification
blaCMY-18	132.97	2.13	Beta-lactam	Enzymatic inactivation
qnrB41	23.98	2.13	Quinolone	Target protection
blaMOX-5	13.87	2.13	Beta-lactam	Enzymatic inactivation
blaOXA-539	13.48	2.13	Beta-lactam	Enzymatic inactivation
cat(pC221)	10.70	2.13	Amphenicol	Enzymatic inactivation
sul4	7.63	2.13	Folate pathway antagonist	Purine synthesis
blaOXA-427	4.10	2.13	Beta-lactam	Enzymatic inactivation
floR	1.16	2.13	Amphenicol	Increased efflux
blaOXA-49	0.79	2.13	Beta-lactam	Enzymatic inactivation

