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INTERCONNECTED RESISTOMES AND THE ACCUMULATIVE ANTIBIOTIC RESISTANCE CRISIS

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DOCTORAL DISSERTATION

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I	Katariina Pärnänen participated in the design of the study and executing the laboratory experiments. She wrote the manuscript in collaboration with other authors.
II	Katariina Pärnänen did laboratory work, bioinformatic and statistical analysis, wrote the manuscript, and participated in conceiving the study.
III	Katariina Pärnänen participated in the design and interpretation of the reported experiments. She participated in the acquisition and analysis of the data. She participated in the writing of the manuscript.
IV	Katariina Pärnänen participated in the interpretation of the results and writing and reviewing of the manuscript
V	Katariina Pärnänen participated in the design of the study. She executed the experiments, developed methodology, analyzed and visualized the data, curated most of the data, interpreted and validated the results, and wrote the manuscript.

ABBREVIATIONS AND VOCABULARY

16S rRNA	16S ribosomal ribonucleic acid
ARB	antibiotic-resistant bacteria
ARG	antibiotic resistance gene
BLAST	basic local alignment tool
ESBL	extended-spectrum beta-lactamase
DNA	deoxyribonucleic acid
MGE	mobile genetic element
IAP	intrapartum antibiotic prophylaxis
IPCR	inverse polymerase chain reaction
ORF	open reading frame
PCR	polymerase chain reaction
qPCR	quantitative polymerase chain reaction
WWTP	wastewater treatment plant

ABSTRACT

Antibiotics were once miracle cures, but because of the spread of antibiotic resistance in bacteria, their effectiveness is threatened. Although antibiotics have only been produced industrially for 70 years, antibiotic-resistant bacteria are a threat to human health. The effects of antibiotic use pass on over generations, and resistance kills an estimated 214,000 infants a year. Antibiotic-resistant bacteria have also become widespread in the environment.

In my dissertation, I used a microbial ecology perspective to study how the antibiotic resistance crisis manifests itself in humans (with a focus on mothers and infants) and in the environment. My main lines of research focused on selection pressures that shape bacterial communities and the effects of the spread of resistant bacteria. I studied the amounts of antibiotic resistance genes in different environments, utilizing methods based mainly on metagenomics.

Mothers pass on antibiotic-resistant bacteria to their children. However, in my study, the resistance load of infants' intestines was most affected by infant formula use. Infants who received formula had a significantly higher proportion of bacteria carrying resistance genes than exclusively breastfed infants. Surprisingly, formula use increased the intestinal resistance load more than the antibiotic regimens given to babies, which could not be shown to have an effect in my dissertation.

Antibiotic selection pressure did not explain the number of resistance genes in the environmental samples I studied either. The results suggested that fecal contamination is almost always behind the resistance load observed in the environment. It was therefore interesting that the treated wastewater discharged from European wastewater treatment plants into the environment corresponded to the types of resistance of bacterial strains isolated from infected patients. The result suggests that inadequate wastewater treatment is part of the resistance problem in Europe as well, and not just in developing countries, and potentially increases the spread of antibiotic-resistant bacteria to humans.

My work shows that the most effective ways to reduce resistance may not be intuitive. Bacterial spread may play a larger role than previously thought. Efficient waste treatment and exclusive breastfeeding may reduce the number of resistant bacteria in society, the environment, and young children more effectively than reducing the use of antibiotics.

TIIVISTELMÄ

Ennen antibiootit olivat ihmelääkkeitä, mutta bakteerien yleistyneen vastustuskyvyn, eli resistenssin, vuoksi niiden tehokkuus on uhattuna. Vaikka antibiootteja on tuotettu teollisesti vasta 70 vuoden ajan, ovat antibiooteille resistentit bakteerit uhka ihmisten terveydelle. Antibioottien käytön vaikutukset siirtyvät yli sukupolvien ja resistenssi tappaakin arviolta 214 000 imeväisikäistä lasta vuodessa. Antibiooteille vastustuskykyiset bakteerit ovat levinneet laajasti myös ympäristöön.

Väitöskirjassani olen tutkinut mikrobiekologian näkökulmasta, kuinka antibioottiresistenssikriisi näkyy ihmisissä (keskittyen äiteihin ja vauvoihin) ja ympäristössä. Päätutkimuslinjani keskittyivät bakteeriyhteisöjä muokkaaviin valintapainesiin ja vastustuskykyisten bakteerien leviämisen vaikutuksiin. Tutkin eri ympäristöjen antibioottiresistenssigeenimääriä hyödyntäen pääosin metagenomiikkaan perustuvia menetelmiä.

Äidit siirtävät lapsilleen myös antibiooteille vastustuskykyisiä bakteereita. Tutkimuksessani vauvojen suoliston resistenssikuormaan vaikutti kuitenkin eniten äidinmaidonkorvikkeen käyttö. Vauvoilla, jotka saivat korviketta, oli huomattavasti suurempi osuus resistenssigeenejä kantavia bakteereja kuin täysimetetyillä. Yllättäen korvike lisäsi suoliston resistenssikuormaa enemmän kuin vauvojen saamat antibioottikuurit, joilla ei väitöskirjassani pystytty osoittamaan olevan vaikutusta.

Antibioottivalintapaine ei selittänyt resistenssigeenien määrään myöskään tutkimissani ympäristönäytteissä. Tulokset viittasivat siihen, että ulostesaaste on lähes aina ympäristössä havaitun resistenssikuorman takana. Olikin kiinnostavaa, että eurooppalaisilta jätevedenpuhdistamoilta ympäristöön päätyvä puhdistettu jätevesi vastasi infektiopotilaista eristettyjen bakteerikantojen resistenssityyppejä. Tulos viittaa siihen, että jätevesien puutteellinen käsittely on osa resistenssiongelmaa myös Euroopassa, eikä vain kehittyvissä maissa, ja mahdollisesti lisää antibiooteille vastustuskykyisten bakteerien leviämistä ihmisiin.

Tehokkaimmat tavat vähentää resistenssiä eivät välttämättä ole intuitiivisia. Bakteerien leviäminen on mahdollisesti suuremmassa roolissa kuin on ajateltu. Toimiva jätevirtojen käsittely ja täysimetys saattavat vähentää vastustuskykyisten bakteerien määrää yhteiskunnassa, ympäristössä ja pikkulapsissa tehokkaammin kuin antibioottien käytön vähentäminen.

INTRODUCTION

1.1 A concise history of the dawn and dusk of the antibiotic era

1.1.1 Manmade antibiotics and discovery of resistant bacteria

We know that antibiotic is an ancient, natural phenomenon, and the origin of many antibiotic resistance genes (ARGs) is environmental (Allen et al. 2009; 2010; D'Costa et al. 2011). Antibiotic compounds have diverse roles in nature. Some bacteria and fungi produce low concentrations of antibiotics released to the environment (Figure 1). One explanation for ARGs' occurrence is that antibiotic producers themselves need protection against the antibiotic (Martinez 2008; Davies and Davies 2010). Thus, there are ARGs also in pristine locations (Allen et al. 2009; Bhullar et al. 2012).

Antibiotics are essential in modern medicine, but their use might be an ancient invention. Possibly, the first evidence of anthropogenic use of antibiotics (tetracycline) dates to 350-550 CE (Nelson et al. 2010). However, it was not until much later when human use of antibiotics became common. The invention of antibiotics that could be produced commercially at the beginning of the 20th century and the start of industrial-scale production of penicillin, discovered by Ian Fleming (1908-64) in the 1920s, marked the beginning of the golden era of antibiotics.

Penicillin saved countless lives during World War II and was a true miracle drug (Davies 1997). New antibiotic inventions followed penicillin's discovery and broadened the range of diseases that could now be cured just by taking a few pills. The use of antibiotics enabled more advanced surgeries and immunosuppressive treatments. However, Fleming himself discovered bacteria that were resistant to penicillin. He postulated, quite rightly so, that there is no antibiotic that bacteria could not become immune to (Levy and Marshall 2004).



Figure 1. Antibiotic resistance in natural environments. Antibiotic-producing bacteria and fungi reside in nature and release antibiotics to their surroundings. Antibiotics serve various ecological functions in bacterial communities. Microbes that produce antibiotics need ARGs to protect themselves from their effects. Many resistance genes also serve other purposes, such as pumping other substances out of bacterial cells.

The discovery of penicillin resistance was one of the first inclinations that antibiotics are not miracle drugs, after all. Now we know that each time a new antibiotic compound enters the market, a clock starts ticking. There is a short honeymoon period before clinical resistance towards the antibiotic spreads to opportunistic pathogens. It took five years for penicillin resistance to emerge, a typical timeframe for all antibiotic classes (Levy and Marshall 2004; Clatworthy, Pierson, and Hung 2007; Davies and Davies 2010). The amount of antibiotics produced after the 1950s could be somewhere in the ballpark of 1 000 000 tons, which is very likely much more than is naturally produced by micro-organisms (Davies 2006). Therefore, it is no wonder that since the commercialization of antibiotics, we have observed a rapid spread of antibiotic resistance. Antibiotic-resistant bacteria have disseminated globally, and ARGs, which have emerged due to antibiotics' anthropogenic use, have contaminated isolated regions, including the Antarctic (Wang et al. 2016).

1.1.2 Anthropogenic antibiotic resistance in the environment and the One Health approach

In the past few decades, a growing awareness of the expanding antibiotic resistance issue has caused scientists from several fields, besides clinical microbiologists following in the footsteps of Fleming, to investigate antibiotic resistance, for example, in animals and the environment. In the early 2000s, studies on zoonotic diseases, such as the avian flu, SARS, and Ebola, led to a transdisciplinary collaboration called One Health between veterinarians, clinicians, and environmental scientists. One Health describes a holistic approach to health, including antibiotic resistance, which considers human and animal health and ecological aspects (McEwen and Collignon 2018; Hernando-Amado et al. 2019).

For example, microbiologists and veterinarians research antibiotic resistance from perspectives that fit under the scope of One Health. These studies done in non-clinical sectors provide information on ARGs and ARB (antibiotic-resistant bacteria) occurring in animal production and environments polluted by antibiotics and ARB. There is a dynamic flow of ARB and ARGs between nature and human and animal compartments (Figure 2). However, it is still largely unknown how exactly ARGs occurring in environmental or animal resistomes impact human health. Therefore, risk assessment for resistance genes found in non-human compartments is challenging and requires multi-sectorial collaboration.

One of One Health's goals is to prevent dire consequences for human health caused by food production practices and poor food hygiene, especially in animal husbandry. One example of what can go wrong is the COVID-19 pandemic caused by a zoonotic virus. The pandemic put the whole world in lockdown, caused nearly two million deaths in a year, and changed how we do business, work, and interact with each other. Antibiotic resistance is a slower, more silent pandemic, and we need to take measures in all aspects of One Health to prevent casualties, currently estimated to be 700 000 annually (WHO World Health Organization 2014; Strathdee, Davies, and Marcelin 2020).

Just as the zoonotic COVID-19 virus, antibiotic-resistant bacteria spread from animals to humans. Industrial animal production began in the 1930s in the United States, and since then, antibiotics have been used to treat production animals. In intensive farming, animals are confined to small spaces in large numbers, and they are exposed to feces (Silbergeld, Graham, and Price 2008).

Antibiotics have been used as growth promoters for over 60 years. Nowadays, many countries have banned the growth promotion use of antibiotics, including the EU (Silbergeld, Graham, and Price 2008). What most people might find

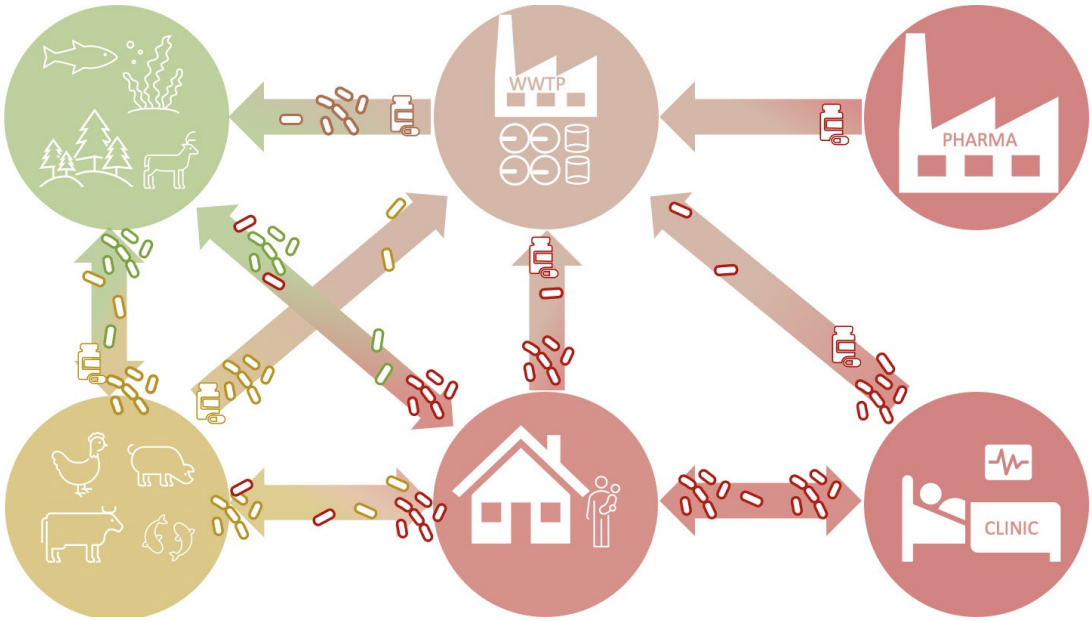


Figure 2. The flow of antibiotic-resistant bacteria and antibiotics between compartments. 1) Antibiotic-resistant bacteria (ARB) flow from human sources (community and hospital) to natural environments through wastewater treatment plants or directly to the terrestrial and aquatic environments. 2) ARB also transfer between the community and the clinic. 3) Selection pressure and the emergence of new ARBs can be caused by the release of antibiotics from drug manufacturing. The drug manufacturing waste should be treated, however in some cases, the runoff ends up to the environment directly. 4) Animal production uses antibiotics, and ARB and antibiotics are released to nature if the waste is untreated or manure is used as fertilizer. Production animals can also receive ARB from the environment, especially if they are kept outside or consume fodder. 5) Humans come in contact with environmental bacteria and consume foodstuffs with ARB. In the figure, green denotes compartments and ARB/antibiotics of primarily non-anthropogenic origin, and brown represents mixed human, environmental, or animal-associated ARB/antibiotics, and red sites with mostly human-associated bacteria and antibiotics used by humans. Yellow indicates ARB/antibiotics from animal sources. Arrows show the dominant direction of ARB/antibiotic flow, and double arrows indicate that there is likely flow in both ways.

surprising is that animals still consume two-thirds of the produced antibiotics. Antibiotics are given to all production animals, including fish, poultry, pork, and cattle. Since animal-based product consumption is predicted to increase globally, animal husbandry will use more antibiotics, despite efforts to limit antibiotic use to animals with bacterial infections and improve hygiene and vaccination coverage.

Antibiotic-resistant bacteria and antibiotic resistance genes are ubiquitous in the animal production chain. A recent study has shown that in low and middle-income countries, antibiotic resistance of animal pathogens has increased significantly, with China and India having the highest AMR levels in animals (Van Boeckel et al. 2019). Fish, dairy, swine, and poultry farms have numerous ARGs conferring antibiotic resistance to antibiotics in human use (Tamminen et al. 2011; Zhu et al. 2013; Muurinen et al. 2017; Van Boeckel et al. 2019). There have been efforts to reserve critically important antibiotics for human use, and the EU has guidelines for what antibiotics are allowed in animal farming. However, animal use of antibiotics is not strictly regulated globally.

Treated wastewater releases ARGs to the environment, and it reflects the microbial community's resistance potential. Treated wastewater typically flows to

a receiving body of water, and it contains ARGs that were not eliminated during the wastewater treatment process (Karkman et al. 2018). Antibiotic resistance gene levels in wastewaters have been studied extensively, and descriptive reports are available from several countries. How much the gene flow from wastewater treatment plants contributes to the resistance in the clinic is unclear. Recently, ARGs in wastewater have been used to predict resistance levels in municipalities, and they correlate with resistant pathogenic strains isolated from the clinic (Karkman et al. 2018). Wastewater treatment plants (WWTPs) are also proposed to be hotspots for antibiotic resistance (Berendonk et al. 2015).

Besides urban wastewater treatment plants, one of the likely hotspots for developing new resistance determinants is aquatic environments that receive untreated or minimally treated wastewater from the antibiotic production industry (Razavi et al. 2017; Bengtsson-Palme et al. 2014). There is debate on how much antibiotics are needed to cause selection in natural settings. Sub-inhibitory concentrations increase the prevalence of antibiotic resistance in in vitro studies, but there is limited concrete evidence that ARGs enrich in environments with low concentrations of antibiotics. However, untreated wastewater from antibiotic-producing factories contains high and clinically relevant antibiotic concentrations and is thus a likely hot spot for selecting new and pre-existing ARGs (Bengtsson-Palme, Kristiansson, and Larsson 2018).

1.1.3 Evolution of mobile resistance and the rise of superbugs

Antibiotic resistance towards all antibiotics is driven by two factors: antibiotic use and the spread of ARGs and antibiotic-resistant bacteria (Collignon 2015). A schoolbook example of the emergence of antibiotic resistance is that under antibiotic selection pressure, a mutation able to confer resistance to the antibiotic occurs, making the strain with the adaptation able to dominate the niche previously occupied by bacteria susceptible to the antibiotic. However, for the mutation to persist after antibiotic selection pressure ceases, the fitness cost of the mutation should be minimal. Thus, persistence is rarely the case for spontaneous mutations. However, new mutations are more likely to prevail and spread to other bacteria if the gene integrates into a mobile genetic element (MGE) that can capture and disseminate ARGs (Hall and Collis 1995; Liebert, Hall, and Summers 1999; Gillings et al. 2008). Japanese scientists Ochiai et al. (Ochiai et al. 1959, original article in Japanese referenced in (Watanabe 1963)) discovered transferable ARGs already in the 1950s, and they have since become ubiquitous. Mobile ARGs are found even in the Antarctic (Wang et al. 2016).

The tremendous human use of multiple classes of antibiotics and other bioactive compounds has led to the rapid evolution of MGEs containing resistance genes and increased ARG dissemination. For several decades, it has not been at all uncommon to find plasmids, the epitopes of MGEs, that carry resistance genes conferring protection against all antibiotics that doctors could use to treat infections caused by the plasmid's host (Perry, Waglechner, and Wright 2016). MGEs are primarily responsible for the rise of so-called superbugs no antibiotic can eradicate (Alanis 2005). Suppose penicillin's discovery marked the dawn of the golden age of antibiotics. In that case, the rise of superbugs is a clear sign that we are seeing the very last moments of the remaining daylight. WHO has warned that due to the lack of available functioning antibiotics, we are heading back to the pre-antibiotic era where even the most trivial infections and medical procedures are life-threatening (WHO World Health Organization 2014).

The most notorious superbugs include multidrug-resistant ESKAPE pathogens (Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae,

Acinetobacter baumannii, Pseudomonas aeruginosa, and Enterobacter spp.), Escherichia coli, and Clostridioides difficile. Extended-spectrum beta-lactamase (ESBL), vancomycin, carbapenem, and methicillin resistances are the most clinically relevant phenotypes in pathogens classified as superbugs.

1.2 The complex dynamics of selection, resistance, horizontal gene transfer, phylogeny, ecology, and socioeconomics

1.2.1 Natural selection and mosaic mobile genetic elements

As mentioned before, antibiotic resistance is ancient and has been around before the invention of commercial antibiotics. However, bacteria have developed more genes to fight antibiotics after their widespread use began because of natural selection. The presence of antibiotics causes a selective pressure leading to natural selection that results in the evolution of new resistance traits. Therefore, the more types of antibiotics are used, and the more bacteria are exposed to them, the more different types of resistant strains and species can emerge. Antibiotics kill or enable the reproduction of bacteria that are susceptible to it, and only strains resistant to the antibiotic survive (Figure 3). Resistance will emerge in all types of bacteria regardless of their pathogenicity and possible hosts when selective antibiotic concentrations are present.

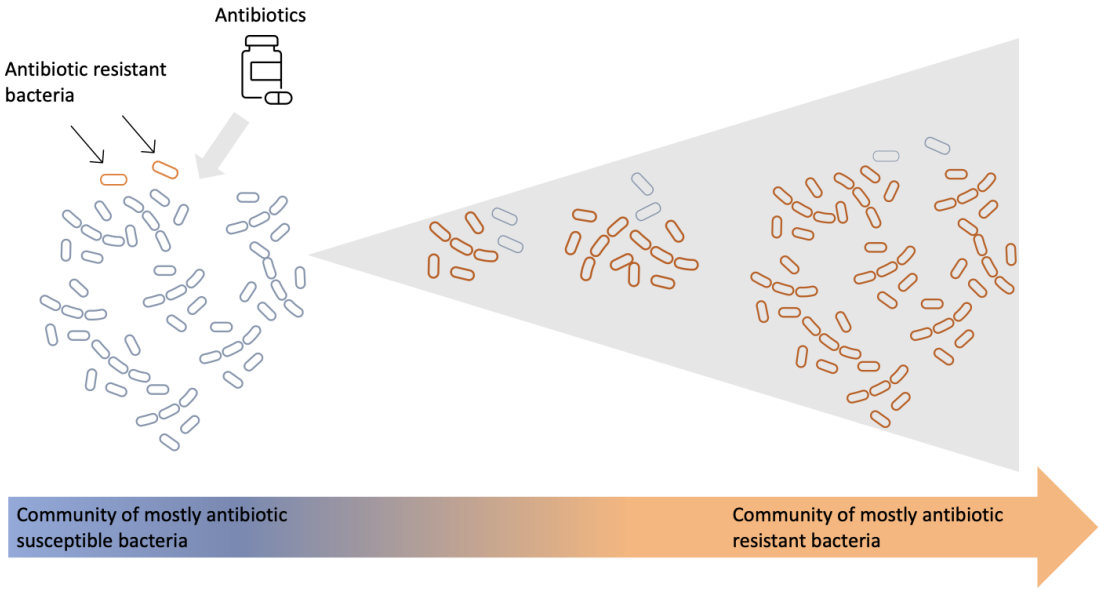


Figure 3. Natural selection of antibiotic-resistant bacteria. In the beginning, the bacterial community consists of the most susceptible bacteria and two resistant bacteria. When the community is exposed to an antibiotic, the antibiotic kills most of the sensitive bacteria, and the resistant bacteria survive. Only the resistant bacteria can proliferate as long as the antibiotic selection occurs and increase in number over time. In the end, resistant bacteria dominate the community.

Pre-existing ARGs are often well adapted to their hosts, very efficient in transferring to new bacteria, and located on MGEs carrying other ARGs that confer resistance to most imaginable antibiotic classes. The MGEs can also harbor genes that give resistance towards metals or biocides (Pal et al. 2015; Li, Li, and Zhang 2015). Due to co-selection, antibiotic resistance is affected by other products used to curb bacterial growth and by heavy metals found in polluted environments, as shown in Figure 4.

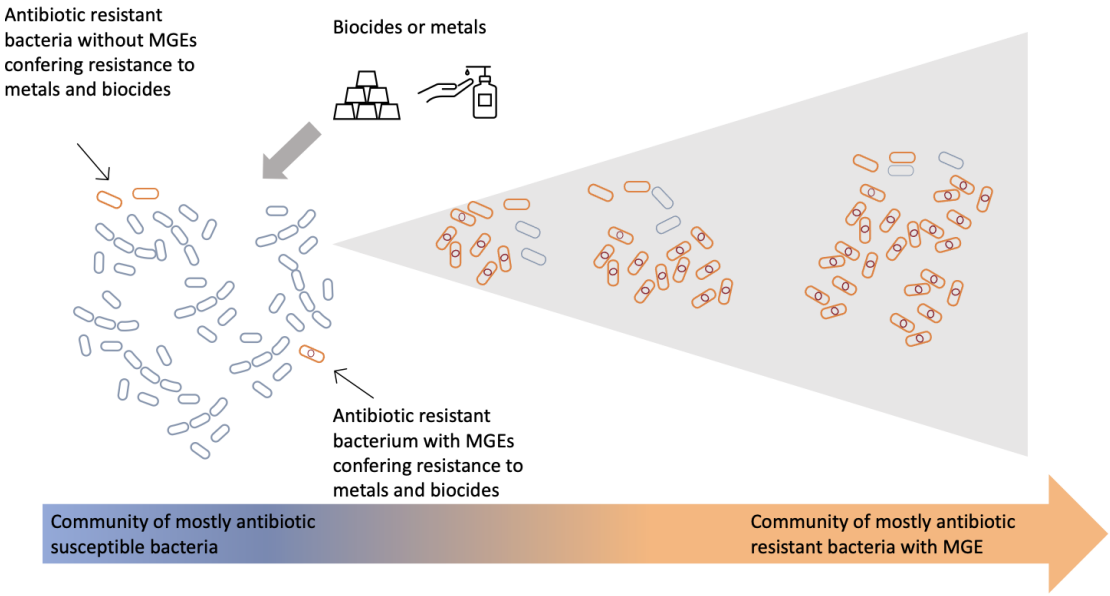


Figure 4. Co-selection of antibiotic-resistant bacteria during metal or biocide selection. In this example, in the beginning, the bacterial community consists primarily of antibiotic susceptible bacteria and two antibiotic-resistant bacteria without MGEs and one with an MGE conferring resistance to antibiotics, metals, and biocides. When the community is exposed to biocides or metals, they inhibit the proliferation of the susceptible bacteria and the antibiotic-resistant bacteria. As a result, only the MGE carrying antibiotic-resistant bacteria can proliferate. As long as there are biocides or metals, natural selection occurs, and the ARG-carrying bacteria increase in numbers over time, even in the absence of antibiotics. In the end, the community is dominated by resistant bacteria, with the MGE encoding antibiotic, biocide, and metal resistance.

All of the other selective genes on the MGE affect the ARG's persistence during varying selection pressures. However, the resistance gene containing elements are not always lost from bacterial genomes when selection pressure towards the compounds that the MGE confers resistance to seizes. For example, mobile sulphonamide and tetracycline resistance genes have spread widely. They persist even in the absence of any apparent antibiotic, metal, or biocide selection pressure, for example, in fish farms (Tamminen et al. 2011; Muziasari et al. 2014).

There are many different types of MGEs, which can transfer between bacteria and replicate independently (Frost et al. 2005). Plasmids are circular DNA molecules, which contain genes that are accessory to the bacterial genome. Typically, they have more genetic material than other MGEs. However, there is considerable variation in the number of genes on plasmids. Plasmid sizes vary from a few kilobases to several hundreds of kilobases. Transposons are MGEs, which can insert into bacterial genomes or plasmids. They are also called jumping genes since some can "jump" between genome sites or between the genome and plasmid.

Bacteriophages are bacterial viruses, but they are also MGEs, as they can insert their DNA into bacterial genomes and transport genes between bacteria. Phages sometimes capture bacterial DNA and thus, can transfer bacterial genes as well. Integrons are genetic elements that are not mobile by themselves. Integrons have a site-specific integrase, a recombination site recognized by the integrase. Integrons also contain a promoter region that directs the transcription of genes found in the integron's gene cassettes. Integrons are often linked to MGEs that can transfer them between bacteria. However, integrons are not mobile themselves, even though they are sometimes misleadingly referred to as MGEs.

Class I integrons are the best examples of ARG carrying elements that have successfully disseminated globally, and consequently, they are suggested to be used as proxies for estimating ARG pollution levels in human-impacted environments (Gillings et al. 2008). Class I integrons have been shown to capture novel ARGs, and thus, they can contribute to the emergence of new mobile ARGs in clinical settings (Hall and Collis 1995; Razavi et al. 2017; Böhm et al. 2020; Baharoglu, Bikard, and Mazel 2010). Class I integrons typically confer resistance to at least two classes of antibiotics. The class I integron encodes sulphonamide resistance via the sul1 gene located in the class I integron's conserved backbone. The most common other types of resistance genes carried by class I integrons confer resistance to aminoglycosides and beta-lactam antibiotics (Hall and Collis 1995; Gillings et al. 2008). Tn21 class transposons, which typically confer resistance to tetracyclines and mercury, are examples of highly efficiently transferring MGEs, which have the potential to capture new resistance-conferring genetic material (Liebert, Hall, and Summers 1999, 9).

What further complicates the picture of mobile antibiotic resistance is that integrons can be captured by transposons, transposons by plasmids, and plasmids can have several transposons and integrons. Thus, MGEs are highly mosaic in their structure, and their genetic material is varying (Frost et al. 2005). Also, MGEs can integrate into several different types of genetic contexts and be carried by several taxonomically distinct bacterial hosts. Considering all the properties of MGEs, it is no wonder that we have multiresistant superbugs. Since MGEs contribute to the emergence of clinical resistance and multiresistant bacteria, it has been proposed that the mobility potential of ARGs should be considered in the risk assessment of antibiotic resistance genes found in different environments (Martinez, Coque, and Baquero 2015).

1.2.2 Horizontal gene transfer barriers link bacterial phylogeny and ecology to the resistome

In the context of antibiotic resistance spread, it is essential to consider how MGEs are transferred between bacteria. Horizontal gene transfer in bacteria is similar to sexual reproduction in higher organisms as it laterally transfers new genetic material between bacteria (which otherwise reproduce asexually via clones). Horizontal gene transfer can occur via several mechanisms. Conjugation is the transfer of DNA between two bacterial cells via a pilus, and plasmid incompatibility groups affect which plasmids can be transferred. Bacteria can capture free DNA from their environment, which is referred to as transformation. Transformation is also used in laboratory settings to create genetically modified bacterial clones with desired properties and genes. Transduction is different from transformation and conjugation. It requires a bacteriophage, which infects the bacterial cell, and the phage DNA integrates into the bacterial genome. Sometimes bacteriophages carry DNA from bacteria they have infected before. Bacteriophages can thus act as vectors that transfer bacterial DNA from one bacterium to another.

Each horizontal gene transfer mechanism has some prerequisites for when the transfer can occur between bacteria and when the transferred DNA is maintained in the recipient bacterial genome (Thomas and Nielsen 2005). First of all, donor and recipient cells need physical contact for conjugation to occur. Transformation is also more likely to occur between bacteria that occupy the same environment as the free DNA from lyzed cells is more readily available to bacteria living nearby. Secondly, conjugation occurs most often between closely related bacteria, and bacteriophages are typically quite host-specific. Also, transformed DNA is more likely to be integrated into the genome and maintained by the recipient if the DNA uses similar codons as the donor cell. Thus, the pre-existing ARGs responsible for most resistant infections are rather host-specific, as most MGEs tend to transfer and be maintained only between closely related bacteria (Thomas and Nielsen 2005), although there are some exceptions. Host specificity of MGE transfer might be one of the reasons why we still have antibiotics that work. Therefore, it is not surprising that antibiotic resistance mirrors bacterial phylogeny (Forsberg et al. 2014).

The linkage of resistomes and microbial phylogeny further affects antibiotic resistance in environments with varying substrate availability, temperature, pH, and oxygen levels. Bacteria thrive in various conditions and prefer different substrates. Thus, for example, substrates can affect the proportion of resistant bacteria, as shown in Figure 5. Horizontal gene transfer is more common between bacteria, which share ecological niches, and ecology even might be a more important driver of horizontal gene transfer than phylogeny (Smillie et al. 2011). Antibiotic resistance follows the principle 'every gene is everywhere, but the environment selects' and resistomes cluster by ecology (Gibson, Forsberg, and Dantas 2015; Fondi et al. 2016). Thus, ecology and phylogeny cause barriers between entirely free exchange of genes between bacteria and profoundly affect the resistome.

Socioeconomics affects the spreading of resistant bacteria and resistance genes

1.2.3 The spread of bacteria is a critical aspect that affects antibiotic resistance prevalence. Resistant bacteria also need to spread between community members or flow between the environment and people to disseminate. Antibiotic use correlates with antibiotic resistance on both individual and country scales, and reduced use causes a decline in resistance (Collignon et al. 2018; 2015). However, there is a common misconception that antibiotic resistance is almost entirely related to the amount of antibiotics used, which is not true. A study shows that there might even be an inverse relationship between antibiotic use and antibiotic resistance, and the spread of ARBs and ARGs caused by poor hygiene and waste management in countries with low socioeconomic markers explains the bulk of the resistance (Collignon and Beggs 2019). The spread of antibiotic-resistant bacteria and genes also depends on infection control, food quality, and travel (Collignon 2015; Olesen, Lipsitch, and Grad 2020).

Global-scale studies have shown that although antibiotic use and misuse are primarily responsible for maintenance and the emergence of new resistance genes, other factors that affect the spreading of genes and bacteria contribute to the increased prevalence of antibiotic resistance (Collignon 2015; Collignon et al. 2018). Recently, antibiotic resistance has been studied from a socioeconomic perspective. These studies demonstrate that governance strongly correlates with antibiotic resistance when comparing countries' resistance levels (Collignon 2015; Collignon et al. 2018). In Europe, the corruption index correlates more strongly with antibiotic resistance than documented antibiotic use (Collignon et al. 2015). Globally, the recorded antibiotic usage does not significantly explain resistance, whereas better governance and infrastructure are linked to lower resistance levels

(Collignon et al. 2018). However, socioeconomic factors do not directly affect antibiotic resistance prevalence. Still, it is a good, relatively easily estimated indicator for multiple combined factors that increase the spread of resistance in the community. Targeted approaches to improve hygiene in homes and everyday life have been suggested to mitigate the spread and emergence of resistant pathogens (Maillard et al. 2020).

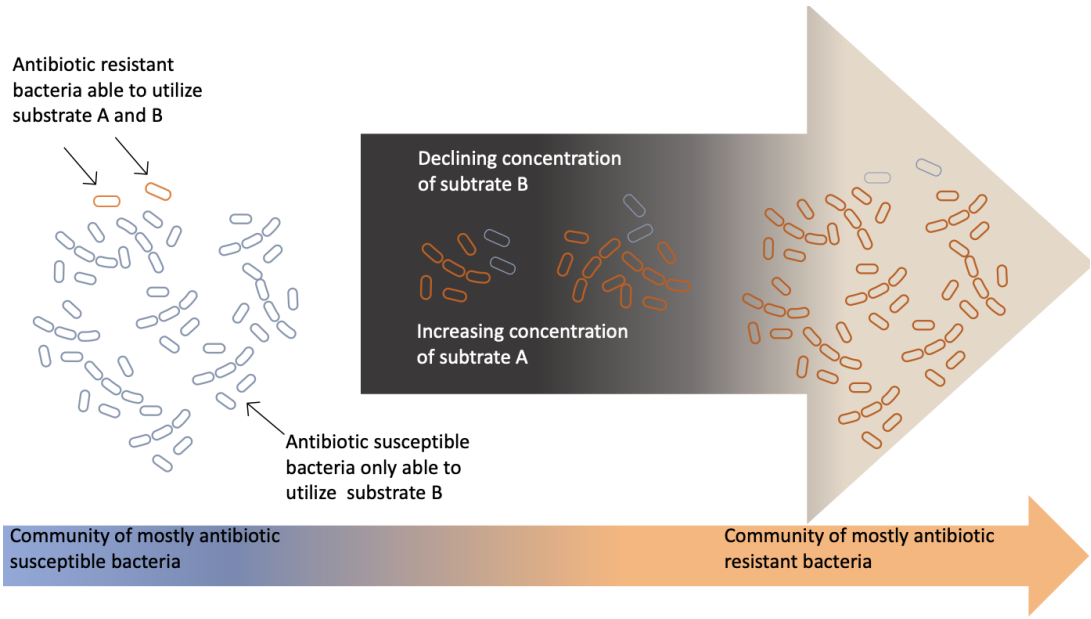


Figure 5. Substrate availability affecting antibiotic-resistant bacteria prevalence. In this example, the community initially consists of bacteria that are susceptible to an antibiotic and can utilize only substrate B and antibiotic-resistant bacteria, which can utilize substrates A and B. In the beginning, both substrates are available. In this example, the concentration of substrate B declines until bacteria have used it up completely, while substrate A is not limiting. In the absence of substrate B, only the antibiotic-resistant bacteria, which can also utilize substrate A, can proliferate. The substrate shift results in a community dominated by substrate A utilizing bacteria, which also happen to be antibiotic-resistant despite that there is no antibiotic present.

1.3 Infant gut microbiota and resistome

1.3.1 Primary succession in the infant gut ecosystem

Diet, gestational age, antibiotic use, and delivery mode have the most substantial role in defining the infant microbiota (Palmer et al. 2007; Bäckhed et al. 2015; Yassour et al. 2016; Korpela et al. 2018; Stewart et al. 2018; Vatanen et al. 2018; Shao et al. 2019). There is debate regarding intra-uterine colonization of infants. However, we can safely say that major bacterial colonization occurs in infants during and after childbirth (Rautava et al. 2012; Collado et al. 2016; Gensollen et al. 2016). The infant gut is (wholly or nearly) void of bacteria before birth, and bacteria colonize it shortly after (Jimenez et al. 2008). The first colonizers are typically facultative aerobes as the neonatal gut conditions are aerobic or microaerophilic. The initial colonizers include enterobacteria, streptococci, and staphylococci. They are replaced in breastfed infants by bifidobacteria or bacte-

roidia, both of which are anaerobic (Bäckhed et al. 2015; Korpela and de Vos 2018; Stewart et al. 2018).

During a vaginal delivery, the infant is exposed to bacteria in the mother's birth canal and fecal and skin bacteria. Vaginally delivered infants typically share many bacterial strains with their mothers, with the mother's gut microbiota contributing most to the infant's microbiota (Bäckhed et al. 2015; Asnicar et al. 2017; Korpela and de Vos 2018). If the infant is delivered by cesarean section, bacteria from non-maternal sources are more common first colonizers in the gut. During the first weeks of life, neonatal microbiotas are distinct in vaginally delivered subjects and infants born by cesarean section (Asnicar et al. 2017; Korpela and de Vos 2018; Shao et al. 2019). Most notably, the colonization of the gut by bacteroidia and bifidobacteria is hindered in cesarean section infants.

Infants and parents share bacteria since they are in intimate contact with each other, also after childbirth. Thus, the microbiota of parents impacts the microbiota of the infant's gut. Bifidobacteria have many properties that benefit infant health (Servin 2004). Many infants are now colonized by bacteroidia instead of bifidobacteria, which has been observed in cohorts from high-income countries (Collado et al. 2008; Korpela and de Vos 2018; Kostic et al. 2015; Stewart et al. 2018). This may partly be due to the possible lifestyle-, antibiotic- and diet-induced reduction of bifidobacteria in adults. The effect of lifetime maternal antibiotic use on the infant microbiota has not been studied to my knowledge. However, antibiotics given during delivery affect the infant gut microbiota (Azad et al. 2016; Mazzola et al. 2016; Nogacka et al. 2017).

Later in infancy, environmental factors increasingly influence the microbiota as they are exposed to more microbes. At the latest, solid foods and drinks besides human milk, such as water, are given to the infant around the half-year mark after birth which further modifies the microbial community of the gut. The microbiota is affected by the living environment, and environment-induced differences in microbial community composition affect children's health (Karkman, Lehtimäki, and Ruokolainen 2017). Children living in rural versus urban areas have differing skin microbiota, contributing to the differences in allergy prevalence in these groups (Lehtimäki et al. 2017).

1.3.2 Human milk and formula shape the microbiota

After delivery, the infant microbiota is exposed to new microbes, substrates from formula, fortifier, or human milk, and various bioactive compounds found in human milk if the child is breastfed. Newborns and infants are fed human milk or formula (or fortifier) exclusively for the first months of life. The infant's diet substantially shapes the microbial community's development.

Formula and human milk are very different in their oligosaccharide composition (Bode 2012), influencing the substrates available to microbes in the infant gut. Human milk oligosaccharides (HMOs) are unique to human milk. They are composed of the five monosaccharides glucose, galactose, N-acetylglucosamine, fucose, and sialic acid. Over a hundred different HMOs have been characterized, with each mother having a slightly different HMO profile (Bode 2012). Interestingly, HMOs also can function as receptor analogs for pathogens, inhibiting pathogens such as rotavirus from binding to, for example, the gut epithelium, and thus, they serve a double role in benefiting infant health (Bode 2012; Yu et al. 2014).

The infant microbiome is enriched in genes needed for HMO utilization (Bäckhed et al. 2015). During formula feeding, the gut microbiota changes – bifidobacteria and lactobacilli specialized in HMO degradation decrease, and clostridia, enterobacteria, and enterococci become more common (Mountzouris,

McCartney, and Gibson 2002; Penders et al. 2006; Bäckhed et al. 2015; Azad et al. 2016; Forbes et al. 2018).

In addition to the unique sugars, human milk contains antibodies not found in formula. The antibodies in human milk are essential for neonates as they lack antibodies of their own and are essentially immunocompromised without them (Mountzouris, McCartney, and Gibson 2002). Besides antibodies, breast milk has antimicrobial peptides that inhibit the growth of certain types of bacteria (Lönnerdal 2003). Altogether breast milk is a cocktail tailored for the infant's needs, which is, at this time, impossible to mimic in an artificial formula.

Human milk is not sterile and contains microbes, which can further guide microbiota development in infants. It is not completely clear where the human milk microbes originate, but the same species are found in the infant gut, skin, mouth, human milk, and on the mother's skin, and mothers pass down their microbes to their infants (Jost et al. 2014; Benito et al. 2015; Asnicar et al. 2017).

Breastfeeding protected infants from infections and was essential for infants' survival before the antibiotic era. Still, in the new millennium, 1.4 million child deaths are attributable to suboptimal breastfeeding, especially non-exclusive breastfeeding during the first six months of life (Black et al. 2008). In addition to child mortality, poor breastfeeding practices also contribute to the morbidity of children — approximately 10% of the disease burden in children under five years is due to poor breastfeeding practices — especially non-exclusive breastfeeding during the first six months of life (Black et al. 2008).

The recommended duration of exclusive breastfeeding is six months, and the WHO recommends continuing breastfeeding until two years of age. Despite the recommendations, in Africa, Asia, Latin America, and the Caribbean, only 47–57% of infants younger than two months are exclusively breastfed (Black et al. 2008). Only 25–31% are solely breastfed at the ages of 2 5 months (Black et al. 2008). Of the children aged 6–11 months, 6% in Africa and 10% in Asia are not breastfed. Respectively, 32% in Latin America and the Caribbean have stopped breastfeeding. Only 9% of Finnish neonates (age<1 month) and 50% of Finnish infants were exclusively breastfed at the time of filling the questionnaire used for the report by Ikonen et al. (Ikonen et al. 2019). In Finland, socioeconomic factors and demographics strongly affect breastfeeding (Ikonen et al. 2019). Low-income, young, and smoking mothers are less likely to breastfeed exclusively.

The World Health Organization promotes breastfeeding. However, it is difficult to change people's perceptions, which are often influenced by formula advertising (Hastings et al. 2020). It is illegal to advertise formula in the EU. However, there is no such regulation in the United States. A recent paper stated that formula marketing is widespread and uses powerful emotional techniques to sell formula to parents (Hastings et al. 2020). Formula consumption is increasing by 8% yearly, which shows that marketing is highly efficient in gaining new customers (Rollins et al. 2016).

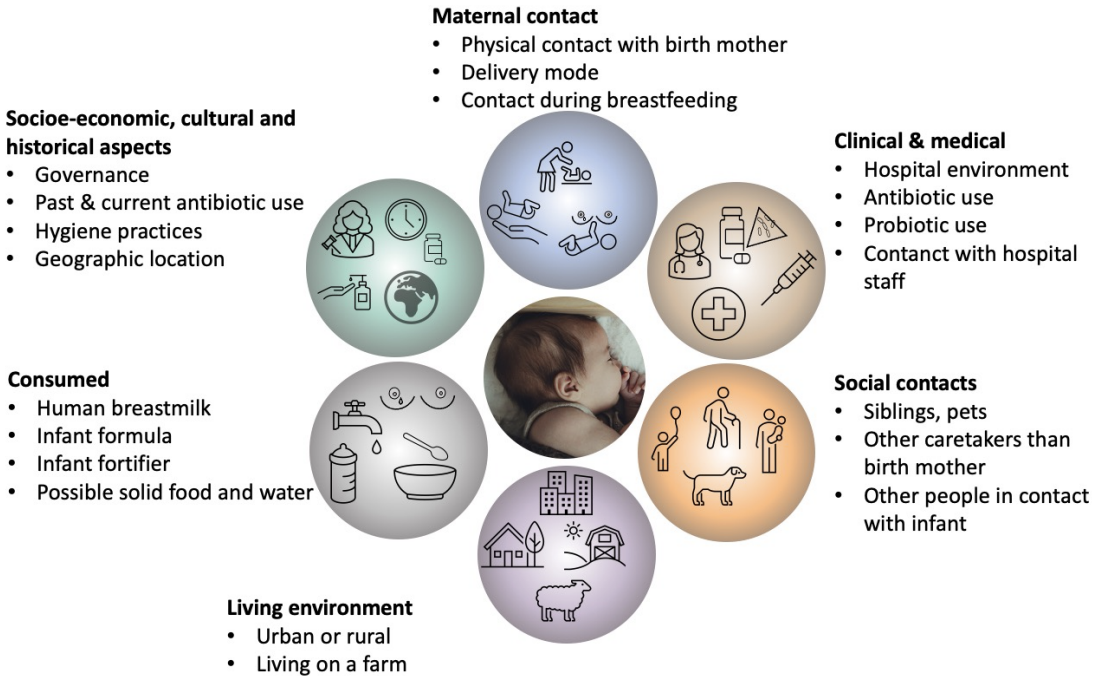


Figure 6. Factors shaping infant gut microbiota and resistome. The first major colonization occurs during birth. Delivery mode (vaginal or cesarean) affects how soon after birth and how much the infant is exposed to maternal bacteria. Clinical factors impact the infant microbiota and resistome. Infants share ARGs with neonatal unit surfaces and are exposed to hospital staff microbiota. Probiotics could be considered a medical contributor as they are used to promote infant health. Their use can affect the infant gut resistome. Infants are exposed to bacteria through physical contact with their mother, during breastfeeding, and contact with other caretakers and people. Siblings and pets can affect the microbes the infant is exposed to. The living environments differ in their microbial communities and therefore impact which bacteria the infant encounters. For example, the microbiota in farms is disparate from the microbiota in urban areas in big cities. Also, farm animal exposure can shape infant microbiota. Infants consume human milk or infant formula, or fortifier, which contain bacteria. Human milk and milk substitutes have different substrates and biological properties causing selection pressures in the infant gut, that affect the microbiome and resistome. Also, antibiotic use during hospital stays and outpatient care causes selective pressure in the infant gut.

1.3.3 The infant gut resistome

The infant's gut has environmental niches that antibiotic-resistant bacterial species can occupy and colonize. The immature gut has low colonization resistance due to the lack of developed host immunity and low microbial community diversity. The infant gut has appropriate concentrations of oxygen and substrates for many opportunistic pathogens. Among others, infant diet, antibiotic treatment history, and delivery mode can affect the environmental conditions and colonization patterns and, therefore, also the resistome.

I have collected resistome studies applying metagenomics and studies of ARG transfer in infants in Table 1. The studies show that antibiotic and probiotic use and breast and formula-feeding significantly affect specific ARGs (specific in the sense that either preselected ARGs were studied or only results for individual genes were reported) with probiotic use decreasing the abundance of ARGs and formula consumption increasing the abundance of ARGs (Gibson et al. 2016; Esaiassen et al. 2018; Hourigan et al. 2018; Casaburi et al. 2019; Gasparrini et al. 2019; Rahman et al. 2018). Delivery mode has been shown to increase the overall

resistance load in C-section-born infants (Bäckhed et al. 2015) and probiotics to decrease the load (Casaburi et al. 2019). Maternal transfer of ARGs has been studied previously in (Zhang et al. 2011; Gosalbes et al. 2016; Moore et al. 2015), but the results were not statistically significant, or no statistical analysis was performed. Hourigan et al. (2018) showed that infants share ARGs with hospital surfaces. There also were significant differences between the two hospital units, which suggests that ARGs are transferred from the hospital surfaces to infants.

ARGs can potentially be transmitted to the infant's gut from the mother, hospital environment, living environment, other people than biological mother, house pets, farm animals in the living environment, and consumed breastmilk, formula, and solids (Figure 6). Maternal antibiotic treatment might affect the infant's resistance load through antibiotic selection caused by antibiotics in breastmilk or by affecting the maternal microbiota and, therefore, transfer.

Human milk is the only source of nutrition for breastfed infants in early infancy. Thus, human milk is one potential source of ARGs and MGEs. Interestingly, probiotic supplementation with bifidobacteria has been shown the decrease ARG abundances when the probiotic strain colonizes the gut efficiently, and high Bifidobacterium counts are associated with lower levels of ARGs (Esaiassen et al. 2018; Casaburi et al. 2019). Parents interact closely with infants during breastfeeding and the transfer of microbiota occurs due to close physical contact and suckling.

Non-maternal sources of ARGS and MGEs are much more challenging to decipher. However, it is known that infants delivered in hospitals share bacteria found on surfaces in the neonatal ward (Adlerberth et al. 1991; Murono et al. 1993; Raveh-Sadka et al. 2016; Brooks et al. 2017) and the resistant bacteria in the hospital environment can be transferred to infants.

Socioeconomic factors could predict aspects of the resistomes in infants by affecting the exposure and composition of bacteria in the infant's milieu. In adults, resistomes and microbiomes vary according to nationality (Yatsunenko et al. 2012; Forslund et al. 2013). However, how socioeconomic factors affect infants' resistomes across demographies has not been studied to my knowledge. However, likely the governance, antibiotic use history, and general hygiene and waste management in the area where the infants live affect exposure to resistant bacteria.

Table 1. 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.

Study	Methodology	Factors affecting specific ARG abundances	Factors affecting total ARG load	ARG transfer
Gibson et al. 2016	Shotgun metagenomics, functional metagenomics	Antibiotic treatment	—	—
Gasparrini et al. 2019	Shotgun metagenomics, functional metagenomics, culture	Antibiotic treatment	—	—
Hourigan et al. 2018	Shotgun metagenomics	Breastfeeding	—	Hospital unit, significant differences between ARG profiles between units
Moore et al. 2015	Shotgun metagenomics, functional metagenomics	—	—	Maternal transfer studied, not significant
Gosalbes et al. 2016	PCR	—	—	Maternal transfer studied, not significant
Moore et al. 2013	Shotgun metagenomics, functional metagenomics	—	—	—
Rahman et al. 2018	Shotgun metagenomics	Formula feeding		—
Bäckhed et al. 2015	Shotgun metagenomics	—	Cesarean section in infants aged < 7 days	—
Zhang et al. 2011	Cultivation and PCR, qPCR	—	—	Same ARGs in mothers and infants, but no statistical testing
Casaburi et al. 2019	Metagenomics, cultivation, and whole-genome sequencing	Probiotic treatment	Probiotic treatment	—
Esaiassen et al. 2018	Metagenomics	Probiotic treatment	—	—

1.3.4 Consequences of the antibiotic resistance crisis for infants and new generations

Antibiotic resistance has complicated the treatment of many infectious diseases. Infants cannot avoid the effects. On the contrary, they are especially vulnerable to fatal infections caused by antibiotic-resistant bacteria (Laxminarayan et al. 2016). Over 200 000 infants die annually due to antibiotic-resistant septic infections (Laxminarayan et al. 2016).

Infants, including newborns never exposed to antibiotics, carry much higher proportions of antibiotic-resistant bacteria in their gut than adults and their mothers (Moore et al. 2015; Bäckhed et al. 2015; Gibson et al. 2016). High resistance gene rates in infants might seem counter-intuitive. Still, since most ARGs are carried by MGEs adapted to be maintained in bacterial genomes without an insuperable fitness cost, resistant bacteria will thrive in environments with suitable niches, including the infant gut, even without antibiotic selection pressure (Zhang et al. 2011; Gibson et al. 2016; Yassour et al. 2016; Gasparrini et al. 2019).

An in vitro study has documented that multi-resistance-conferring ESBL plasmids isolated from neonates gave a competitive advantage over recipients without the plasmids (Hagbø et al. 2020). The plasmid was also abundant in chicken metagenomes suggesting that poultry farms may act ask a reservoir. The ESBL plasmid plausibly originating from chicken farms identified babies is an example of how production animal and human resistomes are interconnected. As meat consumption is on the rise, this is not good news.

With the ever-increasing antibiotic use, lack of sanitation, poorly functioning wastewater treatment plants, and missing financial and regulatory incentives to develop new antibiotics or improve hygiene and waste management, it is likely that resistance rates will continue to rise globally. However, identifying potential intervention opportunities might help mitigate some of the issues related to antimicrobial resistance so that the coming generations can still enjoy the privilege of functioning antibiotics that have been previously taken for granted.

2 STUDY AIMS

In this thesis, I aimed to cover, at least to some extent, all One Health aspects of antibiotic resistance, from environmental ARG pollution to animal farming and humans. I wanted to discover how European countries differ in their wastewater treatment of ARGs and the ARGs found in communal wastewaters. I always aimed to use the best methodology available and to develop new methods and approaches when needed. In the studies included in the thesis, I sought to shed light on MGEs found in the examined environments, as their role in resistance is more obscure and less studied than that of ARGs. To this end, I created a database for MGEs, helping to describe the infant gut and human milk mobilome, which had not been extensively investigated before, and provided the first broad-scale database of MGEs for the scientific community. One of my focuses was aspects affecting ARG loads beyond antibiotic selection pressure, both in the environment and the infant gut.

In **I**, I aimed to decipher how ARG mobility could be investigated without metagenomics using inverse PCR and PacBio long-read sequencing. I desired to develop tools for evaluating the dissemination risk associated with ARGs found in environmental resistomes using fish farms as an example.

In **III**, the aim was to explore the antibiotic resistance potential in European WWTP influents and effluents using a high throughput qPCR array. Our aim was to shed light on how the European countries differ from each other and how different treatment plants release varying ARG loads to the environment, plausibly partly contributing to the differences in the observed clinical resistance patterns.

In **IV**, the aim was to reveal whether environmental ARGs could be linked to human fecal pollution. The objective was to investigate if fecal contamination instead of antibiotic selection pressure or HGT is behind the observed ARGs. An additional aim was to see if the method could be used to detect hotspot environments where antibiotic selection or HGT are more likely to explain resistance than mere fecal contamination.

In **II** and **V**, I wanted to explore what impacts the antibiotic resistome of infants. In addition, I aimed to decipher the resistance potential and transmission patterns in infant and maternal intestinal resistomes and human milk (II). Also, I sought to expose the effect of maternal antibiotic treatment on the infant resistome during childbirth. In V, the aim was to exhaustively analyze the impact of diet in early infancy on infants' antibiotic resistance load. I also wanted to study how other clinical parameters affect the ARG load and how generalizable the results are in a meta-analysis in **V**.

3 SUMMARY OF MATERIALS AND METHODS

The articles in the thesis utilized various methodologies applying several molecular biology and laboratory-based analyses and bioinformatic and statistics approaches outlined in Table 2. The articles in the thesis cover environments listed in Table 3, mirroring the One Health approach for studying antibiotic resistance in animals, humans, and the environment.

3.1 Shotgun metagenomics and identification of resistance genes from short reads and assembled data

In articles **I**, **II**, **IV**, and **V**, shotgun metagenomic sequencing was used. Shotgun metagenomics is the amplification of random DNA fragments from samples. This enables studying the entire community present in the sample and the functional genes encoded by the community members. However, it is impossible to detect whether the genes are expressed with metagenomics, unlike with transcriptomics. There are definite limitations to shotgun metagenomics for ARG studies, in addition to the lack of information for gene expression. It is also impossible to know if the ARGs in the sample confer resistance or if the sequence is similar to a database entry but not functional. This can be overcome by using functional metagenomics.

Shotgun metagenomics produces short reads which fragment the MGEs that carry ARGs into approximately 100 bp to 250 bp segments. As ARGs are typically found in varying contexts, and the MGEs carrying them consist of a mosaic of genes originating from various taxa, the assembly of the genetic environment of ARGs is typically not possible for the current metagenome assembly algorithms. In **I** and **II**, the shotgun metagenomes were assembled, and contigs containing ARGs were identified. The contigs were often short, which could be seen in **I**, where the contigs were truncated at sites where the IPCR products sequenced with PacBio showed diverging contexts.

Shotgun genomics is limited in its sensitivity and does not compare to most PCR-based methods where, at least in theory, it is possible to detect a single copy of a gene in a sample. The typical detection limit for metagenomes is between 10-7 and 10-6 ARG copies per total reads due to the library sizes being in the range of 1M to 10M reads. However, likely sensitivity will increase in the next few years by at least ten-fold as the development of sequencing platforms has been rapid in the past. The price per megabase has decreased from $5 292 to $0. 008, with an almost 700 000-fold decrease from 2001 to 2019. The cost for sequencing the human genome has also dropped from $95 263 072 to $689 and the long-awaited $1000 genome was achieved in February 2019 (National Human Genome Research Institute, https://www.genome.gov/about-genomics/fact-sheets/DNA-Sequencing-Costs-Data, accessed 10.6.2021). Computational capacity and data storage will likely become limiting since the improvements in sequencing technologies have been faster than Moore's law expects the computational efficiency to improve. This means that there is much work for development for less computationally heavy bioinformatic tools, which have traditionally been written by biologists and microbiologists or other experts without extensive coding and high-performance computing training. Thus, many have a lot of overhead in terms of computational resources needed to run them.

In articles **II**, **IV**, and **V**, ARGs and MGEs were identified from short metagenomic reads. In **II** and **V**, the short reads were mapped against ARG and MGE databases. In **II** and **V**, the aim was to identify clinically relevant ARGs from infant gut or human milk samples. Mapping short reads against a nucleotide

database is a computationally efficient way to identify genes of high similarity to the entries in the database. The mapped reads are then typically normalized against library size or the count of reads mapping to 16S rRNA genes. In **II**, and V the reads were normalized against the 16S rRNA genes as the sample types in these studies can have varying amounts of non-bacterial DNA. The aim was to compare the prevalence of ARGs and MGEs in the bacterial community, and therefore the normalization to the total library size would not be meaningful.

In **IV**, the ARGs and MGES were identified from short metagenomic reads as well. However, in **IV**, the reads were first translated to amino acid sequences. The ORFs were identified, and the longest ORF in each read was aligned with DIAMOND (Buchfink, Xie, and Huson 2015) against ARG and MGE databases also translated to amino acid sequences.

The choice of nucleotide versus amino acid sequences to identify ARGs and MGEs depends on the research question and aim. In **II** and **V**, I aimed to study only the ARGs that are clinically relevant in human samples. The hypothesis was that the more closely they are similar to the database entry, the more likely they also confer resistance. In **IV**, the samples were taken from environmental locations and animals. It is expected that the ARG databases less representative of ARGs found in resistomes of non-human environments and that there is more variation in the nucleotide sequences to the database entries than in human samples. This is because the bulk of ARGs in the databases are identified from human origin. Also, using amino acid sequences better ensures that the genes are functional as mapping nucleotide sequences do not identify stop and start codons, unlike amino acid translations which use ORFs.

In metagenome studies of ARGs, it is essential to choose the database and the subset of the ARG database correctly, according to the aim of the study. It is often seen that old versions of ARG databases, such as ARDB (Liu and Pop 2009) that have not been updated in more than ten years, are still used. In addition, it is common that the database version chosen for the study also includes entries that have spot mutations that induce resistance. The spot mutations are meaningless in short read shotgun metagenome studies as it is not possible to have enough coverage to identify spot mutations.

In **II**, **IV**, and **V**, Resfinder database (Zankari et al. 2012) that only includes acquired and clinically relevant ARGs was used. Antibiotic resistance can also be caused by efflux pumps which intrinsically occur in many bacteria and are not mobile. However, it is dependent on the research question whether Resfinder (Zankari et al. 2012) or a database such as CARD (McArthur et al. 2013) that also includes intrinsic ARGs is used. Also, the parameters used in BLAST and DIAMOND alignment or read mapping need to be strict enough to distinguish between functional ARGs and the housekeeping genes from which the ARG has mutated.

Shotgun metagenomics is limited in its sensitivity since the sequencing depth determines how rare genes can be detected. Typically, the least abundant genes are in the range of 10-5 or 10-6 ARG copies/16S rRNA gene copies. This is in a similar range as SmartChip array qPCR but a lot less sensitive than qPCR.

3.2 Long-range inverse PCR adaptations for studying genetic contexts of resistance genes

In article **I**, I improved the existing inverse PCR (IPCR) protocol, a PCR-based method used to amplify circularized DNA surrounding a gene of interest using inverted gene-specific primers. The technique was developed to study the genetic context of ARGs using an alternative method to shotgun sequencing and assembly.

The studied sediment samples were collected from a fish farm located in Turku, Finland. The genes for IPCR were tetM and sul1, as based on qPCR studies conducted previously, they were known to be present at the farm, and their association to MGEs and other ARGs was of interest. The DNA was also sequenced using MiSeq, and the metagenome reads were assembled to compare results with the IPCR protocol.

In I, I combined the existing IPCR protocol with long-range PCR utilizing a long-range polymerase to extend the sequence length captured by the present protocol. Additionally, I further improved the method by including a nested PCR step, which increased sensitivity. PCR products were then sequenced using the Pacific biosciences PacBio sequencing platform, with which it was possible to sequence the long fragments in their entirety. The protocol is outlined in Figure 7.

Next, the genes encoded by the ORFs surrounding the ARG of interest were annotated and identified. The genetic contexts of the ARGs of interest were validated by using both shotgun and IPCR data. The developed IPCR method was compared to shotgun metagenomics in terms of sensitivity. This was done by conducting an experiment in which the target gene was diluted in increasing concentrations of non-target DNA.

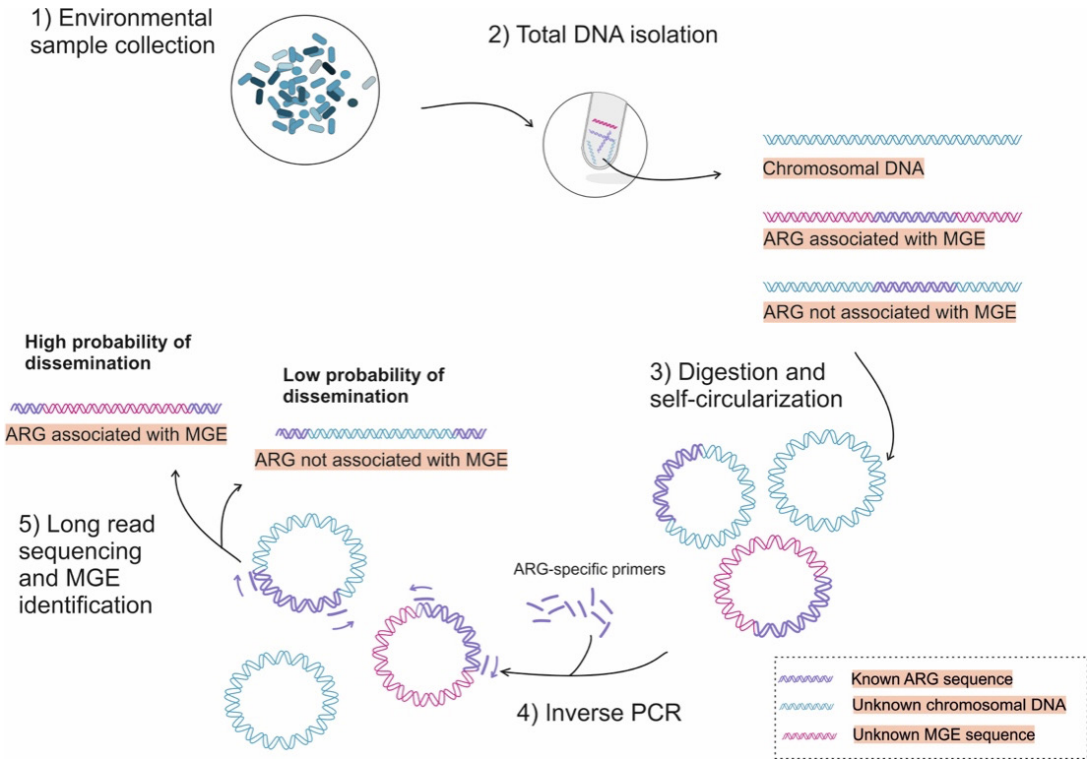


Figure 7. Inverse PCR protocol for studying the genetic environment of ARGs. ARGs can be linked to MGEs, which increases the probability of spreading the ARG to other bacterial strains. Therefore, the risk of dissemination is mainly dependent on the MGE association. Figure adapted from I.

3 SUMMARY OF MATERIALS AND METHODS

Table 2. Summary table of methods used for analysis in articles of the thesis. The methods are divided into molecular biology laboratory-based, bioinformatics, and statistics.

PARAMETER	METHOD	ARTICLE
Laboratory analyses	Shotgun metagenomic sequencing*	I, II, V
	DNA extraction	I, II, V
	DNA quantification	I, II, V
	Long-range IPCR method optimization	I
	Primer design	I
	Bacterial cell culturing	I
	PacBio sequencing*	I
	High throughput qPCR assay	III
Bioinformatics	MGE database creation	II
	kmer profiling of sequence data	II
	Gene annotation from short reads	II, IV, V
	Metagenome assembly	I, II
	ORF prediction	I, II
	BLAST	I, II
	Phylogenetic profiling of metagenomes	II, V
	16S rRNA gene read quantification from shotgun sequencing data	II, V
	Comparison of normalization methods for metagenomic data	V
	Metagenomic data retrieval from public repositories and data curation	IV–V
Statistical analysis	Diversity analysis	II, III, V
	Principal coordinate analysis	II, III, V
	Differential abundance analysis	II, III, V
	Statistical modeling	II, V
	Negative binomial distibution	II, V
	Gamma distribution	II
	Meta-analysis	V
	Statistical model cross-validation	V

3.3 High-throughput array qPCR

In article **III**, a high-throughput SmartChip qPCR array (Takara) was used to identify 384 genes that encode either ARGs or MGE associated genes or were used for normalization of the results. Array qPCR improves manual qPCR as the throughput is larger and the cost of reagents lower. However, unlike in manual qPCR, where standard curves for the studied gene are run along with the samples, and absolute copy numbers are obtained, the results are relative. The quantitation in qPCR is based on fluorescence dyes that bind to double-stranded DNA. Fluorescence is measured after each round of PCR, and the cycle after which the fluorescence raises above the background is relative to the copy number of the target gene in the reaction. The more PCR cycles it takes for the fluorescence to rise above the background, the fewer target copies are in the sample. The benefit of using array qPCR over metagenomics is lower cost and with manual qPCR, the sensitivity is better than with current shotgun metagenomic library depths.

Table 3. Summary of materials and samples used in the articles of the thesis.

MATERIAL	SOURCE	ARTICLE
Human microbial DNA	Mother's feces	II, V*
	Infant feces	II, V*
	Human milk	II
Environmental DNA	Sediment	I, IV*
	Wastewater	III

*In articles I, II, V, the DNA sequencing was outsourced to the Institute of Biotechnology, University of Helsinki. In IV and V meta-analysis cohorts, DNA was sequenced by investigators of the original studies.

4 RESULTS AND DISCUSSION

4.1 New mobile resistance determinants originate from the environment, and clinically relevant ARGs are found everywhere with anthropogenic pollution

In **I**, I developed a long-range IPCR based method for studying the genetic environment of ARGs. Using the IPCR, I examined the genetic background of tetM and sul1 genes in a Finnish fish farm. I found a new ARG possibly encoding beta-lactam resistance. The ARG was not in sequence databases suggesting that it was novel. The ARG was similar to an environmental bacterium's beta-lactamase gene exemplifying that new resistance determinants can emerge in animal farming and the environments receiving farm waste. The developed long-range IPCR enables the investigation of ARGs' genetic background and transfer potential, which can help in the risk assessment of ARGs found in resistomes in all environments. The importance of ARG's mobility in risk assessment has been highlighted by (Martinez, Coque, and Baquero 2015).

Throughout my thesis, I focused on MGEs in addition to ARGs. However, no large-scale MGE databases with several types of MGEs were available. Thus, for studying the mobilomes of infants and mothers in **II**, I gathered an MGE database, which is currently used in several publications and aids in identifying MGEs. In **I**, **II**, **III**, and **V**, I showed that MGEs are abundant in fish farms, wastewater, human gut, and human milk, and they co-occur with ARGs.

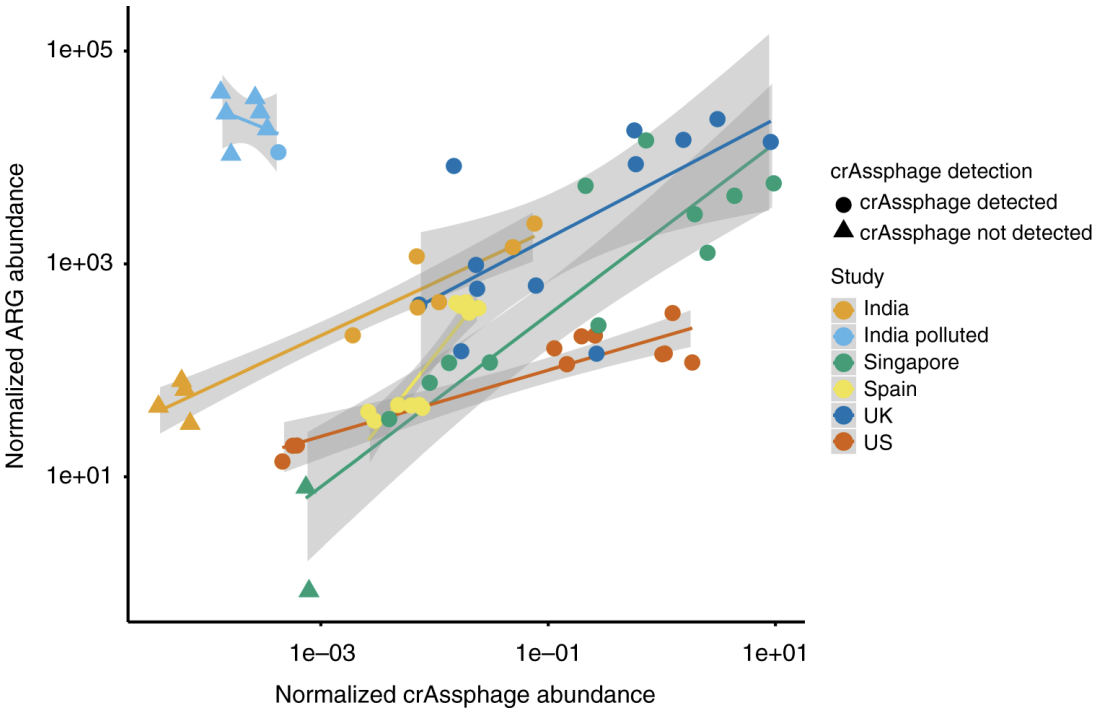


Figure 8. Correlation between ARG abundance and crAssphage abundance in environments with pollution from WWTPs, hospitals, or drug manufacturing. The environments affected by drug manufacturing are polluted with exceptionally high levels of antibiotics, and the analyses show a clear selection for antibiotic resistance as the ARG abundance cannot be explained by fecal pollution. ARG abundance and crAssphage abundance were normalized with total nucleotide count in the metagenomes. For samples where we did not detect crAssphage (indicated by triangles), half of the detection limit (corresponding to one read mapping to crAssphage) was used and normalized to the total nucleotide count. Figure adapted from **IV**.

Article **IV** elucidated the dynamics of ARGs in anthropogenic environments. I observed that fecal pollution correlated with ARG abundances in most studied environments, suggesting that selection for ARGs and horizontal transfer of ARGs did not occur on a large scale (Figure 8). However, in sediments polluted heavily with antibiotics from a drug manufacturing facility, the ARG concentrations did not follow fecal pollution levels, suggesting that active selection or horizontal gene transfer influenced ARG abundances. The drug manufacturing was, thus, possibly a hotspot for selection and ARG transfer.

4.2 Antibiotic resistance in wastewater mirrors clinical resistance patterns in Europe

Finally, in article **III**, I saw that untreated wastewater resistance genes mirrored resistance observed in clinical settings (Figure 9). There were differences with European countries with high (Spain, Portugal, Ireland) and low (Finland, Norway, Germany) antibiotic use, interestingly mainly in the treated wastewaters, possibly reflecting how governance and management in the countries affect waste treatment (Figure 10). Poorly treated wastewater could be a source of clinical infections, and likely it also reflects resistance in the general population.

Collignon et al. have shown that in Europe, the best indicator for antibiotic resistance in clinical isolates is governance metrics, and antibiotic use is not correlated with antibiotic resistance (Collignon et al. 2015). Thus, higher resistance levels in the clinic are likely due to the spread of existing resistant bacteria. Therefore, the less efficient waste management observed in **III** in the countries with high clinical resistance rates (Spain, Portugal, and Ireland), compared to those with efficient WWTPs (Finland, Norway, Germany), might partly contribute to the spreading of these bacteria.

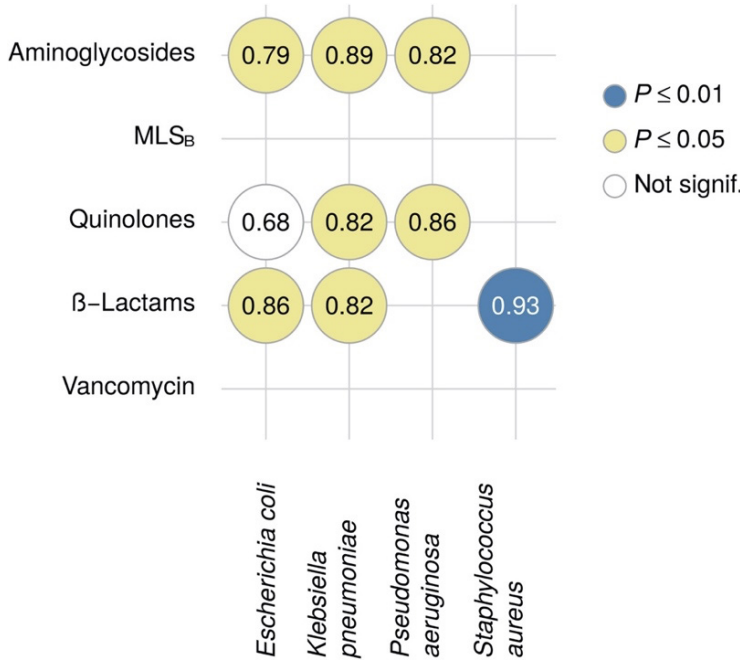
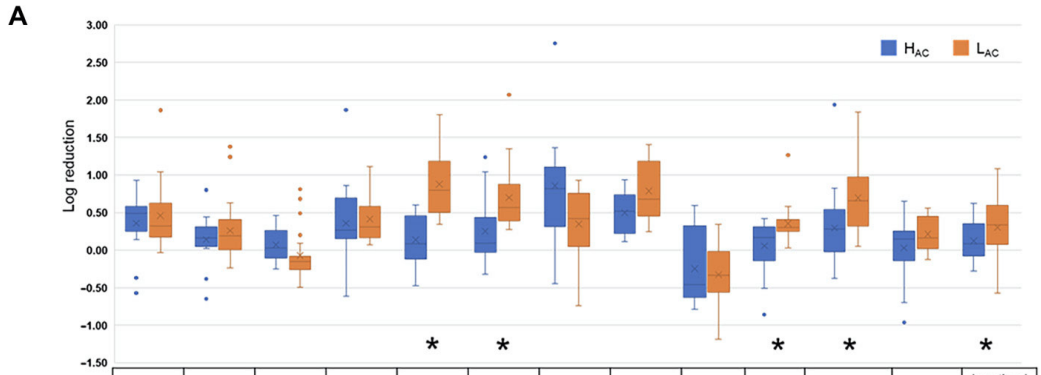


Figure 9. Association between the relative abundance of ARGs in urban WWTP effluents (aggregated by drug classes) and country-level data on phenotypic resistance of clinical isolates (data from EARS-Net). Numbers represent Spearman's rank correlations; color codes indicate statistical significance. No statistically significant correlations were observed for Acinetobacter spp., Enterococcus faecium, or Streptococcus pneumoniae. Figure adapted from III.



	AMG	MDR	SUL	BL	MLS_B	TET	QUI	AMP	VAN	Other	Transposase	Integrase	Insertional sequence
Higher ($P < 0.01$) relative abundance in influent WW	H_{AC}	H_{AC}	H_{AC}	H_{AC}	L_{AC}	L_{AC}	H_{AC}	H_{AC}					L_{AC}

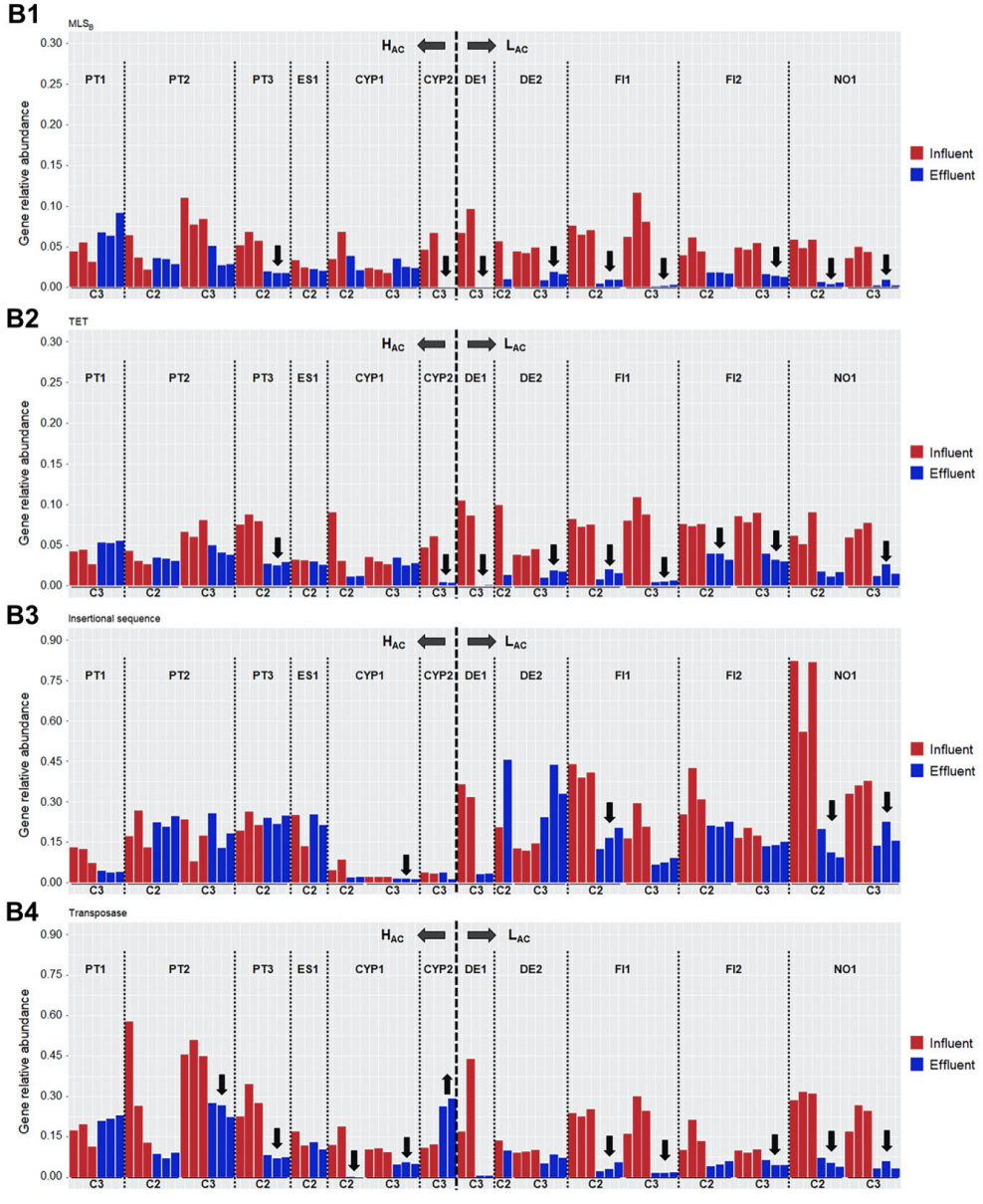


Figure 10. Variation in average ARG prevalence between influent and effluent [log10 (influent – effluent)] in HAC and LAC (high and low antibiotic consumption) countries. (A) Comparison of ARG log reduction values in WWTPs of the HAC and LAC countries, where zero indicates that treatment did not affect the cumulative ARG relative abundance, positive values indicate a reduction, and negative values indicate an increase. The classes for which HAC or LACpresented higher relative abundance in the influent ($P < 0.01$, Mann-Whitney U test) are displayed in the table at the bottom. Asterisks indicate statistically significant differences between HAC and LAC log reduction values, and these cases are detailed in (B). (B) Comparison of average ARG relative abundance in influent and final effluent for the classes with significantly different log reduction values in the HAC and LAC samples: (B1) MLSB, (B2) tetracyclines, (B3) insertion sequences, and (B4) transposase. The arrows indicate significant ($P < 0.01$; Welch's t test) increases (↑) or decreases (↓) after treatment (C1, autumn of 2015; C2, spring of 2016; C3, autumn of

4.3 The maternal and accumulative origins of infant resistomes

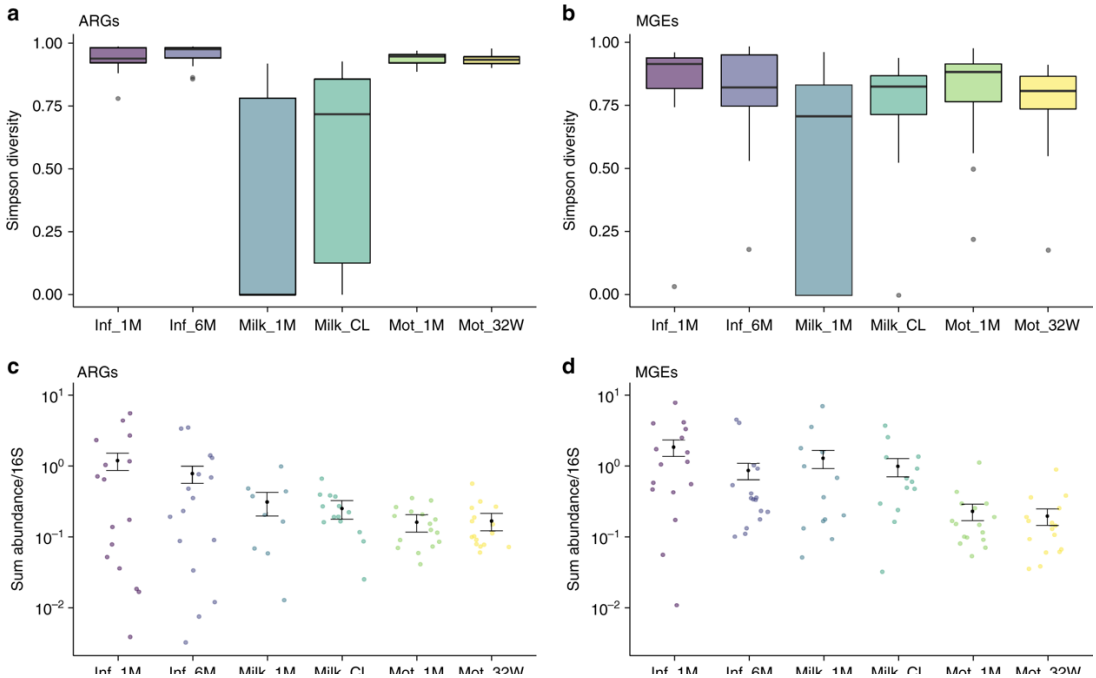


Figure 11. Simpson diversity of ARGs and MGEs and their relative sum abundances. (a) ARG diversity. (b) MGE diversity. (c) ARG sum relative abundances. (d) MGE sum relative abundances. The relative sum abundances are calculated per copy of 16S rRNA gene and normalized by gene lengths. Each colored point represents one sample. The predicted mean and standard error from the negative binomial GLM is drawn in black. Sample names are as follows: Inf_1M=1-month-old infants, Inf_6M=6-month-old infants, Mot_32W=mother fecal samples gestational week 32, Mot_1M=mother fecal samples one month postpartum, Milk_CL=colostrum or milk produced within 7 days after delivery, Milk_1M=milk 1 month postpartum. Each sample type has an (n) of 16. In boxplots a and b, the lower hinge represents 25% quantile, the upper hinge 75% quantile, and the centerline the median. Notches are calculated with the formula median$\pm$1.58 * interquartile range/sqrt (n). Negative binomial general linearized models (GLMs) were used to predict the means and standard errors (SEs) of the relative sum abundances in different sample types. The notches in c and d represent SEs, and means are represented as black points. Figure adapted from II.

Besides studying the effect of antibiotic use on ARGs observed in wastewaters, I investigated the relationship between antibiotic treatment of infants and mothers and ARG abundances in the gut in II. Infants who had never received antibiotics had higher ARG concentrations than their mothers (Figure 11). There was no clear correlation between antibiotic treatment of infants and the resistance potential. However, when mothers were given intravenous antibiotics during delivery, their infants had elevated levels of specific ARGs, suggesting that maternal antibiotic treatment could affect the resistome of infants (Figure 12). Intrapartum antibiotics have been shown to affect the microbiota (Azad et al. 2016; Mazzola et al. 2016). Later the result that IAP increases the abundance of specific ARGs has been corroborated in another study (Nogacka et al. 2017). Interestingly, in II, antibiotic use did not seem to affect resistance gene abundances in the mothers' gut microbiota.

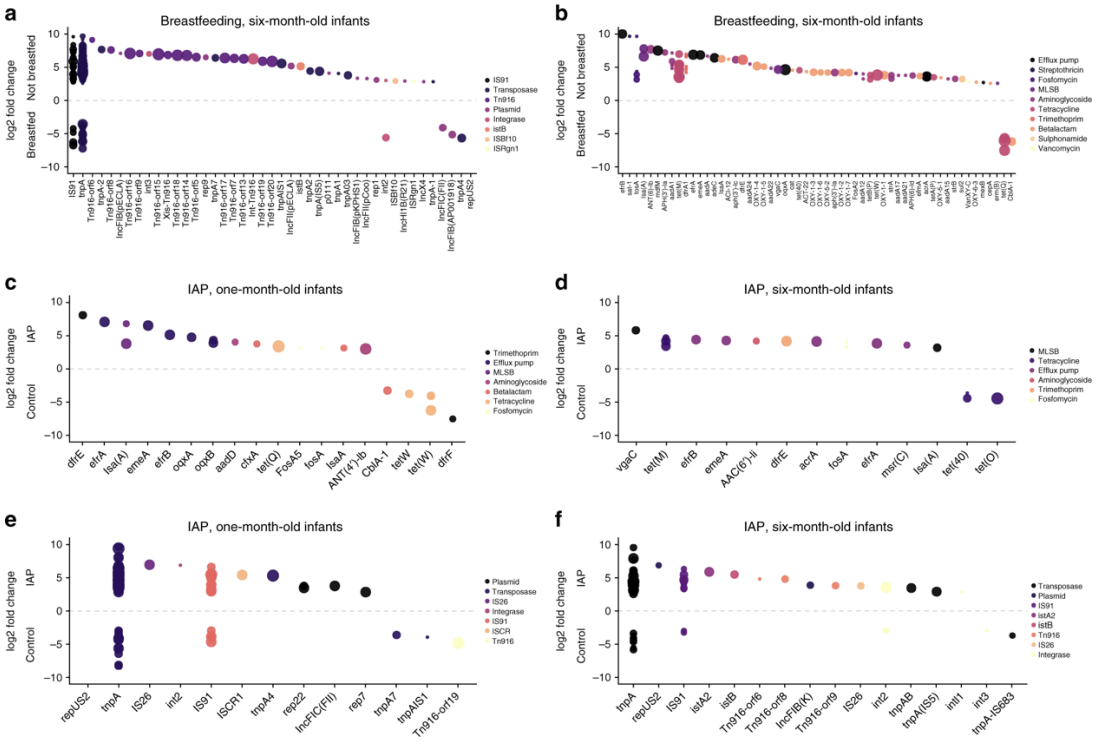


Figure 12. Differentially abundant ARGs and MGEs in 6-month-old infants. (a) MGEs differentially abundant due to breastfeeding in 6-month-old infants compared to non-breastfed infants. (b) ARGs differentially abundant in breastfed infants at 6 months, (c) ARGs differentially abundant in 1-month-old infants due to intrapartum antibiotic prophylaxis (IAP). (d) ARGs differentially abundant in 6-month-old infants due to IAP. (e) MGEs differentially abundant due to IAP in 1-month-old infants. (f) MGEs differentially abundant due to IAP in 6-month-old infants. Genes that have negative fold changes are more abundant in the non-breastfed and IAP groups. Sizes depict the number of samples each gene was found in (n=1–10). Color represents ARG or MGE class. The y-axis shows log2 fold changes, and the x-axis denotes gene names. Figure adapted from II.

The infants' resistomes were similar to their mothers' but not to unrelated mothers' resistomes in II (Figure 13). Thus, there likely is a vertical transfer of ARGs between mothers and infants. The potential transfer occurred with both maternal human milk and maternal feces. In V, the mothers' ARG abundance correlated with their infants, suggesting that the more ARGs the mother has, the more ARGs are transferred to the infant. However, not all ARGs of the infant resistome were observed in their mother's gut or breastmilk in II. It is possible that the genes were very rare in the mothers and not detected in them due to sequencing depth limitations. However, infants likely also carry genes from the environment, other people, or other maternal body sites. Still, the selection for these genes has occurred before them entering the infant gut. Thus, the spread of bacteria and genes is primarily responsible for the elevated ARG levels in the infant gut, not present antibiotic selection as has already been proposed (Zhang et al. 2011; Moore et al. 2013; 2015).

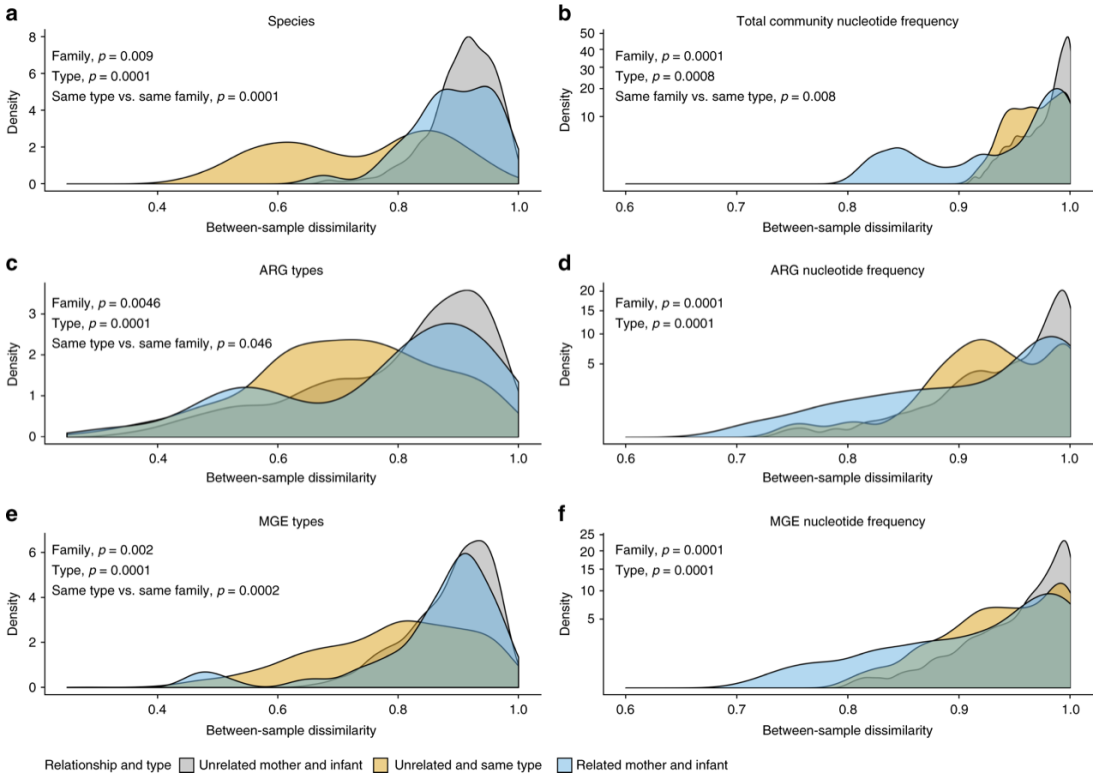


Figure 13. Dissimilarities of infant and mothers' gut microbiota, resistomes, and MGEs. (a) Dissimilarity of the microbial community on species level between infants and mothers. (b) Dissimilarity of microbial communities in infants and mothers based on kmer profiles. (c) Dissimilarity of resistomes between infants and mothers using gene type annotations. (d) Dissimilarity between resistomes using kmer profiles. e Dissimilarity of MGEs between infants and mothers using gene type annotations. (f) Dissimilarity between MGEs using kmer profiles. Type notion indicates that mothers and infants are significantly different from each other, family suggests that infant's feces are more similar to mother's feces than to that of unrelated women, same type vs. same family indicates that infants are more similar to each other than to their mothers. Figure adapted from II.

4.4 Early life formula feeding increases ARG loads in infants

I characterized the maternal human milk resistome in II. I observed that human milk has higher relative abundances of ARGs (reads mapping to ARGs/reads mapping 16S rRNA genes) than mother's feces. However, I saw that infants given human milk had lower abundances of specific ARGs than infants who did not receive human milk, suggesting that human milk does not increase the infant gut microbiota's resistance potential.

The observation that human milk has high concentrations of ARGs was concerning, and I further studied the effect of diet on the infant resistome. In V, focusing on infant diet, I observed that infants on exclusive human milk diets during early infancy had differing microbiotas, which caused them to have significantly less ARGs than infants fed any formula despite that human milk has ARGs (Figure 14). The taxa enriched in formula-fed infants are known opportunistic pathogens which typically carry multiple ARGs. Prematurity was also linked to higher resistance load. The effect of an exclusive human milk diet reducing relative ARG abundance could be replicated using several datasets in V. The infants who had had formula in their early infancy had higher ARG abundances later in infancy after introducing solids.

The formula's effect was greater than the impact of antibiotics, which did not significantly increase ARG abundances. However, during the first week of life, the impact of delivery mode was more prominent, and infants delivered by C-section had more ARGs than infants born vaginally. Based on my results, I propose that medical professionals are equally prudent when making decisions regarding formula use as they are with antibiotics to avoid increasing the ARG burden. A recent review paper has suggested that breastfeeding could help mitigate AMR in infants, especially in low and middle-income countries (Nadimpalli et al. 2020).

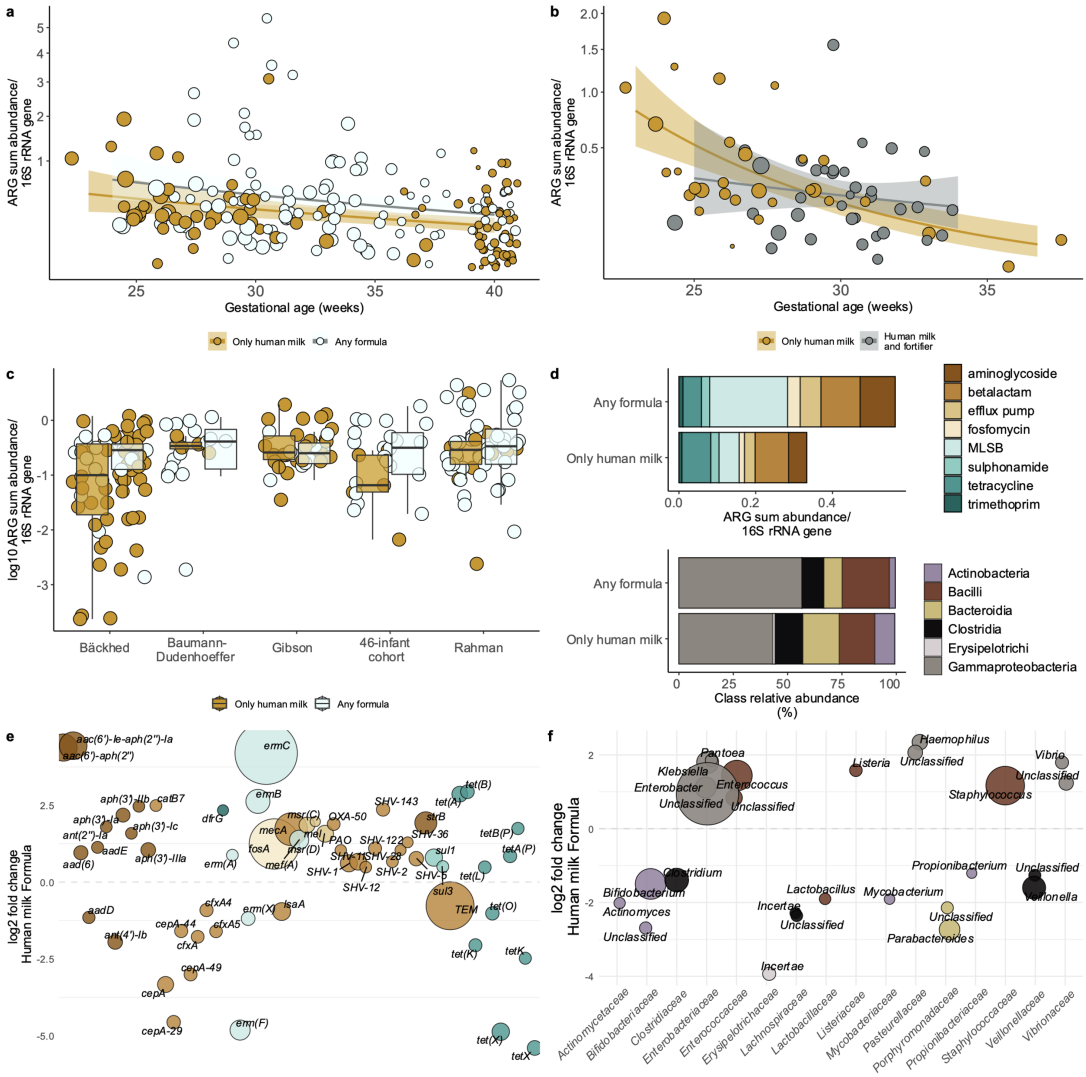


Figure 14. Effect of formula feeding on neonatal gut resistome in meta-analysis cohorts. (a) Differences in relative ARG sum abundance/16S rRNA gene copy numbers in infants only fed human milk or any formula. (b) Differences in relative ARG copies/16S rRNA in infants only fed human milk or any fortifier. In panels a and b, a regression line is fitted using gamma-distributed GLMs. The y-axis represents ARG counts normalized with 16S rRNA gene counts. The y-axes have been square root transformed. Dot size depicts infant age, with larger dots being older infants. (c) Effect of formula on ARG load in meta-analysis datasets by cohort. The x-axis represents cohorts by the first author's last name, with the 46-infant cohort indicating infants sequenced for the present study. The y-axis is the log 10 ARG/16S rRNA. The boxplot hinges represent 25 % and 75 % percentiles and centerline the median. Notches are calculated with the formula median±1.58 ◊ interquartile range/sqrt (n). (d) Differences between most abundant ARG and bacterial classes by diet in meta-analysis datasets. The x-axis represents ARG copies/16S rRNA and relative abundances in %, respectively. (e) Differentially abundant ARGs in infants with different diets. (f) Differentially abundant genera in infants with different diets. Genes with positive values are more abundant in formula-fed infants, and genes with negative values are more abundant in exclusively human milk-fed infants. In panels e and f, genes with positive values are more abundant in formula-fed infants and genes with negative values more abundant in exclusively human milk-fed infants. The point's size represents the mean base level of the gene. The y-axis depicts log2 fold changes observed between the groups. Dot size is proportional to mean abundance. Figure adapted from V.

5 CONCLUSIONS AND FUTURE PROSPECTS

The take-out message of the thesis is that antibiotic resistance is a multifaceted problem. Understanding and solving it requires a holistic and One Health approach to all aspects of resistance, as complicated as it may be. Thus far, as with climate change, we lack local and global legislation, regulation, and governance tools to mitigate the issue in time to avoid a crisis effectively.

My results challenge the idea that current antibiotic selection is the only driver of antibiotic resistance loads in the environment, agri-, aquaculture, or human hosts. Thus, the focus must also be put on the spread of ARGs and bacteria, which brings in governance's importance. In I, III, and IV, it was seen that waste contaminated with feces in fish farms, water bodies receiving treated wastewater, and environmental samples all had ARGs of clinal relevance, even without selection by antibiotics. Based on the results of my thesis, the legislation and regulation of releasement of waste containing human and production animal feces are vital in preventing the spread of ARGs.

At present, there is no requirement for waste management of municipalities or production animal facilities to in any way ensure that ARG contamination is restricted. The importance of limiting pollution with ARG containing DNA and resistant bacteria should be communicated to politicians and decision-makers. Thus far, the importance of controlling the release and use of antibiotics has more or less been made known to legislative and regulative parties. However, in my opinion, it is a much more immediate risk to have antibiotic-resistant bacteria polluting environments than antibiotic compounds. For example, there is a regulation on the amounts of antibiotic residues present in foodstuffs and customer demand for antibiotic-free meat. However, as a scientist studying antibiotic resistance, I am much more afraid of consuming fresh produce irrigated with ESBL bacteria-containing water than having a little bit of residual antibiotics in my food. Infections caused by ESBL bacteria can be lethal, but having low residual concentrations of antibiotics in food is not very harmful to humans. However, it is likely easier to communicate the dangers of chemicals than of genes or resistant bacteria. Also, a major hindrance in having legislation is that it is impossible to determine cut-off values for genes in the same way as it is possible to conduct experiments with a chemical substance's impact on living organisms.

The dogmas and best practices related to chemical substance release regulation need to be critically viewed to forward the legislation process of biological agents. Using proxies for fecal contamination such as coliform cultures can be helpful also when controlling ARG contamination. However, other proxies not relying on culturing are more high-throughput and can be more direct in estimating the polluting resistance burden. For example, crAssphage can be used as a marker for environmental pollution with human feces. For total ARG pollution intI, genes have been shown to act as an excellent indicator (Gillings et al. 2008). I support the proposal of monitoring intI genes in waste streams and setting limits for intI copies, which is commonly suggested by many other scientists working in the field. It is to be noted that the setting of limits for genes is complicated and requires a risk assessment expert's contribution before such recommendations can be proposed to decision-makers and politicians.

Stakeholders could also act before legislation and regulation when the problem is known. However, for stakeholders to act widely, consumer and general public pressure are often needed. Thus, it is essential to communicate the importance of waste management in limiting the resistance crisis and the severity of the problem to the general public. All scientists working in the field can help raise public awareness.



Figure 15. Formula feeding, prematurity, and cesarean section increase ARG loads in infants. Infants fed formula had approximately 70% more antibiotic resistance genes than infants fed human milk exclusively. During the first week of life, birth mode had the most substantial effect on ARG loads, with infants born via section having more ARGs than infants delivered vaginally.

One of the most exciting findings in my thesis was that infant diet so forcefully affects antibiotic resistance loads in infants along with prematurity (Figure 15). This knowledge, coupled with low breastfeeding rates, especially in low and middle-income countries and many high-income countries, opens up an opportunity to mitigate infant ARG burdens by identifying infants whose parents should be supported to continue exclusive breastfeeding. Also, neonatal and maternity wards are essential in breastfeeding initiation and how their staff is trained in supporting breastfeeding varies tremendously, both globally and within hospitals. The impact of formula marketing on consumer behavior should not be neglected, especially in countries that lack regulation for breast milk substitute advertisement. Strict laws should be put to use globally and also monitored more efficiently than now.

In my thesis, I have discovered vital areas where interventions could be made, such as improving breastfeeding recommendation compliance and waste management in wastewater treatment plants, aquaculture and, drug manufacturing. More research, especially multidisciplinary work, is needed to study ecological, socioeconomic, and environmental factors of ARG spread. However, in addition to research, it is essential to efficiently communicate the effects of antibiotic resistance to stakeholders to enforce improvements in legislation and financial incentives. Similar actions that have been made to combat the COVID-19 pandemic and climate change are now needed to reduce mortality and economic losses caused by antibiotic-resistant bacteria in the years to come.

The work I have done gives new tools and methodologies for scientists that study the resistance issue. The MGE database created will help identify mobile genetic contexts and analyze the relationship of the mobilome and resistome further. The improved IPCR method also enables further investigation of the mobility potential of select genes. However, at present, the throughput of IPCR is very low, but in the future, applying an array-based IPCR with long-read sequencing would improve the throughput but still retain the benefits of targeted amplification and long-read sequencing compared to shotgun metagenomics.

I have used shotgun metagenomics in most of the articles in the thesis. It is the most comprehensive single method to study the resistome and resistant bacterial community members. However, shotgun metagenomics presents challenges in identifying the host and the genetic context of the ARG, as mentioned before. The host of the ARG is essential in the sense that ARGs carried by commensal and non-infectious bacteria pose a very different risk to humans compared to ARGs carried by pathogens. epicPCR is a PCR-based method that identifies the host of a gene of interest (Spencer et al. 2016). Single-cell genomics enables the amplification of a single genome from metagenomes. However, both of these methodologies are at present low throughput and need further development to compare to the throughput of shotgun metagenomics.

In the coming years, the sequencing methodologies will improve, and the length of reads and library depth will increase, and correspondingly the error rates and price per base will decrease. When it is economically feasible to produce deep libraries with long-read sequencing platforms, the assembly of metagenome-assembled genomes will enable high throughput single-cell genomics of metagenomes and identifying low abundance genes. The longer the reads are sequenced, the easier it will become for assembly algorithms to assemble complete genomes from metagenomes, given that the depth is adequate and the error rates are low.

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