Resistome composition and dynamics in gut microbiota of humans and animals in rural Bangladesh

10-10-2024

Mieke Brinkman (6918190)
MSc Environmental Biology

Supervisor: Tim Dallman
Institute of Risk Assessment Sciences, Utrecht University

ABSTRACT

Background
The rise of antimicrobial resistance (AMR) in bacteria is a worldwide problem that causes considerable morbidity and mortality, since infections with resistant pathogens may have no therapeutic treatment. People that live in low- and middle-income countries (LMIC) are especially impacted by antimicrobial resistance. However, data on the emergence and spread of antimicrobial resistance in these countries is lacking. Improved AMR surveillance in LMICs is urgently needed, considering that antimicrobial resistance prevalence is increasing in LMIC. People in rural areas often are in close contact with animals and might have poor sanitation, which are risk factors for transmission of antimicrobial resistance genes (ARGs). In this longitudinal study, we characterised ARGs in humans and animals in households in rural Bangladesh.

Methods
Samples were taken at three different timepoints. At the first timepoint, faecal samples from pregnant women, siblings, and household livestock were taken. A week after giving birth, mothers and their infants were sampled. The last timepoint was several months later, and again, mothers, infants, siblings, and household animals were samples. Using a metagenomics approach allowed for identification of the total pool of ARGs (the resistome) in the gut microbiome of humans and animals.

Results
Resistome analysis showed that samples of mothers contained a more diverse set of ARGs around the time of giving birth. The composition of mother resistomes just after giving birth was distinct from mother resistomes while they were pregnant or a several months after giving birth. Moreover, newborn resistomes differed from those of their mothers and siblings, reflecting that their microbiomes and thus, their resistomes are still developing. Differential abundance analysis showed that numerous ARGs were more abundant in mothers and newborns just after birth compared to the other timepoints. These changes in the human resistome were not linked to antibiotic usage. However, a significant correlation was found between the microbiome and the resistome composition, and the microbiome of mothers also changed around the time of giving birth. Thus, co-occurrence networks were made, in which *Klebsiella* was found to correlate with multiple ARGs.

Discussion
The high prevalence of ARGs and possibly pathogenic bacteria in mothers and infants just after birth could increase the risk of people in this study population to obtain difficult to treat infections, or to spread resistant bacteria within their households. Further research could focus on identifying transmission routes of mobile ARGs in rural households, and on determining if the increase in ARGs in mothers and infants is due to exposure to resistant bacteria in the hospitals where the infants were born.

PLAIN LANGUAGE SUMMARY

Antimicrobials are important drugs, since they are used to treat infections with harmful bacteria. Unfortunately, some bacteria have developed methods to evade the effects of antimicrobials, making them resistant. Genetic information that is needed for antimicrobial resistance is encoded in the DNA of the bacterium, in so-called antimicrobial resistance genes (ARG). Human guts contain many bacteria, and some of them carry ARGs. This is not always an immediate problem, because most of the bacteria in the human gut are not harmful and sometimes even beneficial. However, bacteria can share genes with each other. Thus, ARGs from beneficial bacteria in the human gut might be transferred to pathogenic bacteria. This imposes a problem, because antimicrobial resistant pathogens endanger healthcare practices. Since the usual medicines do not work, infections with these bacteria are difficult to treat. People that live in low-income countries are even more severely impacted by resistant bacteria, and there is less knowledge on how these bacteria spread between people in these settings. However, people in these communities are exposed to several factors that increase their chances of obtaining resistant bacteria. This includes close contact with farm animals, and poor hygiene at home and in hospitals. Therefore, research into how prevalent antimicrobial resistant bacteria are and how they spread in low-income countries is needed to prevent their abundance from increasing even more. In this study, we looked at the DNA of bacteria in guts of humans and animals that live in rural Bangladesh. We determined which and how many ARGs were present in the bacterial DNA. The ARGs found in different household members were compared. In mothers, several ARGs increased in abundance just after the mothers had given birth. The ARGs found in newborn babies were different from those found in their siblings or mothers. At the same time, several pathogenic bacteria were more abundant in mothers and infants just after birth. Because the infants were all born in hospitals, it might be possible that mothers and infants both are exposed to pathogens that carry ARGs in their DNA in the hospital. This research is a first step towards a better understanding of the ARGs that exist in guts of people in low-income households. Future research is needed to determine if these ARGs are indeed acquired when people spend time in the hospital, and how resistant bacteria can spread between humans and animals that live closely together.

TABLE OF CONTENTS

INTRODUCTION

The increase in antimicrobial resistance (AMR) in bacteria poses a threat for global health, as many pathogenic bacteria have gained antibiotic resistance, which makes these infections difficult to treat (Prestinaci et al., 2015). This includes common pathogens such as *Klebsiella pneumoniae*, *Escherichia coli*, and several *Staphylococcus* species. The rise in AMR prevalence has led to more expensive and less effective treatments, and longer hospital times for patients that have infections with resistant bacteria (Laxminarayan et al., 2013). Some medical procedures depend on the use of antibiotics, and thus, AMR occurrence impacts morbidity and mortality (Prestinaci et al., 2015). It was estimated that in 2019, 1.27 million deaths globally were attributable to infections with antimicrobial resistant bacteria (Murray et al., 2022). However, AMR affects low- and middle-income countries (LMIC) more severely. Antimicrobial resistant bacteria cause more deaths in low-income regions such as South Asia and sub-Saharan Africa compared to high-income regions such as Europe and North America (Murray et al., 2022).

The World Health Organization (WHO) reported that AMR prevalence has reached alarming heights, and that we are at risk of entering a post-antibiotic era where common infections could kill (World Health Organization, 2014). Therefore, the WHO emphasizes the urgent need for collaboration on global AMR surveillance in order to develop effective strategies to mitigate this worldwide problem. However, there is a significance data gap in AMR surveillance in LMIC (Murray et al., 2022; Oldenkamp et al., 2021; Van Boeckel et al., 2019). The limited availability on AMR prevalence data in LMIC is due to a lack of trained personnel, laboratory facilities and supplies, and to inadequate government health care spending (Gandra et al., 2020; Oldenkamp et al., 2021). Additionally, clinical and epidemiological data is also often not properly or consistently submitted, since this takes significant time and resources (Gandra et al., 2020). High-quality AMR surveillance data with accompanying epidemiological data is essential to be able to identify how AMR spreads in communities in LMIC.

Using a One Health approach could aid in understanding the emergence and spread of ARGs (Kim & Cha, 2021; Robinson et al., 2016). The One Health principle recognizes that people, animals, and their environment are connected, which is especially applicable for AMR (Robinson et al., 2016). This is because antimicrobial resistance genes (ARGs) are able to spread between human and environmental sources. Therefore, recent research has focused on profiling ARG occurrence in different sources (Kim & Cha, 2021). This showed that gut microbiomes are reservoirs of AMR, and usually contain high counts of ARGs. Additionally, ARG-carrying bacteria have been found in the environment, for example in hospitals, farm animals, soil, and waste water (Chng et al., 2020; Lawther et al., 2022; Pehrsson et al., 2016). Spread of ARGs between these sources can occur by transmission of resistant bacterial strains, or by transmission of mobile ARGs between bacteria (Baquero et al., 2019; Crits-Christoph et al., 2022; Hu et al., 2017).

Since ARGs in a microbiome could be shared by commensal, beneficial, and pathogenic bacteria alike, AMR profiling is often done by identifying and quantifying the complete set of ARGs in the microbiome. These ARGs together form the resistome, which is intrinsically part of the microbiome. Research into the resistome has greatly benefited from the advancements made in shotgun sequencing (Crofts et al., 2017). Instead of using culture-based methods and primers for specific known ARGs, metagenomics can be used to sequence all bacterial DNA in a sample and afterwards quantify the ARGs that occur in these microbiomes (Bengtsson-Palme et al., 2017). Using this approach, a comprehensive view of the resistome can be obtained, including sequence variants and the detection of newly emerged ARGs. Metagenomic based resistome profiling therefore makes it possible to research which factors influence the composition of the resistome, and to identify transmission routes of ARGs.

One of the main drivers of AMR emergence and spread is the use or overuse of antibiotics (AB), since this causes a selective pressure on bacterial resistance (Holmes et al., 2016). After treatment with AB, compositional changes can be observed in the gut microbiome and consequently, in the resistome. In healthy adults, a five-day course of antibiotics caused a decrease in the diversity of the microbiome (Anthony et al., 2022). This results in empty ecological niches, providing an opportunity for broadly resistant bacteria to colonize the gut (Baquero et al., 2019). This is reflected in the finding that the total number of ARGs in the gut microbiome increases after AB treatment, an effect that can persist over time (Anthony et al., 2022; Zaura et

al., 2015). Effects of AB usage on the microbiome and the resistome have been detected up to one year afterwards (Li et al., 2023). This study additionally reported an increase in plasmid abundance after AB usage, which could affect ARG horizontal transmission between gut bacteria. Other studies however reported less clear effects of AB usage on the resistome, and suggest that the effects might depend on individual resistome composition, and on the type or duration of the antibiotic treatment (Nielsen et al., 2021; Willmann et al., 2019).

However, the aforementioned studies were carried out in high income countries. There only is a limited number of studies on the effect of AB use on the human gut resistome that are conducted in LMIC. These studies either used qPCR methods to quantify the abundance of selected ARGs, or determined AMR in specific pathogenic bacteria (Auguet et al., 2021; Bich et al., 2019). These studies thus do not provide a complete view of the full resistome in relation to AB use. Hendriksen et al. (2019) did research the complete bacterial resistome, using metagenomic analysis of untreated urban sewage in high-income countries as well as in LMIC. By linking the metagenomic resistome data to available information of global AB usage, they found that only a minor part of the variation in ARG abundances between countries could be explained by AB usage.

Moreover, it has been reported that while less antibiotics are used in LMIC compared to high-income countries, ARG abundances are higher in LMIC (Hendriksen et al., 2019; Klein et al., 2018). Since AB use does not seem to influence AMR prevalence on a large scale, these patterns point to a substantial influence of socioeconomic factors on ARG transmission. Indeed, poor sanitation, poor governance, lack of access to high quality diagnostics, and low government health expenditure all correlated with high AMR prevalence (Collignon et al., 2018; Hendriksen et al., 2019). Thus, it can be concluded that while it depends on the type of antibiotic treatment, AB usage influences the gut resistome on the level of the individual. These findings are probably applicable to people in LMIC as well. However, researching the resistome locally in LMIC communities is crucial to be able to understand how socioeconomic factors contribute to the high abundance and burden of AMR in LMIC, and to identify possible high-risk environments or factors for ARG transmission.

Contact with livestock is one of the factors that facilitate transmission of ARGs from the environment to humans. Gut microbiomes of livestock such as poultry, ruminants, and swine are reservoirs of ARGs, with the highest ARG diversity found in poultry (Lawther et al., 2022; Munk et al., 2018a; Yang et al., 2022). Microbiomes of farmers that are exposed to livestock contain shared microbes with animals (Mahmud et al., 2024). This translates to the resistome composition, which is altered in farmers (Van Gompel et al., 2020). Specific farming practices in LMIC could possibly influence ARG transmission from livestock to humans. For example, the number of small-scale animal production farms is rising in LMIC. Regulations on the veterinary use of AB are loose and thus, AB are often used to treat animals (Hicks et al., 2021; Van Boeckel et al., 2019). As a consequence, high abundance of AMR can be found in poultry held in small-scale farms (Guo et al., 2018). In rural Bangladesh however, animals are usually kept free-roaming around the house (Roess et al., 2013). Poultry is the main livestock that is raised this way, but additionally, several cows, goats, or ducks might also be owned by the household. Families are largely dependent on these animals as a main source of income and food, and therefore spend a relatively substantial part of their resources on treating sick animals with AB and other medicines (Roess et al., 2013). Furthermore, family members often are in close contact with livestock, sharing drinking water sources and living space with their animals. This increases the risk of bacterial pathogen and ARG transmission within the household. Thus, members of these rural households are more vulnerable to obtaining bacterial infections that could be difficult to treat because of AMR.

In these settings, newborns form an especially vulnerable group. The infant gut microbiome and resistome are still developing and thus less stable (Zimmermann & Curtis, 2018). Consequently, the infant resistome is initially sensitive to external disturbances and exposure to AMR in the environment (Bargheet et al., 2023; Leo et al., 2022). AB treatment causes an increase in ARG abundance and diversity in infant gut resistomes (Li et al., 2023). While this study reported a quicker recovery of the gut resistome after AB treatment in infants compared to adults, long-term effects of AB usage in infants have been described as well (Gasparrini et al., 2019). Considering the observed increase in ARGs after AB treatment, it is especially worrying that the majority of the AB prescriptions in LMIC are unnecessary (Fink et al., 2020). Moreover, infants that are born in hospitals are more likely to carry resistant pathogens in their gut microbiome, since hospitals in LMIC are unfortunately

an hotspot of antibiotic resistant bacteria because of a lack of appropriate hygiene (Monjur et al., 2010; Zaidi et al., 2005). Thus, infants in LMIC are vulnerable to exposure to ARG-carrying bacteria and to the risks of inappropriate AB usage (Luchen et al., 2023). However, the AMR burden is also higher for children in LMIC, since neonatal infections occur more frequently compared to industrialized countries (Zaidi et al., 2005). This highlights the importance of characterising the resistome of this understudied population, which simultaneously carries a high AMR burden and is also more likely to acquire resistant bacteria from the environment because of close contact with animals and poor sanitation.

In this longitudinal study, the gut resistomes of animals and humans in 19 households in rural Bangladesh were profiled using metagenomic sequencing of faecal samples. Households were composed of mothers, newborns, and if present, siblings. Additionally, most households owned chickens, and some owned cows, goats, ducks, or pigeons. Samples were taken at three different timepoints, ranging from the last trimester of pregnancy to several months after giving birth. The resistome profiles of samples were compared, to determine if the resistome composition and diversity differs between sample types. The longitudinal design of the study also allowed us to research how the resistome of humans might change over the time course of the study, and if factors such as aging of newborns or giving birth affect the gut resistome composition. Lastly, we asked if antibiotic usage affects the human resistome in terms of ARG abundance and diversity.

METHODS

Sampling and DNA sequencing

Field workers from the Child Health Research Foundation (CRHF) collected stool samples from humans and samples from animal droppings between January 2020 and December 2021. 220 households in the Dhaka district in Bangladesh were sampled in total. In this study, data from 19 households was analysed. The longitudinal sampling per household was conducted within 8 months, which included three timepoints. At baseline, mothers were in their third trimester of pregnancy. At this timepoint, mothers were sampled, and if present, siblings and animals as well. The midline timepoint was 2-3 months later. Sampling was conducted within a week after infants were born and included mothers and newborns. The endline sampling was conducted 3-4 months later, and included mothers, infants, and if present, siblings and animals. Alongside the sample collection, epidemiological surveys were conducted. Surveys contained questions on antibiotic usage in humans and animals, birth mode, the number of animals in the household, if newborns had fevers, and several other factors. Samples were stored at -80 ^{o}C, and shipped to the Quadram Institute Bioscience (QIB; Norwich, United Kingdom). DNA extraction and sequencing were conducted with Illumina sequencing according to QIB protocols.

Read processing and taxonomic profiling

Five samples were excluded from the data analysis since they were repeat samples. These samples were R-02-056-02, R-03-056-01, R-02-056-35, R-02-070-35, and R-03-070-01. One repeat sample was included in the analysis since there was no existing duplicate, this was sample R-02-070-02. An additional four samples were not further analysed since the sequencing depth was insufficient. These were samples 01-038-03, 02-029-31, 02-016-02, and 03-063-01.

Read processing was conducted using taxprofiler nf-core pipeline (v1.10) (Stamouli et al., 2023). The best-practice pipeline performs read-processing (adaptor clipping, quality filtering and host-read removal). The processed reads were then used for the microbiome and the resistome profiling.

Taxonomic classification for the microbiome profiling was performed using Kraken2 with best-practice default options (Wood et al., 2019). Operational taxonomic units (OTUs) uncharacterized at the phylum level were removed, as well as any OTUs with non-microbial origins, like *Chordata*, *Streptophyta*, *Evosea* and *Heterolobosea*. The phylum *Ascomycota*, consisting of 5 total reads in 4 samples, was also removed based on its low read count. Singletons, defined as OTUs present only once across all samples, were defined as noise and removed after a Procrustes analysis with a Mantel test confirmed that the overall composition remained unchanged. The Kraken2 data was subsampled with *rarefy* to a uniform sequencing depth of 1,200,000 reads, removing 9,103 OTUs that were no longer present after random subsampling.

The microbiome data was analysed in a similar manner as the resistome data. Alpha and beta diversity

measures were calculated, and differential abundance analysis was performed. For this, the same R packages and methods were used as described for the resistome analysis.

Resistome profiling

Cleaned metagenomic reads were mapped to the ResFinder database v. 2.0.0, which contains acquired genes that mediate antimicrobial resistance (Bortolaia et al., 2020). Mapping was done using Bowtie2 v. 2.2.5 with default settings (Langmead & Salzberg, 2012). Using SAMtools v. 1.19.2, the mapped reads were sorted, indexed, and quantified by calculating the number of read that mapped to each ARG per sample. ARG counts were converted to FPKM counts to normalize for ARG gene length and the number of bacteria in the sample. Bacterial counts were extracted from the sample report of the taxonomic profiling with Kraken, which lists the number of fragments assigned to the domain "Bacteria". To account for non-specific mapping to ARG variants, ARGs in the ResFinder database were clustered based on 90% nucleotide identity , resulting in FPKM counts for each ARG cluster per sample. Clusters were named after the ARGs in the cluster. Some ARGs with the same name had multiple clusters, which is why the cluster names also contain a number that differentiates clusters that contain the same ARG. The ResFinder database also lists to which antibiotic class each ARG confers resistance. To analyse the resistome on class level, ARG counts were aggregated into antibiotic classes, which leveraged FPKM counts for each class per sample.

Data analysis and visualization

Analysis of the resistome data was performed in R v. 4.2.3. ARG counts, taxonomy table, and meta data were combined into a phyloseq object using the phyloseq package v. 1.42.0 (McMurdie & Holmes, 2013).
When comparing FPKM counts between samples, log-transformed ARG counts were used. This was done because the number of counts per sample varied greatly within sample types, making it more difficult to visualize ARG counts in different sample types figure 1B). Alpha diversity measures were calculated using the R package vegan v. 2.6.4 (Oksanen et al., 2024). Three different alpha diversity indices were calculated, since they quantify alpha diversity in different ways. ARG richness indicates the observed number of unique ARGs, and thus does not take the distribution of the prevalence of genes into accounts. In contrast, the evenness of the distribution of ARGs does contribute in the calculation of Shannon and Simpson alpha diversity. Evenness of ARGs weighs more in the Simpson diversity compared to the Shannon diversity index.
For the beta diversity analysis, Bray-Curtis distances between FPKM ARG counts of samples were calculated with the vegdist function in vegan. NMDS ordinations were made using vegan's metaMDS function, using the following parameters: k= 3, maxit= 999, trymax= 999. PERMANOVA's were conducted on Bray-Curtis distances between samples, using the adonis2 function in vegan with 999 permutations (Arbizu, 2017/2023). For each PERMANOVA, the vegan function betadisper was used to test if the assumption of homogeneity of variances in groups of the tested factors was met. Procrustes analysis was performed to determine if there was a significant correlation between the resistome and the microbiome. The vegan function protest was used to rotate the NMDS ordinations on Bray-Curtis distances for the resistome and the microbiome, and to test if the correlation was significant.
Plots were made with ggplot2 v. 3.4.4, unless stated otherwise. R packages multcompView, rstatix, and RColorBrewer were used for colour palettes and displaying results of statistical tests in plots (Graves & Dorai-Raj, 2024; Kassambara, 2023; Neuwirth, 2022).

Differential abundance analysis

DESeq2 v. 1.38.3 was used to identify ARGs that were differently abundant in human sample types (Love et al., 2014). For the input data for the differential abundance analysis, metagenomic reads that mapped to the clusters of ARGs in the ResFinder database were normalized against the length of the ARG, but not against the number of bacteria in the sample. Instead, normalization was done using the methods provided by DESeq2 (McMurdie & Holmes, 2014). SizeFactors were manually calculated by dividing the number of bacterial counts in each sample by minimal number of bacterial counts found in the samples (Munk et al., 2018b). First, the likelihood ratio test (LRT) as implemented by DESeq2 was used to test for interaction factors, and identify ARGs that could be differently abundant between sample types or timepoints, but only in specific households. This yielded no significant ARGs, and thus, the differential abundance analysis was continued with DESeq2's default two-sided Wald test. The design of the model was defined as follows: ~ sample_BME + HouseholdHHnumber1. Sample_BME is a combined factor of sample type and timepoint, "Mom_M" would for example indicate mom

samples taken at midline. Household number was included in the design of the dds object (since PERMANOVA analysis indicated a significant effect of household number on resistome composition), but differences in ARG abundance between households was not tested. Results were extracted from the DESeq2 test for the comparisons listed in table 3. Differently abundant ARGs were filtered on their significance (BH adjusted p-value < 0.05) and on their abundance. Filtering on abundance was done to prevent genes from being marked as differently abundant while they were not present in the one group that was compared, but were present in only 1 or 2 samples in the other group. While DESeq2 identified these ARGs as differently abundant, they were not deemed as biologically relevant. Therefore, it was determined for each gene in what proportion of the samples in each of the two groups there were more than 0 counts for the gene. Only genes that were present in more than 50% of the samples in one of the compared groups were kept in the list of differently abundant genes for that comparison.
The differential abundance analysis was also performed on antibiotic class level. The design factor was identical to the analysis on ARG level, but the differentially abundant classes were not filtered on prevalence.
Lastly, a separate analysis was conducted on the mom samples at midline, which compared moms who used any antibiotics at midline compared to those who did not. The design of the model included only the factor that described antibiotic usage. The resulting differently abundant ARGs were not filtered on prevalence.

Co-occurrence networks

To determine correlations amongst the abundances of ARGs (FPKM) or between ARGs and microbial genera, co-occurrence networks were constructed with the method used by Munk et al. (2024). Only the ARGs and genera that had a total read count of above 100 were kept and used as input for the network. Count matrices for the resistome and the microbiome were transformed to relative abundances. Correlations between ARG-ARG pairs and ARG-bacterial genus pairs were determined by calculating all pairwise Spearman's correlations, and adjusting for multiple testing using a Bonferroni-Hochberg post-hoc correction. Significant correlations (BH adjusted p-value < 0.01) were additionally filtered on the robustness of the correlation, as indicated by the rho value. First, correlations were filtered on a rho of above 0.8. The networks that were constructed with this rho cut-off value were dominated by microbial genera that correlated with tet(40) and/or tet(O/32/O), suggesting that these tetracycline resistance genes are present in many microbial genera in the human microbiome (figures S10-S13). Therefore, correlations containing tet(40) and/or tet(O/32/O) were removed, and the remaining correlations were filtered on a rho of above 0.7. This provided a more detailed view of the clusters surrounding the large clusters with these two tetracycline genes. The co-occurrence networks with significant correlation were visualised by the Fruchterman-Reingold algorithm using Gephi v. 0.10.1 (Bastian et al., 2009).

RESULTS

ARG abundance

First, total numbers of ARG counts and the average number of counts per sample type were calculated (table 1). The mean and median FPKM counts of ARGs over all samples was, respectively, 103668.1 (SEM= 28777.52) and 201281.3 (IQR= 51322.7 - 197361.4). There was no difference in number of log-transformed ARG counts between sample types (two-way ANOVA, p-value= 0.35) (figure 1).

Table 1: *Number of ARG counts (FPKM) in all samples, per sample type. "n" indicates the number of samples per sample type. Standard error of the mean (SEM) was calculated by dividing the standard deviation by the square root of n.*

Sample type	n	Median number of ARG counts	Mean number of ARG counts	SEM
Mother	50	122297.5	185674.1	42503.92
Newborn	36	138640	231588.5	49829.85
Sibling	17	109102.2	144810.4	27507.77
Chicken	22	79289.56	387764.2	148106
Duck	6	98757.89	87134.5	24002.85
Pigeon	8	51648.28	66926.06	20222.15
Cow	12	78290.18	88474.41	14264.88
Goat	2	31322.86	31322.86	2941.184

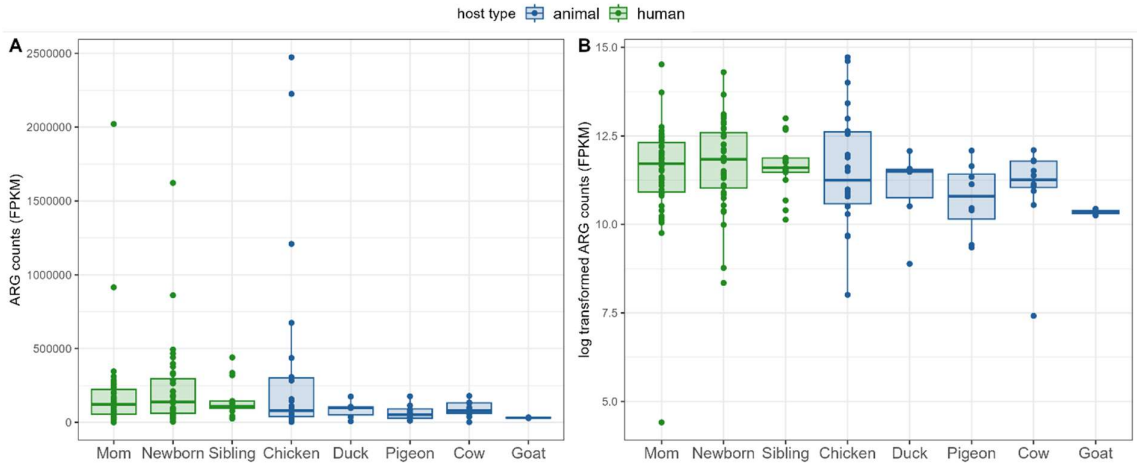

Figure 1: *ARG counts in metagenomic data of microbiomes from different hosts.* ***(A)*** *shows FPKM counts and* ***(B)*** *shows log-transformed FPKM counts. Green and blue coloured boxplots indicate samples from human and animal hosts, respectively.*

Abundance of ARGs on antibiotic class level

The ResFinder database lists to which antimicrobial class each of the ARGs in the database confers resistance to. Therefore, the abundance of ARGs could first be analysed on class level. More than half (53.6%) of the total ARGs found in the metagenomic dataset mapped to genes that confer resistance to tetracycline (figure 2). Resistance to macrolides (17.9%) and to beta-lactams (10.9%) was the next most prevalent.

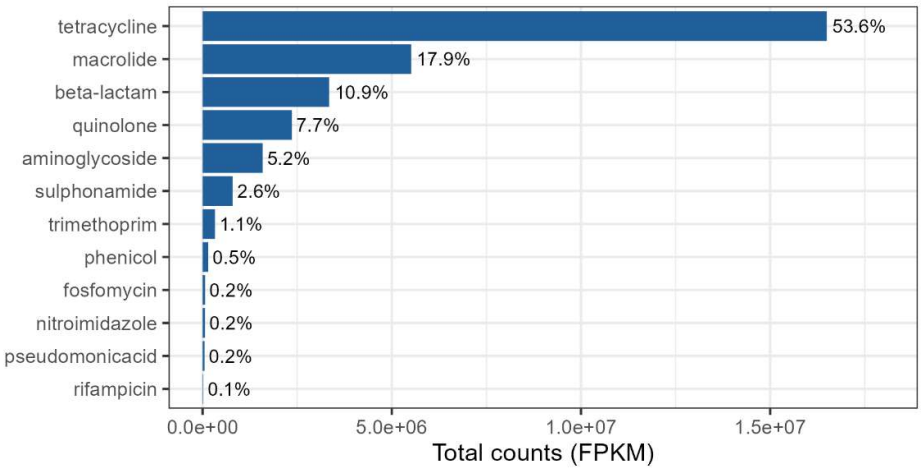

Figure 2: *Total ARG counts (FPKM) in the whole dataset, per antibiotic class. Percentages indicate abundance of resistance genes on class level relative to the total number of ARG counts (FPKM counts for class/total FPKM counts*100). Only classes that contained at least 0.1% of total ARG counts are shown.*

To obtain more detailed information about the abundance of antibiotic classes in different sample types, the relative abundance of ARGs at the class level was calculated for each individual sample. For human samples, this showed that while resistance genes to tetracycline were indeed the most prevalent overall, there was quite some variance in the abundance of resistance genes to tetracycline (figure 3). In 59 out of 103 human samples, tetracycline was the most abundant class. The proportion of tetracycline ARGs in human samples ranged from 0.1% (in a newborn sample) to 97% (in a sibling sample).

For mothers, tetracycline resistance was less prevalent in samples taken around the time of giving birth. The percentage of ARGs conferring resistance to tetracycline decreased from 68.7% at baseline to 29.1% at midline. In endline samples, tetracycline resistance seemed to recover to similar levels (60.8%) as seen in baseline samples. However, differential abundance analysis on class level with DESeq2 showed that there were no significant differences in tetracycline abundances in mother samples taken at different timepoints (figure S1). Furthermore, resistance against less abundant classes such as quinolone and aminoglycoside increased in mothers at midline compared to baseline and endline. Quinolone resistance genes comprised 14.4% of the total ARGs in mothers at midline, compared to 2.1% at baseline and 7.1% at endline. DESeq2 analysis on class level confirmed the increase in quinolone ARGs at midline, and showed that quinolone resistance remained more abundant in mothers at endline compared to baseline (figure S1). The relative abundance of aminoglycoside ARGs also increased, from 1% at baseline to 8.5% at midline (figure S1). At endline, aminoglycoside ARG abundance in mother samples decreased again to 2.5%. DESeq2 analysis on class level confirmed that ARGs that confer resistance to aminoglycosides were not more prevalent in mothers at endline compared to baseline.

The distributions of antibiotic class resistance in newborns seemed different to those in mothers and siblings. Macrolide ARGs were more abundant in newborns (26.9% of the total ARGs) compared to mothers (12.5%) and siblings (19.5%). The same was true for aminoglycoside resistance genes, which comprised 8.1% of the total ARGs in newborns, as opposed to 3.7% in mothers and 1.4% in siblings. Tetracycline ARGs were less abundant in newborns, since they comprised 21.9% of total ARGs found in newborns, compared to 54.8% and 62.2 % in mothers and siblings, respectively. However, no differences in tetracycline ARG abundance was found in the DESeq2 class level comparison between newborns and mothers (figure S2). DESeq2 analysis showed that macrolide and aminoglycoside resistance genes were only more abundant in newborns at endline compared to mothers at endline, and not in newborns at midline compared to mothers at midline. This is consistent with the patterns that can be observed for newborns, where macrolide resistance seems to become more prevalent with time (figure 3). In newborns at midline, 19.4% of the total ARGs were macrolide ARGs, which increased to

34.5% at endline. However, DESeq2 analysis on class level showed no significant differences in the abundance of macrolide resistance in newborns at endline compared to midline.

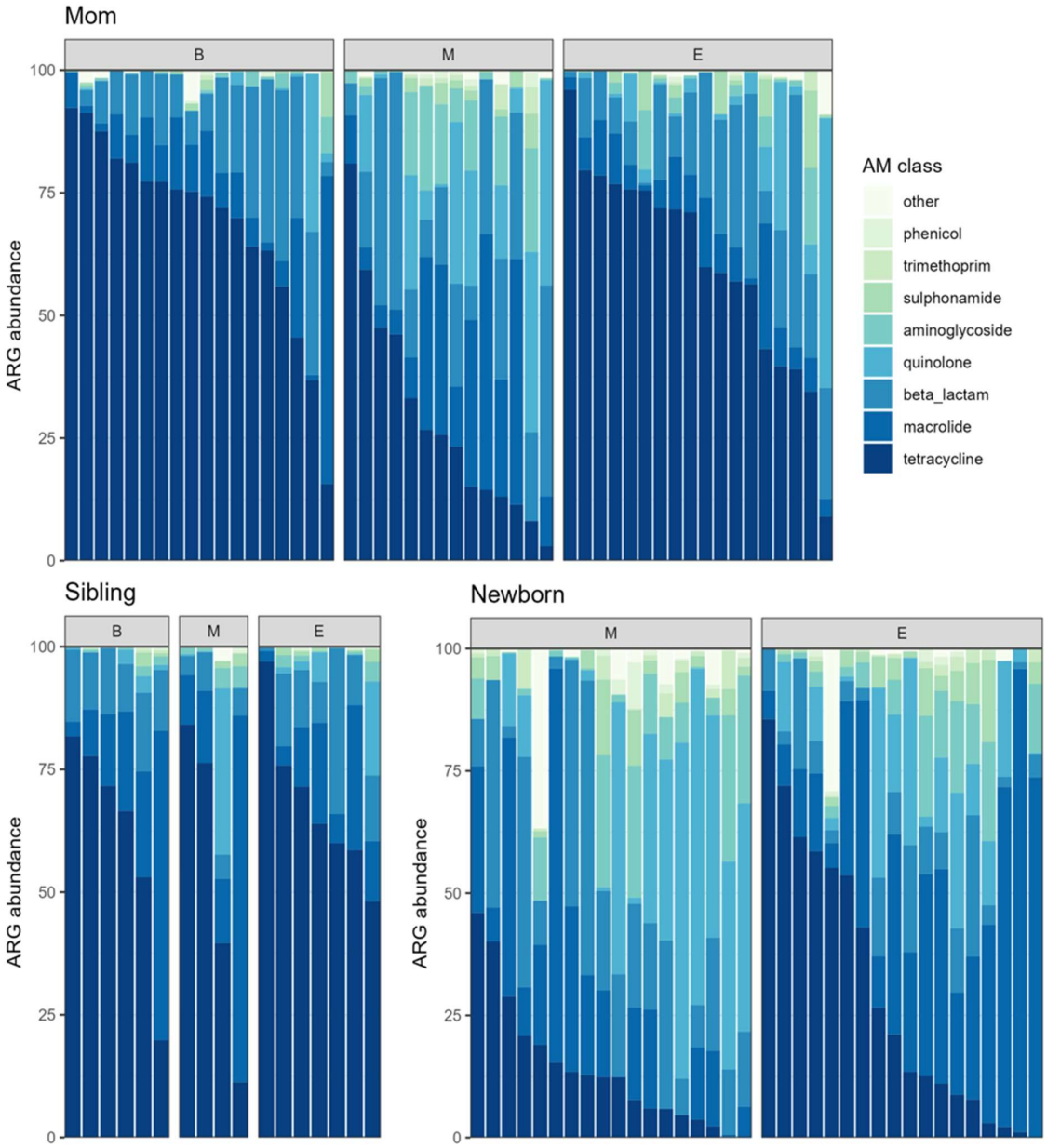

Figure 3: *Relative abundance of ARGs for antibiotic classes in human microbiomes, per sample type and timepoint. Each bar on the x-axis represents one individual sample. Y-axis shows the relative abundance (%) per antibiotic class, as calculated by: FPKM counts for class in sample/total FPKM counts in sample*100%. Timepoints are abbreviated, where B= baseline, M= midline, and E= endline. Different colours represent the antibiotic classes, where the 8 most abundant classes in the whole dataset are shown, and the remaining classes are collapsed into category "other".*

The prevalence of ARGs that confer resistance to different antimicroial classes was also analysed for the animal samples in the dataset. The high abundance of tetracycline resistance genes was apparent for most animal gut microbiomes (figure 4). Cow samples seemed to be an exception, where macrolide ARGs were often more abundant than tetracycline ARGs, and beta lactam ARGs also seemed more prevalent compared to other animal samples.

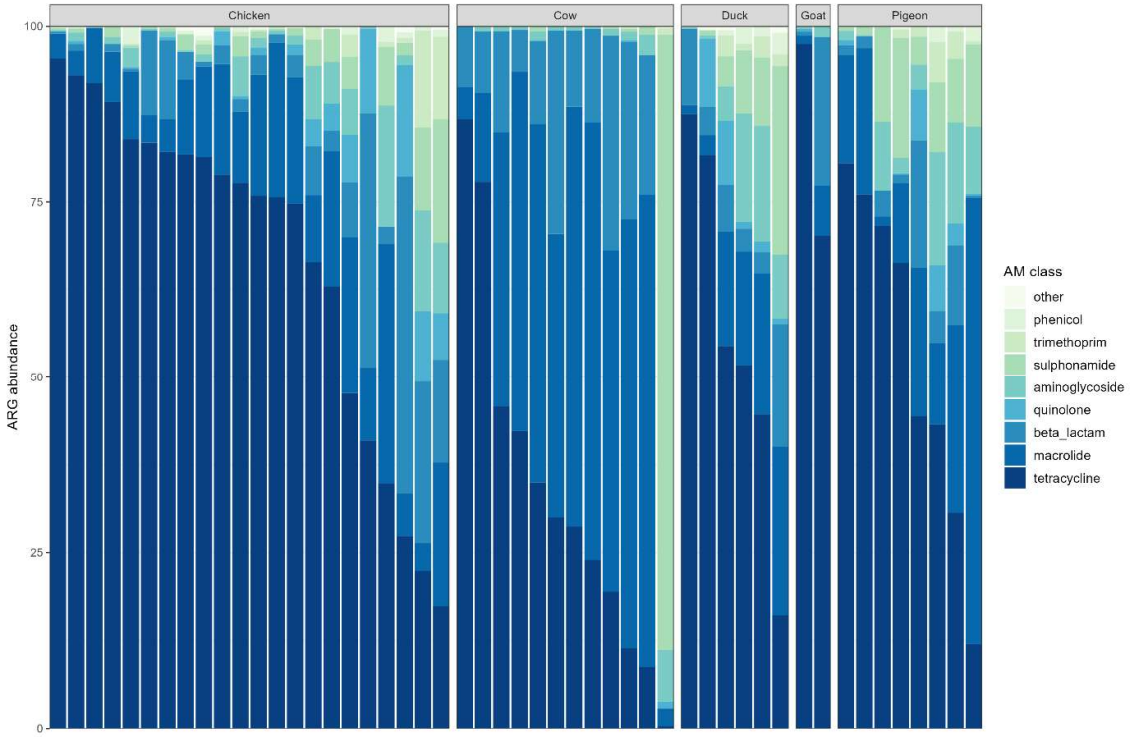

Figure 4: *Relative abundance of ARGs for antibiotic classes in animal microbiomes, per sample type. Each bar on the x-axis represents one individual sample. Y-axis shows the relative abundance (%) per antibiotic class, as calculated by: FPKM counts for class in sample/total FPKM counts in sample*100%. Different colours represent the antibiotic classes, where the 8 most abundant classes in the whole dataset are shown, and the remaining classes are collapsed into category "other".*

Alpha diversity

Since sample types had different prevalences and distributions of ARGs on class level, it was assessed if the alpha diversity of the resistome would differ between sample types. For this, alpha diversity measures for the different sample types were calculated (figure 5)

Chicken microbiomes contained a higher number of observed individual ARGs (mean +- SEM= 76.38 +- 6.89) compared to microbiomes of mothers (55.44 +- 2.79) and newborns (50.89 +- 2.79) (Dunn's test with BH correction, adjusted p-value= 0.03 and 0.01, respectively). The number of observed ARGs was also higher in ducks (96.67 +- 16.79) compared to newborn samples (Dunn's test with BH correction, adjusted p-value= 0.02). Cow resistomes contained less observed ARGs (35.42 +- 4.84) than resistomes of mothers, siblings, chickens, and ducks (Dunn's test with BH correction, adjusted p-value= 0.02, 0.02, 0.0002, and 0.001, respectively). Goat resistomes contained a relatively low number of observed individual ARGs. However, it should be noted that there were only a few samples taken for this group, and therefore these results might not be as reliable.
For the Shannon diversity measure, only a few differences between alpha diversities in sample types were found, and no differences were found in the Simpson diversities of sample types (figure 5).

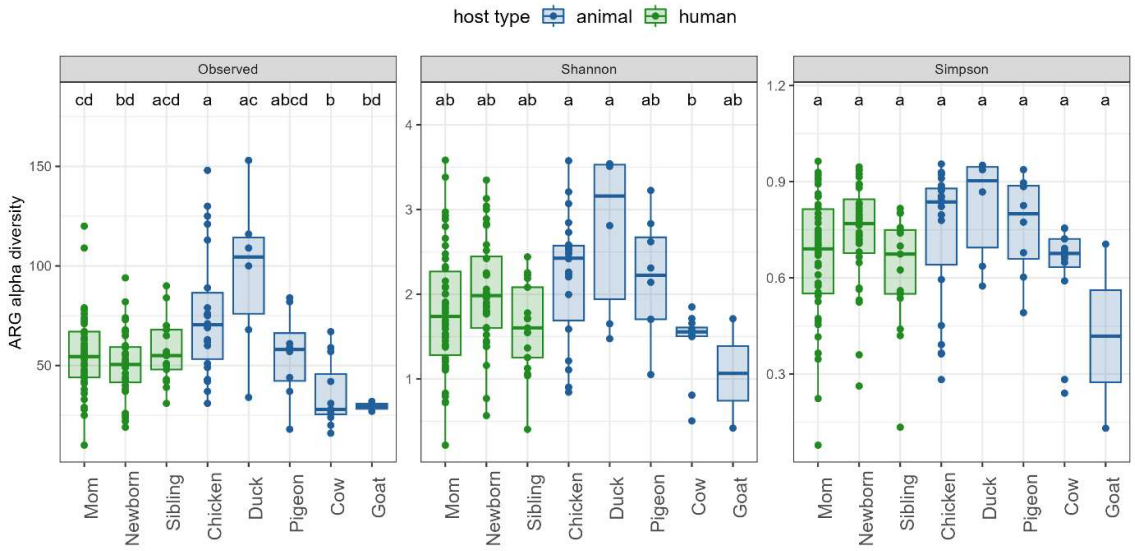

Figure 5: *Observed number of unique ARGs (richness), Shannon, and Simpson diversity measures for the resistomes found in different sample types. A Kruskal-Wallis test was conducted on the alpha diversity measures and the sample type, which yielded significant results for all three diversity measures (p value= 5.21e-05, 0.002, and 0.01, respectively). To test for differences between individual groups, Dunn's test with a BH post-hoc correction was used to compare all groups with each other within one diversity measure. Significant results from the Dunn's test are indicated by letters above the boxplots, where diversity measures of groups with the same letter do not significantly differ (adjusted p-value > 0.05). Green and blue coloured boxplots indicate samples from human and animal hosts, respectively.*

Since figure 3 and the results of the DESeq2 analysis on class level showed differences in resistome composition between human sample types, we decided to look further into the alpha diversity of the human resistome specifically. To test whether the abundance and alpha diversity of ARGs differed between human sample types and timepoints, two-way ANOVA's were conducted on sample type, timepoint, or an interaction of these two factors (value ~ sample type + timepoint + sample type * timepoint). This was done separately for the log transformed ARG FPKM counts, ARG richness, and the Shannon and Simpson diversity measures. For all four, no interaction effect between sample type and timepoint was found (two-way ANOVA, p-value= 0.75, 0.44, 0.12, and 0.31, respectively). For the ARG counts, no effect of sample type or timepoint was found (two way-ANOVA, p-value= 0.73 and 0.58, respectively). This was also true for the number of observed unique ARGs (richness) (two-way ANOVA, p-value= 0.38 and 0.11, respectively).

Shannon alpha diversity did differ between sample types (two-way ANOVA, p-value= 0.049). Thus, pairwise comparisons were conducted between all sample types (figure 6C). Shannon diversity was lower in siblings (mean +- SEM= 1.61 +- 0.13) compared to newborns (2.04 +- 0.11). Furthermore, timepoint also had a significant effect on Shannon diversity (two-way ANOVA, p-value= 0.008). Shannon diversity of human samples had a value of 1.54 +- 0.09 at baseline, and increased to 2.14 +- 0.11 at midline. In endline human samples, Shannon diversity decreased compared to baseline, to 1.77 +- 0.11. Since Shannon diversity in human samples at baseline and endline did not differ, it can be concluded that the Shannon diversity increases in human samples at midline, and at endline has returned to its original levels.
A similar pattern is observable for the Simpson diversity index, where the diversity measure also differed between timepoints (two-way ANOVA, p-value= 0.02). The Simpson diversity increased from 0.61 +- 0.03 at baseline to 0.76 +- 0.02 at midline, and then decreased at endline to 0.66 +- 0.03. The endline Simpson diversity was not different than at baseline. (figure 6D)
Thus, Simpson and Shannon diversity in human resistomes peak around midline, which is around the time of giving birth, and then recover to "normal" levels in endline samples (figure 6).

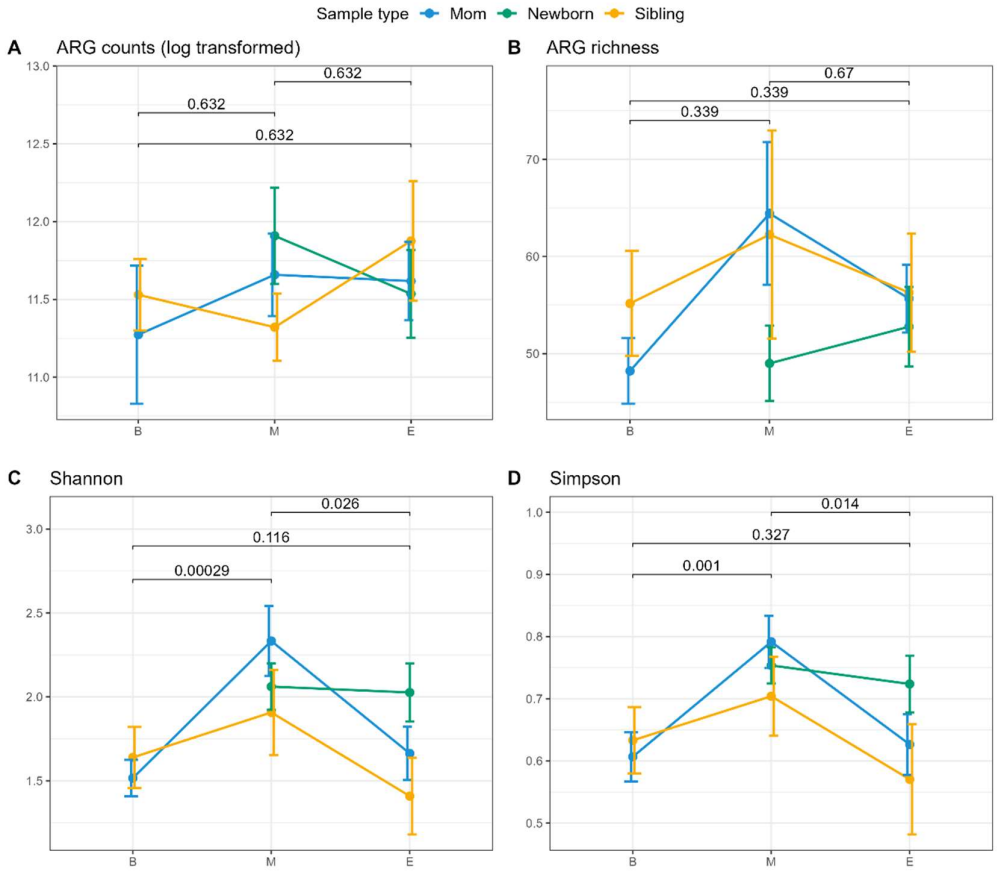

Figure 6: *Mean values and SEM (shown by the error bars) of* ***A)*** *ARG counts (log transformed FPKM values),* ***B)*** *ARG richness,* ***C)*** *Shannon diversity, and* ***D)*** *Simpson diversity, at different timepoints. Timepoints are abbreviated, where B= baseline, M= midline, and E= endline. Adjusted p-values of pairwise comparisons between all human samples at each timepoint are shown, which resulted from performing t-tests with a BH post-hoc correction for the four measures separately. Blue lines and dots indicate mother samples, green indicates newborn samples, and yellow indicates sibling samples.*

Beta diversity

To determine dissimilarity in resistome composition between different samples, Bray-Curtis distances were first calculated between the ARG counts (FPKM) in all samples. Subsequently, an NMDS ordination in three dimensions was conducted on the dissimilarity matrix (figure S3). The ordination shows a separation of sample types on the first NMDS axis (figure S4). Cow and goat samples clustered together, as did chicken and duck samples. Permutational Analysis Of Variance (PERMANOVA) on Bray-Curtis distances was used to determine if sample type, timepoint, household number, or the interaction between these factors had a significant effect on the resistome composition. All three individual factors affected ARG composition (table S1). The effect of household number on resistome composition may be influenced by variation in the composition of households, where not all households had animals or siblings present. The interaction effect of household and sample type on resistome composition was also significant (table S1), indicating that there might be a household specific effect of sample type. However, an important note is that the assumption of homogeneity of variances was not met for the factors sample type and timepoint (function “betadisper”, $p < 0.05$) (Anderson, 2006). As a consequence, the PERMANOVA results may not be reliable, especially considering the unbalanced design of the dataset (Anderson & Walsh, 2013).

Since the composition of the resistome on class level and the ARG alpha diversity were different for human samples taken at different timepoints, beta diversity analyses were performed for a subset of the data that contained all human samples. This allowed for a more detailed analysis on the effect of sample type and timepoint on the human resistome composition on ARG level. In a similar manner as described above, an NMDS ordination was made on Bray-Curtis dissimilarities of ARG counts (FPKM) for human samples only (figure 7, figure S5).
Here, it was clear that on the first NMDS axis, mother and sibling samples clustered together and newborn samples were separated from the other human samples. Moreover, it became apparent that mother samples taken at midline were less similar to mother samples taken at base- or endline, and were more similar to newborn samples. Within the newborn samples, a slight effect of time can be observed, where newborn endline samples become more similar to mother and sibling samples compared to newborn midline samples. This again suggests that 1) the resistome of mothers is impacted after giving birth, but recovers at endline, and 2) resistomes in newborns could develop with age to a similar composition as seen in mother and sibling samples. These two noticeable patterns in the NMDS ordination correspond to the patterns at antibiotic class level seen in human samples (figure 3), and to the results of the alpha diversity analysis (figure 6).

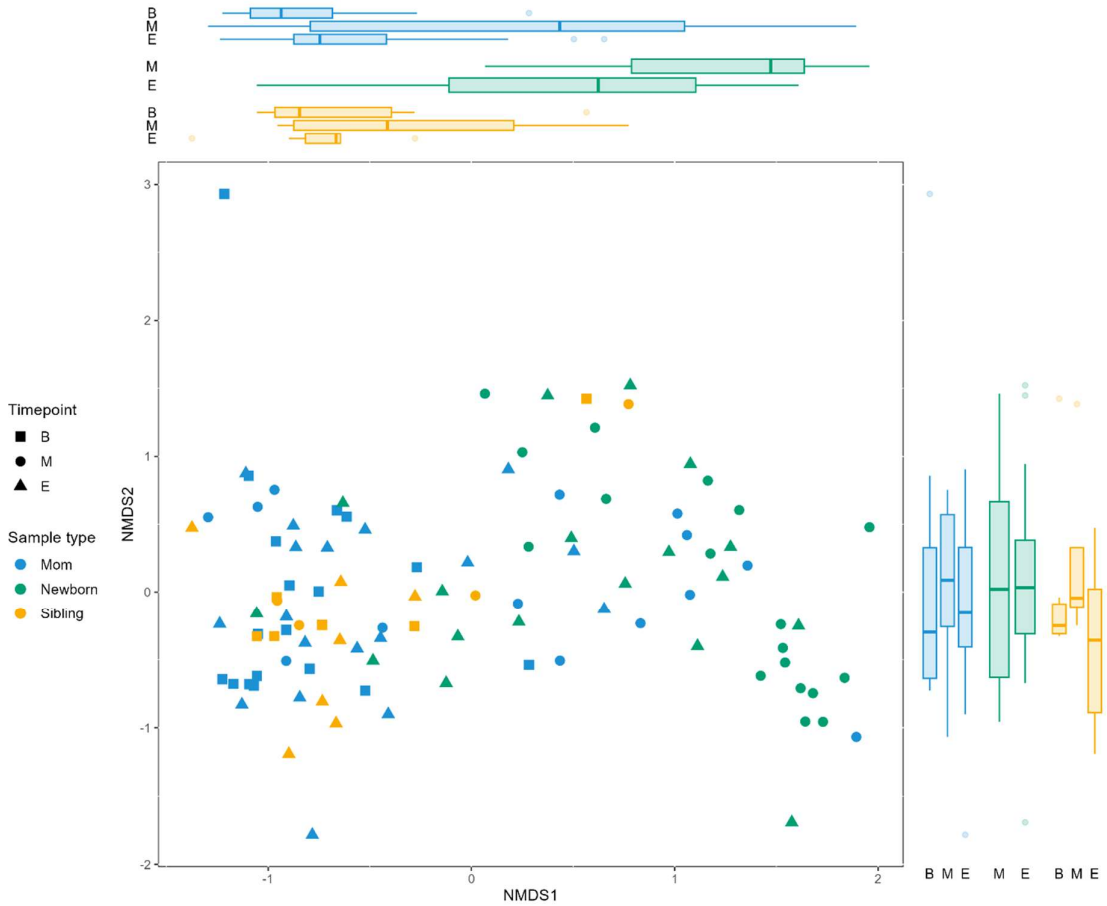

Figure 7: *First two axis of the NMDS ordination on Bray-Curtis dissimilarities between ARG counts (FPKM) in human samples. Each point represents one sample, where sample type is indicated by the colour of the points, and the timepoint is indicated by the shape. Timepoints are abbreviated, where B= baseline, M= midline, and E= endline. The boxplots show the distribution of values on the first two NMDS axes for different sample types.*

Next, a PERMANOVA was conducted on sample type, timepoint, household, and interactions between these factors and the Bray-Curtis distances between ARG counts (FPKM) in human samples. Additionally, antibiotic usage was taken into account. For every human sample, it was noted if the person had used any antibiotics at

the time when the sample was collected (yes/no). The PERMANOVA on the subset with human samples showed similar results to the one that was performed on the whole dataset. Sample type, timepoint, and household number all affected resistome composition, and the interaction between household and sample type was also significant (table 2). Antibiotic usage did not influence the human resistome composition, and there was no interaction effect of antibiotic usage and sample type. In the subset with human samples, the dispersions of the sample types and timepoints again were not equal (function "betadisper", $p < 0.05$) (Anderson, 2006). This means that the significant results from the PERMANOVA test might be due to differences in variance in groups instead of differences in distances between the groups. Combined with the patterns seen in the NMDS plot, this analysis however provides an indication that household, sample type, and timepoint influence the human gut resistome, and that therefore, these factors will be included when modelling the variance in the dataset in further analysis.

Table 2: *Results of the PERMANOVA that was carried out on the Bray-Curtis dissimilarities between ARG counts (FPKM) in human samples. *** p-value < 0.001, ** p-value < 0.01, * p-value < 0.05.*

Factor	R2	p-value
Sample type	0.11	0.001 ***
Timepoint	0.05	0.001 ***
Household	0.19	0.011 *
Antibiotic usage	0.01	0.334
Sample type : timepoint	0.03	0.074
Household : sample type	0.23	0.034 *
Household : timepoint	0.26	0.291
Antibiotic usage : sample type	0.02	0.358

Differential abundance analysis

The NMDS and PERMANOVA analyses showed that the resistome composition was influenced by sample type and timepoint. To determine which ARGs are more or less prevalent at specific timepoints or in specific human sample types, differential abundance analysis was performed with DESeq2. Since the PERMANOVA indicated a potential interaction between household number and sample type, the likelihood ratio test (LRT) offered by DESeq2 was used to assess household-specific effects of sample type on the resistome composition (Love et al., 2015). This showed that no ARGs had household-specific changes in abundance in sample types. Therefore, the differential abundance analysis was continued using the default Wald test in DESeq2. ARG counts for all sample types within one timepoint were compared, and counts for all timepoints for each sample type were compared (table 3). Differently abundant genes (DAGs) were filtered on significance (adjusted p-value < 0.05) and on prevalence (see Methods section).

The largest number of DAGs was found in the comparisons between mother samples at midline versus mother samples at baseline, where 40 DAGs were identified. Notably, 32 of the 40 DAGs (80%) in this comparison became more abundant at midline (genes with a log2 fold change > 0). Only a small number of DAGs were found in the comparisons between siblings and mothers. In the comparison between mothers and siblings at midline, 5 DAGs were found, while no DAGs were found in the same comparison at the other timepoints. A similarly low number of DAG were found in the comparisons between siblings at different timepoints. No DAGs were found when comparing siblings at endline versus baseline, and only 1 and 3 DAGs were found in sibling_E vs sibling_M and the sibling_M vs sibling_B comparisons, respectively. These patterns concur with the class abundance and NMDS plots, where the sibling resistomes appear similar to each other and to mother samples (figure 3, 7).

***Table 3**: Total number of differently abundant genes (DAGs) in each comparison as determined using DESeq2, and the number of increasing and decreasing DAGs per comparison (DAGs with positive and negative log2 fold changes, respectively). DAGs are filtered on significance (adjusted p-value < 0.05), and on prevalence (see Methods section). The group that is mentioned second in the name of the comparison was set as the reference group. Timepoints are abbreviated, where B= baseline, M= midline, and E= endline.*

Comparison	Total nr. of DAGs	Nr. increased DAGs	Nr. decreased DAGs
Sibling_B vs Mom_B	0	0	0
Sibling_M vs Mom_M	5	1	4
Sibling_E vs Mom_E	0	0	0
Newborn_M vs Mom_M	21	8	13
Newborn_E vs Mom_E	25	14	11
Newborn_M vs Sibling_M	22	8	14
Newborn_E vs Sibling_E	18	10	8
Sibling_M vs Sibling_B	3	3	0
Sibling_E vs Sibling_B	0	0	0
Sibling_E vs Sibling_M	1	0	1
Newborn_E vs Newborn_M	24	13	11
Mom_M vs Mom_B	40	32	8
Mom_E vs Mom_B	11	11	0
Mom_E vs Mom_M	29	5	24

In mothers at midline, 32 genes were more abundant compared to mothers at baseline. Among these were multiple aminoglycoside resistance genes, such as the ARGs with the highest log2 fold change: aac(6')-Ii (log2fc= 26.83), aac(6')-Ib (log2fc= 26.25), and aac(3)-IIa (log2fc= 13.88) (figure 9A). This strong increase in several aminoglycoside ARGs is in accordance with the DESeq2 results on class level, which showed an increase in aminoglycoside genes in mother samples at midline compared to baseline (figure 3, S1).
Notably, many of the ARGs that increase in abundance in mother samples at midline compared to baseline, decrease again in abundance in mother samples at endline (figure 9). These are ARGs that have increased in mothers after giving birth.
Out of the 32 ARGs that increase in abundance at midline, 22 decrease significantly at endline compared to midline (table S2). These genes might underlie the patterns seen in the alpha diversity plots for mother samples and the NMDS plot, where midline samples differed in composition and diversity compared to baseline samples. Endline mother resistomes appeared to recover, and were similar in composition and alpha diversity to those at baseline. This could thus be caused by the decrease of the genes that increased in abundance at midline. Additionally, there are 6 ARGs that increase at midline, and then remain high in abundance until endline, since these genes still have a positive log2 fold change in the Mom_E vs Mom_B comparison (figure 9C). These 6 ARGs that remain high in abundance in mothers at endline were blaSHV-2a, aph(6)-Id, qnrS1, OqxA, OqxB, and fosA5. For the remaining 4 ARGs that increase in abundance at midline, it cannot be said that they either decrease or remain highly abundant, since they are not significantly differently abundant in the Mom_E vs Mom_M or the Mom_E vs Mom_B comparisons (table S2).

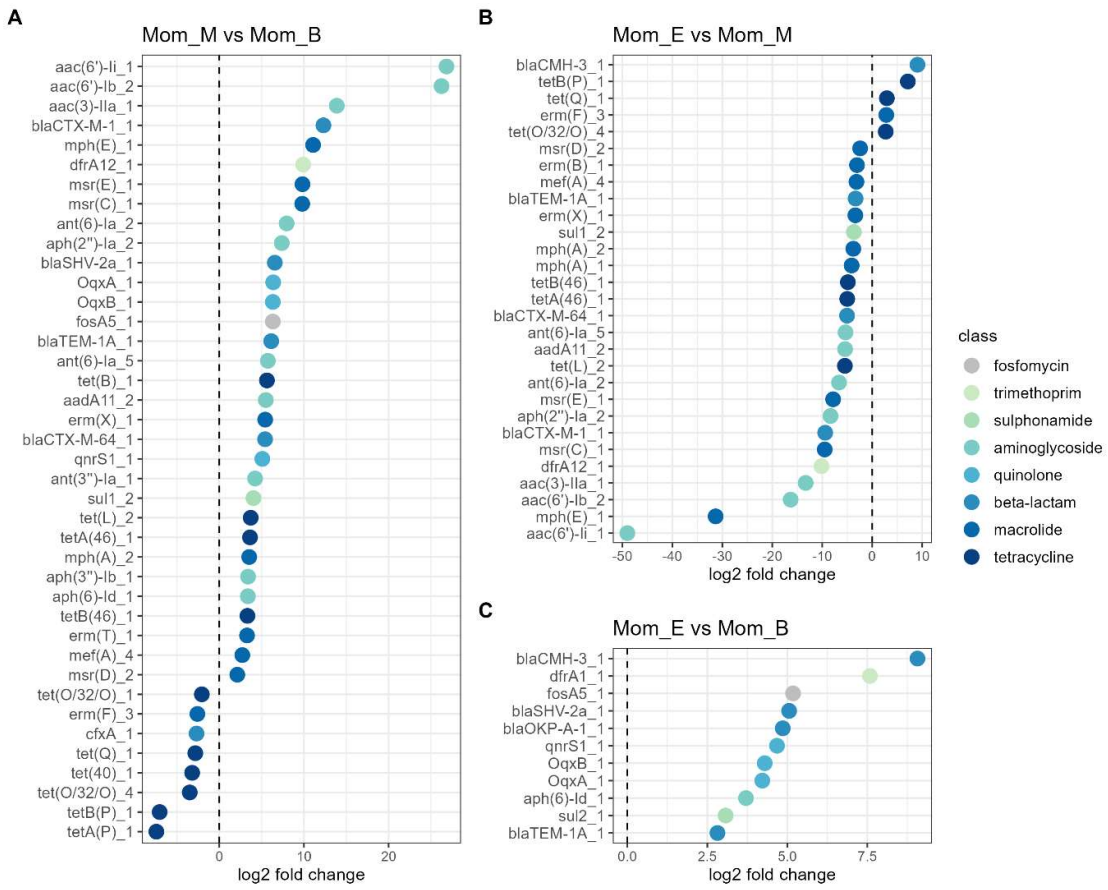

***Figure 8**: Significantly (adjusted p-value < 0.05) differently abundant ARGs in comparisons between mother groups, as determined by using DESeq2. Log2 fold changes of ARGs are shown on the x-axis, where a log2 fold change of 0 is marked by the dashed line. Genes are coloured based on the class of antibiotics that they confer resistance to. In **A-C**, the group that is mentioned second in the name of the comparison was set as the reference group. Timepoints are abbreviated, where B= baseline, M= midline, and E= endline.*

In newborns, ARGs that changed in abundance over time (at endline vs midline) mostly conferred resistance to macrolides, tetracyclines, or aminoglycosides (figure 9). When looking at the relative abundance of antibiotic classes (figure 3), it appeared that macrolide, tetracycline, and aminoglycoside resistance was more abundant in mothers and siblings than in newborns, and that resistance to these classes increased in newborns over time. However, these three classes were not significantly differently abundant in the class level comparison between newborns at endline vs midline, or when comparing newborns at midline with mothers at midline (figure S2). For macrolide and tetracycline ARGs, this might be caused by contrasting patterns in individual ARGs. For example, most of the differently abundant tetracycline ARGs increase in newborns at endline compared to midline, except tet(M) and tet(K). This pattern is also seen when comparing newborns to mothers (figure S6), where tet(K) is more abundant in newborns, while all other differently abundant tetracycline genes are more abundant in mothers. This indicates that generally, tetracycline ARGs are less abundant in newborns at midline, but tet(K) specifically increases in these samples. Aminoglycoside resistance genes show a different pattern in newborns over time, since all differently abundant aminoglycoside genes decrease in newborns at endline compared to midline (figure 9). Notably, the same set of aminoglycoside ARGs were more abundant in mothers at midline, and decreased in mothers at endline (figure 8). Thus, specific aminoglycoside ARGs accumulate in both mothers and newborns at midline. This suggests that these ARGs might be transmitted from mother to newborns when giving birth, or that mothers and newborns are exposed to the same ARGs, for example because these ARGs are prevalent in hospitals.

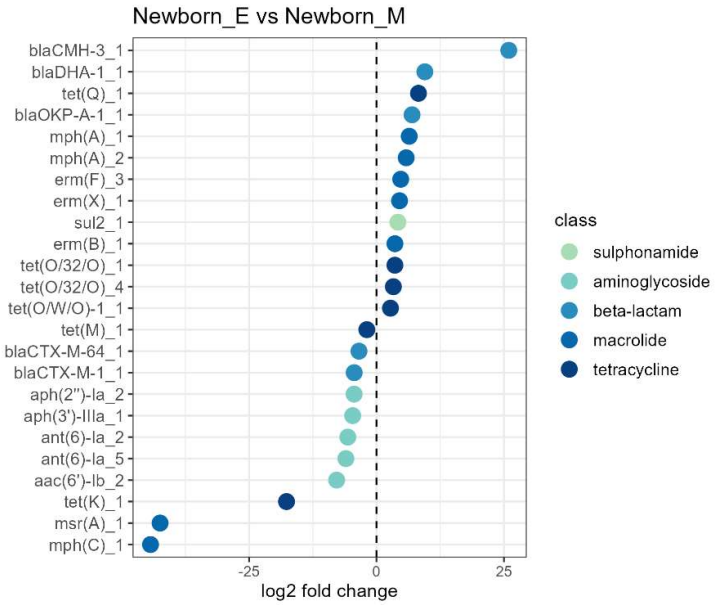

Figure 9: *Significantly (adjusted p-value < 0.05) differently abundant ARGs in newborns at endline (E) compared to midline (M), as determined by using DESeq2. Log2 fold changes of ARGs are shown on the x-axis, where a log2 fold change of 0 is marked by the dashed line. Genes are coloured based on the class of antibiotics that they confer resistance to.*

Effect of antibiotic use on the human resistome

The PERMANOVA analysis on human samples indicated that there was no effect of antibiotic usage on the resistome composition of human samples. However, antibiotic usage was not equally frequent in every sample type or at every timepoint, which might explain why general effects on the human resistome are hard to identify. Therefore, the effect of antibiotic usage on ARG counts and diversity was analysed in more detail.

Antibiotic usage was highest in mothers, especially at midline. Almost 65% of mothers (9/14) had used antibiotics in a time period of a week prior to the sampling at midline. This could be because mothers were administered intrapartum antibiotic prophylaxis. Mothers who gave birth by caesarean section were more common (10/14) in the dataset than mothers who gave birth vaginally (4/14). Out of the 10 mothers who had caesarean sections, 8 had used antibiotics in the week before the sampling at midline. For the mothers who gave birth vaginally, only 1 out of 4 had used antibiotics at midline. Surprisingly, antibiotic usage at endline was mostly attributed to newborns (approximately 28%, or 5/18) and siblings (approximately 14%, or 1/7).

To assess whether antibiotic usage could be a driver of the differences in resistome composition and alpha diversity in human samples, we classified within timepoint antibiotic usage for each sample, e.g. if any antibiotic use was noted for a sample at the timepoint of sampling (yes/no). ARG counts and several measures of alpha diversity did not differ between people who did and did not use antibiotics (figure S7). However, since antibiotic use was higher in mothers, especially at midline, the same analysis was performed using only mother samples (figure 10). This showed that ARG richness at midline was higher in mothers who used any antibiotics at midline (74.44 +- 8.93), compared to those who did not (46.4 +- 8.81) (t-test, df= 10.8, p-value= 0.048). Thus, Shannon and Simpson diversity were not affected by antibiotic use in mothers, and no effects on ARG abundance and diversity were seen when looking at all human samples combined. Within timepoint antibiotic use therefore does not seem to be able to explain the increase in alpha diversity in human samples at midline compared to base- and endline (figure 6). This appears to be in line with the result from the PERMANOVA analysis, where it was found that within timepoint antibiotic usage does not influence the human resistome composition (table 2).

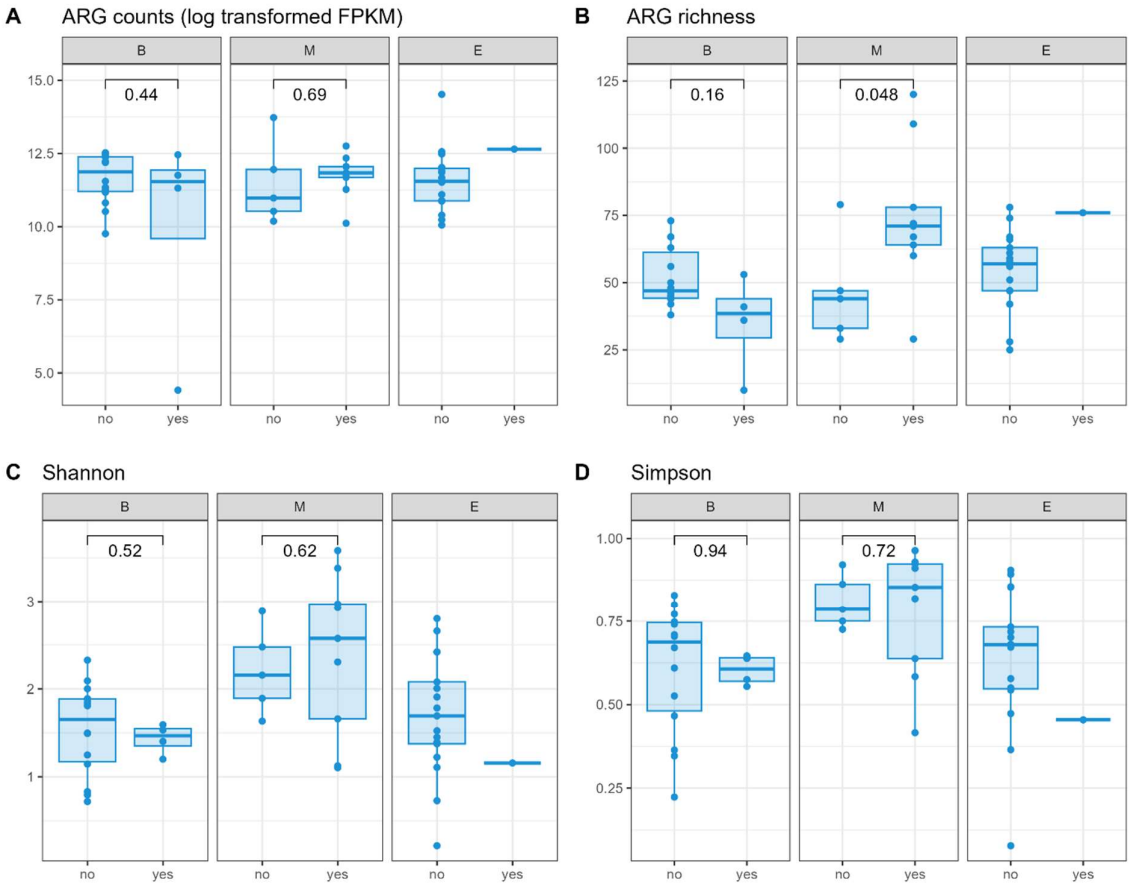

Figure 10: *ARG abundance and alpha diversity of mothers for whom no or any antibiotic use was registered for the timepoint at which the sample was taken.* ***A)*** *ARG counts (log transformed FPKM values),* ***B)*** *ARG richness,* ***C)*** *Shannon diversity, and* ***D)*** *Simpson diversity, at different timepoints. Timepoints are abbreviated, where B= baseline, M= midline, and E= endline. Depicted p-values were obtained by performing two-sided t-tests on the value of the different measures and the within timepoint antibiotic use (value ~ AB usage), which was done separately for each timepoint.*

However, looking at general antibiotic usage, e.g. if a person has used any kind of antibiotics, might hide possible effects of the usage of specific antibiotics on ARG counts that confer resistance to the used type of antibiotics. Therefore, we also looked into which antibiotics were administered to mothers at midline. 10 out of 14 mothers (53%) had used beta lactams at midline, 13 (68%) had used nitroimidazoles, and 1 mother (5%) had used aminoglycosides. While nitroimidazole usage was highest, only 4 out of 14 mother midline samples contained any nitroimidazole ARG counts. Out of these 4 mothers at midline where nitroimidazole ARGs were found, only 2 had used nitroimidazoles. Thus, there did not seem to be a correlation between nitroimidazole usage and the number of nitroimidazole ARG counts in mother samples at midline. Beta lactam resistance genes were more prevalent in the resistomes of mothers at midline, since all 14 samples had beta lactam ARG counts. Therefore, the number of beta lactam ARG counts was compared between mothers who did or did not use beta lactams at midline. However, beta lactam use at midline did not influence beta lactam ARG counts in mother resistomes at midline (two-sided t-test, p-value= 0.81).

Since the previous results from the differential abundance analysis showed that individual ARGs within one class could display opposite dynamics over time, it could be a possibility that only specific ARGs increase in abundance in mothers at midline who used antibiotics. Therefore, a differential abundance analysis was performed on a small subset of the data that only contained mothers at midline. Mothers who used any antibiotics were compared to those who did not. This analysis yielded only 6 differentially abundant genes, that all increased in abundance in mothers who used antibiotics (table S3). Most of these ARGs were also

significantly more abundant in mothers at midline compared to baseline. Thus, their increased abundance in mothers at midline might be caused by antibiotic usage. However, it cannot be excluded that other factors that might influence the resistome of mothers around the time of giving birth also have an effect on this set of ARGs.

Notably, the beta lactam resistance gene blaNDM-19 was significantly enriched in mothers at midline who used antibiotics, but not in the comparison between all mothers at midline vs mothers at baseline. This suggests an effect of antibiotic usage specifically on the abundance of blaNDM-19, since the gene was not prevalent in mothers at midline who did not use antibiotics (figure S8). Of these 5 mothers who did not use antibiotics, none had counts for blaNDM-19. On the other hand, 6 out of 9 mothers who used antibiotics at midline had counts for blaNDM-19. Of these 6 mothers, 5 had used both nitroimidazoles and beta lactams, and 1 had used only nitroimidazoles. None of the mothers at midline had used only beta lactams, which makes it difficult to assess whether the increase in blaNDM-19 counts in mothers at midline who used antibiotics was due to nitroimidazole or beta lactam use.

Correlation between the resistome and the microbiome

As mentioned previously, newborn microbiomes and resistomes still have to develop with time and thus are more easily influenced by external factors just after birth, which is at midline in our data. One could argue that the microbiomes of mothers might also be perturbed around the time of giving birth because of exposure to bacteria in the hospital, antibiotic treatment, and stress. This could be a possible explanation of the observed changes in the mother's resistome around midline. Interestingly, analysis of the gut microbiome of people in our dataset showed similar patterns to the resistome analysis. Microbiomes of mother at midline were different in composition compared to mothers at baseline, and recovered again at endline. Additionally, microbiomes of newborns at baseline differed from the other human microbiomes, and converged towards mother and sibling microbiomes at endline. Similar to the resistome analysis, no clear effect was found of antibiotic usage on the microbiome composition. Because of the recurring patterns in the microbiome and the resistome data, it was assessed if these could be linked. Procrustes analysis showed that the microbiome and resistome composition of human guts were correlated (p-value= 0.001, r= 0.595) (figure S9), meaning that samples with similar microbiomes were likely to also have similar resistomes.

Separate networks were made on a subset of the data that only consisted of mother samples. As input, all microbial taxa and the ARGs that were differently abundant in any of the comparisons between mothers were used (figure 11). Large clusters appeared around erm(F), cfxA, and tet(Q), which are ARGs that decreased in abundance in mothers at midline compared to baseline (figure 8). 19 bacterial genera correlated with more than one of these three ARGs, suggesting that changes in their prevalence might underlie the decrease in multiple associated ARGs. However, only 6 of these taxa were significantly differently abundant in the comparison between microbiomes of mothers at midline and mothers at baseline. These were *Lachnospira, Coprococcus, Simiaoa, Paramuribaculum, Pseudoprevotella,* and *Odoribacter*, which were less abundant in mothers at midline. The decreased prevalence of these taxa might thus explain the simultaneous decrease in erm(F), cfxA, and tet(Q). Moreover, in the network made with DAGs and all taxa in mother samples, *Klebsiella* correlated with blaSHV-2a, fosA5, OqxA, and OqxB, and these genes all correlated with each other as well. This points to the presence of *Klebsiella* bacteria that harbour multiple resistance genes in the guts of mothers. Lastly, the tetracycline genes tetA(46) and tetB(46) were associated with Streptococcus.

The network constructed using all ARGs but only differentially abundant bacterial genera in the mother microbiome comparisons yielded partly similar results (figure S11). Many differently abundant bacteria correlated with erm(F), tet(Q), cfxA, and additionally, cfxA6. In the network with differently abundant genera, the same *Klebsiella* and *Streptococcus* clusters appeared as in the network with differently abundant ARGs, strengthening the result that these ARG-carrying bacteria increase in abundance in mothers at midline. Furthermore, several differently abundant genera were associated with the aminoglycoside resistance gene ant(6)-Ia.

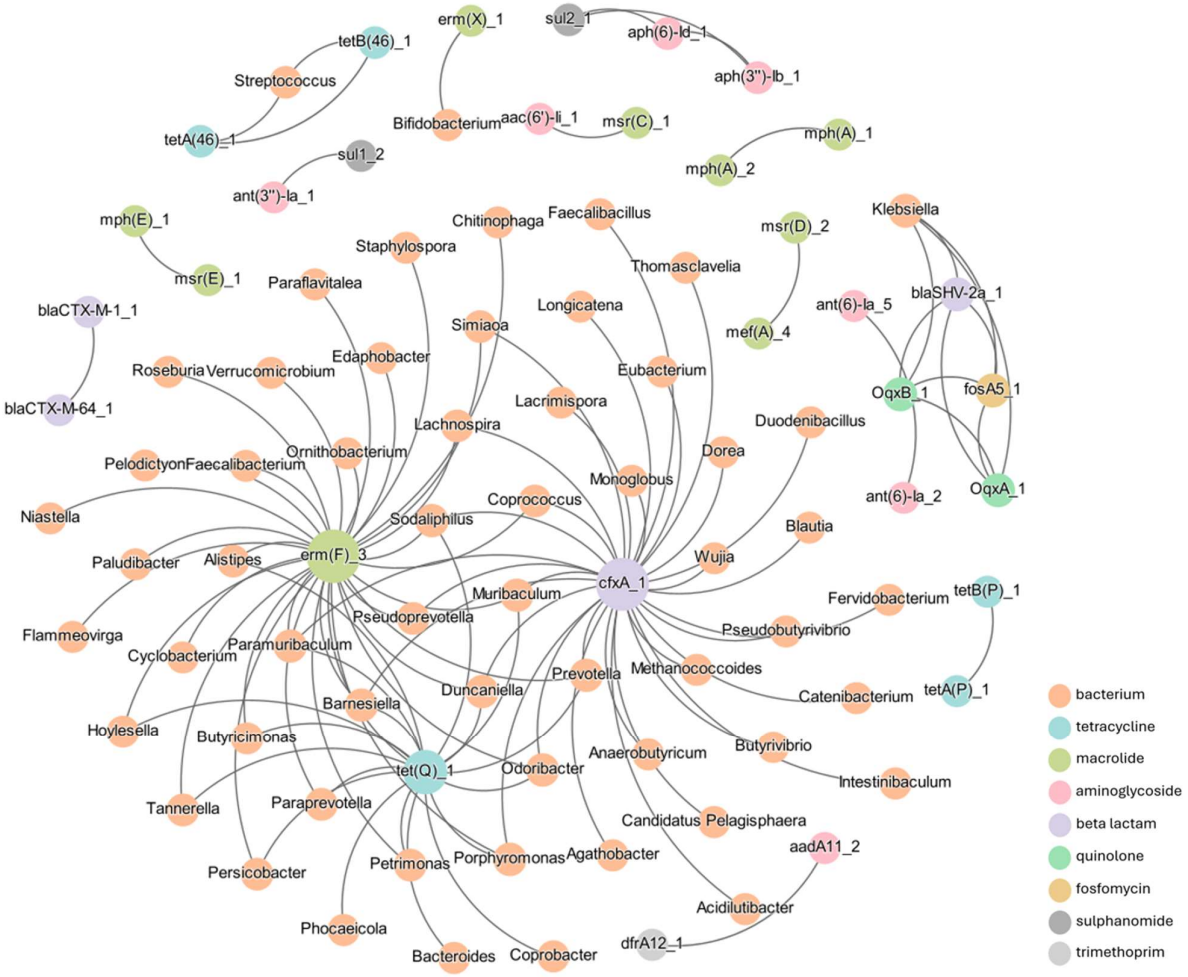

Figure 11: *Fruchterman-Reingold representation of the co-occurrences between differently abundant ARGs and microbial taxa in mother samples. ARG-ARG and ARG-bacteria co-occurrences were determined by calculating Spearman's correlations between ARG and bacterial counts. Correlations were filtered on significance (BH adjusted p-value < 0.01) and robustness (rho > 0.7). Correlations that included tet(40) or tet(O/32/O) were significant in this network, but were filtered out. Node size represents the degree of connectedness, and node colour represents the different antimicrobial classes or a bacterial genus.*

The same co-occurrence analysis was done with a subset that contained only newborn samples. Similar to the co-occurrence networks made for mothers, large clusters of bacteria appeared around erm(F) and tet(Q) (figure S12B). Additionally, a clusters of aminoglycoside genes was visible, which consisted of aph(3')-IIIa and two separate clusters of ant(6)-Ia (which could be the same gene). These three genes all were less abundant in newborns at endline compared to midline.

Secondly, a network was made using all ARGs but only the bacterial genera that were differently abundant in newborns at endline compared to midline (figure S13). This showed similarities with the network with differentially abundant genera that was made for the mother samples. Many differentially abundant bacterial genera in newborn samples contained erm(F), tet(Q), cfxA, or cfxA6. The latter two were not differently abundant in the comparison between newborns at endline vs midline, which seems to contradict the finding that their abundance correlates with several differently abundant bacterial genera.

DISCUSSION

In this study, the gut resistomes of animals and humans in households in rural Bangladesh were characterized. Samples from mothers that were taken within a week after giving birth were distinct from mother samples that were taken during the third trimester. While ARG abundance did not change in mothers around the time of giving birth, the resistome composition and the alpha diversity did change. This could be caused by the several ARGs that increased in abundance in mothers at midline. 3-4 months after giving birth, the resistome of mothers recovered to look similar to the baseline samples. Resistomes of newborns were different from the other human samples. Newborn resistomes developed over time, meaning that the alpha diversity decreased towards endline and that the newborn resistome became more similar to the other human samples. Lastly, ARGs that were differently abundant in mothers or newborns over time were mostly associated with the same bacterial genera, with a large cluster appearing that consisted of multiple ARGs and the *Klebsiella* genus.

In our Bangladesh resistome data, ARGs that confer resistance against tetracycline were the most abundant, followed by macrolide and beta lactam. These antibiotic classes are often reported among the most abundant classes in resistome data for humans as well as for livestock, although the exact degrees of class prevalence can vary (Bargheet et al., 2023; Feng et al., 2018; Li et al., 2023; Ma et al., 2021; Mahmud et al., 2024). This shows that while the people in this Bangladesh study live in different circumstances compared to other studies, a similar ARG profile can be found, where mostly tetracycline ARGs are the dominant class in human and animal gut resistomes.

Contrasting to previous findings, the ARG abundance and diversity was similar in the resistomes of different animals samples in our data. Other studies reported a higher abundance of ARGs in poultry microbiomes compared to ruminants (Lawther et al., 2022; Qiu et al., 2022). The authors argued that this could be attributed to the high usage of AB in intensive poultry farming (Lawther et al., 2022). This might explain why ARG abundance and diversity is not higher in chicken resistomes in our data compared to other sample types. Farming methods in rural Bangladesh are not identical to those in industrialized countries, and animals are mostly treated with AB only when they are sick (Roess et al., 2013). Indeed, it has been reported that ARGs are less abundant in chickens that are kept around the house, compared to those raised in small-scale chicken production farms (Guo et al., 2018). In this study in Ecuador, household chickens fed on food scraps and were not treated with antibiotics. This is highly similar to how most chickens in our Bangladesh dataset were kept. Therefore, it is a possibility that household chickens in rural Bangladesh carry less ARGs in their guts compared to chickens kept in small or large scale production farms. Alternatively, the lack of differences in ARG counts between different animals in our data could be explained by the contact between these animals in one household, which facilitates ARG transmission.

When looking at the human samples separately, we also did not find differences in ARG abundance between mother, sibling, and newborn samples. Contrastingly, other studies that used metagenomic (Bäckhed et al., 2015; Pärnänen et al., 2018) or qPCR-based (Sosa-Moreno et al., 2020) methods found that guts of newborns contained a higher number of ARGs compared to mothers. Moreover, newborns in the Bangladesh study population were exposed to several factors that have been reported to lead to an enriched resistome. This includes intrapartum antibiotic prophylaxis in mothers and C-section births (Bäckhed et al., 2015; Lebeaux et al., 2021; Leo et al., 2022). Based on these studies, it would be expected that newborn resistomes in our data would show a higher ARG abundance, which was not the case. Previous studies reported that the gut resistome composition is largely determined by the phylogeny of the microbiome (Pärnänen et al., 2018). In our data, newborn guts contained an unusually high abundance of opportunistic pathogenic bacteria such as *Escherichia, Enterobacter*, and *Klebsiella*. These pathogens have been found to often carry multiple ARGs (WHO Bacterial Priority Pathogens List, 2024), and thus, the high prevalence of these genera also seems contradictory with the finding that newborns in our data do not contain a higher number of ARGs compared to mothers and siblings. However, *Klebsiella* was also highly prevalent in mothers and siblings. It could therefore be possible that mothers and siblings carry more ARGs compared to studies conducted in high-income countries. The finding that ARG abundance does not differ between newborns and older family members might not be due to

relatively low ARG abundance in newborns compared to other studies, but instead to a higher ARG abundance in mothers and siblings.

While there was no difference in alpha diversity between resistomes of mothers, newborns, and siblings, we did observe significant differences in human resistomes over time. Around midline, which was a week after giving birth, Shannon and Simpson diversity of ARGs in the human gut increased. Since this pattern is not visible in the ARG richness, this increase in alpha diversity at midline probably is not due to the acquirement of new, unique ARGs. Instead, the pattern could be explained by an increase in the abundance of more rare ARGs or a decrease of very dominant ARGs, since this would enhance the evenness of ARGs and thus lead to a higher Shannon and Simpson diversity. An example of highly abundant ARGs that decrease in mothers at midline are tet(Q) and erm(F). These were the two most prevalent ARGs among the differently abundant ARGs in mothers at midline. Furthermore, the ARGs that had the highest positive log2 fold change (e.g. the highest increase) in mothers at midline were aminoglycoside resistance genes that were lowly abundant in the full dataset.

These aminoglycoside genes that increased in mothers around the time of giving birth were aac(6')-Ii, aac(6')-Ib and aac(3)-IIa. Notably, these ARGs have been listed as high-risk resistance genes (Shuai et al., 2024). The risk assessment of ARGs was based on their relative abundance, their mobility, and pathogenicity of the hosts that they are often found in. These aminoglycoside ARGs were denoted as high-priority ARGs. Additionally, some of the highest priority beta-lactam resistance genes such as blaCTX-M-1 and blaCTX-M-64 were more abundant in mothers at midline. Interestingly, these 5 high-risk ARGs were also more abundant in newborns at midline (when they were only a week old) compared to endline. This shows that around the time of giving birth, mothers and newborns both acquired ARGs that are often associated with pathogenic hosts, or that are easily horizontally transmitted between commensal and pathogenic bacteria. At endline, a few months after birth, the abundances of these high-risk ARGs had decreased again, showing that the resistomes of mothers and newborns can recover after exposure to high-risk ARGs.

Furthermore, the co-occurrence networks made with differently abundant ARGs or bacterial taxa for mother samples show recurring patterns. In every network, a cluster is visible that consists of *Klebsiella* and the same ARGs, which were OqxA, OqxB, fosA5, and blaSHV-2a. *Klebsiella* is an opportunistic pathogen, and was more abundant in microbiomes of mothers at midline. Its associated ARGs also increased in abundance at the same time. Both *Klebsiella* and the ARGs in the cluster remained enriched in mother samples at endline. This suggests that mothers might acquire *Klebsiella* bacteria that harbour multiple resistance genes around the time of giving birth. A study into ARGs in genomes of *Klebsiella* isolates in LMIC showed that all strains harboured genes that confer resistance to 3 or more AB classes, with fosA5, OqxA, and OqxB being the most prevalent ARGs (Silvester et al., 2022). The prevalence of multi-drug resistant *Klebsiella* in LMIC and in mothers in our data is worrying, since this increases the chances of obtaining infections that are difficult to treat. *Klebsiella* has been reported to be responsible for the majority of neonatal sepsis cases in LMIC (Sands et al., 2021). Thus, the higher prevalence of *Klebsiella* and its associated ARGs in mothers several months after giving birth might impose a risk for newborns, since these possibly pathogenic bacteria could be transmitted from mother to newborns, or other family members.

Staphylococcus is another major pathogen that causes neonatal infections in LMIC (Zaidi et al., 2005). This was mostly attributed to hospital-acquired infections, as opposed to mother-infant transmission. In the Bangladesh data, *Staphylococcus* abundance was higher in newborns just after birth compared to endline, when they were 3-4 months old. At the same time, among the ARGs of which the abundance decreased the most strongly in newborns at endline compared to midline were multiple genes that are reported to be found in *Staphylococcus* species in the Comprehensive Antibiotic Resistance Database (CARD). This includes the aminoglycoside genes aac(6')-Ii, aac(6')-Ib and aac(3)-IIa, the macrolide genes msrA and mphC, and the tetracycline gene tetK. Moreover, several of these genes were more highly abundant in newborns at midline compared to mothers or siblings (figure S6). While *Staphylococcus* does not appear in any of the co-occurrence networks that were made for newborn samples, these results suggest that newborns were exposed to ARGs that often occur in *Staphylococcus* and to *Staphylococcus* species at the same time. This could point to the presence of multi-drug resistant *Staphylococcus* bacteria in the hospitals where the children were born.

Surprisingly, the analysis showed no clear effect of AB usage in mothers. There could be several reasons for this. Firstly, the resistome composition of mothers showed variance at baseline (figure 3), before antibiotics were administered. The effect of AB usage has been reported to be dependent on the initial composition of the resistome (Nielsen et al., 2021). While mothers used only two types of different AB, the treatment could cause different shifts in resistome composition in each individual. This would not result in a large set of differently abundant ARGs in mothers who used AB compared to those who did not, and this could explain why only 6 differently abundant ARGs were found in this comparison. Secondly, most mothers in our study were administered AB. As a consequence, the group with mothers who did not use AB at midline contained only 5 samples. These small group sizes, combined with the high variance in ARG abundance and diversity that occurs in the whole dataset, make it less likely that significant differences between mothers who did or did not use antibiotics will be found.

Thus, increasing the number of samples could strengthen the research into the resistomes of people and animals in rural Bangladesh. At the moment, we included data from 19 households. In total, samples were taken from 220 households, which are still being sequenced and analysed. With the current 19 households, several groups consist of only a few samples. This is true for siblings, but also for cow, duck, goat, and pigeon samples. Expanding the dataset would thus also be beneficial for comparisons between animal resistomes. This could lead to novel findings, since the limited number of studies that looked at resistomes of multiple livestock species simultaneously were conducted in high-income settings.

Furthermore, the current results show that several ARGs become more abundant in mothers and newborns at midline. Some of these have been described as high-risk ARGs because of their association with known pathogenic bacteria and their mobility (Shuai et al., 2024). The high abundance in mothers and newborns around the time of giving birth thus might be worrying, especially if these ARGs could easily be transmitted to other people. To further assess what the risk is of the ARGs in our data to spread within households, the profile of mobile genetic elements (MGE) in the metagenomic reads could be determined. For this, the reads would be mapped to a database containing MGEs (Pärnänen et al., 2018), after which MGE profiles can be compared in order to identify which factors could influence ARG transmission between bacteria or between individuals. Comparing ARG and MGE profiles in infant-mother pairs could lead to insights into whether newborns are more likely to acquire mobile ARGs from their mothers, or from environmental sources (Pärnänen et al., 2018).

Lastly, while several ARGs were associated with bacterial taxa in the co-occurrence analysis, this was based on a correlation between their abundances. Therefore, this does not give definitive proof on the hosts of the ARGs found in the metagenomic data. However, opportunistic pathogenic bacteria and ARGs that are often found in these bacteria were simultaneously enriched in mothers and newborns just after birth. Post-partum maternal infections with antibiotic resistant bacteria impose a significant burden on mothers in LMIC (Monari et al., 2024). This might be reason to further research the presence of these bacteria in hospitals in Bangladesh, and to take measures to increase sanitation and awareness of AMR spread in these hospitals.

BIBLIOGRAPHY

Anthony, W. E., Wang, B., Sukhum, K. V., D'Souza, A. W., Hink, T., Cass, C., Seiler, S., Reske, K. A., Coon, C., Dubberke, E. R., Burnham, C.-A. D., Dantas, G., & Kwon, J. H. (2022). Acute and persistent effects of commonly used antibiotics on the gut microbiome and resistome in healthy adults. *Cell Reports*, *39*(2). https://doi.org/10.1016/j.celrep.2022.110649

Arbizu, P. M. (2023). *pairwiseAdonis* [R]. https://github.com/pmartinezarbizu/pairwiseAdonis (Original work published 2017)

Auguet, O. T., Niehus, R., Gweon, H. S., Berkley, J. A., Waichungo, J., Njim, T., Edgeworth, J. D., Batra, R., Chau, K., Swann, J., Walker, S. A., Peto, T. E. A., Crook, D. W., Lamble, S., Turner, P., Cooper, B. S., & Stoesser, N. (2021). Population-level faecal metagenomic profiling as a tool to predict antimicrobial resistance in Enterobacterales isolates causing invasive infections: An exploratory study across Cambodia, Kenya, and the UK. *eClinicalMedicine*, *36*. https://doi.org/10.1016/j.eclinm.2021.100910

Bäckhed, F., Roswall, J., Peng, Y., Feng, Q., Jia, H., Kovatcheva-Datchary, P., Li, Y., Xia, Y., Xie, H., Zhong, H., Khan, M. T., Zhang, J., Li, J., Xiao, L., Al-Aama, J., Zhang, D., Lee, Y. S., Kotowska, D., Colding, C., … Wang, J. (2015). Dynamics and Stabilization of the Human Gut Microbiome during the First Year of Life. *Cell Host & Microbe*, *17*(5), 690–703. https://doi.org/10.1016/j.chom.2015.04.004

Baquero, F., Coque, T. M., Martínez, J.-L., Aracil-Gisbert, S., & Lanza, V. F. (2019). Gene Transmission in the One Health Microbiosphere and the Channels of Antimicrobial Resistance. *Frontiers in Microbiology*, *10*. https://doi.org/10.3389/fmicb.2019.02892

Bargheet, A., Klingenberg, C., Esaiassen, E., Hjerde, E., Cavanagh, J. P., Bengtsson-Palme, J., & Pettersen, V. K. (2023). Development of early life gut resistome and mobilome across gestational ages and microbiota-modifying treatments. *eBioMedicine*, *92*, 104613. https://doi.org/10.1016/j.ebiom.2023.104613

Bastian, M., Heymann, S., & Jacomy, M. (2009). Gephi: An Open Source Software for Exploring and Manipulating Networks. *Proceedings of the International AAAI Conference on Web and Social Media*, *3*(1), 361–362. https://doi.org/10.1609/icwsm.v3i1.13937

Bengtsson-Palme, J., Larsson, D. G. J., & Kristiansson, E. (2017). Using metagenomics to investigate human and environmental resistomes. *Journal of Antimicrobial Chemotherapy*, *72*(10), 2690–2703. https://doi.org/10.1093/jac/dkx199

Bich, V. T. N., Thanh, L. V., Thai, P. D., Van Phuong, T. T., Oomen, M., Driessen, C., Beuken, E., Hoang, T. H., van Doorn, H. R., Penders, J., & Wertheim, H. F. L. (2019). An exploration of the gut and environmental resistome in a community in northern Vietnam in relation to antibiotic use. *Antimicrobial Resistance & Infection Control*, *8*(1), 194. https://doi.org/10.1186/s13756-019-0645-9

Bortolaia, V., Kaas, R. S., Ruppe, E., Roberts, M. C., Schwarz, S., Cattoir, V., Philippon, A., Allesoe, R. L., Rebelo, A. R., Florensa, A. F., Fagelhauer, L., Chakraborty, T., Neumann, B., Werner, G., Bender, J. K., Stingl, K., Nguyen, M., Coppens, J., Xavier, B. B., … Aarestrup, F. M. (2020). ResFinder 4.0 for predictions of phenotypes from genotypes. *Journal of Antimicrobial Chemotherapy*, *75*(12), 3491–3500. https://doi.org/10.1093/jac/dkaa345

Chng, K. R., Li, C., Bertrand, D., Ng, A. H. Q., Kwah, J. S., Low, H. M., Tong, C., Natrajan, M., Zhang, M. H., Xu, L., Ko, K. K. K., Ho, E. X. P., Av-Shalom, T. V., Teo, J. W. P., Khor, C. C., Chen, S. L., Mason, C. E., Ng, O. T., Marimuthu, K., … Nagarajan, N. (2020). Cartography of opportunistic pathogens and antibiotic resistance genes in a tertiary hospital environment. *Nature Medicine*, *26*(6), 941–951. https://doi.org/10.1038/s41591-020-0894-4

Collignon, P., Beggs, J. J., Walsh, T. R., Gandra, S., & Laxminarayan, R. (2018). Anthropological and socioeconomic factors contributing to global antimicrobial resistance: A univariate and multivariable analysis. *The Lancet Planetary Health*, *2*(9), e398–e405. https://doi.org/10.1016/S2542-5196(18)30186-4

Crits-Christoph, A., Hallowell, H. A., Koutouvalis, K., & Suez, J. (2022). Good microbes, bad genes? The dissemination of antimicrobial resistance in the human microbiome. *Gut Microbes*, *14*(1), 2055944. https://doi.org/10.1080/19490976.2022.2055944

Crofts, T. S., Gasparrini, A. J., & Dantas, G. (2017). Next-generation approaches to understand and combat the antibiotic resistome. *Nature Reviews Microbiology*, *15*(7), 422–434. https://doi.org/10.1038/nrmicro.2017.28

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

Fink, G., D'Acremont, V., Leslie, H. H., & Cohen, J. (2020). Antibiotic exposure among children younger than 5 years in low-income and middle-income countries: A cross-sectional study of nationally representative facility-based and household-based surveys. *The Lancet Infectious Diseases*, *20*(2), 179–187. https://doi.org/10.1016/S1473-3099(19)30572-9

Gandra, S., Alvarez-Uria, G., Turner, P., Joshi, J., Limmathurotsakul, D., & van Doorn, H. R. (2020). Antimicrobial Resistance Surveillance in Low- and Middle-Income Countries: Progress and Challenges in Eight South Asian and Southeast Asian Countries. *Clinical Microbiology Reviews*, *33*(3), 10.1128/cmr.00048-19. https://doi.org/10.1128/cmr.00048-19

Gasparrini, A. J., Wang, B., Sun, X., Kennedy, E. A., Hernandez-Leyva, A., Ndao, I. M., Tarr, P. I., Warner, B. B., & Dantas, G. (2019). Persistent metagenomic signatures of early-life hospitalization and antibiotic treatment in the infant gut microbiota and resistome. *Nature Microbiology*, *4*(12), 2285–2297. https://doi.org/10.1038/s41564-019-0550-2

Graves, S., & Dorai-Raj, H.-P. P. and L. S. with help from S. (2024). *multcompView: Visualizations of Paired Comparisons* (Version 0.1-10) [Computer software]. https://cran.r-project.org/web/packages/multcompView/index.html

Guo, X., Stedtfeld, R. D., Hedman, H., Eisenberg, J. N. S., Trueba, G., Yin, D., Tiedje, J. M., & Zhang, L. (2018). Antibiotic Resistome Associated with Small-Scale Poultry Production in Rural Ecuador. *Environmental Science & Technology*, *52*(15), 8165–8172. https://doi.org/10.1021/acs.est.8b01667

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

Hicks, J. P., Latham, S. M., Huque, R., Das, M., Newell, J., Abdullah, S. M., Al Azdi, Z., Jahan, I., Rassi, C., Hamade, P., Shafique, M., Islam, M. S., & King, R. (2021). Antibiotic practices among household members and their domestic animals within rural communities in Cumilla district, Bangladesh: A cross-sectional survey. *BMC Public Health*, *21*(1), 406. https://doi.org/10.1186/s12889-021-10457-w

Holmes, A. H., Moore, L. S. P., Sundsfjord, A., Steinbakk, M., Regmi, S., Karkey, A., Guerin, P. J., & Piddock, L. J. V. (2016). Understanding the mechanisms and drivers of antimicrobial resistance. *The Lancet*, *387*(10014), 176–187. https://doi.org/10.1016/S0140-6736(15)00473-0

Hu, Y., Gao, G. F., & Zhu, B. (2017). The antibiotic resistome: Gene flow in environments, animals and human beings. *Frontiers of Medicine*, *11*(2), 161–168. https://doi.org/10.1007/s11684-017-0531-x

Kassambara, A. (2023). *rstatix: Pipe-Friendly Framework for Basic Statistical Tests* (Version 0.7.2) [Computer software]. https://cran.r-project.org/web/packages/rstatix/index.html

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

Klein, E. Y., Van Boeckel, T. P., Martinez, E. M., Pant, S., Gandra, S., Levin, S. A., Goossens, H., & Laxminarayan, R. (2018). Global increase and geographic convergence in antibiotic consumption between 2000 and 2015. *Proceedings of the National Academy of Sciences*, *115*(15), E3463–E3470. https://doi.org/10.1073/pnas.1717295115

Langmead, B., & Salzberg, S. L. (2012). Fast gapped-read alignment with Bowtie 2. *Nature Methods*, *9*(4), 357–359. https://doi.org/10.1038/nmeth.1923

Lawther, K., Santos, F. G., Oyama, L. B., Rubino, F., Morrison, S., Creevey, C. J., McGrath, J. W., & Huws, S. A. (2022). Resistome Analysis of Global Livestock and Soil Microbiomes. *Frontiers in Microbiology*, *13*. https://doi.org/10.3389/fmicb.2022.897905

Laxminarayan, R., Duse, A., Wattal, C., Zaidi, A. K. M., Wertheim, H. F. L., Sumpradit, N., Vlieghe, E., Hara, G. L., Gould, I. M., Goossens, H., Greko, C., So, A. D., Bigdeli, M., Tomson, G., Woodhouse, W., Ombaka, E., Peralta, A. Q., Qamar, F. N., Mir, F., … Cars, O. (2013). Antibiotic resistance—The need for global solutions. *The Lancet Infectious Diseases*, *13*(12), 1057–1098. https://doi.org/10.1016/S1473-3099(13)70318-9

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

Leo, S., Curtis, N., & Zimmermann, P. (2022). The neonatal intestinal resistome and factors that influence it—A systematic review. *Clinical Microbiology and Infection*, *28*(12), 1539–1546. https://doi.org/10.1016/j.cmi.2022.07.014

Li, X., Brejnrod, A., Thorsen, J., Zachariasen, T., Trivedi, U., Russel, J., Vestergaard, G. A., Stokholm, J., Rasmussen, M. A., & Sørensen, S. J. (2023). Differential responses of the gut microbiome and resistome to antibiotic exposures in infants and adults. *Nature Communications*, *14*(1), 8526. https://doi.org/10.1038/s41467-023-44289-6

Love, M. I., Huber, W., & Anders, S. (2014). Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biology*, *15*(12), 550. https://doi.org/10.1186/s13059-014-0550-8

Luchen, C. C., Chibuye, M., Spijker, R., Simuyandi, M., Chisenga, C., Bosomprah, S., Chilengi, R., Schultsz, C., Mende, D. R., & Harris, V. C. (2023). Impact of antibiotics on gut microbiome composition and resistome in the first years of life in low- to middle-income countries: A systematic review. *PLOS Medicine*, *20*(6), e1004235. https://doi.org/10.1371/journal.pmed.1004235

Ma, T., McAllister, T. A., & Guan, L. L. (2021). A review of the resistome within the digestive tract of livestock. *Journal of Animal Science and Biotechnology*, *12*(1), 121. https://doi.org/10.1186/s40104-021-00643-6

Mahmud, B., Vargas, R. C., Sukhum, K. V., Patel, S., Liao, J., Hall, L. R., Kesaraju, A., Le, T., Kitchner, T., Kronholm, E., Koshalek, K., Bendixsen, C. G., VanWormer, J. J., Shukla, S. K., & Dantas, G. (2024). Longitudinal dynamics of farmer and livestock nasal and faecal microbiomes and resistomes. *Nature Microbiology*, *9*(4), 1007–1020. https://doi.org/10.1038/s41564-024-01639-4

McMurdie, P. J., & Holmes, S. (2013). phyloseq: An R Package for Reproducible Interactive Analysis and Graphics of Microbiome Census Data. *PLOS ONE*, *8*(4), e61217. https://doi.org/10.1371/journal.pone.0061217

McMurdie, P. J., & Holmes, S. (2014). Waste Not, Want Not: Why Rarefying Microbiome Data Is Inadmissible. *PLOS Computational Biology*, *10*(4), e1003531. https://doi.org/10.1371/journal.pcbi.1003531

Monari, C., Onorato, L., Coppola, N., Raviglione, M. C. B., & Gon, G. (2024). Burden of Antimicrobial Resistance Among Women with Post-Partum Infections in Low-Middle Income Countries: A Systematic Review. *Journal of Epidemiology and Global Health*, *14*(2), 274–290. https://doi.org/10.1007/s44197-024-00222-8

Monjur, F., Rizwan, F., Asaduzzaman, M., Nasrin, N., Ghosh, N., Sarker Apu, A., & Haque, F. (2010). Antibiotic sensitivity pattern of causative organisms of neonatal septicemia in an urban hospital of Bangladesh. *Indian Journal of Medical Sciences*, *64*, 265–271. https://doi.org/10.4103/0019-5359.99605

Munk, P., Knudsen, B. E., Lukjancenko, O., Duarte, A. S. R., Van Gompel, L., Luiken, R. E. C., Smit, L. A. M., Schmitt, H., Garcia, A. D., Hansen, R. B., Petersen, T. N., Bossers, A., Ruppé, E., Lund, O., Hald, T., Pamp, S. J., Vigre, H., Heederik, D., Wagenaar, J. A., … Aarestrup, F. M. (2018). Abundance and diversity of the faecal resistome in slaughter pigs and broilers in nine European countries. *Nature Microbiology*, *3*(8), 898–908. https://doi.org/10.1038/s41564-018-0192-9

Munk, P., Yang, D., Röder, T., Maier, L., Petersen, T. N., Duarte, A. S. R., Clausen, P. T. L. C., Brinch, C., Van Gompel, L., Luiken, R., Wagenaar, J. A., Schmitt, H., Heederik, D. J. J., Mevius, D. J., Smit, L. A. M., EFFORT Consortium, Bossers, A., & Aarestrup, F. M. (2024). The European livestock resistome. *mSystems*, *9*(4), e01328-23. https://doi.org/10.1128/msystems.01328-23

Murray, C. J. L., Ikuta, K. S., Sharara, F., Swetschinski, L., Aguilar, G. R., Gray, A., Han, C., Bisignano, C., Rao, P., Wool, E., Johnson, S. C., Browne, A. J., Chipeta, M. G., Fell, F., Hackett, S., Haines-Woodhouse, G., Hamadani, B. H. K., Kumaran, E. A. P., McManigal, B., … Naghavi, M. (2022). Global burden of bacterial antimicrobial resistance in 2019: A systematic analysis. *The Lancet*, *399*(10325), 629–655. https://doi.org/10.1016/S0140-6736(21)02724-0

Neuwirth, E. (2022). *RColorBrewer: ColorBrewer Palettes* (Version 1.1-3) [Computer software]. https://cran.r-project.org/web/packages/RColorBrewer/index.html

Nielsen, K. L., Olsen, M. H., Pallejá, A., Ebdrup, S. R., Sørensen, N., Lukjancenko, O., Marvig, R. L., Møller, K., Frimodt-Møller, N., & Hertz, F. B. (2021). Microbiome Compositions and Resistome Levels after Antibiotic Treatment of Critically Ill Patients: An Observational Cohort Study. *Microorganisms*, *9*(12), Article 12. https://doi.org/10.3390/microorganisms9122542

Oksanen, J., Simpson, G. L., Blanchet, F. G., Kindt, R., Legendre, P., Minchin, P. R., O'Hara, R. B., Solymos, P., Stevens, M. H. H., Szoecs, E., Wagner, H., Barbour, M., Bedward, M., Bolker, B., Borcard, D., Carvalho, G., Chirico, M., Caceres, M. D., Durand, S., … Weedon, J. (2024). *vegan: Community Ecology Package* (Version 2.6-8) [Computer software]. https://cran.r-project.org/web/packages/vegan/index.html

Oldenkamp, R., Schultsz, C., Mancini, E., & Cappuccio, A. (2021). Filling the gaps in the global prevalence map of clinical antimicrobial resistance. *Proceedings of the National Academy of Sciences*, *118*(1), e2013515118. https://doi.org/10.1073/pnas.2013515118

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

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

Prestinaci, F., Pezzotti, P., & Pantosti, A. (2015). Antimicrobial resistance: A global multifaceted phenomenon. *Pathogens and Global Health*, *109*(7), 309–318. https://doi.org/10.1179/2047773215Y.0000000030

Qiu, T., Huo, L., Guo, Y., Gao, M., Wang, G., Hu, D., Li, C., Wang, Z., Liu, G., & Wang, X. (2022). Metagenomic assembly reveals hosts and mobility of common antibiotic resistome in animal manure and commercial compost. *Environmental Microbiome*, *17*(1), 42. https://doi.org/10.1186/s40793-022-00437-x

Robinson, T. P., Bu, D. P., Carrique-Mas, J., Fèvre, E. M., Gilbert, M., Grace, D., Hay, S. I., Jiwakanon, J., Kakkar, M., Kariuki, S., Laxminarayan, R., Lubroth, J., Magnusson, U., Thi Ngoc, P., Van Boeckel, T. P., & Woolhouse, M.

E. J. (2016). Antibiotic resistance is the quintessential One Health issue. *Transactions of The Royal Society of Tropical Medicine and Hygiene*, *110*(7), 377–380. https://doi.org/10.1093/trstmh/trw048

Roess, A. A., Winch, P. J., Ali, N. A., Akhter, A., Afroz, D., El Arifeen, S., Darmstadt, G. L., & Baqui, A. H. (2013). Animal Husbandry Practices in Rural Bangladesh: Potential Risk Factors for Antimicrobial Drug Resistance and Emerging Diseases. *The American Journal of Tropical Medicine and Hygiene*, *89*(5), 965–970. https://doi.org/10.4269/ajtmh.12-0713

Sands, K., Carvalho, M. J., Portal, E., Thomson, K., Dyer, C., Akpulu, C., Andrews, R., Ferreira, A., Gillespie, D., Hender, T., Hood, K., Mathias, J., Milton, R., Nieto, M., Taiyari, K., Chan, G. J., Bekele, D., Solomon, S., Basu, S., … Walsh, T. R. (2021). Characterization of antimicrobial-resistant Gram-negative bacteria that cause neonatal sepsis in seven low- and middle-income countries. *Nature Microbiology*, *6*(4), 512–523. https://doi.org/10.1038/s41564-021-00870-7

Shuai, X., Zhou, Z., Zhu, L., Achi, C., Lin, Z., Liu, Z., Yu, X., Zhou, J., Lin, Y., & Chen, H. (2024). Ranking the risk of antibiotic resistance genes by metagenomic and multifactorial analysis in hospital wastewater systems. *Journal of Hazardous Materials*, *468*, 133790. https://doi.org/10.1016/j.jhazmat.2024.133790

Silvester, R., Madhavan, A., Kokkat, A., Parolla, A., B.m., A., M., H., & Abdulla, M. H. (2022). Global surveillance of antimicrobial resistance and hypervirulence in *Klebsiella pneumoniae* from LMICs: An *in-silico* approach. *Science of The Total Environment*, *802*, 149859. https://doi.org/10.1016/j.scitotenv.2021.149859

Sosa-Moreno, A., Comstock, S. S., Sugino, K. Y., Ma, T. F., Paneth, N., Davis, Y., Olivero, R., Schein, R., Maurer, J., & Zhang, L. (2020). Perinatal risk factors for fecal antibiotic resistance gene patterns in pregnant women and their infants. *PLOS ONE*, *15*(6), e0234751. https://doi.org/10.1371/journal.pone.0234751

Stamouli, S., Beber, M. E., Normark, T., Christensen, T. A., Andersson-Li, L., Borry, M., Jamy, M., Community, N.-C., & Yates, J. A. F. (2023). *nf-core/taxprofiler: Highly parallelised and flexible pipeline for metagenomic taxonomic classification and profiling* (p. 2023.10.20.563221). bioRxiv. https://doi.org/10.1101/2023.10.20.563221

Van Boeckel, T. P., Pires, J., Silvester, R., Zhao, C., Song, J., Criscuolo, N. G., Gilbert, M., Bonhoeffer, S., & Laxminarayan, R. (2019). Global trends in antimicrobial resistance in animals in low- and middle-income countries. *Science*, *365*(6459), eaaw1944. https://doi.org/10.1126/science.aaw1944

Van Gompel, L., Luiken, R. E. C., Hansen, R. B., Munk, P., Bouwknegt, M., Heres, L., Greve, G. D., Scherpenisse, P., Jongerius-Gortemaker, B. G. M., Tersteeg-Zijderveld, M. H. G., García-Cobos, S., Dohmen, W., Dorado-García, A., Wagenaar, J. A., Urlings, B. A. P., Aarestrup, F. M., Mevius, D. J., Heederik, D. J. J., Schmitt, H., … Smit, L. A. M. (2020). Description and determinants of the faecal resistome and microbiome of farmers and slaughterhouse workers: A metagenome-wide cross-sectional study. *Environment International*, *143*, 105939. https://doi.org/10.1016/j.envint.2020.105939

Wood, D. E., Lu, J., & Langmead, B. (2019). Improved metagenomic analysis with Kraken 2. *Genome Biology*, *20*(1), 257. https://doi.org/10.1186/s13059-019-1891-0