ANTIBIOTIC USE DURING PREGNANCY AND ITS EFFECT ON MATERNAL AND INFANT FECAL RESISTOME: A COHORT STUDY

By

Andrea Romina Sosa Moreno

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ABSTRACT

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Nearly 90% of pregnant women in the US take at least one medication during pregnancy, and in more than 40% of cases, that prenatal medication is an antibiotic. Prenatal exposure to antibiotics could shape the total number of antimicrobial resistance genes in stool samples - the fecal resistome - in women, and also in their infants, who acquire his or her initial microbiome by vertical transmission.

We examined 51 pregnant women enrolled during their third trimester of pregnancy in Lansing and Traverse City, MI, and in 42 6-month-old infants to evaluate the association between prenatal antibiotic use and fecal resistome patterns. Prenatal antimicrobial exposure in mothers was assessed using clinical and questionnaire data. Antibiotic resistance gene (ARG) and mobile genetic element (MGE) richness and abundance were assessed using multiplex qRT-PCR. Alpha and Beta diversity were measured. Wilcoxon non-parametric test was used for comparisons.

Infants had both significantly greater relative abundance and higher diversity of MGE than their mothers (Shannon diversity and Inverse Simpson $p<0.05$). We found a high variability of shared patterns between women and their infants, with an average of 29% ARG being shared between dyads. Mother and infant samples are different in terms of ARG and MGE relative abundance and absence/presence data (Adonis $p<0.0001$). We found differences in specific ARGs diversity among antibiotic exposed vs. non-exposed groups using medical records.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

KEY TO ABBREVIATIONS

ARCH	The Archive for Research in Child Health
ARG	Antibiotic Resistant Gene
BMI	Body Mass Index
CI	Confidence Interval
DNA	Deoxyribonucleic Acid
GBS	Group B *Streptococcus*
LN	Logarithm
MDR	Multi-Resistant Drug
MG	Milligrams
MGE	Mobile Genetic Element
MLSB	Macrolide-Lincosamide-Streptogramin B
OTU	Operational Taxonomic Unit
PCoA	Principal Coordinate Analysis
RNA	Ribonucleic Acid

INTRODUCTION

The emergence and spread of antibiotic resistance is a major global public health concern since it presents as an obstacle in treatment and control of infections [1]. Antibiotic resistance often occurs as a spontaneous event driven by mutations in bacterial targets or changes in efflux pumps [2]. However, the overuse of medications in humans and animals can speed up the emergence of antibiotic resistance, creating a selective pressure on bacteria with antibiotic resistance genes (ARG). ARGs are responsible for conferring resistance to antibiotics. Infections caused by resistant strains are more difficult to treat due to the absence of alternative treatments which can also increase the cost. Such is the case for tuberculosis, gonorrhea and pneumonia [3].

In developed countries, the use of antimicrobials among pregnant women is frequent and it has been rising in the past decades. The number of drugs used during pregnancy, as assessed by self-report questionnaires, has increased 68% from 1976 to 2008 [4]. Moreover, 88.8% of pregnant women reported previous use of specific medications at any point in pregnancy, 70% of those during the first trimester [4]. Prescription records, on the other hand, showed that more than half (64%) of pregnant women received a drug other than a vitamin during pregnancy [5].

Antibiotics and antifungals are among the most frequently used medications among pregnant women. It is estimated that more than 40% of pregnant women have received antibiotics before delivery usually in order to avoid prenatal and postnatal complications in both the mother and the newborn [4]. Also, prescription medical records have shown that nearly 70% of women used at least one antibiotic at some time during pregnancy, 48.8% being in the first

trimester [4]. Therefore, the data would suggest that a high percentage of newborns are being exposed to antibiotics during gestation, parturition and neonatal stages.

Antibiotic exposure is associated with changes in the resistome, the collection of all ARGs in the genome of an individual's microbiome [6]. Previous research has shown that populations with low antibiotic exposure have lower number of ARGs [7]. Moreover, overuse of antibiotics has been associated with a larger pool of antibiotic resistance genes in vitro and in hospital settings [8, 9].

Previous studies assessing resistome patterns had some limitations: a) small number of ARGs [7, 10-11], b) used culture-dependent techniques [10] or c) had non-targeted metagenomic approaches [13-15]. Studies screening for a small number of ARGs cannot report comprehensive resistome data. Culture-dependent techniques capture only a small percentage of the gut microbiome, those microbes that are culturable, reducing the possibility of identifying ARGs associated with un-culturable microbes [11]. Non-targeted metagenomics approaches are time-consuming and labor-intensive techniques. In contrast, targeted metagenomics approaches, such as real-time PCR, provide relative abundance data of already established ARGs. Here, we studied the effect of antimicrobial exposure during pregnancy in a wide set of ARGs using Wafergen Smartchip technology, a high-capacity quantification technique, based on a targeted metagenomics approach.

BACKGROUND: Antibiotic use during pregnancy and its effect on infant's resistome – A review

Human gut microbiota as a reservoir of ARGs

The gastrointestinal (GI) tract is an open system that connects the mouth to the anus. The GI tract has a dynamic and individual-specific microbiome, accessible to environmental bacteria or pathogens from food and other ingested substrates. Some of those bacteria can harbor ARGs in their genome [11, 17]. Moreover, gut microbiota can also harbor mobile genetic elements (MGEs) which facilitate genomic transfer among bacteria [12]. Therefore, ARG horizontal transfer from pathogens to the normal gut microbiome and vice versa is possible. Those opportunistic pathogens that acquire ARGs represent a public health concern [11].

Use of medications during pregnancy

In developed countries, pregnant women are frequently prescribed medications to prevent prenatal or postnatal complications. The use of prescribed medications during the first trimester of pregnancy increased 60% among pregnant women since 1976. Overall, 89% of all pregnant women in the US take at least one medication during pregnancy [4]. Among all prescriptions, antibiotics and antifungals are the most frequent medications used in pregnant women [5]. It is estimated that more than 40% of women received antibiotics before delivery, usually to avoid intrapartum fever, for the prevention of neonatal Group B *Streptococcus* fever (GBS), and because of cesarean section [13]. Besides applications to avoid prenatal and postnatal complications, antibiotics are also prescribed as treatment in cases of respiratory infections (i.e. sinusitis and upper respiratory infections) [14] and genital infections (i.e. vaginal infections) in pregnant women [22-23].

Antibiotics are given to pregnant women prenatally to prevent early-onset of Group B Strep disease in newborns. Approximately, 1 in 4 pregnant women harbors GBS increasing 25 times the risk of delivering a baby with GBS infection compared to non-harboring women [15]. In those cases, GBS bacteria is part of the maternal vaginal or fecal microbiota. Women who test positive for GBS bacteria between 35 to 37 weeks pregnant are prescribed with antibiotics to avoid vertical transmission. Additionally, anti-GBS treatment is prescribed to pregnant women who are at high risk for GBS bacteria colonization which includes history of positive GBS bacteria screening, fever during pregnancy, labor complications and preterm birth [16]. Therefore, a high percentage of infants in developed countries are likely to have been exposed to antibiotics during gestation. For this reason, it is important to understand the effects of antibiotic therapy during pregnancy on the infant resistome.

Emergence of ARGs

The use of antibiotics has increased and with it there is a growing trend in the emergence of ARGs. Antibiotic exposure selects for gut microbes with resistant genes, increasing the abundance of the resistome [17]. The presence of microbiota with high ARG frequency increases the probability that the infection becomes problematic to treat [3, 27]. On the other hand, MGEs modify the resistome by transporting genes within same and between different species among the microbial community [18]. Antibiotic use promotes genetic information transfer through MGEs in bacteria [19], providing another explanation for the increased diversity and richness of ARGs. The group of MGEs - including transposons, integrons, plasmids and insertion sequences - in the genome is known as mobilome.

Early life is a critical stage in the establishment of the gut microbiome

Prenatal and postnatal factors such as mode of delivery and breastfeeding are involved in infant microbiome and resistome acquisition and have been studied extensively [10]. For instance, C-section delivered newborns have a higher richness of ARGs compared with vaginally delivered newborns [20]. Antibiotic use during the first days of life of pre-term babies also affects the colonization of the newborn's gut microbiota [21] and leads to lower bacterial diversity in the first weeks after birth [22]. One possible explanation is that antibiotics cause alterations on the infant indigenous microbiota, those alterations could play a role in childhood development and risk of disease as an adult [13]. Additionally, infant microbiota was found to be vastly resilient which would suggest a long-time effect of alterations [23].

ARG vertical transmission between women and their infants

Resistome studies in neonates have shown ARGs' presence in early life, suggesting vertical transmission from their mothers [10, 31]. However, possible contamination of infant samples with the hospital environment should not be discarded. Even in the case of an authentic ARG vertical transmission between mother and their infants, there is still controversy about the timing of this event. The traditional approach suggested that this transmission happens after birth or during the delivery based on the premise that the womb was a sterile environment. However, recent studies have reported ARGs presence in meconium – the earliest stool from an infant – suggesting *in utero* transmission [24]. Regardless, newborns are at higher risk of harboring pathogens containing antibiotic resistance due to their unstable microbiota and naïve immune system. This makes newborns prone to becoming reservoirs of ARGs and MGEs [25, 11] and therefore prone to infections caused by resistant bacterial strains.

Maternal gut and breast milk have been proposed as mechanisms for ARGs transmission from mother to infant [33-34]. Parnanen, et al. found that ARG patterns in infants were more similar to their own mothers than to other unrelated mothers [26]. Parnanen, also found that MGE patterns in women's milk were similar to those found in infant's fecal samples. Additionally, mode of delivery and prematurity are other factors that play a role in the vertical transmission of ARGs [15, 35].

Prenatal antibiotic use effect on infant's microbiota

Antibiotics may have an effect on the commensal microbiota in pregnant women which would also influence the newborn's microbiota. Maternal GBS prophylaxis is associated with higher percentage of Enterobacter and lower diversity in infants [32, 36]. Those results are limited to anti-GBS treatment and excluded other antibiotic exposures. Additionally, animal studies presented evidence of microbiome alterations in offspring from mothers with history of antibiotic exposure during pregnancy [37-38].

Rationale

The use of antibiotics during pregnancy has been rising over decades due to its beneficial effect reducing the incidence of prenatal and postpartum complications such as GBS infections, preterm births and severity of infection after cesarean [20, 23, 39]. In spite of the high use of antibiotics during pregnancy, there is scarce information about the effects of prenatal antibiotic exposure shaping pregnancy fecal and vaginal resistome before delivery and, therefore, its vertical transmission to newborns [19, 40]. Therefore, more studies assessing this problem are needed to fill current knowledge gaps.

The present study provides much needed, prospectively-collected information about the characteristics of the pregnancy and infancy resistomes and mobilomes through the use of highly multiplexed and targeted analysis of ARGs and MGEs. Additionally, it provides information about the association between antibiotic use and changes in resistome patterns.

Research Question and Hypotheses

The present study characterized resistome data from pregnant women and 6-month-old infants. Additionally, we compared women and infants who were exposed to antibiotics during pregnancy with women and infants non-exposed. Data from two cohorts of pregnant women in Lansing and Traverse City, MI were used [27]. Additionally, mobile genetic elements (MGE) were targeted to understand mobilization of genes within the fecal community. Resistome vertical transmission patterns between women and infants were also studied.

We proposed the following:

1. Analyse resistome vertical transmission patterns between pregnant women and their infants. We hypothesized that:
    a. There are ARGs being shared between pregnant women and their infants.
2. Determine if antibiotic use during pregnancy affects pregnancy resistome. We hypothesized that:
    a. Antibiotic exposed pregnant women have higher resistome diversity and richness compared to non-exposed.
3. Determine if antibiotic use during pregnancy affects the infancy resistome. We hypothesized that:

a. Infants whose mothers where exposed to antibiotics during pregnancy have higher resistome diversity and richness compared to non-exposed.

MATERIALS AND METHODS

Study Population

All samples were collected as part of the $ARCH_{GUT}$ and $BABY_{GUT}$ cohorts in Lansing and Traverse City, MI. $ARCH_{GUT}$ was nested within a larger cohort study called The Archive for Research in Child Health (ARCH) which mainly aimed to collect data from pregnant women to understand biological, clinical and epidemiological risk factors for childhood disease and problems in development in that population. Women younger than 18 years, underweight (BMI<18.5) and unable to complete an interview in English, were excluded in both cohorts. Participants provided written consent at enrollment.

A total of 51 samples from pregnant women and 42 samples from 6-month-old infants, including a set of twins, were available for analysis (Figure 1). Women completed a questionnaire at enrollment with demographic and other information. Once the infant was 6 months old, their mothers completed a second questionnaire with information about the infant. There were 37 mother-infant dyads. $ARCH_{GUT}$ and $BABY_{GUT}$ cohorts were approved by Michigan State University Institutional Review Board (IRB 15-1240 and 14-170M).

Dependent Variable – Antibiotic use during pregnancy

We reviewed clinical records regarding history of infections and antibiotic use among 174 women with available data recruited as part of the ARCH cohort. Subsequent analyses were done from a subset of $ARCH_{GUT}$ and $BABY_{GUT}$ cohorts including 51 pregnancy samples and 42 infancy samples from which we got resistome data.

Information assessing antibiotic use in women (n=51) one-year prior recruitment was collected using a standardized questionnaire at enrollment.

Additionally, antibiotic use during pregnancy was assessed through medical records in 29% (n=15) of women with medical records available. We assembled 6 comparison groups: 1) any antibiotic treatment during pregnancy (yes/no), 2) any antibiotic treatment in the third trimester (yes/no), 3) any antibiotic treatment in the second trimester (yes/no), 4) any antibiotic treatment in the first trimester (yes/no), 5) total dose of all antibiotic treatments combined (<1000mg vs. ≥1000mg), 6) number of antibiotic treatments (<2, ≥2). We calculated pregnancy trimesters by defining pregnancy from the beginning of each woman's last menstrual period (LMP) and assuming three 90-day trimesters [5]. Antibiotic use by trimester was analyzed to assess any relevant exposure window.

Only systemic antibiotics taken before the sample collection were considered in the evaluation of antibiotic exposure in pregnant women; we excluded antibiotic exposure during labor and delivery. On the other hand, infants whose mothers received anti-GBS prophylaxis or antibiotics during pregnancy were classified into the exposed group.

Cohen's Kappa test was used to compare antibiotic exposure status measured by questionnaire and medical records.

Antimicrobial Resistance Genes and Mobile Genetic Elements

Fecal samples from pregnant women were collected during their third trimester of pregnancy. Fecal samples from infants were collected near 6 months of life. MoBio Powersoil DNA Isolation kit (Qiagen MoBio, Carlsbad, CA) was used for DNA extraction. More information

can be found elsewhere [27]. WaferGen SmartChip Real-time PCR was used to perform high-capacity quantitative PCR to identify a broad group of antibiotic resistance genes, clinically relevant, in a single assay. Methods regarding the PCR can be found elsewhere [28]. Overall, 116 and 27 genes were targeted as ARG and MGE respectively (Table 1). ARGs belonged to 8 antibiotic classes: Aminoglycoside (n=21), beta-lactamase (n=16), fluoroquinolone (n=4), multi-resistant drug (MDR) (n=21), Macrolide-lincosamide-streptogramin B (MLSB) (n=18), sulfonamide (n=5), tetracycline (n=13) and vancomycin (n=8) groups. There was one additional class called Other (n=10) which included miscellaneous genes resistant to Amphenicol and Phenicol. A Ct of 30 was used as threshold cutoff at assessing absence/presence of genes. In other words, samples with detected amplified sequences before 30 cycles of PCR were considered positive.

Assessment of covariates

Additional covariates that have been associated with changes in resistome patterns were collected in this study. We assessed maternal race, age, pre-pregnancy BMI (normal: $18.5 \leq BMI < 25$, overweight: $25 \leq BMI < 30$, obese: $BMI \geq 30$), history of smoking, and parity using the questionnaire at enrollment. Infant's sex, mode of delivery (vaginal / C-section), breastfeeding status (percentage of human milk in infants' diet: <50% / ≥50%), and sample shipping time was assessed using the questionnaire at 6 months after birth.

Statistical Analysis

ARG and MGE composition and structure

To assess resistome composition, we used a Real-time PCR threshold of 30 cycles to assess presence (≤30 cycles) and absence (>30 cycles) of genes. Sample richness, a measure of microbiome health, was defined as the total number of ARGs/MGEs present in each sample. Additionally, we calculated richness within each of the eight ARG classes. We used Wilcoxon, also called as Mann-Whitney U test, a non-parametric test to test for significant differences between independent groups.

To assess resistome structure, we first calculated the number of genetic copies for each gene based on Real-time PCR Ct values using the following formula:

$$GC = 10^{((30-CT)/3.3333))}$$

Then, we calculated relative abundance by normalizing the number of genetic copies using 16S rRNA gene. This extra step is needed to correct for between sample DNA variation.

$$RA = GC_{ARG\ or\ MGE\ gene}/GC_{16S\ rRNA\ gene}$$

Therefore, relative abundance was defined as the average coverage of ARG/MGE in each sample (ARG/MGE copy number per 16S rRNA copy number). Additionally, we calculated relative abundance within each of the eight ARG classes.

Similarities in ARG and MGE patterns between pregnancy and infancy resistome

To understand the influence of maternal gut resistome on infants, we compared presence/absence patterns in infants and their mothers.

Shannon and Inverse Simpson indexes were calculated to assess Alpha-diversity using diversity function from vegan package in R. Sorensen (community composition) and Bray-Curtis (community structure) dissimilarity indexes were calculated to assess Beta-diversity using vegdist function from vegan package. Adonis function was used to test statistically significant differences in Beta diversity by permutational multivariable analysis of variance (Permanova: 9999 permutations). We used Benjamini and Hochberg methods for p value correction [29].

Resistome and mobilome Bray-Curtis and Sorensen Beta diversity indexes, using relative abundance and presence/absence data respectively, were calculated between related and unrelated dyads. Diversity matrixes were divided into: 1) women and infants from different families, 2) women-women or infant-infant from different families, 3) women and infants from the same family, for all 37 available dyads. Kernel density plots using ggplot2 package in R were performed [26]. Anova test was performed in R to test statistical significance (n permutations= 9999).

Ordination analysis

Ordination analysis weas performed to study data clustering. We used the cmdscale command in R with Bray-Curtis dissimilarity index to draw Principal Coordinate Analysis (PCoA) graphs. Permanova analysis – using Adonis function - was used to test for statistically significant differences between clusters. Additionally, Operational Taxonomic Units (OTU) data from fecal samples were available for this study [27] and PCoA graphs were used to visualize this data.

Co-occurrence patterns among ARGs and MGEs

We explored ARG/MGE co-occurrence within both the pregnancy and the infancy samples using Spearman correlation in R. ARG and MGE relative abundances were log transformed for this analysis. Correlation values higher than 0.60 were selected for networking analysis using Gephi v.0.9.1 layout Fruchterman Reingold.

Correlation matrix between resistome and microbial taxa

To understand if the fecal microbial taxa are responsible for the resistome structure, we performed a Procrustes analysis using the command protest in vegan based on PCoA results from the frequency of OTUs at phyla level and ARGs relative abundances.

Additionally, to understand the specifics of the association between resistome and microbial taxa, we identified those OTUs at Genus, Family and Phyla level that were correlated with ARG relative abundances using Pearson correlation in R. For this analysis, we choose only those ARGs present in at least 50% of the samples to reduce bias due to less frequent ARGs. Correlations higher than 0.80 were selected for further exploration using networking approaches.

P values lower than 0.05 were considered as statistically significant, while p values lower than 0.1 were considered as trends.

Analysis plan

Aim 1: Analyze resistome vertical transmission patterns between pregnant women and their infants.

ARG and MGE vertical transmission were assessed using a multi-approach analysis. First, we calculated the total number of genes shared between pregnant mothers and their infants normalized by the number of genes detected either in the infant or mother sample. Beta diversity was calculated to understand vertical transmission.

Aim 2: Determine if antibiotic use during pregnancy affects the pregnancy resistome.

The association between antibiotic use during pregnancy and women's resistome structure (relative abundance) and composition (absence/presence) was assessed by comparisons in alpha and beta diversity indexes between those exposed to antibiotics and those non-exposed.

Aim 3: Determine if antimicrobial use during pregnancy affects the infancy resistome.

The association between antibiotic use during pregnancy and infants' resistome structure (relative abundance) and composition (absence/presence) was assessed by comparisons in alpha and beta diversity indexes between those exposed to antibiotics and those non-exposed.

RESULTS

High antimicrobial use in pregnant women from the ARCH cohort

Overall, 174 women recruited as part of the ARCH cohort, had medical records available. Of those, 110/174 (63%) were prescribed with antimicrobials to treat infections throughout pregnancy. Nitroimidazole (28%) was the most frequent antimicrobial used, follow by nitrofurans (18%), cephalosporin (16%) and antifungals (15%) (Figure 2). Including all infections, median duration of antimicrobial treatment in women was 7 days (range: 1-30 days). We could not find any difference in the median time of antimicrobial use between the first to the fifth infections. Moreover, 43/163 (27%) women received anti-GBS prophylaxis before delivery. Overlap between antimicrobial use and anti-GBS treatment was found in 30 women, whereas the remaining 13 women reported only anti-GBS prophylaxis. Use of antibiotics during labor were recorded in 67/170 (39%) women with available data.

Study population characteristics

Resistome information from 37 dyads, and 15 women and 5 infants who were not part of dyads, was available for this study (total n=93). Characteristics of the study population with resistome data is available in Table 2. Most women were Caucasian (87%) and had given birth to fewer than 3 infants (80%). In total, 69% of all infants were male, 60% were delivered by vaginal route, and 40% by caesarean. Additionally, 80% of infants had incorporated solids as part of their diets (Table 2).

Abundance of screened genes among samples

All of the 143 genes being screened were present in at least one of the 93 samples from women and infants combined. Overall, the number of detected ARGs per sample ranged between 11 and 81 per individual, while MGEs ranged between 1 and 20. Exclusively in pregnancy samples we found all screened genes with ranges between 11-81 and 1-17 number of detected genes for ARGs and MGEs respectively. In contrast, in infancy samples we detected 98% (140/143) of genes being screened with ranges between 39-70 and 9-20 for ARGs and MGEs respectively.

Regarding sum of relative abundance, overall, aminoglycosides and MGEs were the most abundant class of genes, followed by MLSB, MDR and Betalactamase genes. Sulfonamide, fluoroquinolone and vancomycin relative abundance were low. This distribution would reflect the number of genes being screened in those specific antibiotic classes.

High variability in shared ARGs/MGEs patterns between pregnant women and their infants

Information from the 37 dyads were analyzed. We found a broad variability regarding the overlapping of genes between infants and their mothers (Figure 3) with nearly 29% and 24% of all ARGs and MGEs being shared respectively. Tetracycline genes, numerically, were the most shared (50%) between the dyads (Figure 4). Intl2 and IncN_rep, both MGEs, were found exclusively in infancy samples. Similarly, aac(6)-im (aminoglucoside), NDM new (beta-lactamase), vanTG (vancomycin) and catQ (phenicol) were found exclusively in pregnancy samples.

Infancy resistome is more diverse than pregnancy resistome: Alpha diversity

The sum of relative abundances, based on all infancy and all pregnancy data (n=93), revealed that pregnancy gut resistome was significant enriched with aminoglycoside (p value

$1.76x10^{-7}$) and vancomycin (p value=$7.19x10^{-10}$) ARGs. In contrast, MDR (p value=0.01), MLSB (p value=$5.67x10^{-4}$), sulfonamide (p value=$1.71x10^{-5}$), and fluoroquinolone ARGs ($1.36x10^{-4}$) and also MGEs (p value=0.002) were significantly more abundant in infant's resistome (Figure 5).

Total abundance (Figure 6) and the mean Ln of MGE relative abundance (Figure 7) was higher in the infancy resistome compared with the pregnancy resistome (Wilcoxon test, p value 0.0015 and 0.00014 respectively). ARG total abundance and relative abundance did not differ between pregnancy and infancy samples. Also, the number of ARGs/MGEs were not significantly different between pregnancy and infancy samples (Figure 8A & 8B); Wilcoxon test, p value 0.81 and 0.83 respectively).

Infants have significantly higher diversity in MGE relative abundances compared with pregnant women (Shannon diversity p=0.0015, Inverse Simpson p=0.0032). Similarly, ARG relative abundance was higher in infants than in pregnant women (Shannon diversity p=0.029, Inverse Simpson=0.087) (Figure 8C & 8D).

Relative abundance differences between pregnant women and infants were also analyzed within antibiotic classes. Infants have significantly higher diversity of resistance genes such as betalactamase, fluoroquinolone, MDR, and sulfonamide compared with pregnant women samples. However, pregnant women have significantly higher diversity of vancomycin resistant genes compared with infants. There were no differences in gene diversity within aminoglycoside, MLSB or tetracycline resistant genes (Figure 9).

Pregnancy resistome differs from infancy resistome: Beta diversity

In this analysis we used all women and infants' samples (n=93). Data were clustered based on Bray-Curtis (structure) and Sorensen (composition) dissimilarity indexes. Infancy ARG and

MGE relative abundances were significantly different from pregnancy samples (Figure 10). This clustering was consistent with the Sorensen index when using absence-presence data (Figure 11).

Density plots were performed using Beta diversity index. Based on Figure 11, Type p values represented that infancy and pregnancy resistomes are different. Family p values represented that related dyads are more similar than unrelated dyads. Type & family p values represented that infants are more similar to other infants than with their own mothers. Results showed that MGE relative abundance patterns (Figures 12A & 12B) in infants' samples were more similar to those from their own mothers during pregnancy than to those from unrelated pregnant women. Furthermore, infants' MGEs absence-present patterns were more similar to other infants than to their own mothers. ARG presence/absence Beta diversity values showed a bimodal distribution (Figures 12C & 12D).

Self-report and clinical data: resistome patterns differ in antibiotic exposed vs. non-exposed

Among women with available questionnaire data and medical records (n=12), 6/12 (50%) women have inconsistent answers regarding their antibiotic exposure (Cohen's Kappa=-0.028, p value=0.921). Therefore, we analyzed the data using both sources of information assessing antibiotic exposure.

Questionnaire data: self-report

Antibiotic exposure was assessed using data from the questionnaire provided to women at enrollment (n=51). Among 49 women with available antibiotic exposure status, 19 (39%) reported been exposed to antibiotics one-year prior to completing the third-trimester questionnaire. Neither alpha nor beta diversity of ARGs/MGEs differed between women exposed

to antibiotics and non-exposed except for differences in total relative abundance and diversity indexes in MLSB resistance genes. Unexposed women have higher diversity (Shannon index p=0.04191, Inverse Simpson p=0.04408) among genes resistant to MLSB compared with women exposed to antibiotics. We did not find significant differences between infants whose mothers were exposed to antibiotics during pregnancy and those non-exposed.

Medical records data: clinical data

Additionally, we used medical records from 15 women to assess antibiotic exposure. We compared alpha and beta diversity between those women who used antibiotics during pregnancy (n=6) and those who did not use (n=9). No significant differences in ARGs/MGEs pregnancy samples existed, except that exposed pregnant women had higher relative abundance of vancomycin ARGs compared with non-exposed (p value=0.015). Additionally, we compared alpha and beta diversity between infants with prenatal antibiotic exposure and those non-exposed. Prenatal antibiotic exposure included antibiotics during pregnancy or anti-GBS treatment if reported. Non-exposed infants had higher abundance of beta-lactamase ARGs compared with non-exposed (p value=0.025). This association was also found when analyzing antibiotic exposure exclusively during the third trimester (p value=0.033), but not during second or first trimester.

Exposed infants had higher MGE diversity compared with non-exposed (Shannon diversity, p value=0.025). This association remained constant when comparing infants whose mothers received less than two antibiotic treatments during pregnancy with those exposed to two or more than two antibiotic treatments (p value=0.042). Also, when comparing infants whose mothers received < 1000mg in all infections with the $\geq$ 1000mg group (p value=0.042).

Relative abundance patterns in exposed infants differed from non-exposed (Bray-Curtis, p value=0.0103). Also, ARG/MGE absence/presence data from infants whose mothers received < 2 antibiotic treatments during pregnancy differed from the ≥ 2 antibiotic treatments group (p value=0.012). Relative abundance patterns from infants whose mothers received < 1000mg differed from the ≥ 1000mg group (p value=0.033).

Other covariates also have effects on the resistome

We also assessed the effect of additional factors on the pregnancy and infancy resistome. Breastfeeding status, measured as the percentage of breastfeeding in the infant's diet, explained differences in resistome patterns between infants with <50% & ≥50% breastfeeding. We did not find significant differences, but trends, in resistome among infants those delivered by C-section and by vaginal route. Data also suggested that ethnicity may play a role in the resistome. We found differences in resistome patterns between white and non-white women.

Co-occurrence of ARGs/MGEs in fecal samples

Networking analysis graphs nodes represented a gene and each edge represented quantitative co-occurrence. We found two major clusters. The largest cluster was found in both pregnancy and infancy samples and included MDR ARGs: tolC, acrB, mdth, acrF, mdtA, mdtE/yhiU (Figure 13 & 14). The smaller cluster was found only in infants' samples and included aminoglycoside ARGs: sat4, sphA3, aadE, aph3-III (Figure 13). Several ARGs were correlated with MGEs; for instance, pica (MLSB) and IS630, tetW (tetracycline) and intlF165 in pregnancy samples; tetQ (tetracycline) and IS613, msrC (MLSB) and IS256 in infancy samples.

Gut microbiome is associated with ARGs

We found 17 phyla in both infancy and pregnancy samples. Infants' phylum patterns are significant different from women's samples (Permanova, p value < 0.0001) (Figure 14).

Additionally, ARGs patterns had a significant association with microbial taxa patterns (Procrustes, p value = 0.001). This would suggest that microbiome structure is responsible for the ARGs patterns in fecal samples. We used correlation between microbial patterns and ARGs/MGEs relative abundances to identify potential associations between phyla, family or genus with specific genes. We found several genes highly correlated with 4 phyla: Fusobacteria, Lentisphaerae, Synergistetes, and Tenericutes in pregnancy and infancy samples (Table 3 & 4). Results from the correlation at Family (Tables 5 & 6) and Genera (Tables 7 &8) levels are available. For instance, Pseudomonas genera was associated with czcA from the MDR antibiotic class, and Staphylococcus is associated with IS1247 (MGE) and vanHD (vancomycin).

DISCUSSION

Antibiotic use is associated with colonization of antibiotic resistant bacteria. The exposure to antibiotics creates a selective pressure for those drug-resistant strains that are already part of the fecal microbiome, increasing the number and diversity of ARGs in exposed groups. This overrepresentation of ARGs can persist over time even in the absence of a selective pressure and therefore it can become a potential threat to the host [30]. Information about the effects of prenatal exposures on infant's microbiome and resistome is scarce, particularly effects of maternal antibiotic use. The current study describes an exploratory analysis characterizing the resistome of pregnant women and 6-month-old infants and it also assesses the effect of antibiotic exposure during pregnancy on the infant's resistome in a subset of samples from the $ARCH_{GUT}$ and the $BABY_{GUT}$ cohorts.

Studies have focused previously on postnatal factors, such as diet and lifestyle, affecting infants' microbiome patterns. Several postnatal factors, such as delivery mode and breastfeeding status, have been associated with effects on the acquisition of microbiota during early life [30, 35, 41]. Blackhed et al. found differences between infants with exclusive breastfeeding compared with exclusive formula-feeding [20]. On the other hand, Fallani et al., found that the effect of country of birth on the infant's microbiome was more pronounced than the effects caused by delivery mode and feeding methods [31]. Forslund et al. also found differences in gut resistance between countries [32].

We found a high frequency of medication use in the ARCH cohort with 63% of pregnant women being prescribed with medications. Our results from the ARCH cohort coincided with Andrade and colleagues' study where, through medical records, they found 64% of 98,182

pregnant women being prescribed with a drug other than vitamin during pregnancy [5]. Furthermore, we showed that 27% of pregnant women received anti-GBS treatment which is also consistent with previous estimates reporting 30% [33]. Therefore, given the high antibiotic use among this particular population, we decided to study the impact of pre-natal antibiotic exposure on the infant's resistome.

We found high variability in shared ARGs/MGEs patterns between pregnant women and their infants. Every infant sample contained ARGs that were different from their own mother's resistome, suggesting that environment also plays an important role in the acquisition of resistant genes at early age [10, 12]. Additionally, external factors regarding diet and environment could also be important in the transmission of ARGs from mothers to infants, especially at 6 months when infants start to add solids into their diets. The distribution of dietary habits among our sample could explain the large diversity regarding the proportion of genes shared between mother and infants, which was also found previously in 4-month-old children [21].

Resistance to aminoglycosides and MGEs were the most abundant in infant/women samples. Additionally, beta-lactamase, MDR and tetracycline relative abundance were also high. Those antibiotics have been prescribed for many decades, so it is understandable that resistance is well spread among gut microbiota [26, 47]. Tetracycline, in particular, is one of the most frequent antibiotics used as treatment in several infections and its high abundance within fecal samples have been found by Parnanen and others [10-11, 13-14]. Moreover, in our study, Tetracycline was the most frequent antibiotic class being shared between mothers and their infants, which would suggest that those genes are prone to vertical transmission in line with

previous studies [24]. However, we cannot rule out independent acquisition of tetracycline genes in pregnancy and infancy samples. Interestingly, Moore et al. found that infants' tetracycline ARGs were different from those found in their mothers [10].

Vancomycin is the last resource to treat Gram positive bacteria resistant to most other antibiotics [34]. Therefore, it is concerning that we found the presence of vancomycin ARGs in samples from pregnant women infants. Vancomycin ARGs have been detected previously in human fecal samples [13, 47]. Besides vancomycin, we also found fluoroquinolone resistant genes in both women and infants. Here, we want to point out that the spread of clinically important ARGs may not be related to exposure to specifics antibiotics, but it could be the result of horizontal gene transfer within and between bacterial species [25]. Known mechanisms for horizontal gene transfer include MGEs, plasmids and prophages [17].

We found that infants have a higher relative abundance of ARGs compared with pregnant women which is supported by other studies [12, 14, 30]. Additionally, we found a more diverse resistome and mobilome compared with pregnant women, while previous research has not found differences in diversity between those two groups [26]. On the other hand, Moore et al. found differences comparing 1-2-month-old infants with their mothers [10]. Higher ARG abundances and diversity could be explain by the fact that the infants' gut is dominantly colonized by the bacterial class Gammaproteobacteria, known to habor high loads of ARGs [35]. Moreover, infants have higher abundance of MGEs which could facilitate transfer of ARGs within and between species [26].

In line with previous results, we found differences between 6-month-old infant and pregnancy resistome, suggesting that the reported divergence remains persistent at 6-month

[26]. Clustering of ARG patterns between women and infants suggests that despite the variability in ARGs among different samples, specific ARGs were enriched across both the pregnancy and the infancy resistome. Clustering can be explained by dietary factors such as breastfeeding status because 6-month-old infants start to add solid food into their diets. This transition in food intake could lead to shifts in gut microbiota as an adaptive response to a new environment [24]. Other factors may be involved in differentiating resistome patterns between women and infants.

Infants MGE relative abundance patterns were more similar to their own mothers compared with unrelated mothers, which would suggest that the maternal component and the environment shared between mother and their infants could play a role in the acquisition of the mobilome [10]. We also found that infants' fecal mobilome was more similar to other infants than to their own mothers' samples, suggesting that at 6 months-of-age, the influence of the environment is more important in the acquisition of the mobilome compared to maternal influence.

We found changes in diversity and relative abundances of specific ARGs between antibiotic exposed groups (measured by questionnaire and medical data) and non-exposed. Medical data explained much more variation in diversity between exposed and non-exposed groups even with a small sample size (n=15) in contrast with questionnaire data (n=51).

Studies assessing the effect of prenatal antibiotic on the infant's microbiome had inconsistent results. Regarding vertical ARG transmission between mothers and their children, Gomez et al. found that infants who shared similar oral resistome patterns with their mothers were less likely to have received intrapartum antibiotics [21]. Arboleya et al. found that Intrapartum antimicrobial prophylaxis had the same effect or stronger on the resistome than the

direct administration of antibiotics to the infants at the first month of life [36]. Parnanen, et al. reported that intrapartum prophylaxis increased ARGs and MGEs in 6-months-old infants [26]. Other researchers have focused on perinatal exposure to antibiotics and how it can affect the early microbiome colonization. Arboleya et al. reported that microbiome of newborns exposed to perinatal antibiotic use, which included antimicrobial prophylaxis, have higher frequency of the Enterobacteriaceae family. In the same study, the microbiome of 1-month pre-term and full-term infants whose mothers received antimicrobial prophylaxis had different patterns compared to non-exposed groups [36], however the observed effects disappeared after 90 days of life. Rose, et al. did not found significant differences in taxonomic diversity and amount of antibiotic between exposed and non-exposed preterm infants, which was attributed to the small sample size and heterogeneity of the sample [37].

Dose of antibiotic during pregnancy may affect the infancy resistome. We could not find any other study reporting dose of prenatal antibiotic use on the infants' resistome, however those studies assessing duration of treatment reported a decrease in microbial diversity among pre-term infants exposed to brief treatments [22].

Co-occurrence analysis identified two defined clusters among ARGs. Cluster 2 included aminoglycoside resistant genes aadE, aph3-III, apha3 and sat4 which have been reported previously by Werner, et al. and other studies, as part of Tn5405 - a transposon in isolates of *Enterococcus faecium* [51-53].

Several ARGs were associated with MGEs which would allow their dissemination through horizontal transfer among different taxa. Co-occurrence analysis supports the idea of the gut

microbiota functioning as a reservoir of ARGs, with several genes with the ability to be transferred in mobile elements [11].

Interestingly, fecal microbial taxa explained resistome patterns. Significant Procrustes analysis between OTUs and ARGs patterns have been reported previously by Moore et al. Feng, et al. and Forsberg, et al. [12-13, 54]. Specific ARGs were also correlated to some bacterial genera. Although, Feng et al., using a different panel of genes, found *Escherichia coli* correlated to 45 ARGs, we could only find the genus Escherichia / Shigella, in infancy samples correlated with 5 MDR ARGs: acrF, mdth, mdtE, tolC, and acrB. We could not find any correlation within pregnancy samples.

The present study has several strengths such as the use of a culture-independent technique, high-capacity quantitative Real-time PCR with Wafergen smartchip to detect ARGs/MGEs. Wafergen Smartchip allowed us to screen for 116 ARGs and 27 MGEs. This wide coverage captured a more representative sample of the resistome compared with previous research that studied a limited number of ARGs in infants and their mothers [24].

On the other hand, high-capacity quantitative PCR arrays brought limitations to the study including amplification bias, false-negative and false-positive results due to amplification complications [38]. To reduce those biases, we set a low Ct value of 30 cycles which tried to capture strong positive reactions in the samples. Furthermore, all ARGs and MGEs screened via PCR are limited to previously known genes, underestimating real resistant load within the samples. Another limitation is the absence of maternal fecal samples before antibiotic treatment. Therefore, we cannot be sure that differences between exposed and non-exposed groups are due to antibiotic treatment per se.

We found inconsistencies between questionnaire and clinical data assessing history of antibiotic use during pregnancy which is predictable given the fact that both methods assessed antibiotic exposure in different time periods. Questionnaire data assessed antibiotic use one-year prior questionnaire, while clinical records assessed antibiotic exposure during pregnancy. Additionally, both methods to assess antibiotic exposure might have introduced bias through misclassification. Questionnaire data relies on self-reporting antibiotic exposure which means that women had to be aware of the type of medication they received. In contrast, clinical records rely on prescription information which may differ from the authentic use of the medication [39]. In other words, it is possible that physicians prescribe the antibiotic and that the patient decline to take the medication.

CONCLUSION AND FUTURE RESEARCH

Our data show that a high percentage of pregnant women are exposed to antibiotics. The use of antibiotics during pregnancy may disrupt maternal gut microbiota which later will be transferred to the newborn. Therefore, information regarding prenatal exposure to antibiotics is needed in order to predict potential threats to the efficacy of future antibiotic treatments for infants.

Our results highlight the importance of understanding possible effects of antibiotics not only in the pregnant women but also effects in newborns. We found that antibiotic exposure assessed by medical records explained some resistome differences between in exposed versus non-exposed infants and women. Even with a small sample size, our results suggesting that prenatal antibiotic use has a role in the acquisition of ARGs and MGEs in 6-month-old infants. However, for future studies, we strongly recommend increasing sample size to verify if the associations reported here remain. Furthermore, other variables not considered in this study due to the small sample size should be added into future analysis such as the spectrum of antibiotics (broad vs. narrow spectrum), duration of treatment, pharmacodynamics, and pharmacokinetics properties of antibiotic.

Additionally, future analysis should focus in building a generalized linear mixed effect model to determinate if the use of antibiotics during pregnancy affects the infant microbiome while controlling for other variables. For instance, delivery mode, breastfeeding, ethnicity, and sex [23].

Effects of prenatal antibiotic use should be considered when prescribing antibiotics to pregnant women. The results of this study, besides contributing to the general knowledge,

provide further information about the possible role of prenatal exposure to antibiotics in the infancy resistome. Further results are needed to draw solid conclusions that could guide public health decisions about antibiotic use in pregnant women.

APPENDIX

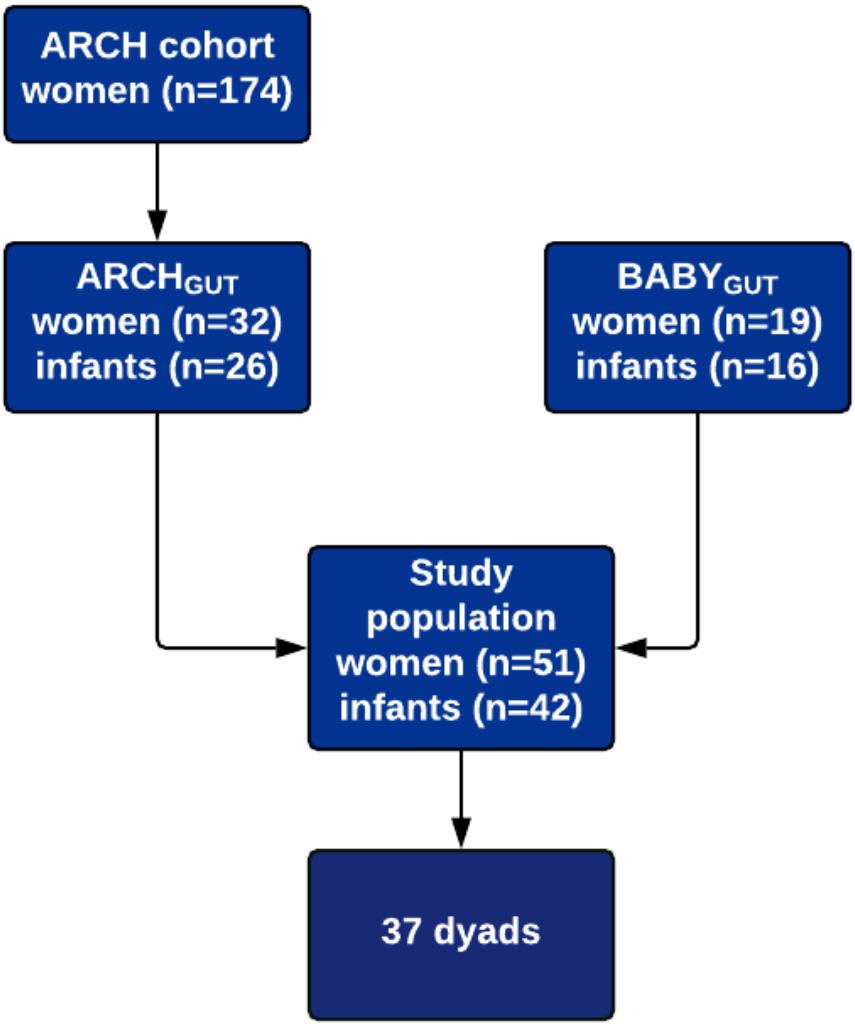

Figure 1 Flowchart of the selection of the study population

Table 1 ARGs and MGEs screened in the Wafergen Smartchip.

N °	Name	Functional classification	Target antibiotics
1	aacA/aphD	deactivate	Aminoglycoside
2	acrB	efflux	MDR
3	acrF	efflux	MDR
4	aphA3	deactivate	Aminoglycoside
5	IS613	MGE	MGE
6	blaOXY-2	deactivate	Beta-lactamase
7	cphA	deactivate	Beta-lactamase
8	sat4	deactivate	Aminoglycoside
9	ceoA	efflux	Other
10	tet(32)	protection	Tetracycline
11	emrD	efflux	MDR
12	mdtE/yhiU	efflux	MDR
13	mexA	efflux	MDR
14	erm(36)	protection	MLSB
15	aph(2')-Id	deactivate	Aminoglycoside
16	cfxA	deactivate	Beta-lactamase
17	cepA	deactivate	Beta-lactamase
18	blaCMY	deactivate	Beta-lactamase
19	blaSFO	deactivate	Beta-lactamase
20	sul2	protection	Sulfonamide
21	ermT	protection	MLSB
22	msr(C)	msr(C)	MLSB
23	Pbp5	protection	Beta-lactamase
24	blaCTX-M	deactivate	Beta-lactamase
25	aadE	deactivate	Aminoglycoside
26	strB	protection	Sulfonamide
27	tetA	efflux	Tetracycline
28	tetB	efflux	Tetracycline
29	tetQ	protection	Tetracycline
30	tetW	protection	Tetracycline
31	tetX	deactivate	Tetracycline
32	tetS	protection	Tetracycline
33	tnpA	MGE	MGE
34	tnpA	MGE	MGE
35	tnpA	MGE	MGE

Table 1 (cont'd).

36	folA	protection	Sulfonamide
37	ermX	protection	MLSB
38	VanB	VanB	Vancomycin
39	vanD	protection	Vancomycin
40	vanHD	protection	Vancomycin
41	vanHB	protection	Vancomycin
42	vgaB	efflux	MLSB
43	pica	protection	MLSB
44	oprD	efflux	MDR
45	penA	protection	Beta-lactamase
46	pmrA	deactivate	Other
47	mepA	efflux	MDR
48	mexE	efflux	MDR
49	sulA/folP	protection	Sulfonamide
50	ermA/ermTR	protection	MLSB
51	oleC	efflux	MLSB
52	tetbP	efflux	Tetracycline
53	tolC	efflux	MDR
54	vanRB	protection	Vancomycin
55	vanRD	protection	Vancomycin
56	vanTG	protection	Vancomycin
57	vanYD	protection	Vancomycin
58	qnrB-bob_redesign	efflux	Fluoroquinolone
59	merA-marko	unknown	MDR
60	int1-a-marko	MGE	MGE
61	intl2	MGE	MGE
62	IncN_rep	MGE	MGE
63	IncP_oriT	MGE	MGE
64	marR	regulator	MDR
65	intI1F165_clinical	MGE	MGE
66	NDM new	deactivate	Beta-lactamase
67	sul1 NEW	protection	Sulfonamide
68	orf39-IS26	MGE	MGE
69	ISSm2-Xanthob	MGE	MGE
70	ISEfm1-Entero	MGE	MGE
71	IS1111	MGE	MGE
72	aph4ib	aph4ib	Aminoglycoside

Table 1 (cont'd).

73	aph6ic	aph6ic	Aminoglycoside
74	spcN	deactivate	Aminoglycoside
75	aac(3)-iid_iii_iif_iia_iie	aac(3)-iid_iii_iif_iia_iie	Aminoglycoside
76	Aac6-Aph2	Aac6-Aph2	Aminoglycoside
77	aac(6)-im	aac(6)-im	Aminoglycoside
78	aadA7	aadA7	Aminoglycoside
79	aadA17	aadA17	Aminoglycoside
80	aadB	aadB	Aminoglycoside
81	ant6-ia	ant6-ia	Aminoglycoside
82	aph3-ib	aph3-ib	Aminoglycoside
83	acc3-iva	deactivate	Aminoglycoside
84	tetR	tetR	Tetracycline
85	tetG_F	tetG_F	Tetracycline
86	dfra21	dfra21	Other
87	dfrA22	dfrA22	Other
88	fosb	fosb	Other
89	mcr-1	mcr-1	Other
90	ere(A)	ere(A)	MLSB
91	erm(B)	erm(B)	MLSB
92	erm(E)	erm(E)	MLSB
93	erm(Q)	erm(Q)	MLSB
94	mphA	deactivate	MLSB
95	erm(35)	erm(35)	MLSB
96	erm(F)	erm(F)	MLSB
97	lsa(C)	lsa(C)	MLSB
98	catQ	catQ	Other
99	cmlV	cmlV	Other
100	blaCTX-M-1,3,15	blaCTX-M-1,3,15	Beta-lactamase
101	blaOXY-1	deactivate	Beta-lactamase
102	blaMIR	bla_MIR	Beta-lactamase
103	norA	norA	Fluoroquinolone
104	qepA_1_2	qepA_1_2	Fluoroquinolone
105	mdth	mdth	MDR
106	mdtg	mdtg	MDR
107	pcoA	pcoA	MDR
108	arsA	arsA	MDR
109	bacA_F	bacA_F (bacitracin)	Other

Table 1 (cont'd).

110	aph6ia	deactivate	Aminoglycoside
111	bexA/norM	efflux	MDR
112	ampC	deactivate	Beta-lactamase
113	tetPA	efflux	Tetracycline
114	mdtA	efflux	MDR
115	mefA	efflux	MLSB
116	blaTEM	deactivate	Beta-lactamase
117	tetM	protection	Tetracycline
118	lnuC	deactivate	MLSB
119	fabK	protection	Other
120	intl3	MGE	MGE
121	ISCR1		MGE
122	czcA	czcA	MDR
123	tet44	tet44	Tetracycline
124	aph3-III	aph3-III	Aminoglycoside
125	ant6-ib	aph3-III	Aminoglycoside
126	bla-ACT	bla-ACT	Beta-lactamase
127	aac(3)-Xa	aac(3)-Xa	Aminoglycoside
128	IS26	IS26	MGE
129	IS3	IS3	MGE
130	IS256	IS256	MGE
131	sugE	sugE	MDR
132	IS200_1	IS200_1	MGE
133	IS1247	IS1247	MGE
134	IS630	IS630	MGE
135	TN5403		MGE
136	IS200		MGE
137	IS21-ISAs29		MGE
138	Tn3		MGE
139	IncI1_repI1		MGE
140	IS91		MGE
141	terW		MDR
142	pbrT		MDR
143	oqxA		Fluoroquinolone

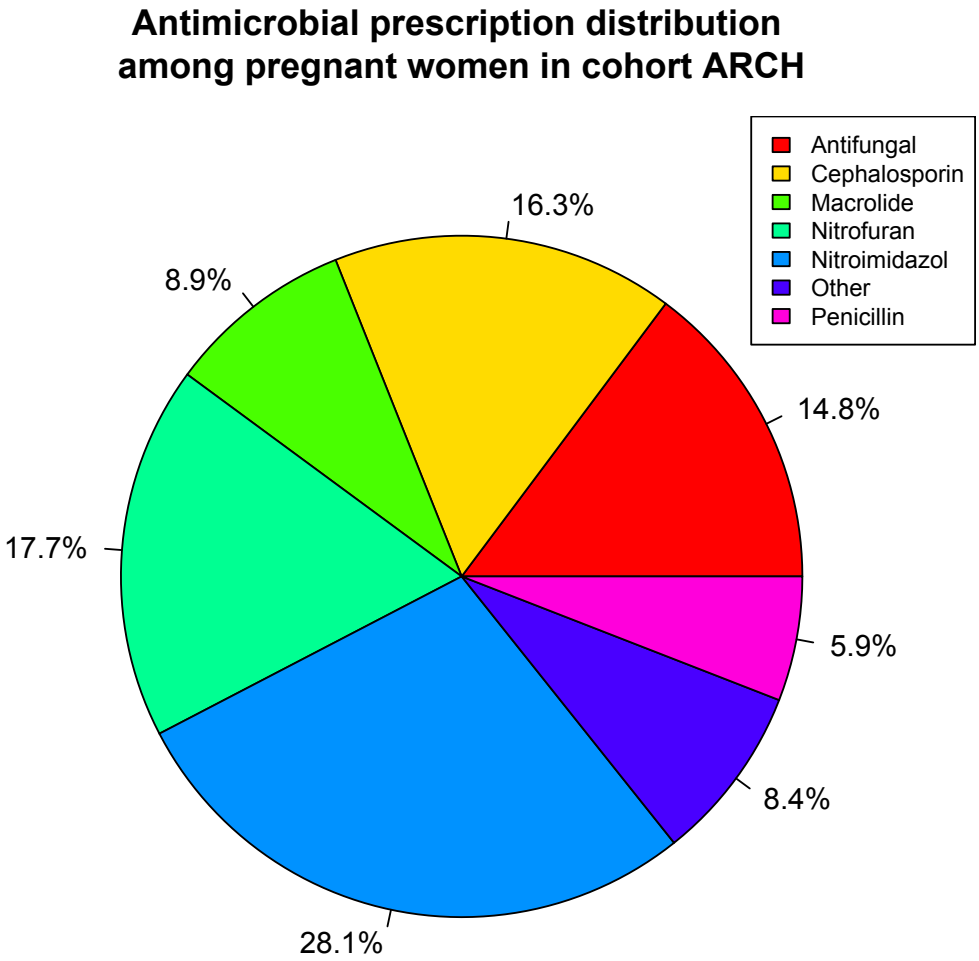

Figure 2 Distribution of antimicrobial drugs used to treat infections in pregnant women.

We used data from 110 women enrolled as part of the ARCH cohort with available antimicrobial treatment records.

Table 2 Clinical characteristics from pregnant women and infants with resistome data (n=93).

Women (n=51)		
Age (years)[1]		30.8 (22.9-37.5)
Race[2]		
	Non-white	6/45 (13)
	White	39/45 (87)
BMI[2]		
	Normal or underweight (<25)	18/50 (36)
	Overweight (25 - <30)	13/50 (26)
	Obese (≥30)	19/50 (38)
Smoking status[2]		
	Ever	22/49 (45)
	Never	27/49 (55)
Parity[2]		
	1-2	40/50 (80)
	≥3	10/50 (20)
Sample Shipping Time (days)[3]		4 (0-12)
Infants (n=42)		
Sex[2]		
	Female	13/42 (31)
	Male	29/42 (69)
Birth delivery[2]		
	Caesarean	17/42 (40)
	Vaginal	25/42 (60)
Breastmilk percentage[2]		
	<50%	17/42 (40)
	>=50%	25/42 (60)
Food [2]		
	No solid food	8/40 (20)
	Solid food	32/40 (80)
Sample Shipping Time (days) [3]		4 (0-22)
Birth weight (grams) [3]		3520 (2268-4940)

[1] median – 95 CI

[2] n(%)

[3] median - range

*Missing data Women: Race (n=6), BMI (n=1), Smoking (n=2), Parity (n=1), Sample Shipping Time (n=1).

**Missing data Infants: Type of food (n=2), Birth weight (n=3)*

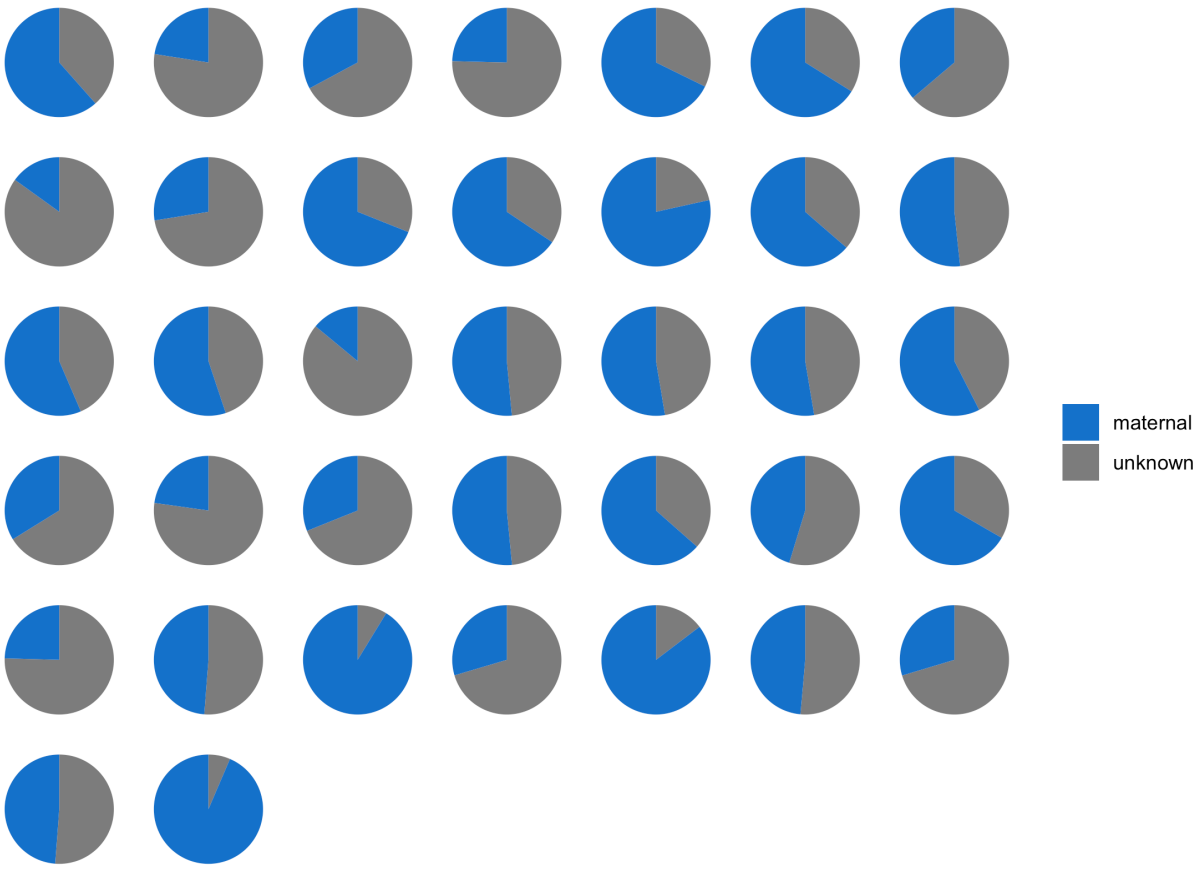

Figure 3 Proportion of pregnancy ARGs/MGEs (blue) and unknown source of environment (grey) in each infant sample's fecal resistome

We used data from all dyads (n=37). This graphic represents the high variability of shared ARG/MGE patterns between pregnant women and their infants.

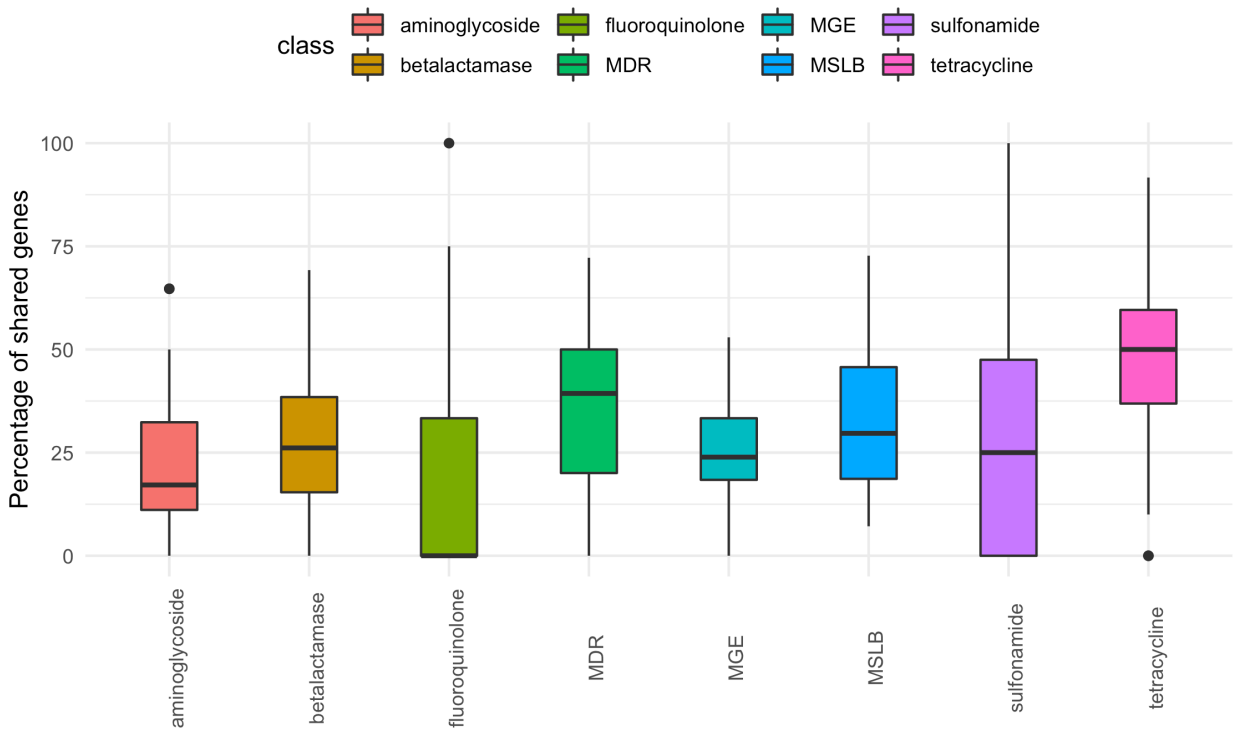

Figure 4 Percentage of shared genes between pregnant women and their infants.

Based on dyads (n=37) data.

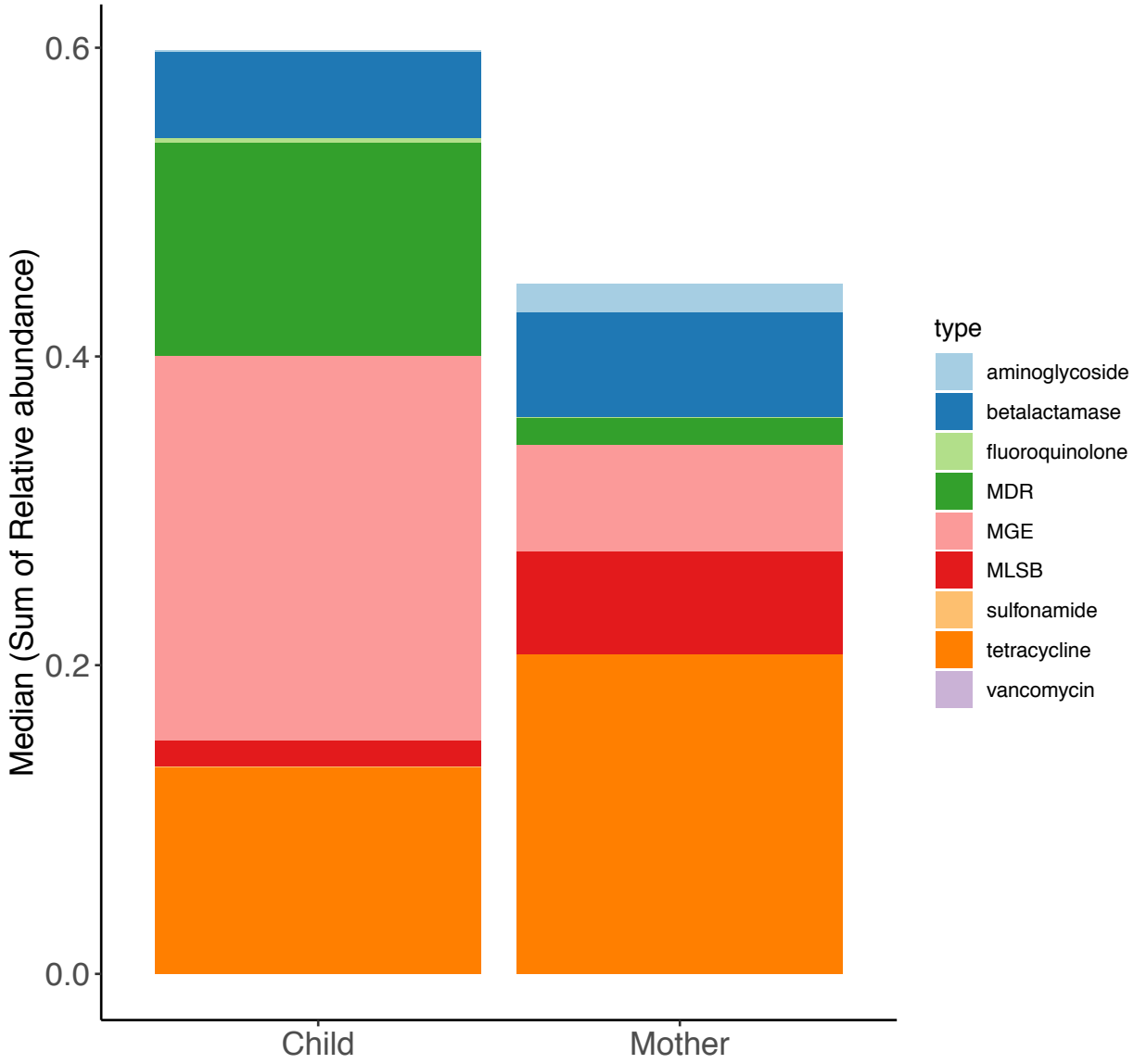

Figure 5 Aggregate ARG relative abundance in infancy and pregnancy samples

Based on all infancy (n=42) and pregnancy (n=51) samples.

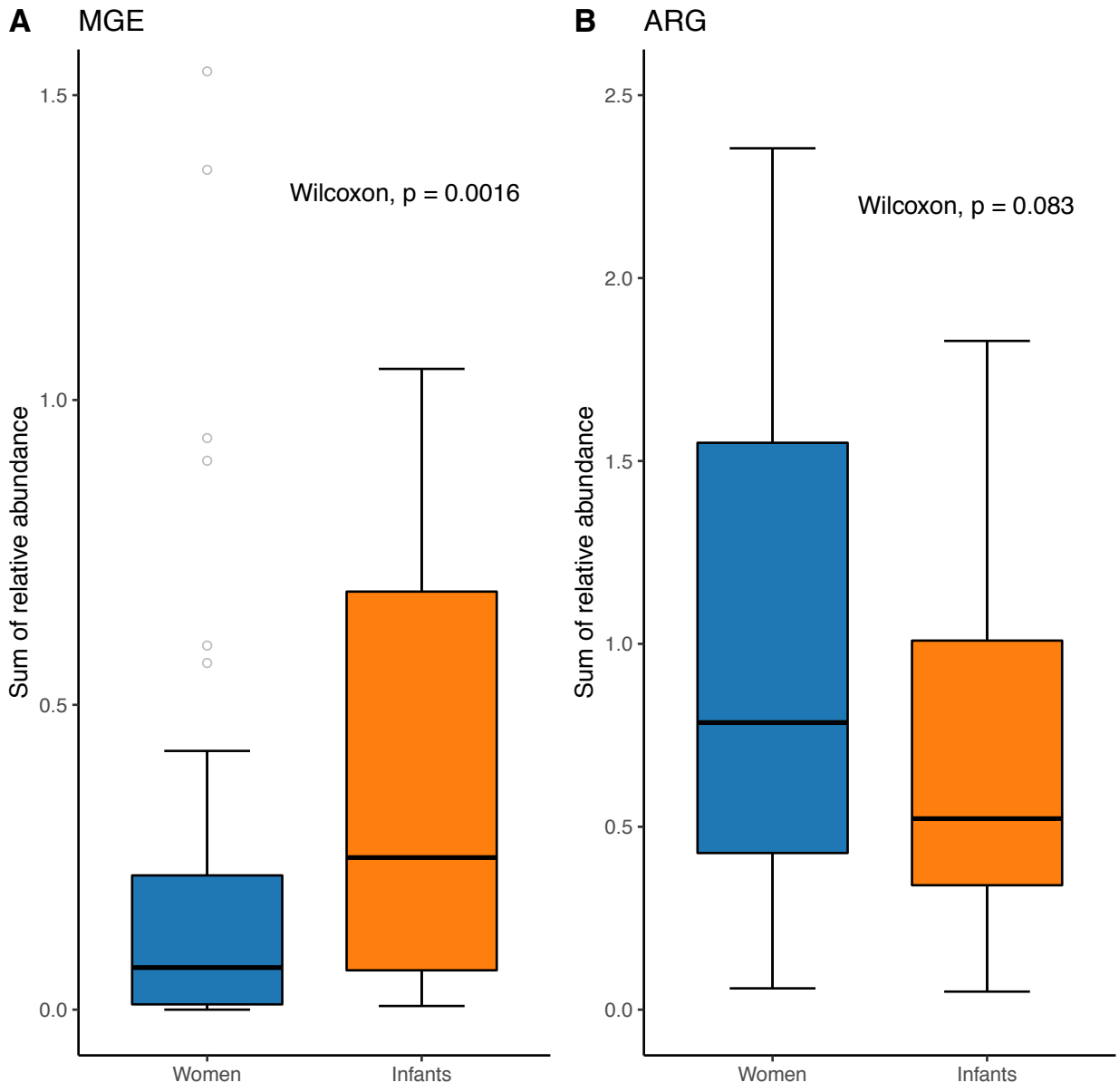

Figure 6 Sum of relative abundances in pregnant women and infants.

Based on all infancy (n=42) and pregnancy (n=51) samples. Wilcoxon parametric test was calculated to test for differences between pregnancy and infancy' samples A) MGE relative abundances, B) ARG relative abundances

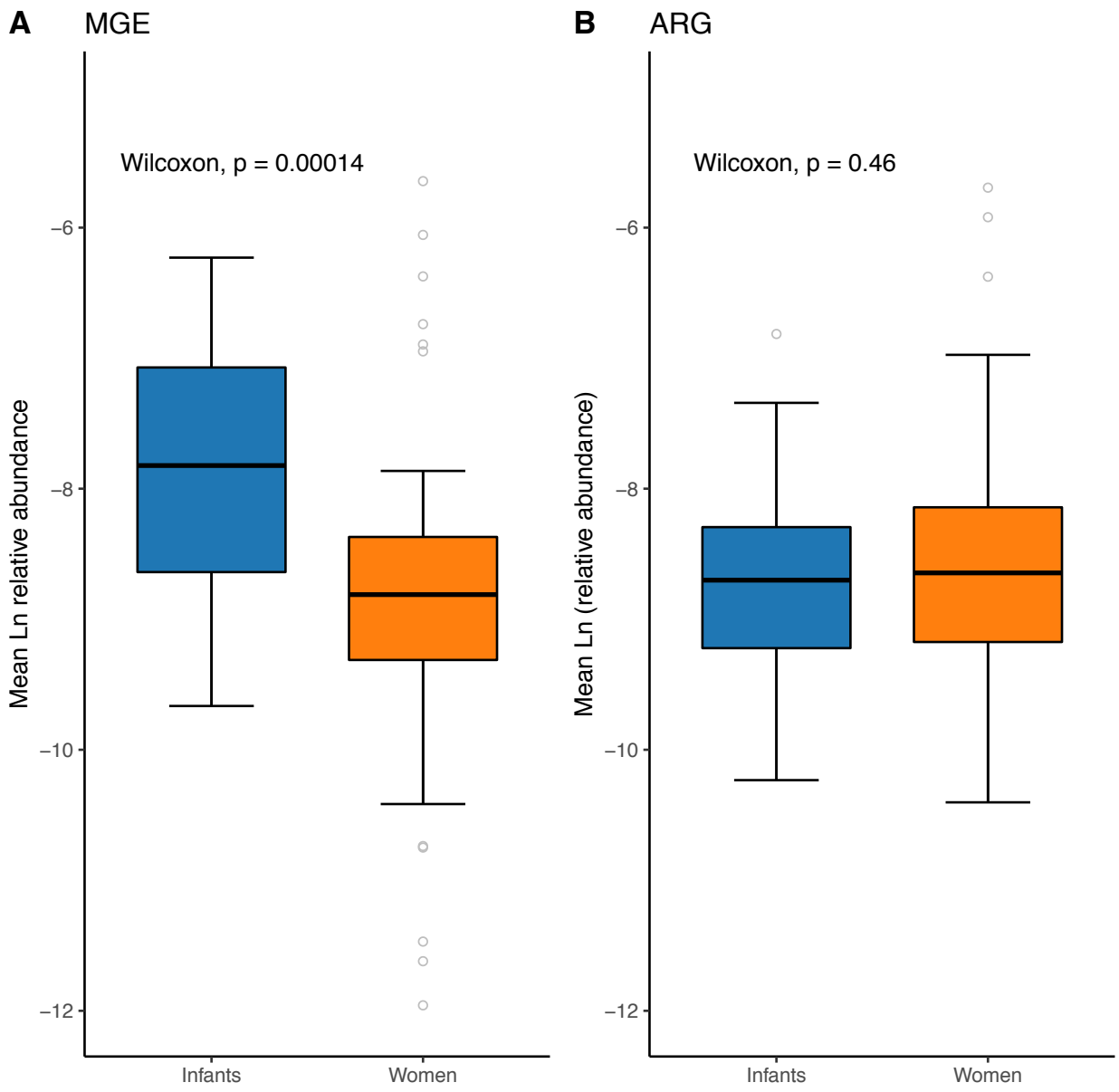

Figure 7 Mean of Ln relative abundances in pregnant women and infants.

Based on all infancy (n=42) and pregnancy (n=51) samples. Wilcoxon parametric test was calculated to test for differences between pregnant women and infants' samples A) MGE relative abundances, B) ARG relative abundances

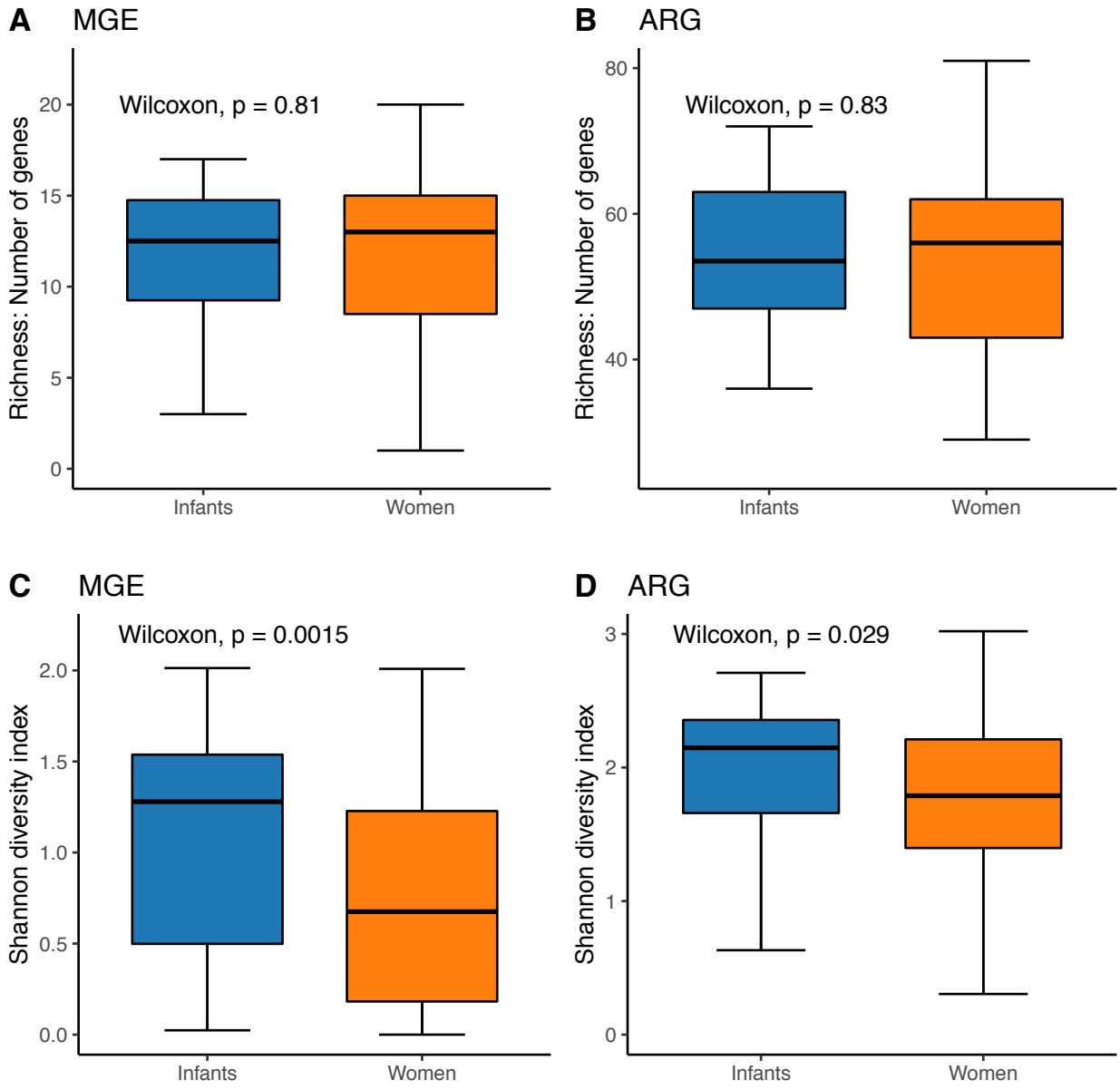

Figure 8 Richness and diversity index comparisons between infants and pregnant women.

Based on all infancy (n=42) and pregnancy (n=51) samples. Wilcoxon test was calculated to test for differences.

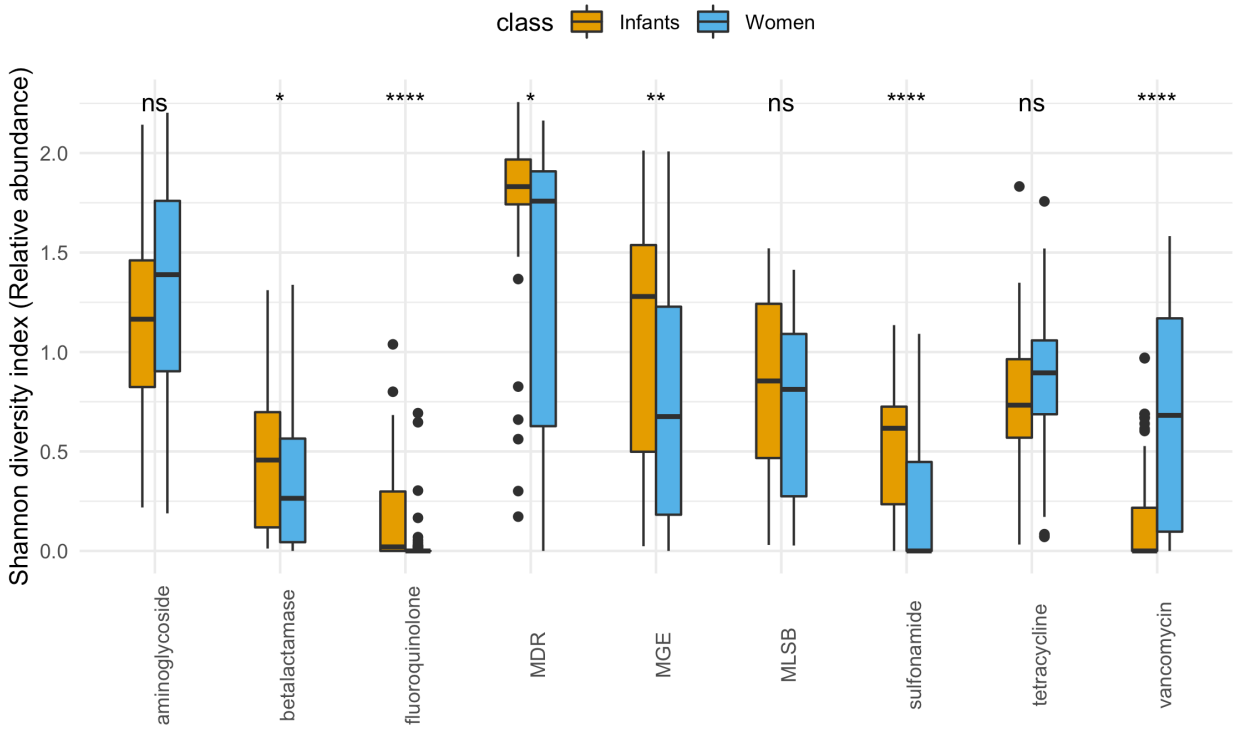

Figure 9 Differences in Shannon diversity index by antibiotic class comparing infants and pregnant women samples.

Based on all infancy (n=42) and pregnancy (n=51) samples. Wilcoxon test was calculated to test for differences: ns= not significant p value > 0.05, * = p value < 0.050, ** = p value < 0.01, *** = p value < 0.001, **** = p value < 0,0001

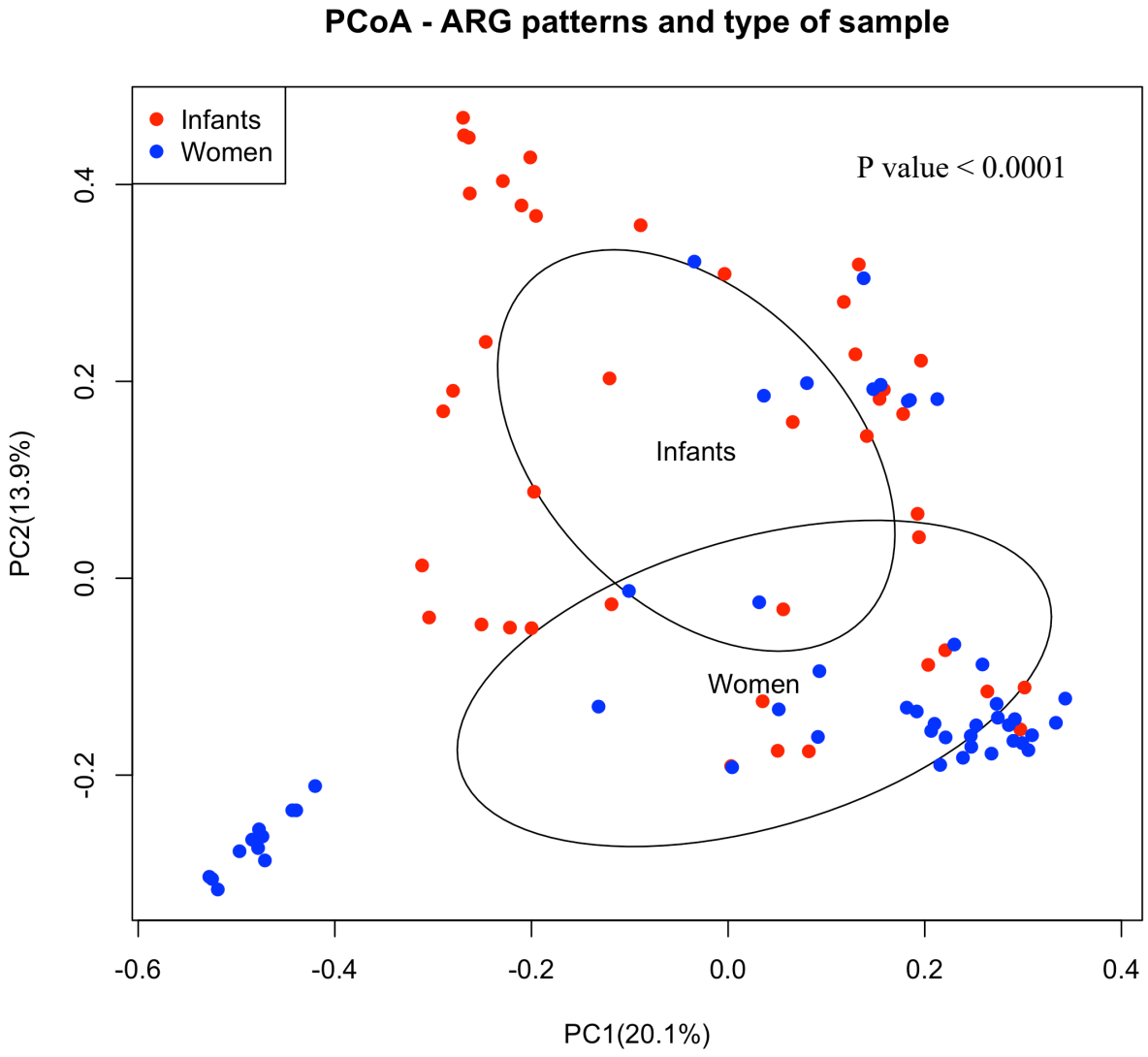

Figure 10 Beta diversity analysis comparing Bray-Curtis dissimilarity between ARG/MGE in pregnant women and infant samples.

Based on all infancy (n=42) and pregnancy (n=51) samples. Permanova test was calculated to test for differences between groups.

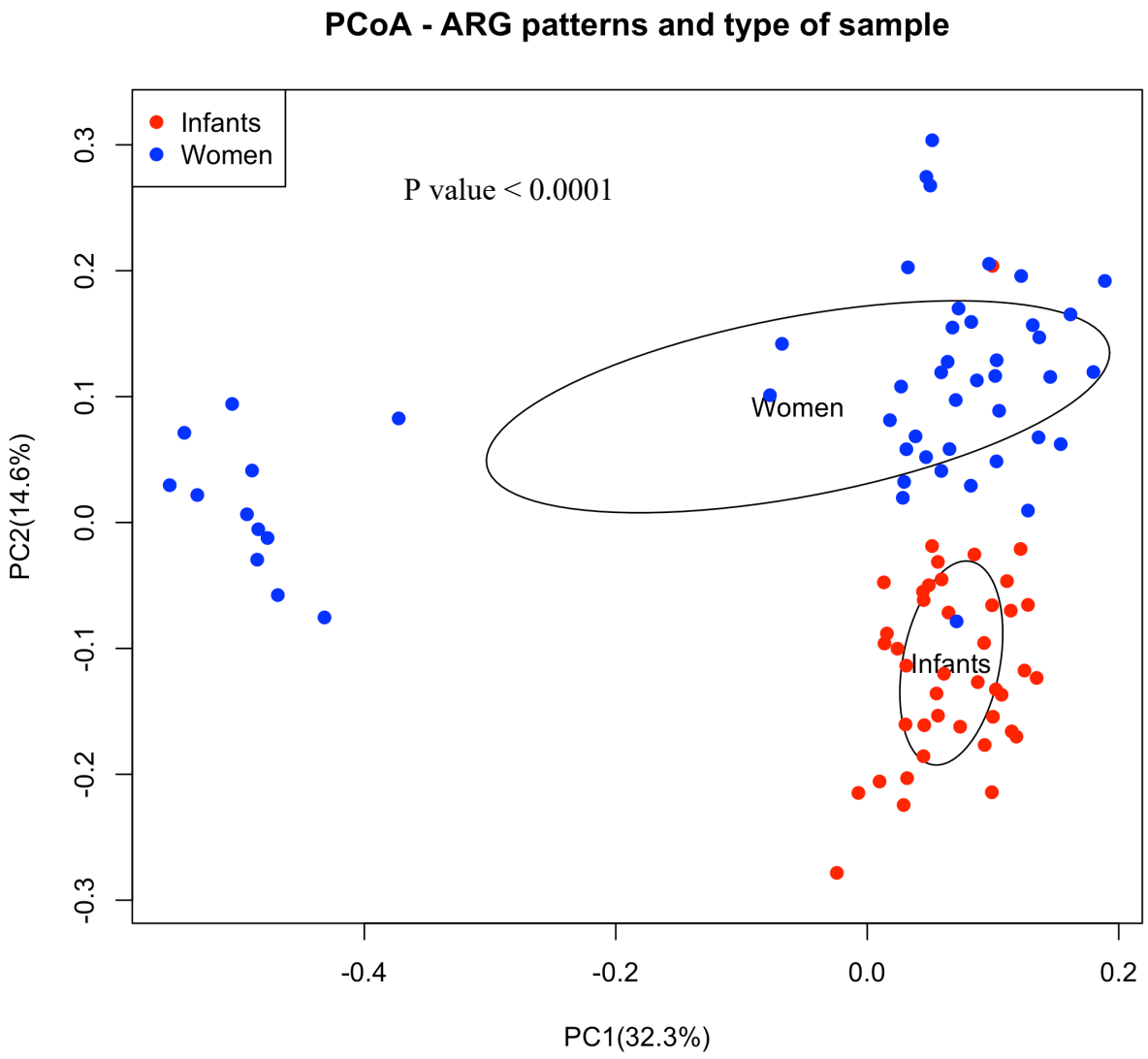

Figure 11 Beta diversity analysis comparing Sorensen dissimilarity between pregnant women and infant samples.

Based on all infancy (n=42) and pregnancy (n=51) samples. Permanova test was calculated to test for differences between groups.

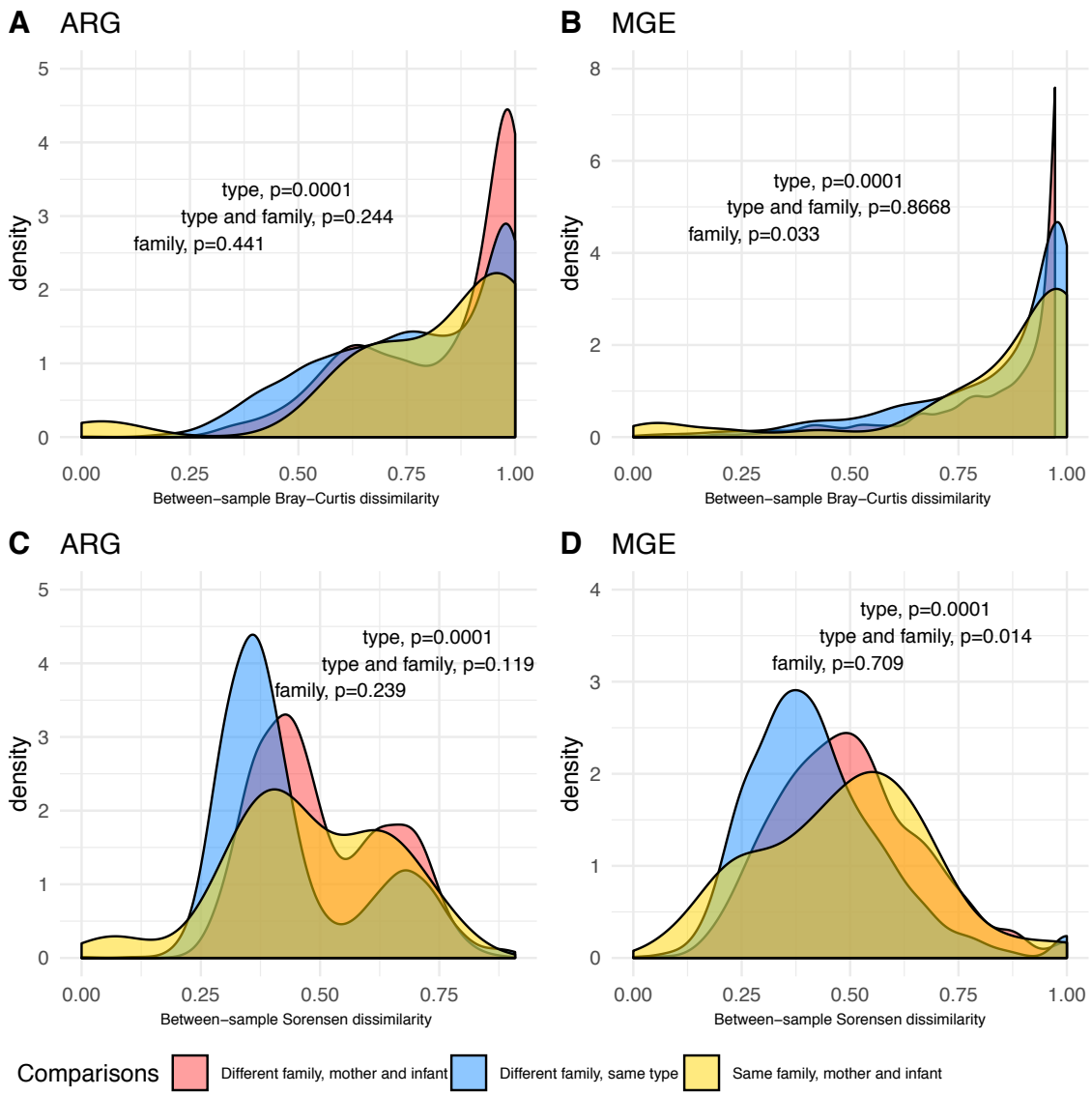

Figure 12 Beta diversity dissimilarities between infants and pregnant women resistomes.

Based on all infancy (n=42) and pregnancy (n=51) samples. Permanova test was calculated to test for differences between groups. A) Bray Curtis (structure) ARG dissimilarities. B) Bray Curtis (structure) MGE dissimilarities. C) Sorensen (composition) ARG dissimilarities. D) Sorensen (composition) MGE dissimilarities.

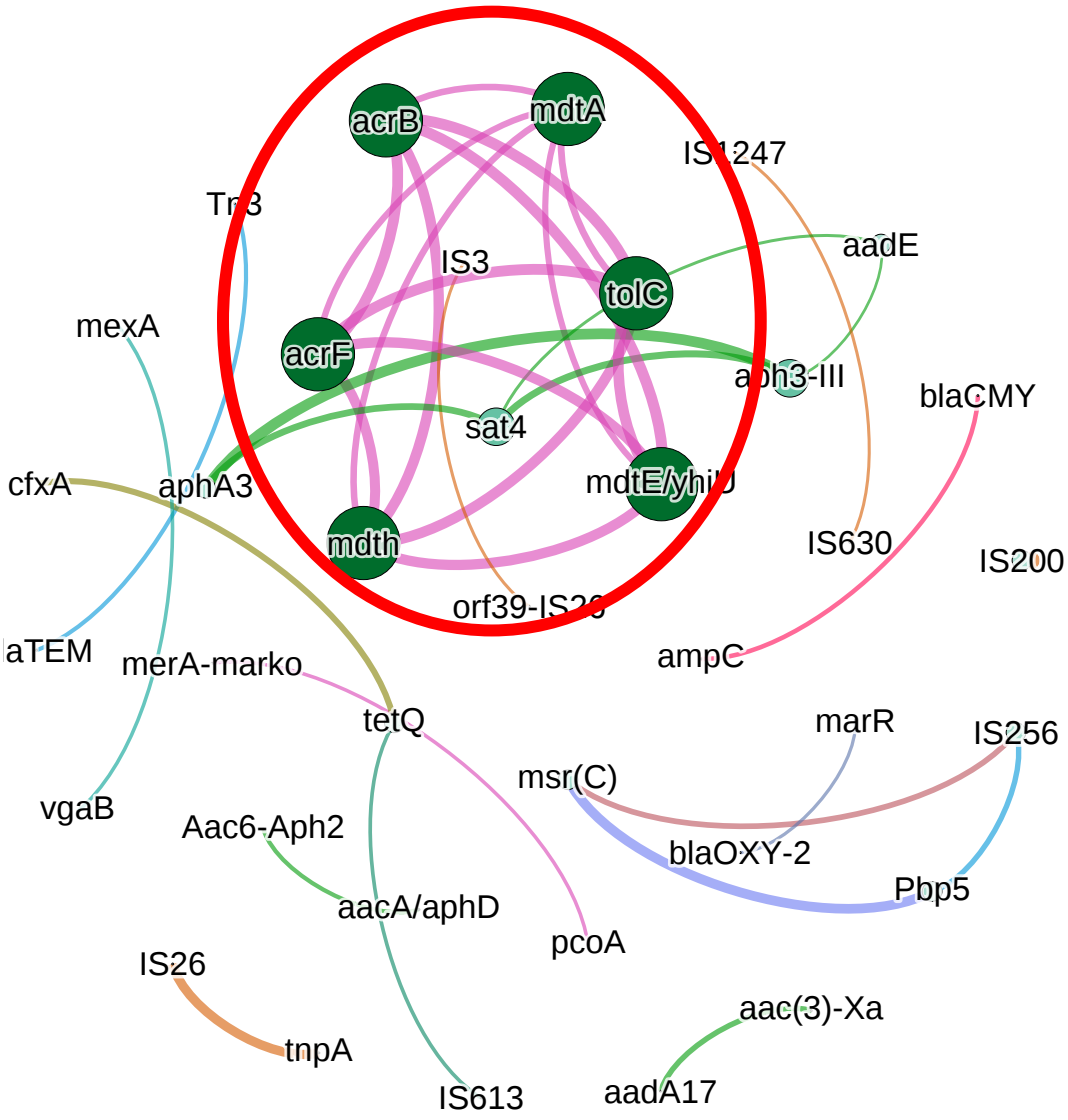

Figure 13 Co-occurrence networking analysis using ARG /MGE log (relative abundances) from infant's samples.

Based on all infancy (n=42) samples. Nodes (genes) connected by edges represent Spearman correlations higher than 0.6. Node sizes represent degree of centrality (number of connections). The color of the edges represents an antibiotic resistant class and their thickness represent greater correlation coefficients.

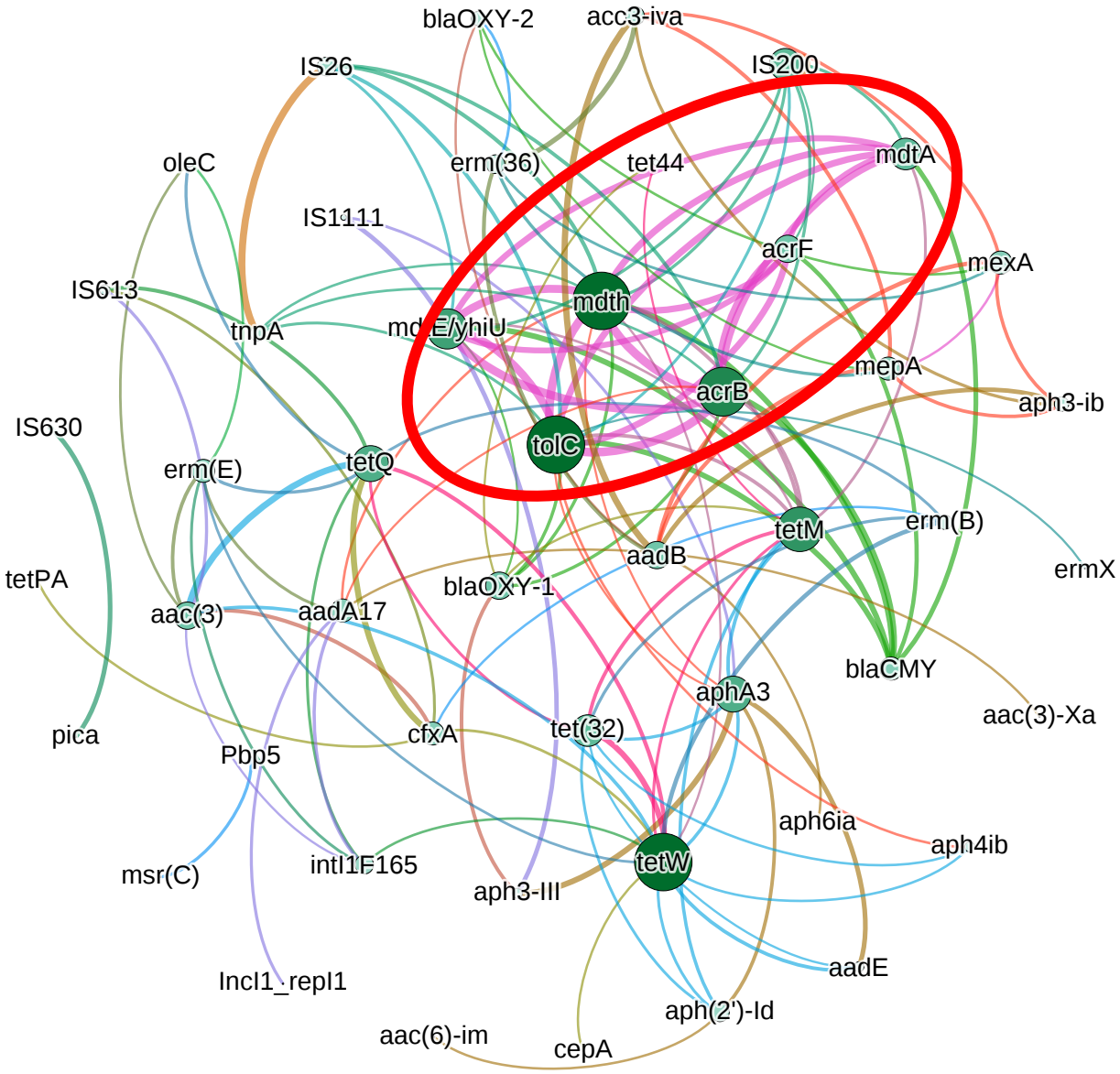

Figure 14 Co-occurrence networking analysis using ARG/MGE log (relative abundances) from women samples.

Based on all pregnancy (n=51) samples. Nodes (genes) connected by edges represent Spearman correlations higher than 0.6. Node sizes represent degree of centrality (number of connections). The color of the edges represents an antibiotic resistant class and their thickness represent greater correlation coefficients.

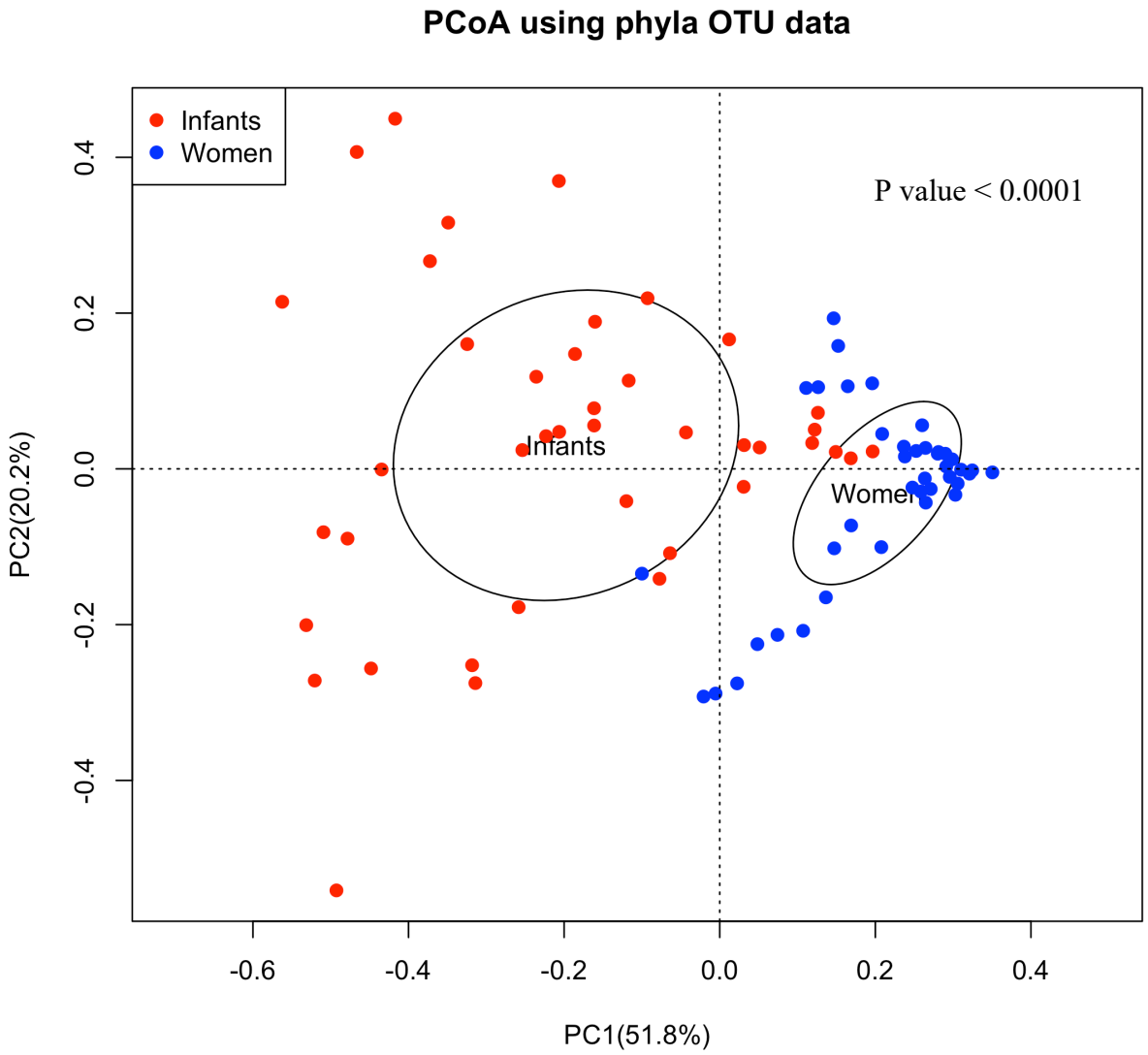

Figure 15 PCoA using OTU data classified into phyla.

Based on all infancy (n=42) and pregnancy (n=51) samples. Permanova test was calculated to test for differences between.

Table 3 Pearson correlation between ARGs/MGEs and bacteria at phyla level ($\rho > 0.8$): Infancy samples (n=42)

Name	OTU	Pearson correlation
blaTEM	Fusobacteria	0.99
ISCR1	Lentisphaerae	0.97
aac(3)-Xa	Fusobacteria	0.98
IS26	Fusobacteria	0.99
sugE	Fusobacteria	0.98
Tn3	Fusobacteria	1.00
IncI1_repI1	Fusobacteria	1.00
IS91	Tenericutes	1.00
tetB	Fusobacteria	0.99
tnpA	Fusobacteria	0.95
tnpA	Fusobacteria	0.99
vanTG	Lentisphaerae	0.93
int1-a-marko	Fusobacteria	0.99
intI1F165_clinical	Fusobacteria	0.97
sul1 NEW	Fusobacteria	0.99
orf39-IS26	Synergistetes	0.93
aph6ic	Synergistetes	0.91
aadA17	Fusobacteria	0.99
tetR	Fusobacteria	0.99
dfra21	Fusobacteria	0.99
erm(E)	Tenericutes	0.99

Table 4 Pearson correlation between ARGs/MGEs and bacteria at phyla level (ρ > 0.8):

Pregnancy samples (n=51)

Gene	OTU	Pearson correlation
blaOXY-1	Lentisphaerae	0.87
mefA	Lentisphaerae	0.89
blaTEM	Fusobacteria	0.99
aac(3)-Xa	Fusobacteria	0.98
IS26	Fusobacteria	0.99
sugE	Fusobacteria	0.98
tetA	Fusobacteria	0.88
tnpA	Fusobacteria	0.95
oprD	Lentisphaerae	0.87
sulA/folP	Synergistetes	0.86
intI1F165_clinical	Fusobacteria	0.97
aph6ic	Synergistetes	0.91
erm(E)	Tenericutes	0.99
mphA	Fusobacteria	0.87

Table 5 Pearson correlation between ARGs/MGEs and bacteria at family level ($\rho > 0.8$): Infancy samples (n=42)

Gene	OTU	Pearson correlation
blaCTX-M-1,3,15	Neisseriaceae	0.83
blaCTX-M-1,3,15	Leptotrichiaceae	0.99
blaTEM	Leuconostocaceae	0.83
tetM	Planococcaceae	0.89
tetM	Bacilli_unclassified	0.88
bla-ACT	Caulobacteraceae	0.99
bla-ACT	Flavobacteriaceae	0.99
bla-ACT	Acidaminococcaceae	0.85
ermX	Actinobacteria_unclassified	0.87
ermX	Thermoanaerobacteraceae	0.87
ermX	Opitutae_unclassified	0.87
oprD	Gammaproteobacteria_unclassified	1.00
oprD	Moraxellaceae	0.84
oprD	Comamonadaceae	1.00
pmrA	Lactobacillales_unclassified	0.81
ermA/ermTR	Rhodospirillaceae	1.00
intI1F165_clinical	Gammaproteobacteria_unclassified	0.84
intI1F165_clinical	Comamonadaceae	0.84
aac(3)-iid_iii_iif_iia_iie	Gastranaerophilales_fa	1.00
aac(3)-iid_iii_iif_iia_iie	Rikenellaceae	0.82
ere(A)	Staphylococcaceae	0.81
ere(A)	Eubacteriaceae	0.86

Table 6 Pearson correlation between ARGs/MGEs and bacteria at family level (ρ > 0.8):

Pregnancy samples (n=51)

Gene	OTU	Pearson correlation
blaOXY-1	Victivallaceae	0.87
aph6ia	Puniceicoccaceae	0.95
mefA	Victivallaceae	0.90
blaTEM	Corynebacteriaceae	0.85
blaTEM	Fusobacteriaceae	0.99
czcA	Pseudomonadaceae	0.80
aph3-III	unclassified.Burkholderiales	0.84
aac(3)-Xa	Corynebacteriaceae	0.83
aac(3)-Xa	Fusobacteriaceae	0.98
IS26	Corynebacteriaceae	0.85
IS26	Fusobacteriaceae	0.99
sugE	Corynebacteriaceae	0.84
sugE	Fusobacteriaceae	0.98
IS1247	Staphylococcaceae	0.82
tetA	Fusobacteriaceae	0.88
tnpA	Corynebacteriaceae	0.82
tnpA	Fusobacteriaceae	0.95
ermX	Pasteurellaceae	0.81
ermX	Streptococcaceae	0.82
vanHD	Staphylococcaceae	0.85
oprD	Victivallaceae	0.88
mexE	Puniceicoccaceae	0.99
sulA/folP	Synergistaceae	0.86
intI1F165_clinical	Corynebacteriaceae	0.82
intI1F165_clinical	Fusobacteriaceae	0.97
ISEfm1-Entero	Enterococcaceae	0.82
aph6ic	Synergistaceae	0.91
aadA7	Bacteroidales_S24.7_group	0.96
tet(32)	unclassified.Burkholderiales	0.84
erm(E)	Mollicutes_RF9_fa	0.99
erm(E)	Clostridiales_vadinBB60_group	0.81
mphA	Fusobacteriaceae	0.87

Table 7 Pearson correlation between ARGs/MGEs and bacteria at genus level (ρ > 0.8): Infancy samples (n=42)

Gene	OTU	Pearson correlation
cfxA	Tyzzerella	0.90
acrF	Escherichia/Shigella	0.83
blaCTX-M-1,3,15	Ruminococcaceae_UCG.014	0.99
blaCTX-M-1,3,15	Butyricicoccus	0.83
blaCTX-M-1,3,15	Leptotrichia	0.99
blaCTX-M-1,3,15	Neisseria	0.85
blaCTX-M-1,3,15	uncultured	0.99
blaOXY-1	Anaeroglobus	0.92
blaOXY-1	Cellulosilyticum	0.97
mdth	Escherichia/Shigella	0.83
pcoA	Atopobium	0.98
pcoA	Scardovia	0.98
blaTEM	Weissella	0.83
tetM	Rikenellaceae_RC9	0.89
tetM	Rummeliibacillus	0.89
bla-ACT	Desulfovibrio	0.98
bla-ACT	Candidatus_Soleaferrea	0.99
bla-ACT	Neorhizobium	0.99
bla-ACT	Lactococcus	0.87
bla-ACT	Brevundimonas	0.99
bla-ACT	Phascolarctobacterium	0.91
bla-ACT	Empedobacter	0.99
IS26	Scardovia	0.91
IS26	Atopobium	0.91
sugE	Dielma	0.85
sugE	Ruminococcaceae	0.88
sugE	Fusicatenibacter	0.82
IS200_1	Dielma	0.97
IS200_1	Ruminococcaceae	0.97
IS200_1	Fusicatenibacter	0.96
IS200	Hungatella	0.88
IS200	Providencia	1.00
oqxA	Atopobium	0.96

Table 7 (cont'd).

oqxA	Scardovia	0.95
ermX	Ruminococcaceae_UCG.003	0.87
ermX	Gelria	0.87
ermX	Ruminococcaceae_UCG.002	0.83
oprD	Acinetobacter	0.84
ermA/ermTR	Dialister	0.80
tolC	Escherichia/Shigella	0.84
merA-marko	Atopobium	0.99
merA-marko	Scardovia	0.99
orf39-IS26	Atopobium	0.97
orf39-IS26	Scardovia	0.97
aac(3)-iid_iii_iif_iia_iie	Gastranaerophilales_ge	1.00
aac(3)-iid_iii_iif_iia_iie	Actinotignum	1.00
aac(3)-iid_iii_iif_iia_iie	Varibaculum	0.91
aac(3)-iid_iii_iif_iia_iie	Peptoniphilus	0.98
aac(3)-iid_iii_iif_iia_iie	Negativicoccus	0.92
aac(3)-iid_iii_iif_iia_iie	Alistipes	0.82
aac(3)-iid_iii_iif_iia_iie	Peptostreptococcus	1.00
emrD	Cellulosilyticum	0.91
emrD	Anaeroglobus	0.85
ere(A)	Ruminiclostridium_9	0.92
ere(A)	Staphylococcus	0.81
ere(A)	Eubacterium	0.85
mdtE/yhiU	Escherichia/Shigella	0.86
acrB	Escherichia/Shigella	0.87

Table 8 Pearson correlation between ARGs/MGEs and bacteria at genus level (ρ > 0.8):

Pregnancy samples (n=51)

Gene	OTU	Pearson correlation
blaOXY-1	Victivallis	0.87
blaOXY-1	Alloprevotella	0.92
blaMIR	Coprococcus_2	0.82
aph6ia	Pseudobutyrivibrio	0.95
mefA	Victivallis	0.89
mefA	Alloprevotella	0.96
blaTEM	Mobiluncus	0.99
blaTEM	Lawsonella	0.89
blaTEM	Moryella	0.99
blaTEM	Porphyromonas	0.94
blaTEM	Fastidiosipila	0.99
blaTEM	Fusobacterium	0.99
blaTEM	Ezakiella	0.97
blaTEM	Corynebacterium_1	0.99
blaTEM	Prevotella_6	0.99
blaTEM	Hungatella	0.90
blaTEM	Tyzzerella_4	0.98
blaTEM	Anaerococcus	0.88
blaTEM	Parvimonas	0.99
blaTEM	uncultured.9	0.99
blaTEM	Finegoldia	0.99
blaTEM	Peptoniphilus	0.99
blaTEM	Jonquetella	0.99
blaTEM	Prevotella	0.91
blaTEM	Murdochiella	0.93
intl3	Chroococcidiopsis	0.83
czcA	Chroococcidiopsis	0.94
czcA	Pseudomonas	0.80
aac(3)-Xa	Tyzzerella_4	0.96
aac(3)-Xa	Fastidiosipila	0.98
aac(3)-Xa	Fusobacterium	0.98
aac(3)-Xa	Anaerococcus	0.87
aac(3)-Xa	Mobiluncus	0.98

Table 8 (cont'd).

aac(3)-Xa	Parvimonas	0.98
aac(3)-Xa	Jonquetella	0.98
aac(3)-Xa	Ezakiella	0.95
aac(3)-Xa	Lawsonella	0.88
aac(3)-Xa	Peptoniphilus	0.97
aac(3)-Xa	Finegoldia	0.98
aac(3)-Xa	Moryella	0.98
aac(3)-Xa	Murdochiella	0.92
aac(3)-Xa	Hungatella	0.91
aac(3)-Xa	Porphyromonas	0.92
aac(3)-Xa	uncultured.9	0.98
aac(3)-Xa	Prevotella	0.94
aac(3)-Xa	Corynebacterium_1	0.98
aac(3)-Xa	Prevotella_6	0.98
IS26	Anaerococcus	0.88
IS26	Prevotella_6	0.99
IS26	Fastidiosipila	0.99
IS26	Parvimonas	0.99
IS26	Fusobacterium	0.99
IS26	Murdochiella	0.95
IS26	Tyzzerella_4	0.99
IS26	Moryella	0.99
IS26	Corynebacterium_1	0.99
IS26	Ezakiella	0.97
IS26	Prevotella	0.93
IS26	Peptoniphilus	0.99
IS26	Porphyromonas	0.94
IS26	Jonquetella	0.99
IS26	Mobiluncus	0.99
IS26	Hungatella	0.94
IS26	Finegoldia	0.99
IS26	Lawsonella	0.89
IS26	uncultured.9	0.99
sugE	Fastidiosipila	0.98
sugE	Moryella	0.98
sugE	Anaerococcus	0.88
sugE	Tyzzerella_4	0.97
sugE	Jonquetella	0.98

Table 8 (cont'd).

sugE	Mobiluncus	0.98
sugE	Finegoldia	0.98
sugE	Fusobacterium	0.98
sugE	Parvimonas	0.98
sugE	Murdochiella	0.92
sugE	Prevotella	0.90
sugE	Peptoniphilus	0.98
sugE	Ezakiella	0.96
sugE	Prevotella_6	0.98
sugE	Hungatella	0.89
sugE	Porphyromonas	0.93
sugE	Lawsonella	0.89
sugE	Corynebacterium_1	0.98
IS1247	Staphylococcus	0.81
IS1247	Faecalicoccus	0.95
IS1247	Mogibacterium	0.87
aadE	Ruminococcaceae_UCG.011	0.84
tetA	Finegoldia	0.88
tetA	Jonquetella	0.88
tetA	Prevotella	0.81
tetA	Murdochiella	0.82
tetA	Tyzzerella_4	0.87
tetA	Fusobacterium	0.88
tetA	Fastidiosipila	0.88
tetA	Corynebacterium_1	0.88
tetA	Prevotella_6	0.88
tetA	Mobiluncus	0.88
tetA	Porphyromonas	0.84
tetA	Parvimonas	0.88
tetA	Ezakiella	0.88
tetA	Peptoniphilus	0.88
tetA	Moryella	0.88
tnpA	Corynebacterium_1	0.95
tnpA	Moryella	0.95
tnpA	Jonquetella	0.95
tnpA	Fusobacterium	0.95
tnpA	Porphyromonas	0.91
tnpA	Prevotella_6	0.94

Table 8 (cont'd).

tnpA	Anaerococcus	0.84
tnpA	Tyzzerella_4	0.95
tnpA	Peptoniphilus	0.94
tnpA	Ezakiella	0.93
tnpA	Mobiluncus	0.95
tnpA	Prevotella	0.91
tnpA	Finegoldia	0.95
tnpA	Parvimonas	0.95
tnpA	Fastidiosipila	0.95
tnpA	Hungatella	0.94
tnpA	Murdochiella	0.94
tnpA	Lawsonella	0.85
ermX	Klebsiella	0.98
ermX	Paeniclostridium	1.00
ermX	Haemophilus	0.82
ermX	Alloscardovia	0.81
ermX	Lactococcus	0.99
ermX	Aggregatibacter	1.00
vanHD	Faecalicoccus	0.98
vanHD	Staphylococcus	0.84
vanHD	Mogibacterium	0.89
oprD	Victivallis	0.87
oprD	Alloprevotella	0.93
oprD	uncultured_ge	0.91
mexE	Pseudobutyrivibrio	0.99
sulA/folP	Dysgonomonas	0.90
sulA/folP	Cloacibacillus	0.81
sulA/folP	Pyramidobacter	0.90
ermA/ermTR	Coprococcus_2	0.82
intI1F165_clinical	Peptoniphilus	0.96
intI1F165_clinical	Fastidiosipila	0.97
intI1F165_clinical	Tyzzerella_4	0.95
intI1F165_clinical	Jonquetella	0.97
intI1F165_clinical	Fusobacterium	0.97
intI1F165_clinical	Prevotella	0.88
intI1F165_clinical	Corynebacterium_1	0.97
intI1F165_clinical	Hungatella	0.90
intI1F165_clinical	Anaerococcus	0.86

Table 8 (cont'd).

intI1F165_clinical	Ezakiella	0.94
intI1F165_clinical	Mobiluncus	0.97
intI1F165_clinical	Porphyromonas	0.91
intI1F165_clinical	Parvimonas	0.97
intI1F165_clinical	Murdochiella	0.91
intI1F165_clinical	Moryella	0.97
intI1F165_clinical	Finegoldia	0.97
intI1F165_clinical	Lawsonella	0.87
intI1F165_clinical	Prevotella_6	0.96
ISEfm1-Entero	Enterococcus	0.85
aph6ic	Cloacibacillus	0.86
aph6ic	Ruminiclostridium_1	0.87
aph6ic	Dysgonomonas	0.96
aph6ic	Pyramidobacter	0.96
aac(6)-im	Olsenella	0.86
aac(6)-im	Mitsuokella	0.93
aadA7	Bacteroidales_S24.7	0.96
aadA7	Ruminococcaceae_UCG.008	0.86
tetG_F	Faecalicoccus	0.85
erm(E)	Peptococcus	0.98
erm(E)	Enterorhabdus	1.00
erm(E)	NB1.n_ge	0.98
erm(E)	Anaerotruncus	0.95
erm(E)	Mollicutes_RF9_ge	0.99
erm(E)	Clostridiales_vadinBB60	0.81
erm(E)	Holdemanella	0.84
mphA	Prevotella	0.93
mphA	Prevotella_6	0.89
mphA	Moryella	0.87
mphA	Fusobacterium	0.87
mphA	Tyzzerella_4	0.85
mphA	Porphyromonas	0.82
mphA	Jonquetella	0.87
mphA	Murdochiella	0.80
mphA	Corynebacterium_1	0.87
mphA	Finegoldia	0.87
mphA	Mobiluncus	0.87
mphA	Ezakiella	0.84

Table 8 (cont'd).

mphA	Parvimonas	0.87
mphA	Fastidiosipila	0.87
mphA	Peptoniphilus	0.86

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