Investigating Early Life Dynamics of Human Gut Antibiotic Resistome and Its Potential Links with Health and Disease

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CERTIFICATE OF APPROVAL

This is to certify that the research work presented in this thesis, entitled "**Investigating Early Life Dynamics of Human Gut Antibiotic Resistome and Its Potential Links with Health and Diseases**" was conducted by **Miss. Saima Bilal** under the supervision of **Dr. Masood Ur Rehman Kayani**.

No part of this thesis has been submitted anywhere else for any other degree. This thesis is submitted to the **School of Interdisciplinary Engineering & Sciences** in partial fulfillment of the requirements for the degree of Master of Science in the Field of **Computational Sciences & Engineering** Department of the National University of Sciences and Technology, Islamabad.

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I hereby state that my MS thesis titled "**Investigating Early Life Dynamics of Human Gut Antibiotic Resistome and Its Potential Links with Health and Diseases**" is my work and has not been submitted previously by me for taking any degree from the National University of Sciences and Technology, Islamabad, or anywhere else in the country/ world.

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Date: Jan 20, 2025

PLAGIARISM UNDERTAKING

DEDICATION

I dedicate this thesis to my exceptional parents, siblings, friends, and teachers whose unconditional love, support, and guidance led me to this world of accomplishment.

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All praise is for **Almighty Allah**, the ultimate source of all knowledge. By His grace, I have reached this stage of knowledge with the ability to contribute something beneficial to His creation. My deepest respects are to the **Holy Prophet Hazrat Muhammad (PBUH)**, the symbol of guidance and fountain of knowledge.

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ABSTRACT

The human gut microbiome plays a critical role in health and diseases. This complex community of microorganisms is established early in life and undergoes significant changes during infancy, a period crucial for long-term health. In infants, the gut microbiome is particularly dynamic, with the mode of delivery being one of the initial factors influencing it. Despite ongoing research, there are gaps in understanding the dynamics of ARG acquisition, evolution and associations to environmental factors such as diet, mode of delivery, maternal antibiotic exposures, infant's antibiotic exposure, and water type used by infants. This thesis aims to characterize ARGs in infants during first year of life and investigate their associations with metadata variables including mode of delivery, maternal antibiotic consumption, and environmental factors.

One way to explore these resistome dynamics is through the study of antibiotic resistome analysis and how these ARGs are associated with mode of delivery and environmental factors. This study combine metagenomics techniques with gene prediction and gene catalogue construction tools like Prodigal and MMseqs2. ARGs were identified through CARD database by using DIAMOND, according to the results infants delivered via C-section delivery had a higher abundance of ARGs which clear pattern of ARG prevalence associated with delivery techniques. Notably, infants exposed to antibiotics whether directly or through maternal source exhibited a disrupted microbiome and increased risk of harboring ARGS potentially lead them to immunological dysregulation and illness like obesity, inflammatory bowel disease, and cardiovascular disorders. This study emphasizes the complex interactions between environmental variables and infant gut microbiome

underscoring the gut microbiota, underscoring the necessity for focused approaches to slow the spread of ARGs. This study will help to prevent antibiotic resistance and its effects on human health by deepening our understanding of antibiotic resistome dynamics their relationship to early life exposures.

Keywords: Infant gut microbiome, Antibiotic Resistance Genes (ARG), C-section delivery, vaginal delivery, Environmental factors, Health and Disease.

CHAPTER 1: INTRODUCTION

1.1 Human Gut Microbiome

Human gut microbiota is a diverse environment made up of bacteria, fungi and other mutualistic microorganisms of the gut, mucosa and skin. The digestive tract is a complex micro ecosystem, which contains around 100 trillion microbes of different types. The most important component of preserving our health is the gut microbiota, which includes the gut bacteria serve several functions, such as fermentation of food, protection against pathogens, boost up the immune system, and produce vitamins [1]. Microorganisms, until recently considered to be pathogenic, have been regarded as acquired symbionts within the host [2]. The intestinal tract is a complex micro ecosystem, which contains around 100 trillion microbes of different classes. The largest population of intestinal microbes is in the large intestine and the distal small intestine, most especially in the colon where the bacteria population is up to 1012 CFU/mL. During the first year of life, the composition of the gut microbiota is relatively simple and shows wide interindividual variations [3]. Over the past few decades, the microbial community that lives in the gut collectively known as the human gut microbiome, has developed a mutualistic relationship with the host and been investigated in relation to human health [4].

1.1.1 Human gut microbiome health and diseases

In healthy humans, the gut microbiome has coevolved to exist in a state of mutually beneficial symbiosis with its host, gut microbiomes are known to express catalytic pathways for the metabolism of complex carbohydrates that result in short chain fatty acids

(SCFA). The metabolism of carbohydrates, amino acids, fermentation and oxidative phosphorylation are among the primary functioning pathways in the adult gut microbiota. Numerous other functions of these SCFA include signaling, metabolic activities, and promoting the development of specific microorganisms [5]. In terms of the immune system, the human microbiota play a crucial role in the development of the intestinal mucosa and immune system in addition to creating antimicrobial compounds that protect the host from external infections [6]. The exact molecular mechanisms are still unclear; however, recognition of commensal microorganisms by the innate immune system is thought to be central for immune system development [7]. Numerous diseases are linked to changes in the gut microbiomes composition, abnormalities, or disturbed hemostasis. Recent studies have demonstrated that gut microbiome influences immunological, mucosal, and distal organ function. The associations between gut microbiome composition and diseases have been reported extensively. Many autoimmune diseases have been associated to functional and compositional abnormalities in the gut microbiota and immunological pathogenesis is influenced by altered gut microbiota [8]. The microbiome is believed to actively promote the etiology of a disease, mostly through inflammatory pathways including colorectal cancer [9] Crohn's disease [10], and autism [11]. Microbial community may be altered by excessive adiposity. Resulting in a response that makes obesity worse. Because the gut microbiomes produce long chain fatty acids(LCFA), it also affects the host metabolism. A human symbiotic bacterium that is closely linked to obesity is *fusimonas intestine* [12].

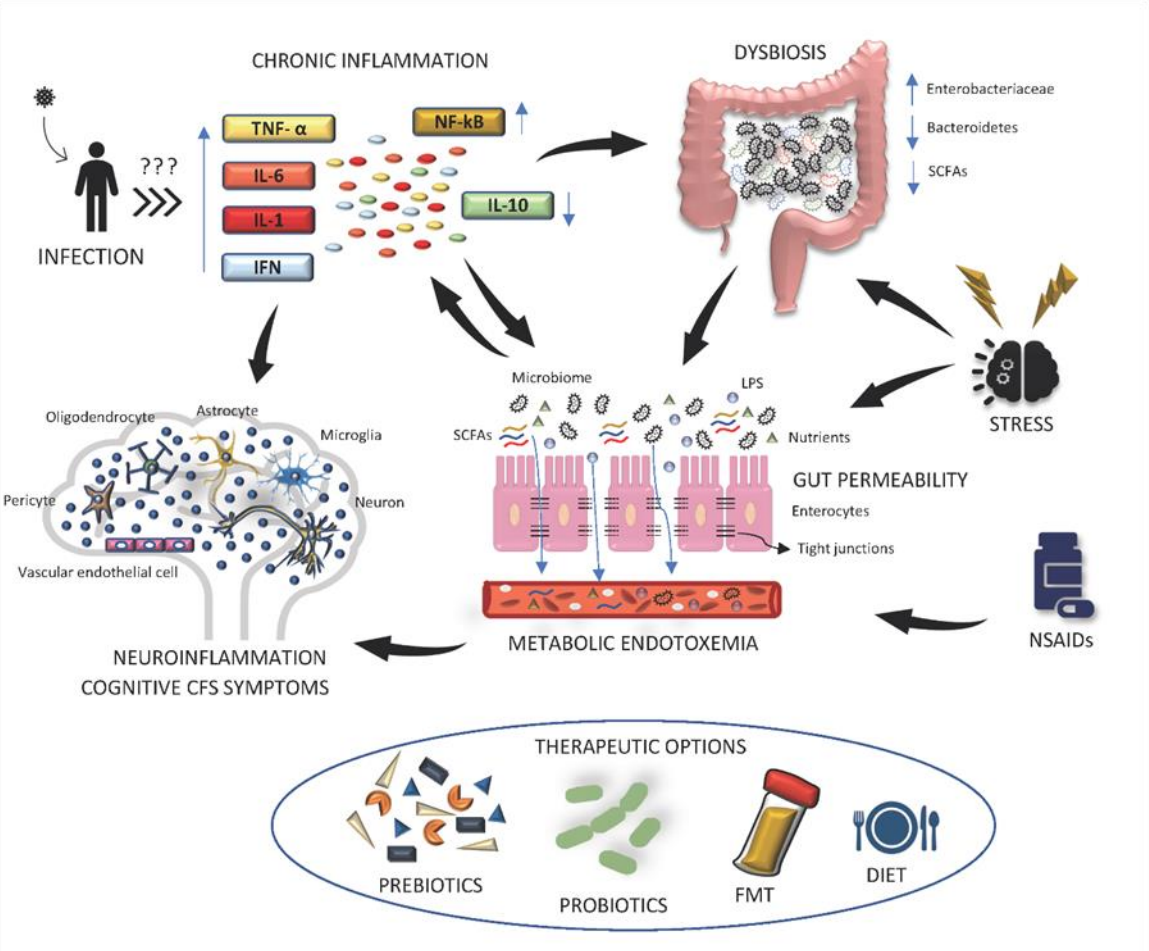

Figure 1.1: Dysbiosis in the human microbiome and its role in disease development [13].

1.1.2 Importance of infant gut microbiome

The development of both the immune system and gut microbiome in infants commences during the initial days following conception, encompassing the periods of pregnancy and the initial two years of life. This "window of opportunity" is a crucial period for ensuring the healthy development of infants at birth, both beneficial and harmful microbes can colonize the infant's gut and can confound the infant's health [14].

1.1.3 Composition, function and importance of infant gut microbiome

The acquisition of gut microbiome gut microbiome by infants typically begins at birth. The microbes encountered by infants at birth, particularly those from the maternal source, May significantly impact the infant's health and disease, or lead to long-lasting consequences [15]. Important members of the core composition of the infant gut microbiome include *Bifidobacteriales, Lactobacillales, Clostridiales, Prevotella, Bacteroidales, and Bacteroides Fragilis*, among others, although these may differ based on what the dominant populations and compositions are. The development and maturation of immunity acquired and innate in infancy are significantly aided by the presence of commensal or beneficial bacteria; for example, *Lactobacillus and Bifidobacterium [16]*. The acquisition of Clostridia species prevents infant colonization by bacterial pathogens. *Clostridiales* raise the colonization resistance of the gut and are considered a defensive mechanism against the attacks of certain pathogens on the gut of the infant [17]. The gut microbiota contributions to human physiology are also significantly influenced by other bacteria particularly those involved in microbial metabolism [18]. *Prevotella* plays a crucial role in glucose metabolism, because it can break down pyruvate into acetate and formate. Furthermore, in the presence of carbohydrates and human milk oligosachrides (HMOs) *Bacteroides thetaiotaomicron* that promotes the growth of bacteria that produce SCFA [19]. Additionally, it also plays an important role in a number of pathways linked to the metabolism of drugs, carbohydrates, and vitamins.

1.2 Factors influencing the infant gut microbiome

Factors influencing infant gut colonization include mode of delivery, vaginal delivery (VD) and C-section delivery (CSD), breastfeeding, gestational age, maternal and infant diets, antibiotic use, environmental exposures, and maternal stress levels, all of which contribute to the establishment of the infant gut microbiome. Most importantly, the mode of delivery significantly influences the infant's gut microbiome [20]. Breast milk carries a unique set of microorganisms along with HMOs that are passed on to infants. These HMOs, which act as prebiotics, encourage the growth of beneficial gut bacteria, prevent harmful pathogens from establishing themselves in the infant's gut, and promote overall health benefits. The composition of an infant's gut microbiome is influenced by exposure to various external environments during early development outside the uterus. Having siblings is associated witan increased *Bifidobacterium* and reduced abundance of *Peptostreptococcus* bacteria in infants. Breast milk fosters the growth of the gut microbiome by supplying probiotics and prebiotics [21]. Most importantly, primary factor influencing the formation of gut microbiota is antibiotics. Whether or not, antibiotics are frequently used to treat infections in both humans and animals. They are also frequently used to enhance animal growth [22]. According to reports, the first ten years of the 21 century experienced a 35% increase in the world comsumption of antibiotics [23].

This resulted into accumulation of accumulation of antibiotic residues and antibiotic-resistant bacteria in the human gut and caused antibiotic resistance genes (ARGs). The primary reason of bacterial resistance is the existence of ARGs. Through plasmid exchange at gene level, pathogenic bacteria acquire ARGs and become highly

resistant to antibiotics. Certain antibiotics can cause bacteria to become intrinsically resistant. The intrinsic resistance of a bacterium, results from its innate structural and functional characteristic, which offer defense against antibiotics effects. While some antibiotics cannot penetrate through outer membrane and cannot reach their target locations, others can be efficiently removed by efflux once they have entered the cells through porin [24]. In addition to mode of delivery, breastmilk and antibiotics, there are many other environmental factors like diet, drinking water, maternal lifestyle, gestational age and maternal stress level all of which contribute to the establishment of the infant gut microbiome as given in (Figure 1.2).

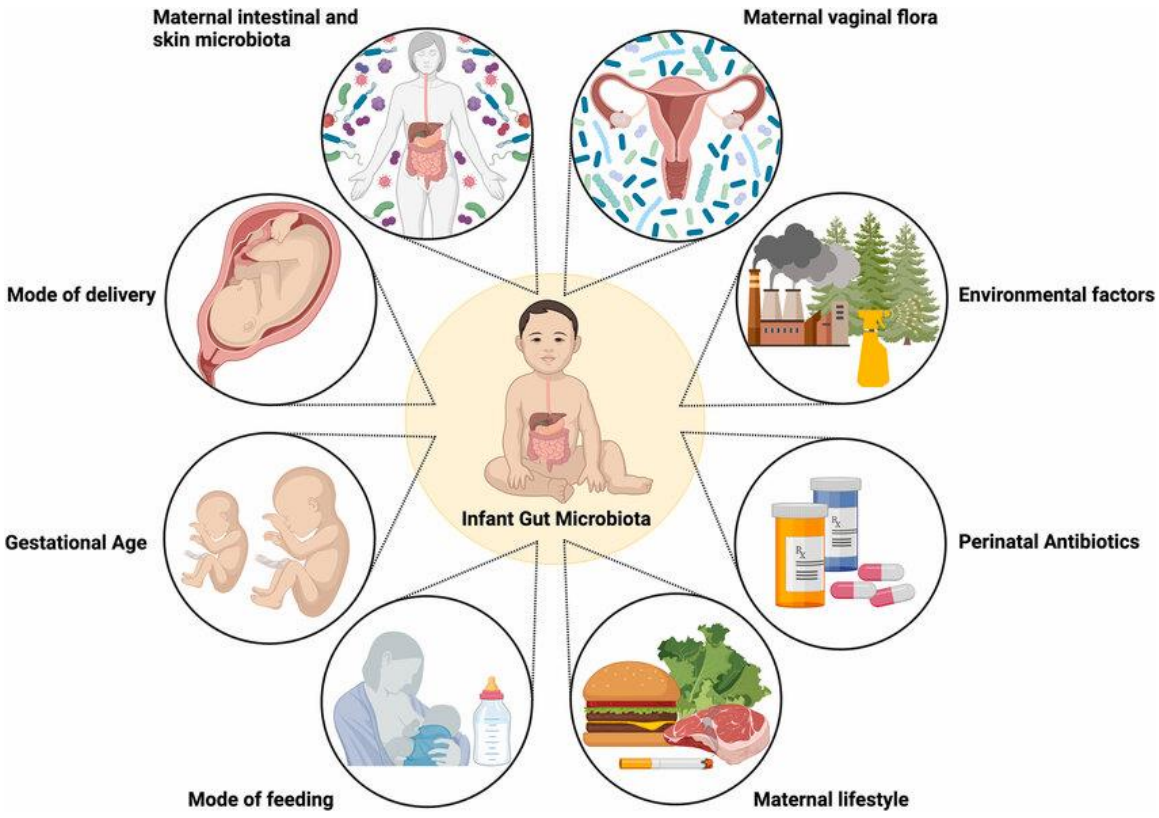

Figure 1.2 Factors influencing the development of the infant microbiome and immune system. [25].

1.2 Research Gap and Problem Statement

Despite growing recognition of presence of antibiotic resistance genes in the infant gut microbiome, there remains a significant gap in understanding the dynamics of their acquisition and evolution during early childhood. Previous studies mostly focused on the abundance of ARGs and determined their types, but the direct associations of these genes to different environmental factors such as delivery mode, diet, maternal antibiotic exposure and other metadata is not fully investigated. In addition, the effects on long term health or the impact of ARGs on the developing immune system and vulnerability to certain diseases in infants are not clearly known. Modern studies have also failed to explain how the ARGs enter the gut and how they are maintained in the developing microbiota.

Despite recognizing the presence of ARGs in infant gut microbiome, there is limited understanding of how these genes are acquired and how their presence evolves during early childhood. Further investigation is needed to find how ARGs are associated with mode of delivery, diet, maternal antibiotic exposure and other metadata variables influence the developing gut microbiome, how these genes are characterized and how they are associated with health and neonatal diseases are not fully understood. Metagenome analysis could be used to characterize ARGs in gut microbiome of infants during early life and to assess their potential links between the antibiotic resistome and infants' health.

1.3 Objectives

- To characterize the antibiotic resistance genes in infants during the first year of life.
- To associate antibiotic resistance genes with the mode of delivery, maternal antibiotic consumption, and other metadata variables.

CHAPTER 2: LITERATURE REVIEW

2.1 Antibiotic Resistance Genes, infant Gut Microbiome and Mode of Delivery

ARGs are naturally present in microbes, especially bacteria. Antibiotic resistance occurs when microorganisms resist the effects of antibiotics, making them no longer effective in treating bacterial infections. Antibiotic-resistant bacteria carry a set of ARGs, thus allowing the bacteria to overcome the antibiotic effects. The set of ARGs present in a population of bacteria is known as the resistome [26]. These ARGs can be easily shared by microbes through horizontal gene transfer (HGT), ultimately making microbes resistant to antibiotics. The vertical transfer of the gut microbiota from mother to infants occurs during pregnancy, childbirth, and other practices. [27]. The mode of delivery is a significant factor which can cause ARGs in infants' gut microbiome. Globally, the prevalence of CSD is continuously rising. A 2015 estimate states that 29.7 million biths were through CSD, making up all 18% of all births in 169 countries. Reduced gut microbiome diversity decreased the transmission of bacterial strains mother to neonates, so altered immune system, functional impairments are short terms hazards associated with CSD [28].

According to Shao et al., infants with CSD are more vulnerable to colonization by opportunistic infections, such as those that contains ARGS [29]. New born are exposed to variety to microbes from mother during VD and certain fecal microbes colonize the infants stomach. Even in the absence of antibiotic exposure, the gut microbiota of infants has more influence of genes that confer resistance to antibiotics than that of

adults [30]. In their study, Dzidic et al. similarly observed that the gut of vaginally delivered infants is mostly colonized with vaginal bacteria, and they found species such as *Bifidobacterium, Klebsiella, Lactobacillus, Escherichia,* and *Prevotella* among them [31]. A less widespread resistome and a healthier microbiome are supported by VD promotion of spontaneous microbial colonization.

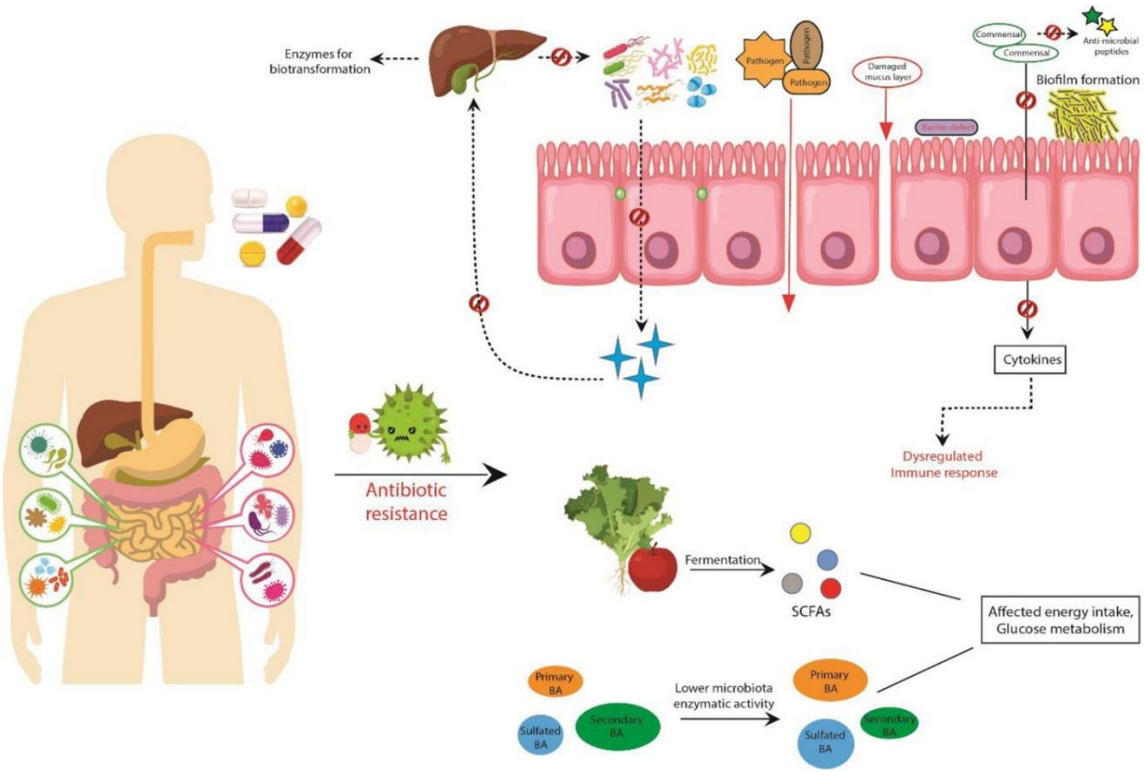

Figure 2.1: Antibiotics impacts on host health; both direct and indirect effects. [32].

By interfering with microbial transmission, CSD increases the burden of resistance and favours infections that carry ARGs. ARGs are prevalent in early gut microbiota of CSD infants, which is dominated by pathogens caused by *klebsiella, enterrococcus*.[33]. Furthermore, studies by Dominguez-Bello et al. identified evidence supporting the direct transmission of bacteria from mother to infant, genes conferring glycopeptide, phenicol,

pleuromutilin, bacitracin, sulfonamide and diaminopyrimidine resistance were significantly increased in CSD compared to VD at day 5 after birth [34]. The effect of birth mode, CSD or VD, on the transmission and occurrence of AMR remains different. These variations in delivery mode have been associated with differences in the gut microbiome of infants (Figure 2.2).

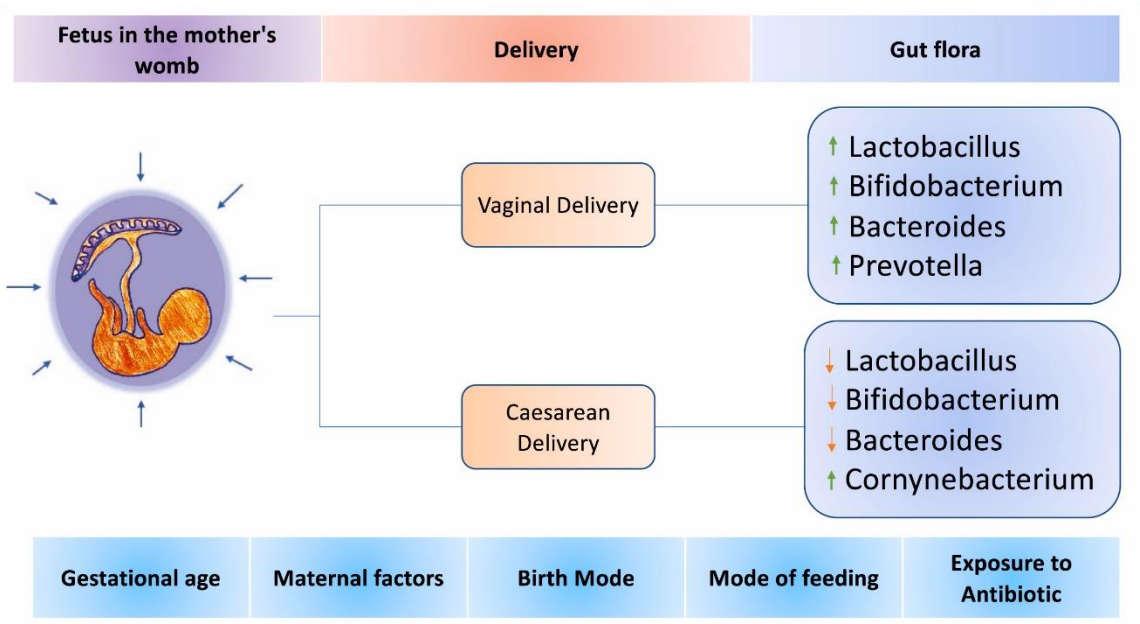

Figure 2.2 The Influence of Delivery Mode on the Formation of the Infant Gut Microbiome [35].

According to the study of Pärnänen et al., there are different variables including mode of delivery associated to affect the infants microbiota, consequently, the resisitome including breastfeeding, mother antibiotic exposure, gestational age, and early life antibiotic exposure are also included. Although evolution og gut microbiota are also influenced by other factors including lactation and breast milk transfers ARGs. Some are also transferred through breastfeeding and maternal gut microbiota [36].

The work of Z. Zhang et al., demonstrates that as soon as infants are admitted to neonatal intensive care unit (NICU), they experience exposure to different microbes in the hospital and have limited exposure to the maternal microbiota but increased exposure to microbes of NICU staff. They are frequently found on NICU surfaces and are among the most common sources of nosocomial infections. Antibiotic use in the NICU admitted infants is higher and this increases the chances of the children developing asthma, obesity, and inflammatory bowel disease and conditions in the future, these infants acquire more ARGs due to antibiotics use in NICU [37].

According to Ramirez et al. hospital born preterm infants and those admitted to NICU have immature gut microbiota thus, they are easily affected with hospital acquired microbes and antibiotics given in NICU. Antibiotic exposure in the NICU plays a specific and irreplaceable role in the selection of resistant bacteria, and often used antibiotics, broad-spectrum coverage, as well as frequent use, contribute to the formation of resistance in the gut microbiota of the neonate. Many mechanisms of HGT such as plasmids, transposons, and integrons are quite active in NICU hospitals thereby making it easier for the ARGs to spread [38]. Other than mode of delivery, breastmilk and NICU, there are variety of environmental factors which are crucial for gut development and antibiotic resistome. In aquatic environment, ARGs can be transmitted to other microbes through horizontal gene transfer (HGT), which facilitates the spread of antibiotic resistome [39]. Human activities such as use of antimicrobials in fertilizers, wastewater discharge and agricultural land use increases ARGs in soil. These ARGs transmitted to plants and can enter the food chain [40].

2.2 Association with health and disease

Several research studies have highlighted the potential influence of the human gut antibiotic resistome on infants' health, either through immune dysregulation or the abnormal microbiome-gut-brain axis [41].The development of antibiotic resistance bacteria (ARB) due to people excessive use of antibiotics is one of the major challenges to human health [42]. The availability of antibiotics altering gut microbial communities leads to enhanced mutation, expression, and transfer of resistance genes [43]. Doan et al. studied the gut resistome in more than 500 children who received azithromycin treatments over four years and found that widespread azithromycin use may lead to an increased abundance of ARGs [44].Early therapeutic-dose pulsed macrolide treatment led to shifts in the gut microbial composition as well as increased the four ARGs' (*acrA, acrB, ant3Ia,* and *ant2Ia*) associated with macrolide resistance. Hence, antibiotics can also promote the dissemination of the ARGs either by escalating the mutations of the genes or by increasing their expression [45]. Jutkina et al. found that very low concentrations (10 μg/L) of tetracycline drive the transfer of diverse ARGs [46].

Wu et al. proved that levofloxacin increased the efficiency of plasmid mediated transformation and therefore the spread of antibiotic-resistant *Escherichia coli* [47]. Alterations in the gut microbiota are also associated with the development of cardiovascular disease; study of Tang et al. has also established that antibiotic resistance genes within the gut microbiota contribute to increased cardio metabolic risk factors. Moreover, trimethylamine (TMA) associated with metabolic gut bacteria facilitate chronic disease associated with cardiovascular diseases owe to reactive oxygen species and endothelial dysfunction [48]. The four ARGs *norB, emrE, ermX, and vanX* were found to

be positively associated with an elevated risk of type 2 diabetes (T2D) by genome-wide association analysis. Additionally, as diabetes worsened, the study found that *norB* and certain β-lactam ARGs became more abundant [49]. And another research pointed out an imbalance in gut microbiota in patients who had Inflammatory Bowel Disease (IBD), they also found that higher amount of pathogenic *E. coli* was associated with multiple ARGs including *mdtO, mdtP, emrK*, which potentially contribute to IBD progression [50]. Kovtun et al. used metagenomic analysis to examine the distribution of ARGs in the gut microbiomes of healthy children aged 3 to 5 and children with autism spectrum disorder (ASD). They discovered that children with ASD had significantly different gut microbiomes, which were marked by higher levels of ARGs. The three distinct ARGs in ASD are the genes *tet(40)* from *Megasphaera, cepA-49* from *Bacteroides* (b-lactams), and *aac(6')-aph(2")* from *Enterococcus* [51].

CHAPTER 3: MATERIALS AND METHODS

In this study, we conducted bioinformatics analysis on metagenomics samples obtained from infants of USA. The analysis involved several important steps to extract meaningful insights from the data. First, we retrieved and preprocessed the metagenomics data, ensuring its quality and integrity. Subsequently, we performed metagenome assembly to reconstruct the genetic material present in the samples. We predicted genes by using prodigal from metagenome contigs. Additionally, we identified genes and constructed a non-redundant gene catalog and estimated their abundance to explore potential functional attributes. After that, we performed a metagenomics analysis for the alignment of protein against a comprehensive Antibiotic Resistance Genes Database. To gain deeper insights into our findings, we conducted statistical analyses to identify associations between ARGs and metadata variables. This comprehensive approach enables a more profound understanding of the identification and associations of ARGs and their relevant antibiotics for which they confer resistance and cause different types of diseases within the metagenomics data obtained from infants.

3.1 Data Description and Acquisition

The metagenome data was obtained NCBI Sequence Read Archive (SRA) database using accession number PRJNA473126. The original study involved a total of 402 gut metagenome samples collected from infants delivered via either of the delivery mode (VD: n = 181, and CSD: n = 221) across different timepoints (M) starting from M0 till M8. We were interested in understanding how the gut microbiome of infants evolves after acquisition from mothers at birth, thus, this dataset allowed us to answer our research

question. Further details, including the metagenome sequencing along with relevant metadata is provided in Table 3.1 and Table 3.2.

Table 3.1: Major characteristics of the participants involved in study

Mode of delivery		CSD	VD
Host Antimicrobial In Last 7days	FALSE	199	199
	TRUE	13	13
Host Lifetime Amoxicillin exposure	NO	187	146
	YES	25	11
Host Lifetime Antibiotics Exposure	NO	185	139
	YES	27	18
Host Lifetime Azithromycin Exposure	NO	210	157
	YES	2	0
Host Maternal Diabetes	FALSE	188	157
	TRUE	24	0
Host Special Care Nursery Admission	FALSE	189	133
	TRUE	23	24

Table 3.2 Comparison of sample sizes for CSD and VD across time points

Time points (Months)	CSD *(n)*	VD *(n)*
0	13	11
1	21	18
2	29	19
3	22	14
4	23	21
5	28	25
6	22	23
7	31	24
8	26	21

3.2 *De novo* Metagenome Assembly

After the preprocessing, the metagenome assembly of the clean reads was performed. The process of reconstructing the contigs using the short reads is known *as De novo* metagenome assembly. In this study, we used metaSPAdes (v3.15.3) [52] to perform the

metagenome assembly through the KBase online server (https://www.kbase.us). Default parameters were applied for the assembly, except for the *k* list, which was set as 31, 59, 87, 115, 143, and a minimum contig length of 500. The output from metaSPAdes included assembled contigs, representing the reconstructed genomic sequences from the metagenomic data. The quality of the metagenome assembly was assessed by using a Quality assessment tool for genome assemblies (QUAST).

3.2.1 Quality Assessment

Quality assessment tool for genome assemblies (QUAST) was used to access the quality of metagenome assembly. The "quast.py" script from the QUAST (v5.2.0) [53]was employed to generate the quality assessment reports. The QUAST report generated statistics like distribution of contigs, largest contig, N50, assembly length, etc for each sample.

3.3 Gene Prediction

For gene prediction, Prodigal (v2.6.3) [54]was employed. Contigs were retrieved from the metagenome assembly in fasta format and were used as input. Three types of output were obtained for gene sequences, nucleotide sequences and protein sequences respectively. These outputs were used for further analysis.

3.4 Gene Catalogue Construction

The gene catalogue was constructed through MMseqs2 [55] to cluster genes predicted from prodigal. MMseq2 was employed to group genes at nucleotide similarity level. Gene catalogues were constructed by running clustering.sh. The sequences were

clustered through linclust command by using sequence identity threshold with following parameters minimum coverage of (--cov-mode 1, -c 0.8), minimum sequence identity of 90 (--min-seq-id 0.9), with the clustering mode to focus on both sequence identity and k-mer composition (--cluster-mode 2), fast nucleotide alignment mode (--alignment-mode 3). After clustering, all representative sequences were extracted and saved in FASTA format and were used to construct gene catalogues for further subsequent analysis. This clustering process enabled the grouping of all closely related genes creating a comprehensive gene catalogue for Antibiotic resistome analysis.

3.5 Identification of Antibiotic Resistance Genes

After gene catalogue construction, Antibiotic Resistance genes were identified by aligning the gene sequences with curated Comprehensive Antibiotic Resistance database (CARD) through DIAMOND (v2.1.9) [56]. The **diamond makedb** tool was used to download and convert the CARD database into a DIAMOND compatible format. As the reference, the resulting CARD database was used to identify ARGs in the gene sequences. The genes sequences obtained from gene catalogues were used against the CARD database through DIAMOND blastx. Following parameters were used for this alignment; the E-value cutoff of 1e-5 to exclude weak hits, identity threshold was 70% for relevant matches and minimum query coverage was also 70% only retaining the top hit for each query sequence.

The results were filtered to keep the best alignment sequence based on high bit score, containing relevant ARGs containing sequence IDs with identified ARGs, e-value and

sequence length. These ARGs sequences were extracted and used for further analysis like relative abundance.

3.6 Antibiotic Resistome Analysis

Antibiotic resistome analysis was carried out in the following three steps:

3.6.1 Antibiotic resistance genes identification

Antibiotic resistance genes obtained from CARD [57] were 2685 in counts against all contigs. According to study, these ARGs confer resistance to antibiotics like *acrA, acrB, ant3Ia, ant2Ia, norB, emrE, ermX, and vanX* were found to be positively associated with diseases. The nucleotide sequences of these genes were retrieved from metagenome assembly, and database was constructed through DIAMOND (v2.1.9).

3.6.2 ARGs contigs extraction

This step involved the identification of ARGs contigs from DIAMOND results. These contigs were then retrieved from the metagenome assembly in fasta format. These ARGs sequences were extracted and used for further analysis like relative abundance.

3.6.3 Antibiotic resistance gene quantification

Following the ARGs extraction, the CoverM (v0.6.1) [58] was used to determine the read counts of each gene. The genes were mapped with metagenomic reads using specific parameters, with "--min-read-percent-identity 95", and "--min-read-aligned-percent 50". The 'Count' method was utilized to determine the percentage of total metagenomic reads mapped to each gene. A customized Python script was prepared to

concatenate multiple CoverM output files into a unified matrix format for subsequent analysis.

3.7 Statistical Analysis

The study employed different statistical techniques to evaluate the findings and compare the results among different data groups.

3.7.1 Median Comparison Using Wilcoxon Test

Wilcoxon test was employed to assess statistical significance, providing a robust method to evaluate differences within and between groups without relying on strong distributional assumptions. To evaluate and compare the contig length, N50, and largest contig distribution in each data group, the Wilcoxon test was applied using the QUAST report. A p-value of < 0.05 was considered to indicate statistically significant differences.

3.7.2 Associations Using MaAsLin2

This study used MaAsLin2 to find associations of ARGs with diet, mode of delivery, maternal antibiotic consumption, NICU admission effects and other metadata variables. For associations of ARGs, we subjected the ARGs matrix and a metadata file having the sample names and corresponding data group to MaAsLin2 [59]. Association analysis between control groups and case samples was carried out. The control group was used as a reference along with the default parameters except for the ‘normalization’ was set to ‘NONE’. The same parameters were also used for determining associations of ARGs with the sample metadata.

CHAPTER 4: RESULTS AND DISCUSSION

4.1 Metagenome Assembly

Metagenomes of both case were assembled by metaSPAdes de novo assembler and their qualities were evaluated using QUAST. The main parameters evaluated for determining assembly quality include the total number of contigs, total assembly length, largest contig, and N50 length. These are discussed in detail below:

4.1.1 Total Number of Contigs In Metagenome Assemblies

The mean and std for the CSD are 0.0179±0.0097 million base pairs (Mbp), respectively. For the VD, the mean and std are 0.016±0.011 Mbp, respectively. This indicates that the number of contigs in both cases was relatively consistent, with the CSD having a relatively higher mean and low std compared to the VD (Figure 4.1). However, significant differences were observed between the cases, with p-value of 0.023.

4.1.2 Total Length of Metagenome Assemblies

Total length of metagenome assemblies for the CSD was 30.75±14.89 Mbp, whereas for the VD, it was 27.30±17.23 Mbp. This suggests that the total length of the metagenome assembly was comparatively higher for the CSD compared to the VD. However, significant differences were observed between the cases, with p-value of 0.0032. (Figure 4.1). This means that mode of delivery has significant has significant impact on metagenome assembly.

4.1.3 Largest Contig Size

Largest contig size of metagenome assemblies for the CSD was 0.1786± 0.1281 Mbp, whereas for the VD, it was 0.2110± 0.1629 Mbp. However, no significant differences were observed between the cases, with p-value of 0.08. (Figure 4.1).

4.1.4 N50 Length

The N50 length for the CSD was 8.96±30.61 Kbp, whereas for the VD, it was 7.62±21.057 Kbp. This suggests that the N50 is slightly higher for the CSD compared to the VD (Figure 4.1).

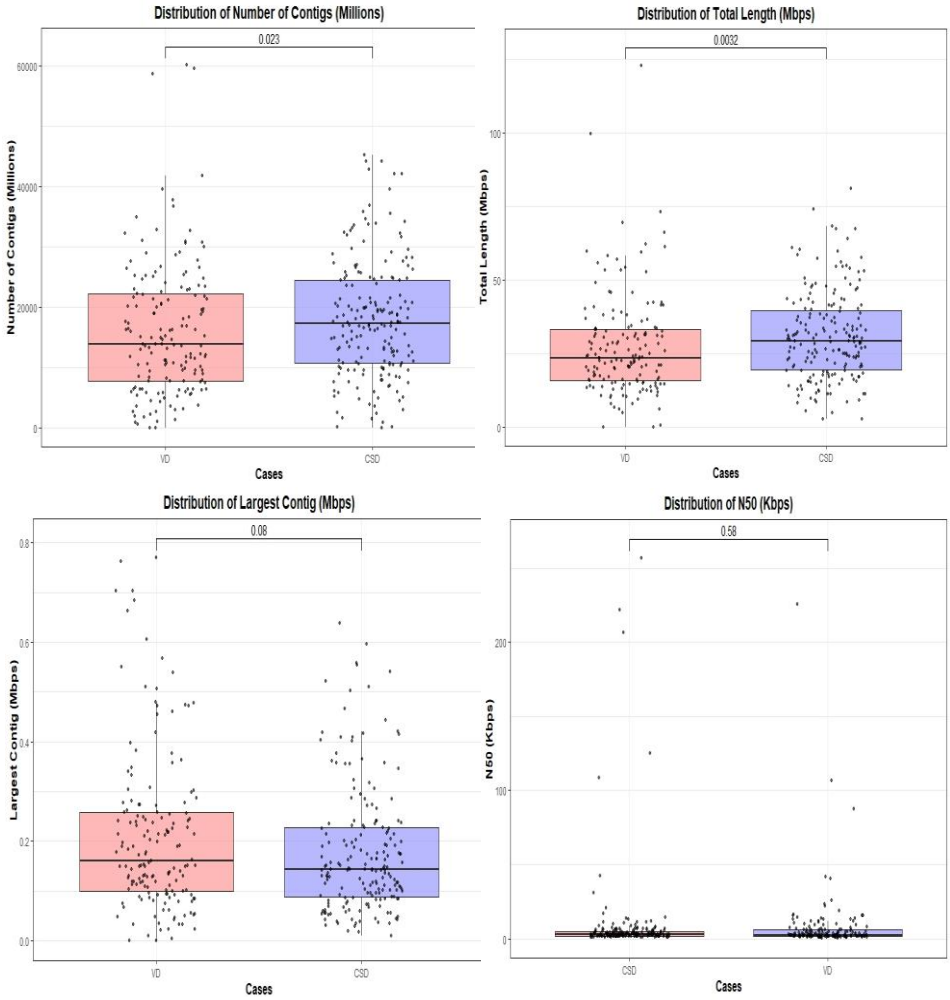

Figure 4.1: Visualization of quality assessment of metagenome assembly

However, no significant differences were observed between the cases, with p-value of 0.58. The results indicate that the metagenome assemblies for the CSD and VD were of similar quality, with no significant differences in the assembly metrics. This suggests that both cases were adequately represented in the assemblies. The assembled metagenomes of infants also enabled the identification of ARGs involved in early life dynamics and their impact on infant health and disease.

4.2 Gene Predictions and Catalogue Construction

After metagenome assembly, accurate and efficient genes were predicted in metagenomics dataset. Gene sequences represented the predicted gene regions which were identified, nucleotide sequences in which DNA sequences for corresponding identified genes and proteins sequences for translated amino acids sequences of the predicted genes were present.

4.2.1 Gene counts for CSD and VD

Gene catalogues were constructed to have a non-redundant collection of genes identified in the dataset, and after that the distribution of gene counts between the CSD and VD groups were analysed. The both groups showing the similar distribution of gene counts, but CSD group showing a narrower interquartile range as compared to VD having similar median gene counts for both with no statistical significant difference given in (Figure 4.2).

Figure(4.2) Gene catalogue construction and gene counts for CSD and VD

4.2.2 Gene Counts across Different Timepoints

The observed pattern of gene counts across different timepoints in the infant gut microbiome as depicted in (Figure 4.3) shows no significant difference between CSD and VD having p-values for comparision of gene counts across timepoints from M0,M1,M2,M3,M4,M5,M6,M7 and M8 months ranges from 0.19 to 0.85 ,there is no significant difference for any timepoints across both groups CSD and VD.

Both groups have consistent trends for gene counts with slight fluctuations across timepoints, so mode of delivery either through CSD or VD has no significantly impact on gene counts during metagenomics. The p-value for M0 is 0.85, for M1 is 0.19, for M2 is 0.68 , for M3 is 0.65 ,for M4 is 0.39 , for M5 is 0.27 ,for M6 is 0.74 , for M7 is 0.27 and for M8 is 0.44, showing no significance difference. The p-values for M5 and M7 is 0.27

having equal distribution of genes at these timepoints, while others have very slight differences, with no significant difference having almost similar gene counts across all timepoints as shown in (Figure 4.3).

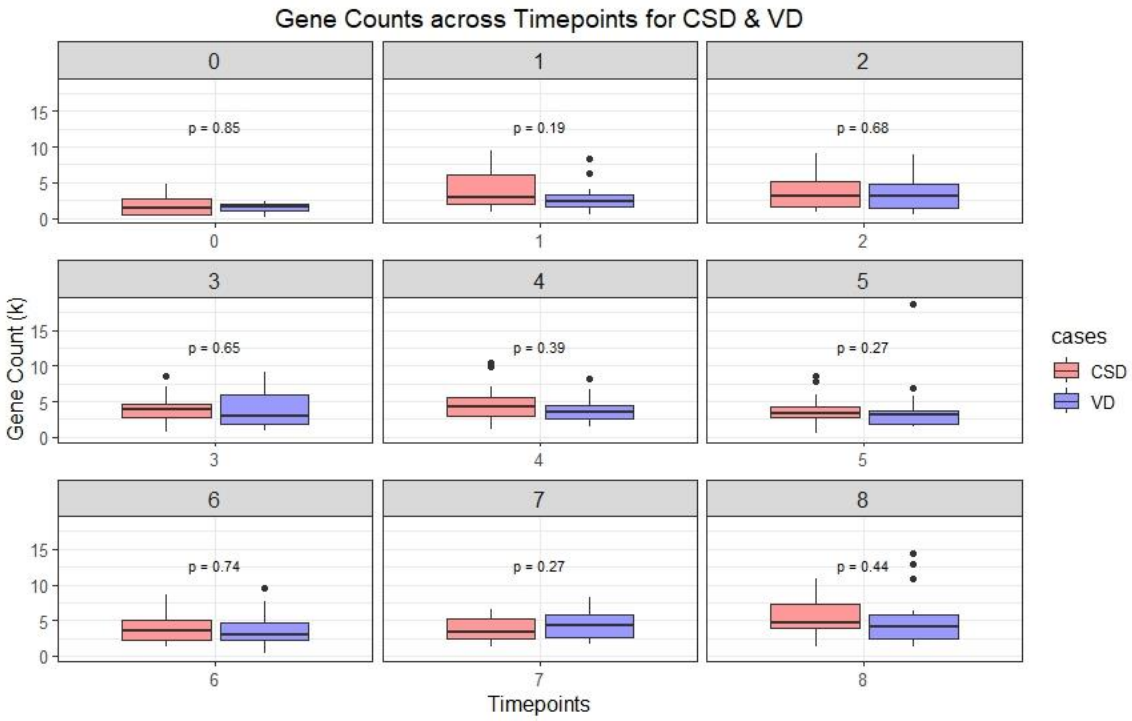

Figure (4.3) Gene counts across timepoints for CSd and VD

4.3 Antibiotic Resistome Analysis

Resistome analysis were analyzed by delivery mode which refers to CSD and VD to determine the relationship between delivery mode and ARGs prevalence. Total 2685 ARGs were identified by analyzing the results CSD group was found to have overall count of more than 1550 genes as compared to VD group having approximately more than 1200 ARGs across the infant microbiome. This dataset has been processed to relate ARGs with other information including metadata variables.

The ARGs were subjected to sequence identity comparison, alignment length and functional categorization using classification provided by CARD. For each gene of interest, hsps in CARD database were searched with its corresponding reference sequence using parameters such as alignment identity of greater than 80%, and e values cut off at $10^{-30.}$ Most identified ARGs showed e-values lower than $1e^{-100}$ indicating statistically significant matches to the CARD database. These ARGs counts for CSD and VD are shown in Figure (4.4).

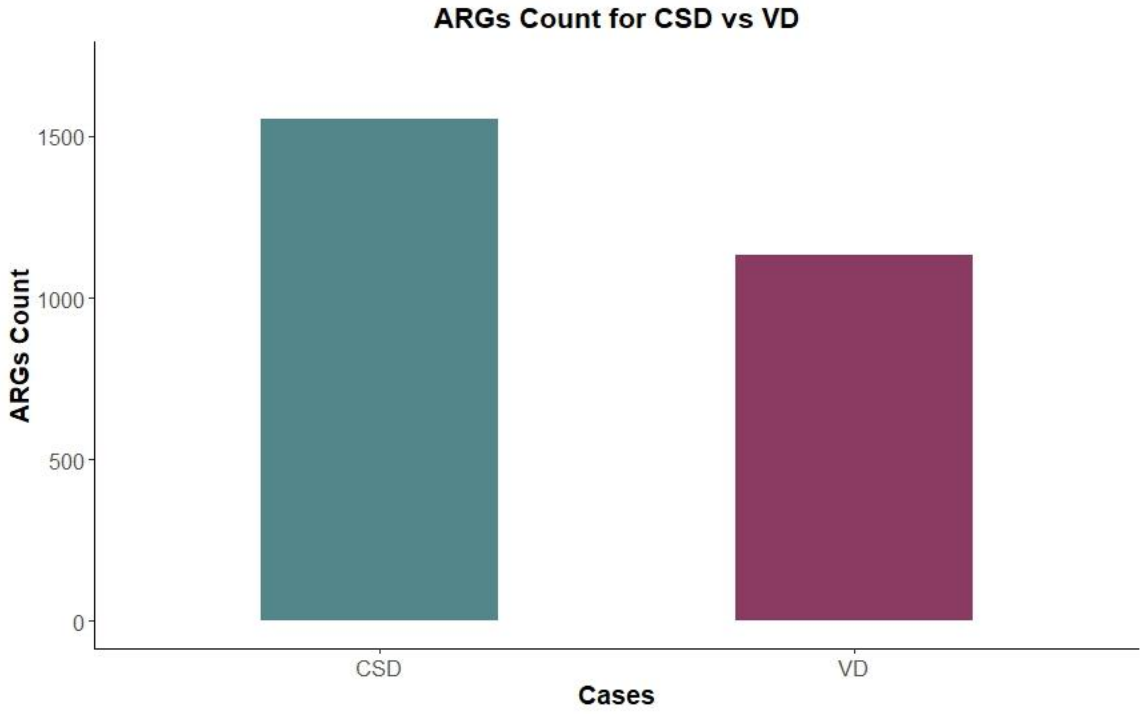

Figure (4.4) ARGs counts for delivery route (CSD & VD)

4.3.1 ARGs counts across different timepoints

The count of ARGs appears to fluctuate between the two groups across different timepoints. The ARGs count seems to vary across timepoints within each group. Some

timepoints show higher counts compared to others for both CSD and VD. The distribution of ARGs in infants during early life across different timepoints is given in Figure (4.5), which shows there is no significant difference for ARGs between the groups for CSD and VD at any timepoint having p-value 1 for all timepoints due to ARGs counts were similar or identical for both groups at every time point. The distribution of ARGs in infants during early life across different timepoints is given in Figure (4.5).

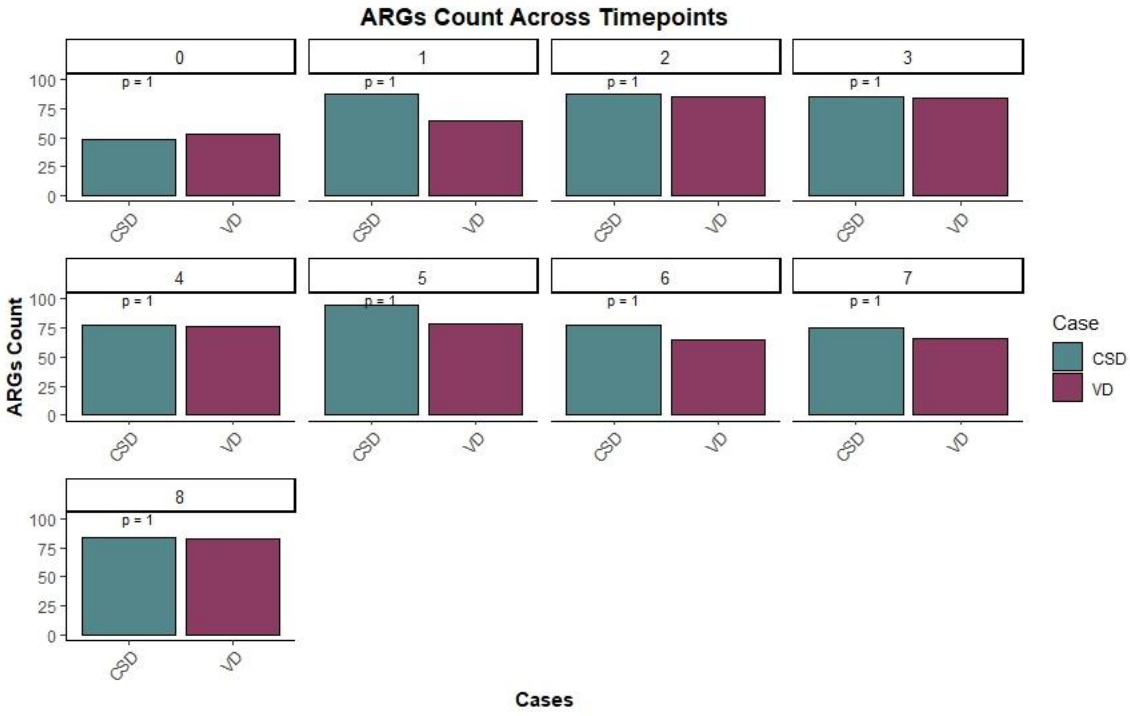

Figure (4.5) Distribution of ARGs across different timepoints

4.4 ARGs Abundance Analysis

To access the dynamics of ARGs abundance in infants, we analyzed relative abundance of ARGs at different timepoints (M0 to M8) for both CSD and VD groups. This suggests that the infant gut microbiome, and consequently ARG abundance undergoes significant changes during the first year of life. While general trends were observed ,the

specific timepoints at which ARG abundance peaked or declined differed between the two groups, like CSD infants showed a higher ARG abundance at timepoint M5 (p=0.0093) shows a significant difference between both the groups. While VD infants exhibited a higher abundance at timepoint M6 (p=0.0045) shows significant difference for both groups. A p-value of 0.056 or lower than it is considered as statistically significant, so for timepoint M7 (p=0.056) shows a significant difference in ARGs abundance for both CSD and VD groups. While others timepoints M0, M1, M2, M3, M4, and M8 has p-values greater than 0.05 showing there is no any significant difference between both groups. The observed variations in ARG abundance across time points reflect the dynamic of nature of infant gut microbiome. Factors such as diet, exposure to environmental microbes, antibiotic consumption, maternal influence on infants, and mode of feeding, mode of delivery can influence the composition and function of gut microbiome in early life and in turn can impact the ARG abundance. The ARGs abundance across different time points are given in Figure (4.6).

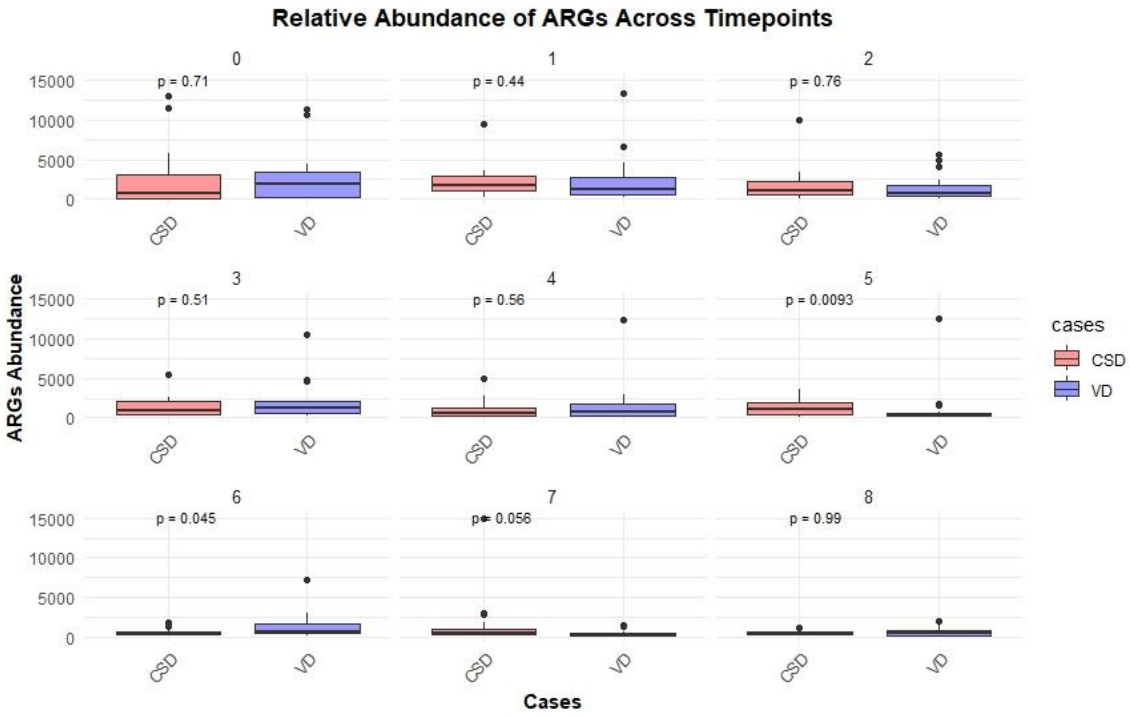

Figure (4.6) Relative abundance of ARGs across different timepoints

4.4.1 Shared and exclusive ARGs for CSD and VD

The dynamic nature of infant gut microbiome ARGs profiles for both groups vary time to time. The composition of ARGs shifts over time, some ARGs acquired early in life and persist, and while others can be transient. The Figure (4.7) shows the 136 ARGs are common for both CSD and VD groups, 60 ARGs were unique for only CSD group and 48 unique ARGs were present in VD group only, these groups have horizontal bars which shows CSD and VD set sizes having pairwise intersection which shows connection for both groups either thry have common ARG or unique ARGs.

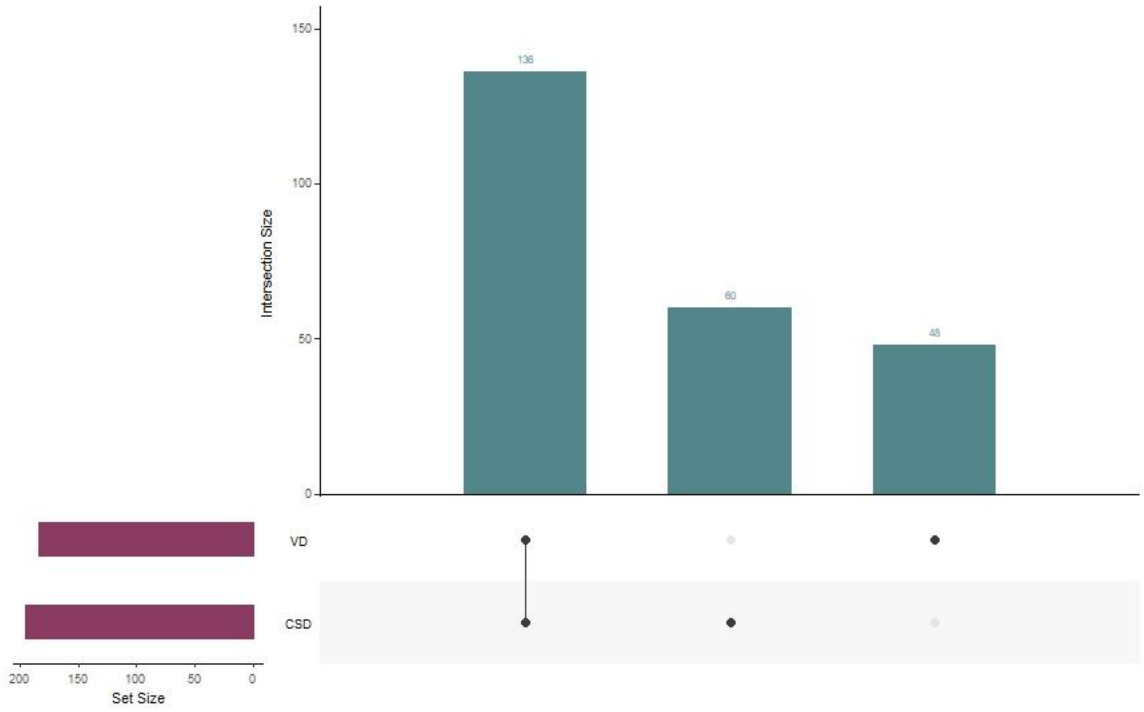

Figure (4.7) shared and exclusive ARGs for CSD and VD

The dynamic nature of infant gut microbiome ARGs profiles for both groups vary time to time. The composition of ARGs shifts over time, some ARGs acquired early in life and persist, and while others can be transient. The presence of unique ARGs across different timepoints emphasizes the temporal process of ARG acquisition in infants. Certain ARGs are more consistent in the gut microbiome while others appear and disappear over time. For CSD group 11 ARGs are unique present only in timepoint M3, M1, M5. 9 ARGs are present for M4, 8 ARGs are present for only M8, 7 unique ARGs are present for M2, and 6 unique ARGs are present for M7 given in Figure (4.8). Similarly, for group VD, some genes are common present in many timepoints and some are unique present in different timepoints like 16 ARGs are unique present only in M0 timepoints, 7 ARGs are

present at M1, 6 ARGs are present in M6 timepoint and 5 ARGs are present in M7 and M4 separately.

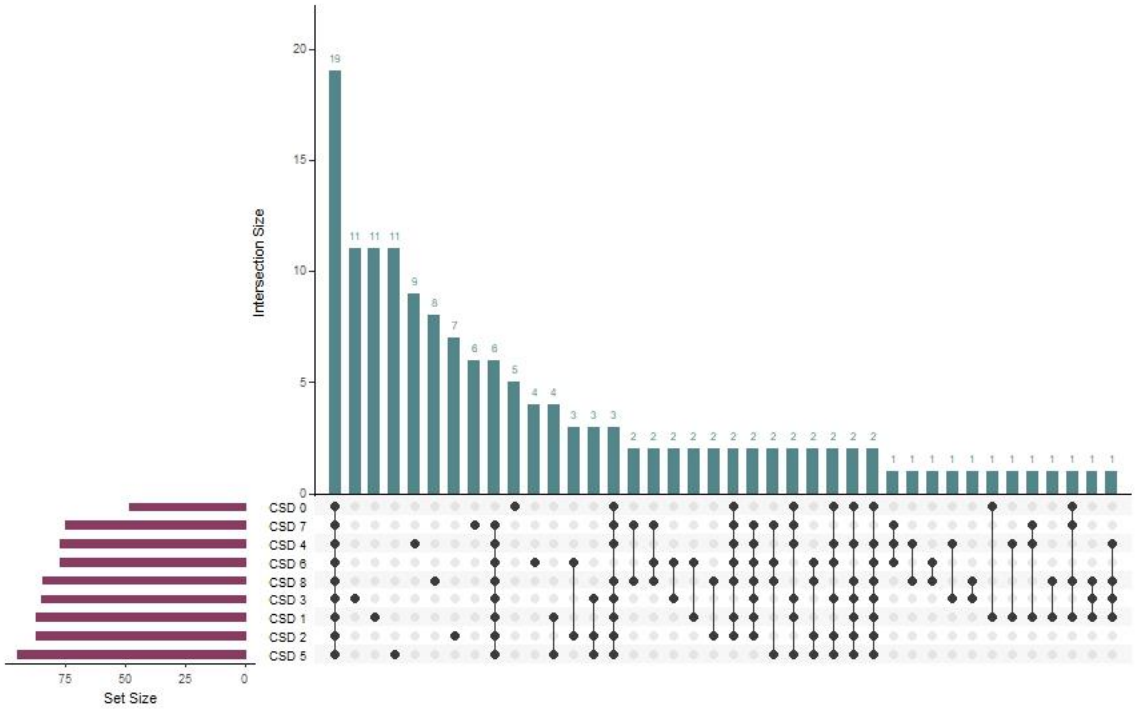

Figure (4.8) shared and exclusive ARGs for CSD only

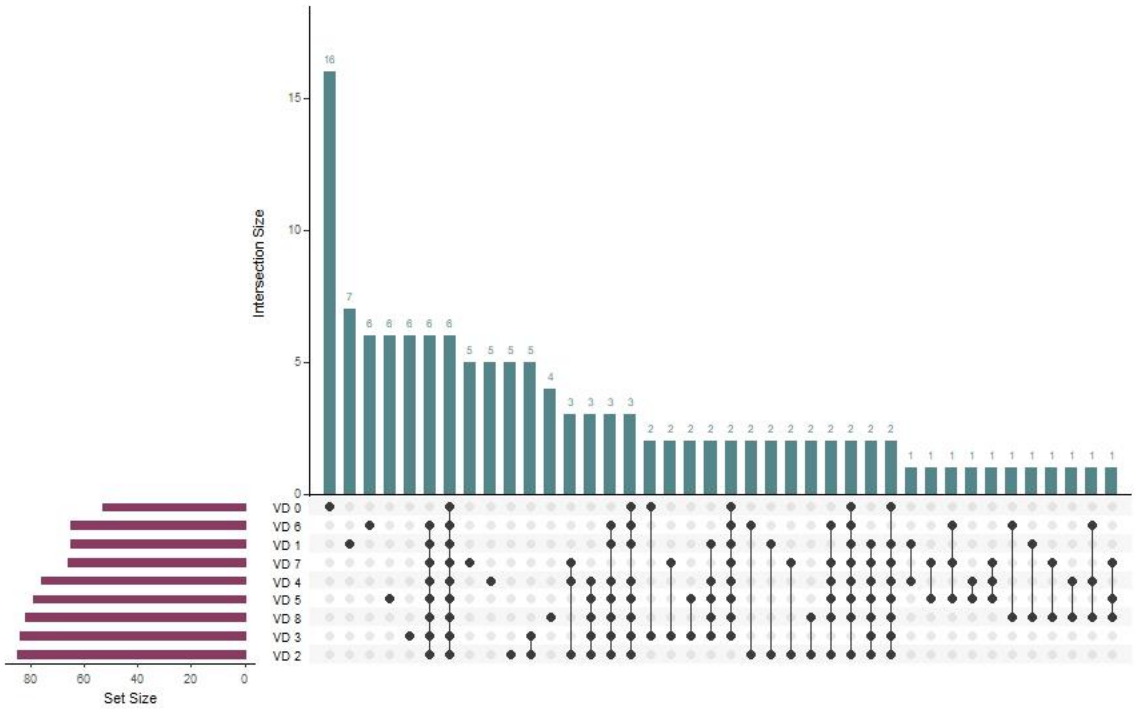

Figure (4.9) shared and exclusive ARGs for VD only

So the Figure (4.10) shows the compared ARGs acquisition for both CSD and VD groups in infants and give insights to show how mode of delivery might influence the acquisition and persistence of ARGs.

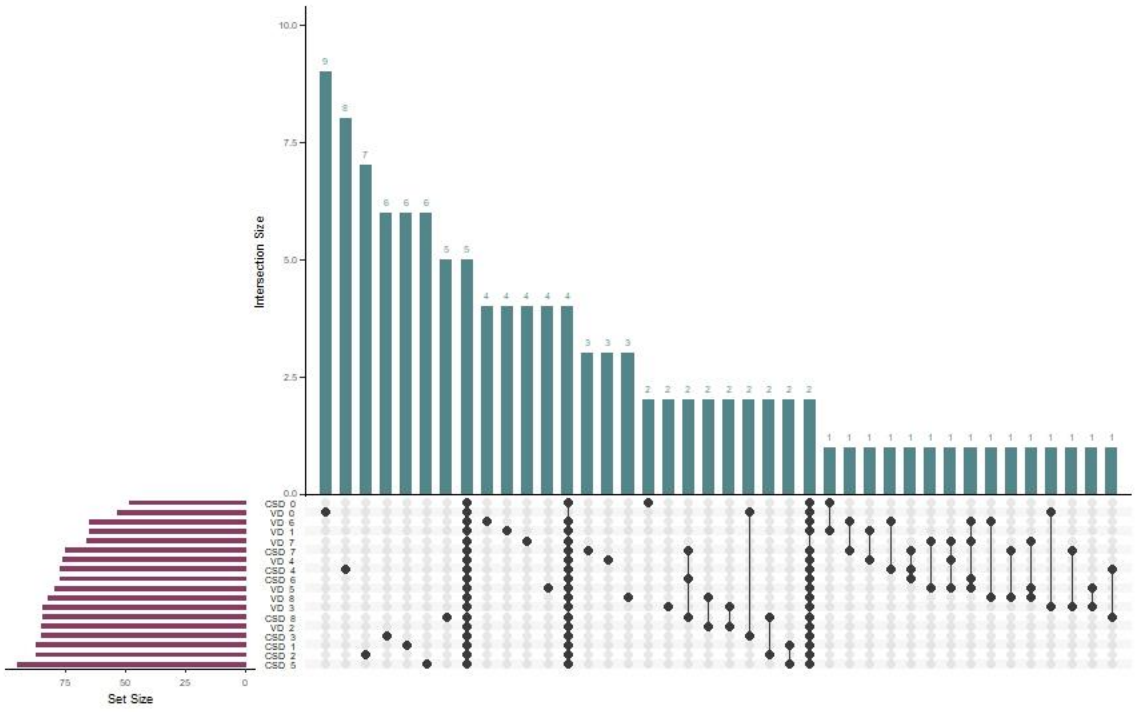

Figure (4.10) shared and exclusive ARGs for both CSD and VD

4.5 Associations of ARGs with metadata

The ARGs were associated with metadata variables like diet, age, birthweight, delivery route, host lifetime amoxilin exposure, host lifetime antibiotic exposure, host special care nursery admission and host water type by using MaAsLin2.

4.5.1 Mode of delivery

The analysis using MaAsLin2 revealed a significant positive association ($p < 0.05$) between the abundance of 10 specific ARGs in which 10 ARGs *mdtE, sdiA, eptB, Ecol_acrA, Yoji, emrR, bacA, msbA, rosB, mdtN, AcrF, MdtQ, kdpE, and sul1* are positively associated with CSD, while these 5 ARGs *CfxA3, Mef.En2, mdtE, tet.X* are positively associated with VD infants. These associations are given in Figure (4.11).

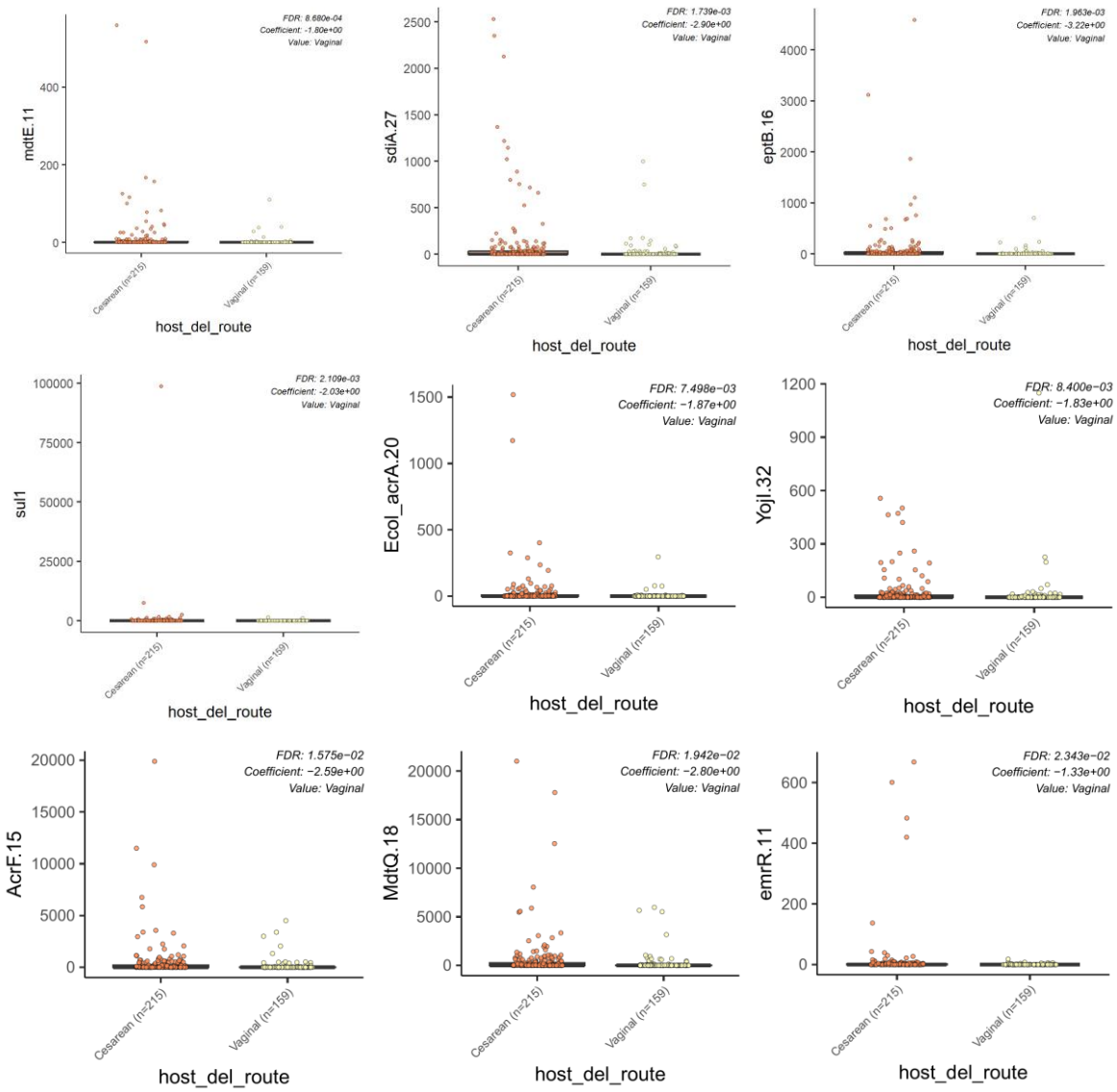

Figure (4.11) Associations of ARGs with mode of delivery

4.1.1 Associations of ARGs with metadata

The analysis using MaAsLin2 revealed a significant positive association ($p < 0.05$) between the abundance of 10 specific ARGs in which *emeA, ugd, APH(6)-Id, tet.A, rpoB2, , dfrA17, mphA, , APH(6)-Id, AcrF,* and *sul1* are positively associated with NICU admitted infants given in Figure (4.12).

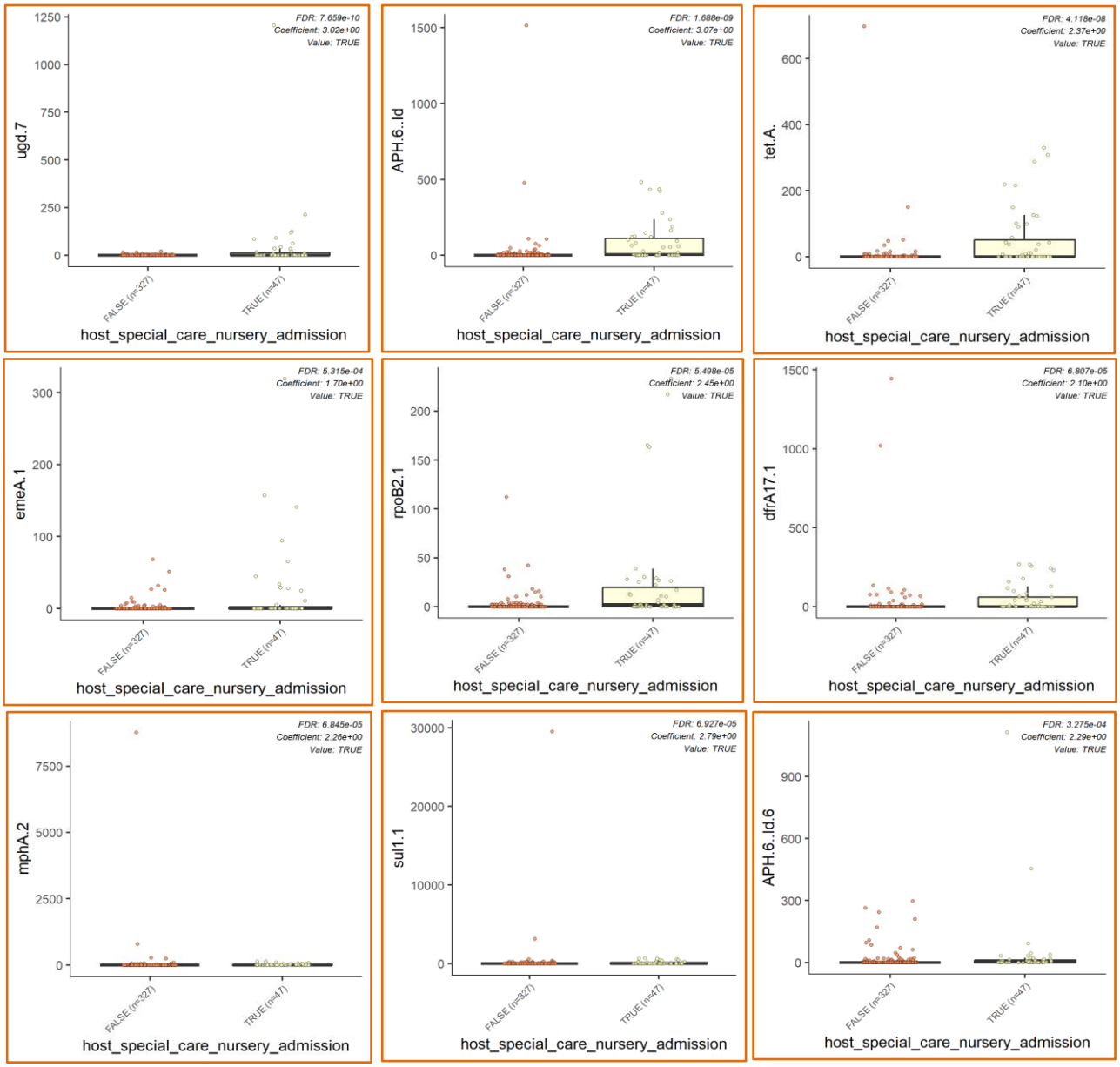

Figure (4.12) Association of ARGs with host special care nursery admission

These ARGs are associated with important variable, type of water used by host, different categories of host water type were used, and these categories include:

- Boiled/distilled water
- Bottled water
- Filtered water
- Tap water

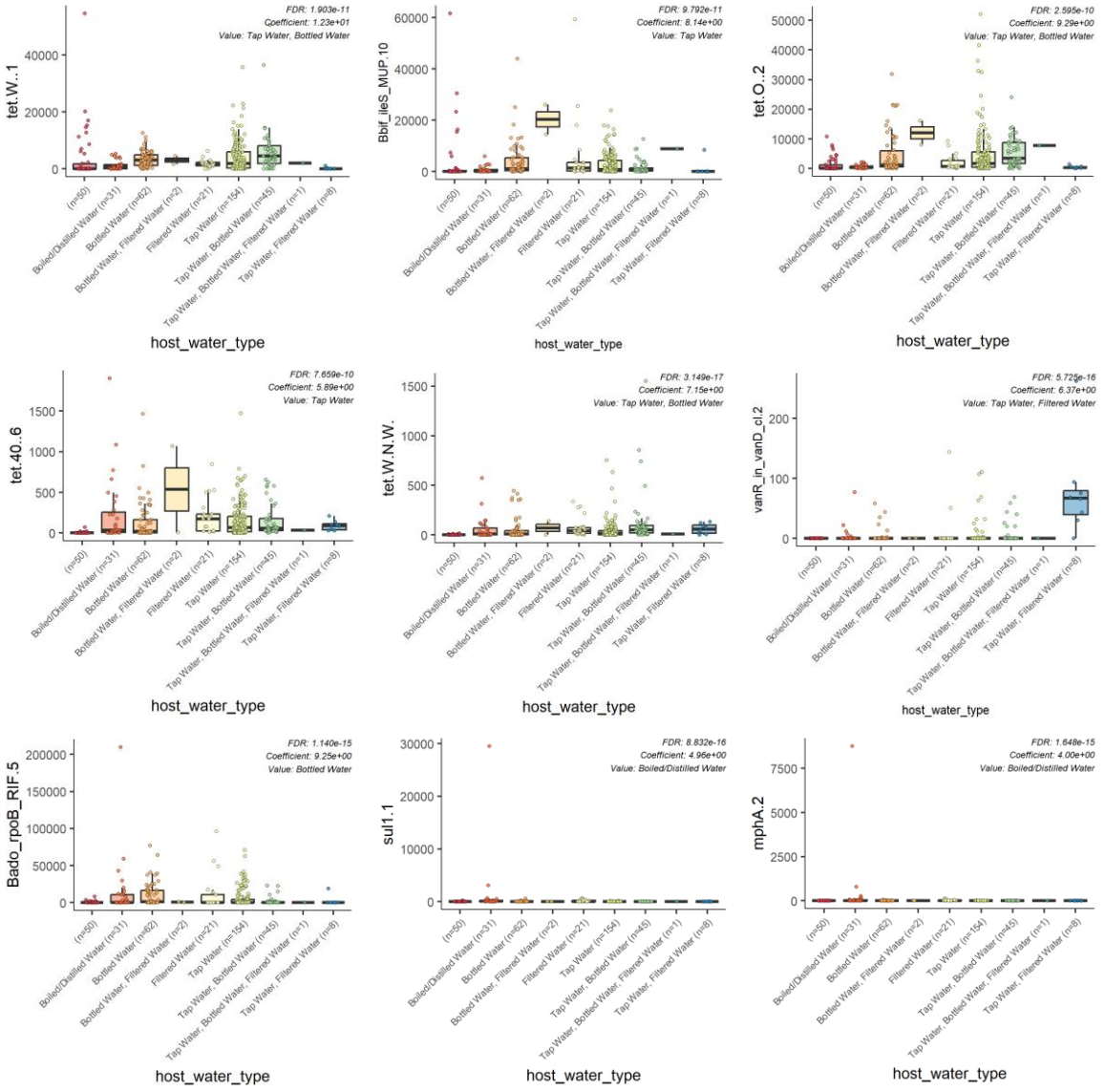

Figure (4.13) Associations of ARGs with host water type

The abundance of ARGs were positively associated with tap water and bottled water categories having highest median and largest spread and having low FDR values closer to 0 and positive coefficients shows significant results. There are 10 ARGs which are significantly associated with tape water and bottled water shown in Figure (4.13).

These ARGs *tet(w)*, *tet(40)*, *tet(O)*, *Bado_rpoB_RIF, Bbif_iles_MUP, vanR_in_vanD_cl* were associated with tape water and bottled water and 2 ARGs *sul1 and mphA* with boiled/distilled water.

Other factors like host lifetime antibiotic exposure has been positively associated with ARGs prevalence in infants. These ARGs were *AxyY, aads, Mef.En2, ErmF, mexY,cepA.* These are given in Figure (4.14).

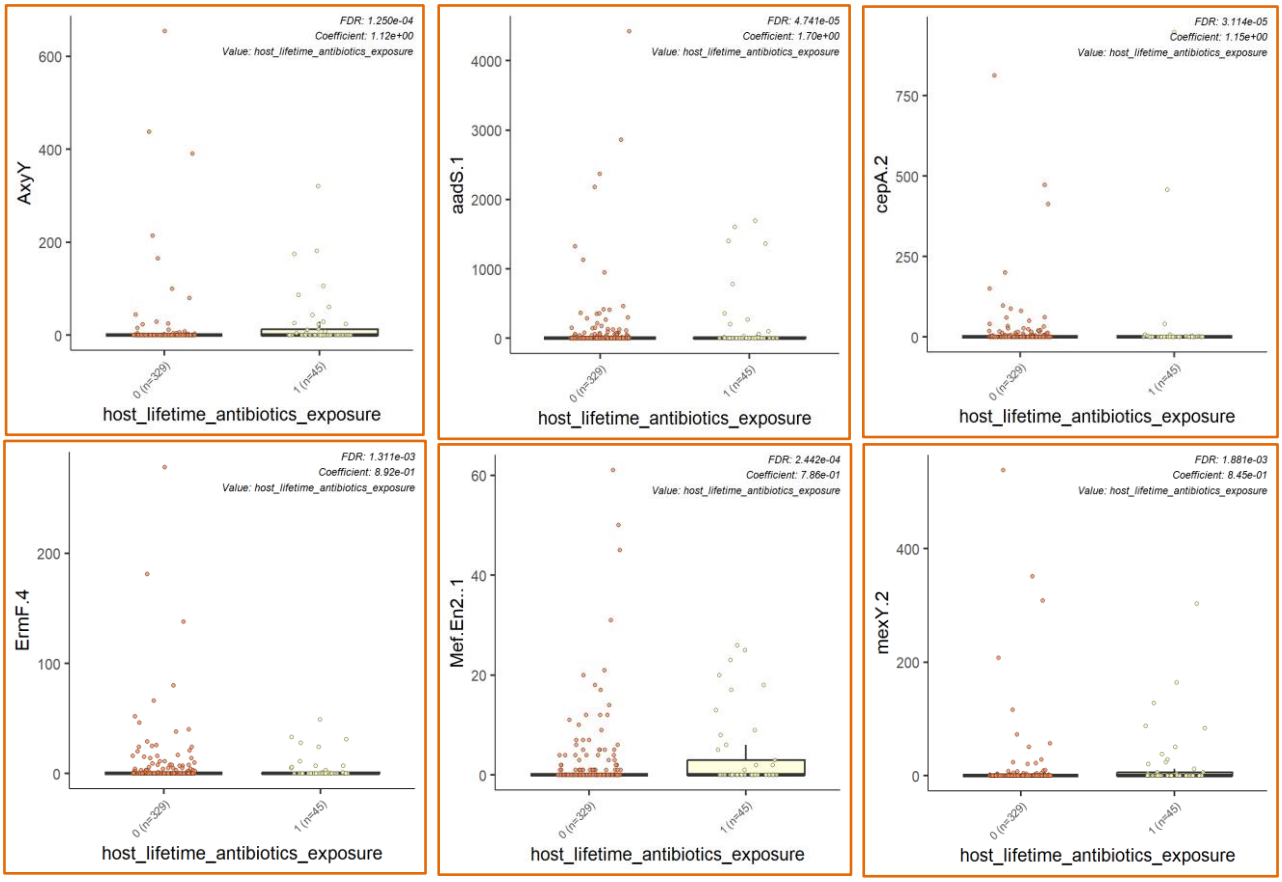

Figure (4.14) Associations of ARGs with host lifetime antibiotic exposure

Similarly, other important factor which is diet, has no associations with ARGs, different type of food was used for infants like formula-fed, breastfed, cow's milk, lactose, cereals, starch and other diet types. The associations can be seen in given heatmap which shows ARGs association with different metadata factors.

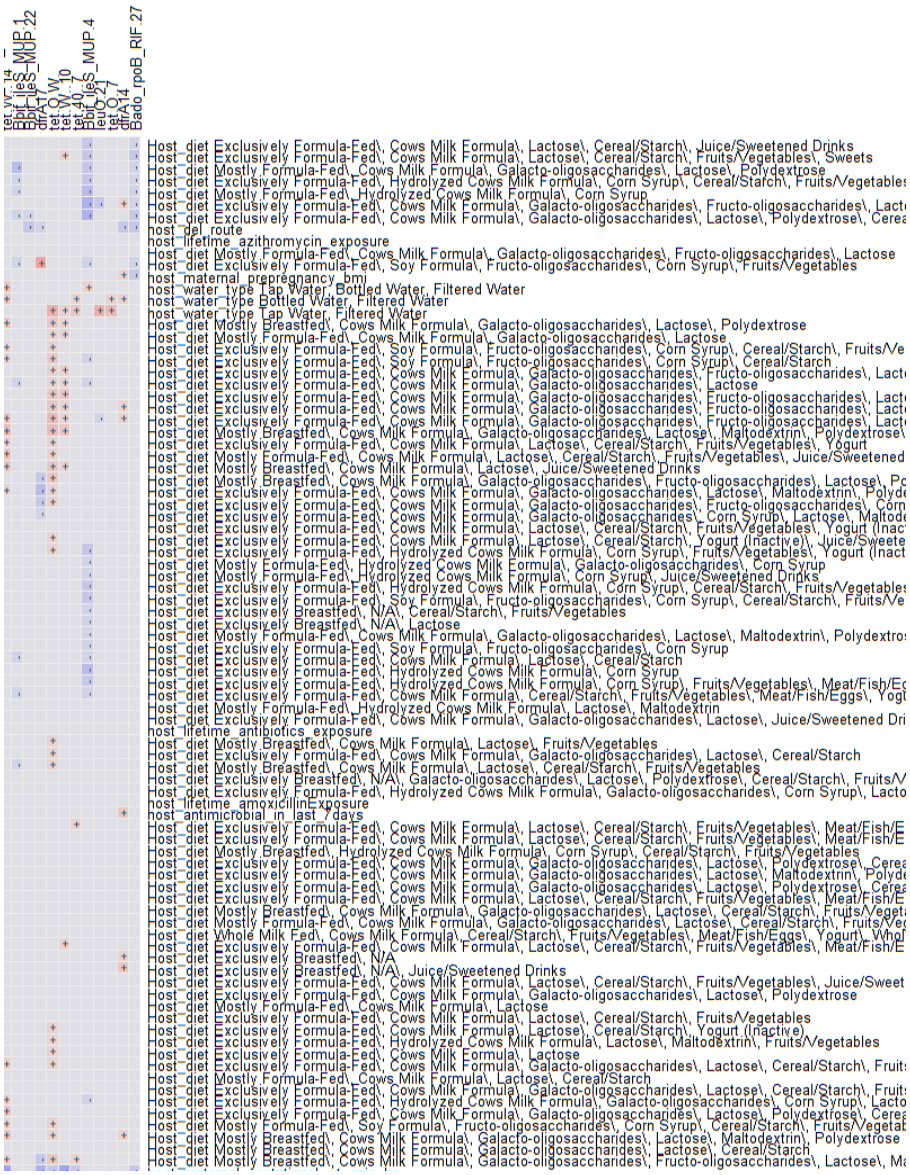

Figure (4.15) Associations of ARGs with Diet

The graph shows wide variety of diets ranging from exclusively formula-fed to mostly breastfed with various combinations of ingredients. Based on the analysis, there is no statistically significant association between the different hosts diets, all diets are above the conventional threshold of 0.05.

CHAPTER 5: CONCLUSIONS

In conclusion, the findings highlight the dynamics of early life antibiotic resistome and its potential implications for health and diseases. Delivery mode was identified as very significant in determining the behavior and dynamics of early life gut microbiome and resistome. The above variations in ARG abundance and associations with metadata factors provide valuable insights and its potential implications for infant health.

ARGs prevalence were observed between CSD and VD infants, shedding light on the intricate interplay between delivery mode, dietry factors, NICU admission, host lifetime antibiotics exposure and water type used by these infants. During infants birth the ARGs prevalence was higher for CSD as compared to VD, ARGs like mdtE, *sdiA, eptB, Ecol_acrA, Yoji, emrR, bacA, msbA, rosB, mdtN, AcrF, MdtQ, kdpE, and sul1* are positively associated with CSD, while ARGs *CfxA3, Mef.En2, mdtE, tet.X* are positively associated with VD infants. Throughout the first year of life different abundance of resistome were observed across different timepoints highlighting the associations of delivery mode with ARGs prevalence.

The distribution of ARGs was higher for CSD infants than for VD infants, suggesting that delivery mode impacts the acquisition and persistence of ARGs. Descriptive analysis by time indicated differential abundance levels of ARG across different timepoints, these variations demonstrate how environmental and developmental impacts affect the nature of a resistome. The relationship between ARGs and the metadata variables including water type and maternal antibiotic use, NICU admission of infants at time of birth, and infants' antibiotics exposure and diet also play significant role in the influence of dynamics of

antibiotic resistome. The temporality of the resistome and the significant roles played by delivery method and exposures to the acquisition of resistance genes are indicated by the comparison of the collection of ARGs identified. This temporal flexibility of the resistome is highlighted by the discovery of shared and exclusive ARGs across timepoints indicating that environmental exposures and delivery mode both have major impact on the acquisition of resistance genes.

In light of these findings, future recommendations must encompass a comprehensive approach to leverage the potential of the early life antibiotic resistme for infant health optimization. Firstly, continued exploration of ARGs interventions tailored to specific ARGs abundance could offer promising avenues for therapeutic intervention. Secondly, longitudinal studies examining the intricate interplay between ARGs abundance, environmental factors and health outcomes are imperative for informing targeted resistome strategies. Furthermore, the development and refinement of microbiome-base therapies, such as fecal microbiota transplantation (FMT), hold promise for restoring microbial diversity and function in infants at risk of symbiosis or associated health conditions. By examining the microbial diversity and ARG prevalence fostering a diverse microbial community will reduce the probability of ARGs spread. By analyzing resistome profiles in newborn admitted to NICU to find relevant ARG associations that can guide antibiotic usage recommendations and infection control procedures.

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