Low Concentration Antibiotic Exposure and Horizontal Gene Transfer in Zebrafish Gut Microbiome

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Islamabad, Pakistan

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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

Antibiotic resistance is a major global health concern driven by horizontal gene transfer (HGT) of antibiotic resistance genes (ARGs). Oxytetracycline (OTC) and low concentrations of other antibiotics in the ecosystem can subtly affect microbial environment and perhaps stimulate the HGT events. This study highlights the impact of low concentrations of OTC on HGT within the zebrafish gut microbiome. Zebrafish were exposed to low concentrations of OTC, and metagenomics analysis was conducted, including assembly using MEGAHIT, HGT identification by WAAFLE, and functional annotations with eggNOG-mapper. Significant increase in HGT frequency and shifts in microbial community structure were observed in OTC group. Key findings include the identification of the *catB2* gene, associated with chloramphenicol resistance, with 73.4% similarity. Comparative analyses revealed distinct HGT patterns, including the transfer of ARGs, virulence factors, and metabolic pathways. Additionally, analyses explored significant increase in the opportunistic pathogens, such as that belong to the Proteobacteria phylum. These pathogens penetrate and cause dysbiosis in the host's gut microbiome via HGT, rendered it easier to acquire virulence factors. These findings highlight the thoroughly impact of low concentrations of antibiotics on HGT and microbial ecology within the zebrafish gut microbiome, emphasizing the necessity to encompassed the environmental antibiotic pollution to mitigate the propagation of antibiotic resistance.

Keywords: zebrafish gut microbiome, OTC, horizontal gene transfer, ARGs, WAAFLE.

CHAPTER 1: INTRODUCTION

1.1 Human Microbiome

Human microbiota, which is all the microorganisms present in and on the human body, is associated with the state of health and disease of the host organism. According to an estimate, the evaluated microorganisms are known to be many more than the total unique human cells within the body [1]. Some microorganisms, until recently, were regarded solely as pathogens, have been considered as ingested symbionts within the host [2].

1.1.2 Gut microbiome

The microbial community residing in the gut, collectively referred to as the human gut microbiome, has established a mutualistic association with the host and has been studied in the context of human health over the past few decades [3].

1.1.3 Major functions of gut microbiome

Whereases there are several functions that are performed by gut microbiome has been known like assistance in digestion process by the production of several enzymes, carbohydrates cleavage is done by carbohydrate-active enzymes (CAZymes) [4], phosphate acetyltransferase for the degradation of dietary fibers, and Malate L of lactase dehydrogenase that play a key role in butanoate metabolism [5]. The gut microbiome is well known for its ability to generate short chain fatty acids (SCFAs) as it's produced by *Bacteroides, Bifidobacterium* and *Ruminococcus*. These SCFAs are important for

metabolism, communication and signaling of the growth of some bacterial species [6]. Additionally, energy harvesting and synthesis of beneficial B vitamins are linked to the microorganisms as well. These vitamins are useful to control metabolic regulation through bile acid endocrine system [7].

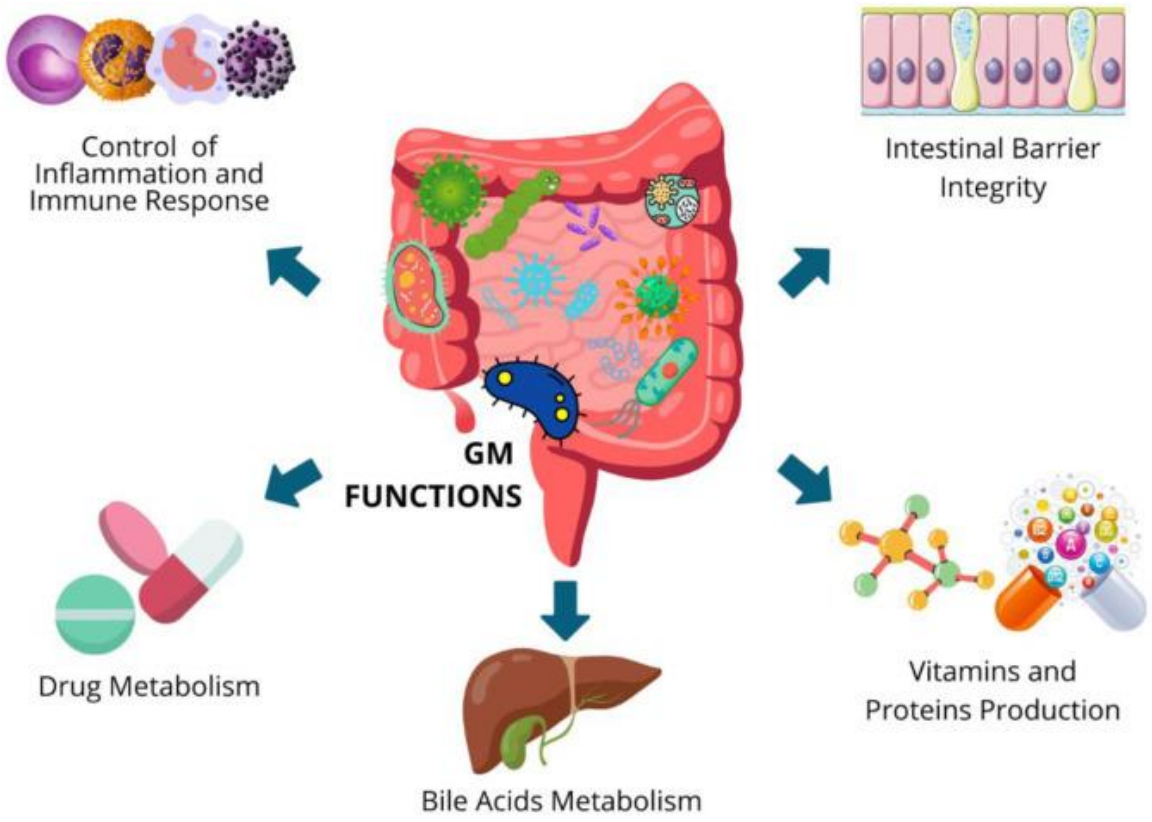

Figure 1.1 The functions of the Gut microbiota [8]

1.1.4 Interaction with the immune system

The ability to influence immune system by the gut microbiota and the ability of the host's immune system to shape the microbiota is mutual exchange and it controls the development and regulatory mechanism of the immune response. The gut microbiome is

also playing an active role in the defense of the host from pathogenic invasion [9]. The exact molecular mechanisms are still ambiguous; however, the recognition and clearance of commensal microorganisms by the innate immune system is considered pivotal for immune system development [10]. Microbiome signals, which include SCFAs, affect myeloid-cell differentiation, innate lymphoid cell maturation, and the mucosal epigenome, influencing disease exposure and treatment responses [11].

1.1.5 Gut microbiome dysbiosis and diseases

Mutations of gut microbiome their shift, or dysbiosis (change in the concentration of gut microbiome) are also linked with different diseases. In some-but not all-cases, the microbiome is intuitive, particularly through inflammation include colorectal cancer [12] Crohn's [13], and autism [14].

Certain bacteria that are typically found in the commensal microbiome can change into opportunistic pathogens in specific situations. For instances *Helicobacter pylori*, which is normally a commensal in the stomach for the majority of the host's life, can turn into a significant risk factor for gastric adenocarcinoma in specific situations [15]. The gut microbiome has also been linked to obesity and metabolic syndromes. Excessive adiposity may alter the microbial community, creating a feedback loop that reinforces obesity [[16], *Lachnospiraceae* were found to play a part in the development of hyperglycemia [17]. Inflammation plays a key role as it does with other diseases given that obesity is associated with a process of chronic low levels inflammation [18]. A comprehension metagenome-wide association study is the one that found specific characteristics of microbiome that may be related to type-2 diabetes, and including reduced butyrate production, increased

abundance of pathogenic bacteria, and enrichment in sulfur reduction and oxidative pressure resistance [19]. Also, chronic inflammatory diseases like Inflammatory bowel disease (IBD) have long been associated with the gut microbiota. Research shows differences between people with IBD and healthy people. For example, *F.prausnitzii* is less common in Crohn's disease and IBD, while severe ulcerative colitis (UC) has more Enterobacteriaceae and β-lactamase-producing bacteria [20].

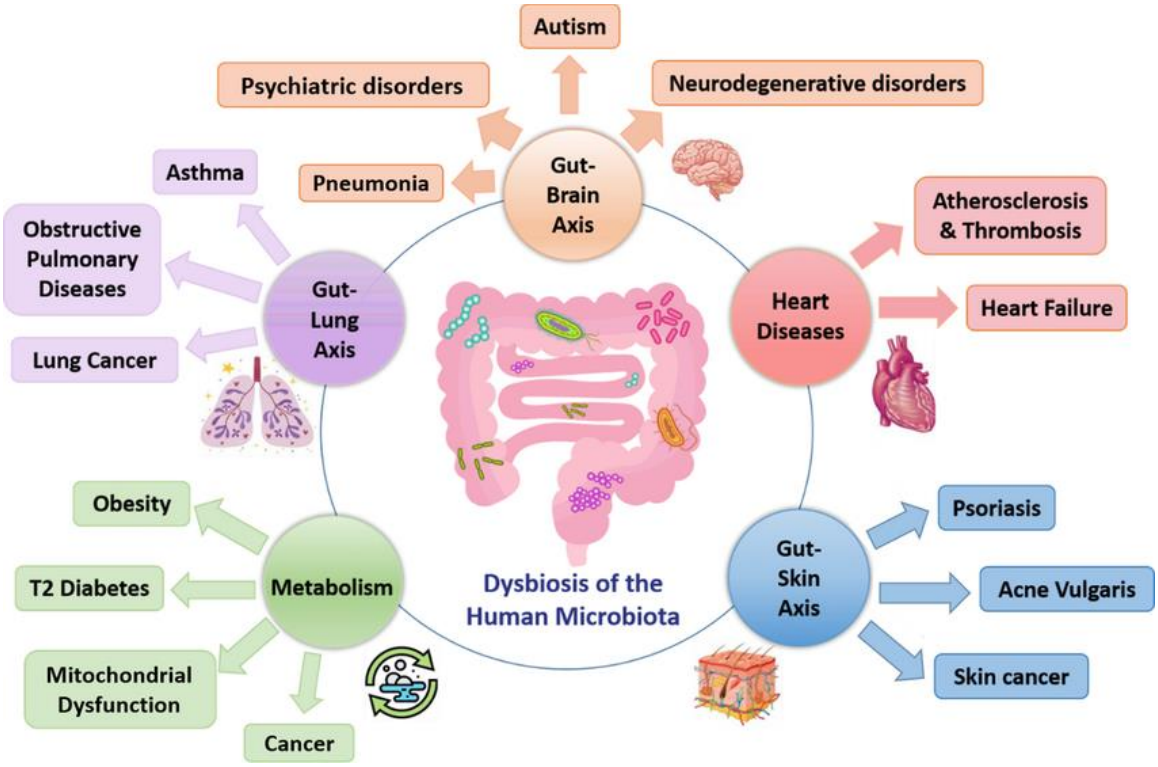

Figure 1.2 The human microbial dysbiosis in human diseases [21]

1.1.6 Zebrafish Gut Microbiome: A Model for Understanding Human Gut Microbiome

Complex organization of bacterial communities and their functions make gut microbiota an essential component of host health, regulating metabolic and immune functions, as well as susceptibility to diseases. In the past few year zebrafish (*Danio rerio*) has emerged as the model of choice for dissecting gut microbiome because it is easily genetically manipulated, has a transparent larva and it has high degree of similarity in gut architecture and function between zebrafish and humans. By comparing the microbial and functional composition of the zebrafish gut microbiome with that of the human gut microbiome, the studies demonstrate that zebrafish is an advantageous model organism to study host-microbe interactions, microbial colonization and perturbation, as well as microbial adaptations to various environmental conditions in the gut.

Just like any other vertebrate the zebrafish gut microbiome comprises of bacteria, archaea, fungi as well as viruses that interact in a complex way with the host organism. Current literature suggests that gut microbiota in zebrafish maintain immune, metabolism, and epithelial barrier functions [22]. Zebrafish larvae are especially useful in the study of gut microbiota because of their ability to rear them in axenic conditions, thus making it easy to manipulate the organism’s microbiota and investigate host-microbiota interactions [23].

1.2 Zebrafish as Model Organism to Study Human Gut Microbiome Dynamics in Health and Disease

Zebrafish's relevance to human microbiome is because both share core microbiota and metabolic pathways. A comparison between the zebrafish and human gut microbial features shows that there is a difference in specific taxa, but they have similar functional

redundancies, which means both have similar biochemical processes carried out by different microbial species [24]. Thus, Zebrafish are a suitable model for studying microbial interactions and an efficient model for exploring the environmental stressors for gut microbiota including diet, inflammation, oxidative stress and antibiotic concentrations. This model enables scientists to study aspects of horizontal gene transfer (HGT) of antibiotic resistance genes (ARGs)) within the gut microbiota. Furthermore, observations made in zebrafish can be considered as translated to humans, providing an understanding of the adaptation of microbial communities and the emergence of antibiotic resistance [25].

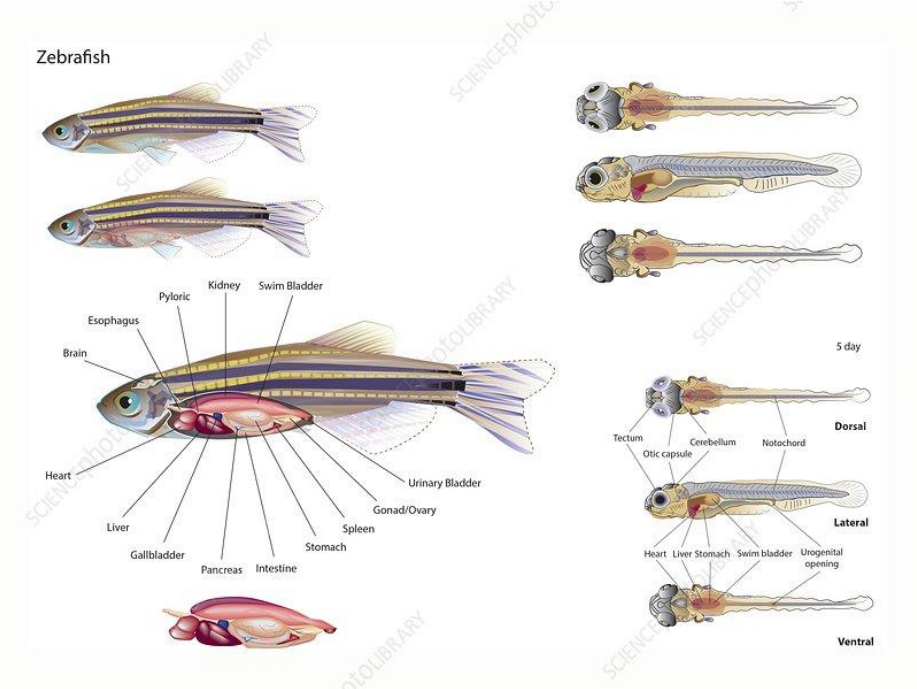

Figure 1.3 Illustration showing anatomy of a zebrafish (Danio rerio) [26]

1.2.1 Antibiotic use and rise of antibiotic resistance

The abuse of antibiotics in treating most diseases has been a great cause of developing antibiotic resistance, a leading public health concern. These antibiotics, even the low concentrations can impose pressure on microbial communities that promote the exchange of resistance genes among bacteria [27],[28], antibiotic resistance represents the modern major problem affecting populations worldwide with the appearance of bacterial strains that no longer succumb to the action of an antibiotic. The emergence of antibiotic resistance mainly occurs because of the increased use of antibiotics, which tend to choose for resistant bacterial sub-populations. This is further compounded by the irrational use and abuse of antibiotics in medical sectors, fishing and in agriculture which disperse resistance genes incomparably. As described by World Health Organization (WHO), Antimicrobial resistance (AMR) is a global health risk since it limits the potent treatment of infectious diseases; a risk to food security since it threatens the use of antibiotics in food-producing animals; and a risk to economic development since it hinders growth of sectors reliant on effective medicines and animal protein production [29].

Even worse, hospital-associated methicillin-resistance *Staphylococcus aureus* (AMR) infections are projected to lead to 10 million deaths each year by the year 2050 if no corrective measures will be taken [30]. The gut microbiome is essential to the spread of antibiotic resistance. ARGs, often referred to as resistome, are conserved in the gut microbiome, which additionally promotes the HGT of these genes between bacterial species. The dissemination of ARGs to hazardous microbes is made possible by mobile genetic elements (MGE) such as integrons, transposons, and plasmids. Evidence has demonstrated that antibiotic-induced alterations in the gut microbiota might encourage the exchange of ARGs [31],[32]. Therefore, for countermeasures, it is crucial to identify the

mechanisms that have led to antibiotic resistance. One of them is low concentrations of antibiotics that have a major impact on the bacteria by inducing stress response, biofilms formation, and HGT events [33].

1.2.2 Silent Threat: Low Concentrations Antibiotics in the Environment

Low antibiotic exposure can be defined as the use of antibiotics at concentrations that are below their minimum inhibitory concentrations (MIC) within the environment or host system. These concentrations are adequate for imposing selection pressure on microbial communities, but they fall below the MIC to stop bacterial growth. Low concentrations of antibiotics are prevalent in natural ecosystems due to environmental contaminants such as wastewater effluents, agriculture runoff, and inappropriate antibiotic disposal, which leading to hotspots for the emergence of antibiotic resistance [34]. Even in the absence of overt bacterial death, low concentration of antibiotics can influence the stress responses, bacterial gene expressions and HGT which may contribute the dissemination of ARGs [35].

1.2.3 Low Concentration Antibiotic Exposure to HGT

Low concentration antibiotic exposure has a substantial effect on the dynamics of HGT in addition to changing the gut microbiome's makeup. It has been demonstrated that sub-inhibitory antibiotic concentrations cause stress reactions in bacteria, which promotes the propagation of genetic material including genes that bestow resistance in microbial communities [36]. This event emphasizes how important HGT is to the efficient propagation of resistance in microbial communities, especially in settings where antibiotics are consistently accessible at sublethal levels.

1.3 Mechanisms of HGT

There are three primary mechanisms of HGT:

1.3.1 Conjugation

Direct transfer of genetic from one bacterial cell to another by cell-to-cell contact, often facilitated by plasmids, is known as conjugation. ARGs can be carried out by these plasmids, which are extrachromosomal DNA pieces that can be passed from one bacterial species to another or even between genera. Since conjugation promotes the rapid development of multidrug resistance, it is particularly challenging in clinical and environmental settings [37].

1.3.2 Transformation

It is the process by competent bacterial cells absorb free DNA from the environment. Environmental variables like low concentrations antibiotics can cause this process or it may unfold haphazardly. Bacteria can directly acquire new chromosomal features, such as resistance genes from lysed cells in their environment through transformation [38].

1.3.3 Transduction

Bacteriophages, which are viruses that invade bacteria, employ transduction to transmit genetic material from one bacterial cell to another. Primarily, in environments with high phage activity, this method aids in the HGT of virulence factors and ARGs [39].

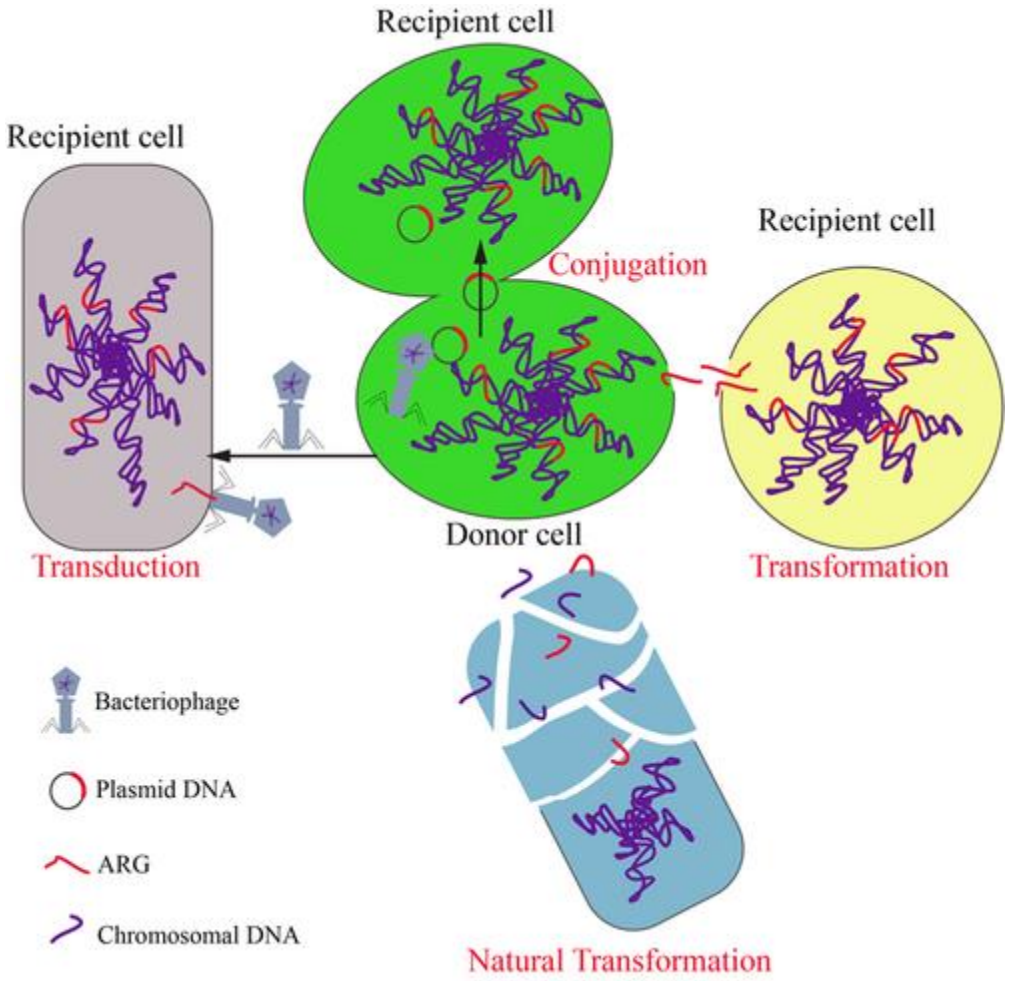

Figure 1.4 Horizontal transfer of antibiotic resistance genes [40]

1.4 Role of HGT in dissemination of ARGs

HGT contribution to the development of antibiotic resistance bacteria can swiftly adjust to the selective pressure imposed by antibiotic use via HGT, which is essential in the spread of ARGs. Microbial communities can develop reservoirs of resistance due to the transferability of ARGs through MGE [41].

For development of strategies to limit the HGT and hence the spread of antibiotic resistance is crucial to understand the specific mechanisms and the environments in which this process occurs. Therefore, the Zebrafish model considering these advantages is suitable for analyzing the HGT process in this organism under specific conditions, such as low antibiotic concentrations.

1.5 Research gap and problem statement

The present literature also has a deficit in respect of further questioning in which direction HGT will steer the metabolic pathways and functions and what is the effect of low concentration on HGT process. This is a very vital area to look at the spread of resistance not only in the form of acquiring genes, but also the consequences of some changes in microbial functions, which enhance the development of resistance. Thus, the study by Kayani et al., and Raita et al., provided important background information about the changes induced in the microbial communities under the exposure of antibiotic.

However, no study has investigated how these low-level antibiotics impact the HGT of antibiotic resistance genes within the zebrafish gut microbiome. HGT allows bacteria to bypass the slow process of evolving resistance through mutations. Instead, they can directly acquire ready-made antibiotic-resistance genes from other bacteria – making the challenge of antibiotic resistance even greater. This research has the potential to unlock crucial insights into the emergence and spread of antibiotic resistance.

1.6 Objectives

1. To explore the influence of low concentrations of antibiotics on HGT of antibiotic resistance genes.

2. To identify and characterize major taxa, genes, and metabolic pathways that are involved in HGT.

CHAPTER 2: LITERATURE REVIEW

2.1 Impact of Antibiotics in Modulating the HGT in Gut Microbiome

Antibiotics have been substantially and continuously used to help regulate infections and enable essential medical treatments in both human and veterinary healthcare. However, the phenomenon of antibiotic resistance mechanisms in bacteria is posing an increasingly serious risk to the efficacy of antibiotics. An international health concern is the rise of antibiotic resistance bacteria, which cause more persistent infections, greater mortality rates, and more expensive medical costs [42]. Even at low concentrations antibiotics can negatively impact microbial ecosystems and promote the dissemination of ARGs in the environment which leads to the current scenario. In this context, it is crucial to know how low concentrations of antibiotic exposure impact the HGT in microbial populations, especially in the gut microbiome.

2.1.1 Environmental Antibiotics Exposure and Gut Dysbiosis

Low concentration of antibiotics is continuously released into the environment through agriculture drainage, wastewater effluent, and other activities for years are potential threats to microbial sub-populations, probity, and the human microbiome. Acute and chronic effects of sub-lethal antibiotic concentrations are the disruption of microbial community structure and functions accompanied by dysbiosis, the condition when healthy bacteria are outcompeted by pathogens [38]. It is argued that taking antibiotics leads to dysbiosis, which alters the metabolism and immune activity of the microbiome and hence provides ground for antibiotic resistance microbes. Frost et al., provided early insights on

how ARGs can spread in microbial communities. The study showed that even low concentrations can cause dysbiosis and change the interactions to promote the emergence of resistance strains [43]. A further study supported this conclusion that the intricate relationship between gut microbes may intensify the impact of low concentrations of antibiotics exposure, fostering the propagation of resistance genes [44].

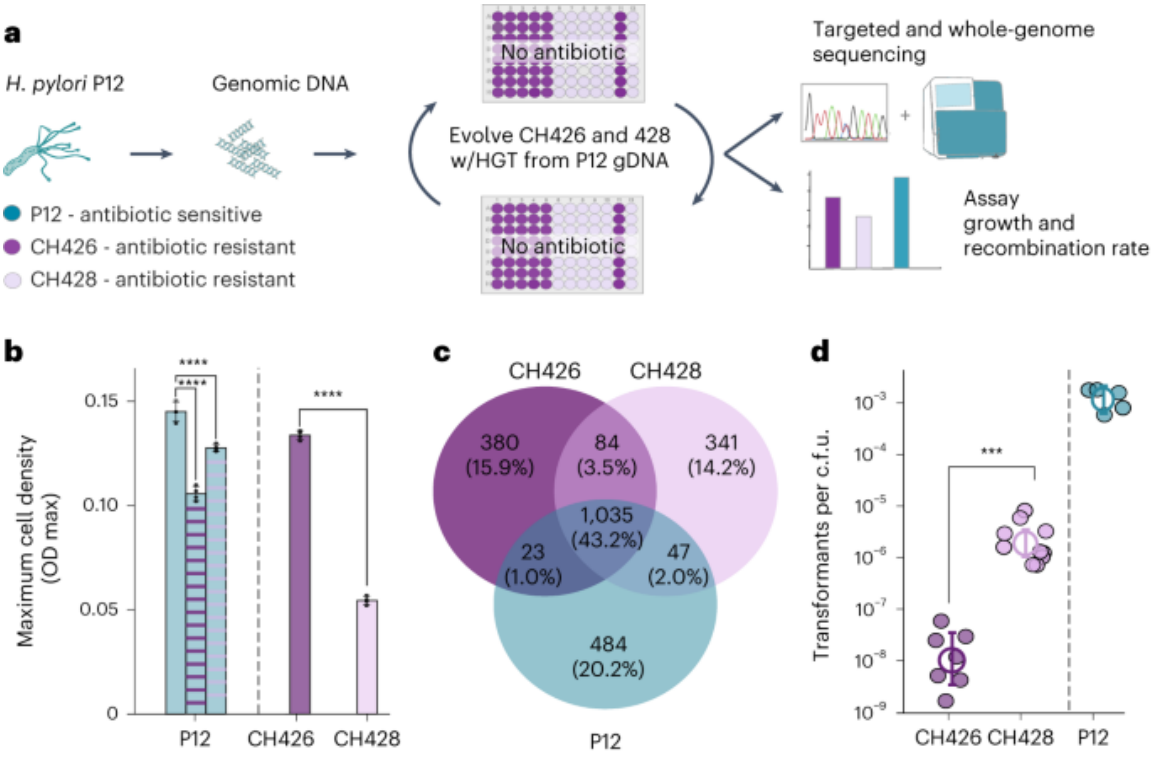

Figure 2.1 Horizontal gene transfer facilitates the molecular reverse-evolution of antibiotic sensitivity in experimental populations of H. pylori [45]

2.2 Zebrafish Gut Microbiome as a Model in Antibiotic Resistance

Zebrafish has recently been used in the investigation of the gut microbiome and the impact of antibiotics given that it is genetically like humans, breeds frequently and is transparent during early development [35]. For instance, zebrafish are used to dissect how

a given antibiotic regimen modulates the structure of the gut microbiome to support the development of antibiotic-resistant bacteria. As discussed by Liu et al., zebrafish treated with a low concentration of antibiotics for weeks experienced perturbation of gut microbiota where antibiotics-resistance strains were favoured. However, the mechanisms of this process or how such low concentrations of antibiotics can enhance the transfer of ARGs between species within the gut of zebrafish are not well understood [35].

2.1.2 Antibiotic Resistance and HGT

Bacteria have been a significant menace to the lives of many people; however, antibiotics have helped to eliminate bacterial infections around the world. However, their continued use has led to a critical public health issue: antibiotic resistance. This is a situation where bacteria developed the ability to counteract effects of antibiotics such that bacterial infections, which used to be relatively easy to cure, are becoming even harder to eliminate [46]. The emergence of multidrug resistance *Acinetobacter buamanii* and *Mycobacterium tuberculosis* makes it even more important to address such a problem. These pathogens resist treatment leading to longer infections, higher prices to health facilities, and higher mortality rates [47]. Another process through which antibiotic resistance arises HGT by which genes are traded between bacteria, thus spreading stably fixed resistance genes among microbes[48]. Despite a large quantity of work devoted to the study of the part played by HGT in the dissemination of resistance, still the authors do not fully understand how, for instance, low concentrations of antibiotics, affect HGT.

In essence, bacteria in environment such as through discharge from agriculture and untreated sewage have been subjected to low concentrations of antibiotics which enhance

resistance through looming sub-lethal effects. In their study, Kayani et al., explained that even low concentrations of antibiotics in water bodies distort the gut microbial composition of zebrafish and increase ARGs of oxytetracycline (OTC) and sulfonamides. However, this study mostly concerned the microbial structure and did not investigate the detailed process of HGT in the microbiome [49].

2.3 Mechanisms of HGT Under Antibiotic Exposure

This concern however was not well addressed in Kayani et al., study, although, the study offered important information about antibiotic-induced dysbiosis in the gut microbiome. This deficit was supplemented by Raita et al., who also extemporized by studying the effect of antibiotics and their relationship to HGT in aquatic organisms inclusive of zebrafish. Their work illustrated how antibiotic use promoted the spread of ARGs across microbial species; however, their research remained mainly focused on how low concentrations affect the ARGs and not the taxa, functional effects of the transference alteration of bacterial metabolic functions, for example. However, their study theory of HGT did not explore the effect under low concentrations and their impact on the host immune system and the microbial community during such transfers [43].

CHAPTER 3: MATERIALS AND METHODS

3.1 Data Description and Acquisition

The raw data was obtained from NCBI sequence Read Archive (SRA) database using accession number PRJNA781418. The original study involved a total of 64 gut metagenome samples collected from zebrafish exposed to OTC or control across different timepoints (days) starting from baseline (0) to 30 days [50]. We were interested in understanding how the gut microbiome of zebrafish evolves ARGs through HGT, thus, this dataset allowed us to answer our research. Further details, including the metagenome sequencing along with the relevant metadata are provided in Table 3.1.

Table 3.1: Major characteristics of the participants involved in this study

Timepoints (Days)	**Control (*n*)**	**OTC *(n)***
Baseline	3	3
3	3	3
6	3	3
9	3	3
12	3	3
18	3	3
24	3	3
30	3	3

This SRA-Toolkit is a collection of libraries and tools created by NCBI for communication with the SRA database [51] utilizing the *prefetch* command, the dataset mentioned above was retrieved from NCBI SRA. Then, the *fastq-dump* command was used to convert the metagenome samples from SRA to FASTQ format. For storage convenience, *gzip* command was used to compress these FASTQ paired-end files.

3.2 Data Preprocessing

Raw metagenome data can typically have compromised sequence quality and must be preprocessed before downstream analysis. The steps taken during preprocessing are described in the subsequent sections.

3.2.1 Quality Control

The raw sequencing data's quality was initially examined using FastQC [52], a widely used tool that provides a modular set of analysis methods to assess the quality of raw reads. We generated quality reports for paired-end files using the "*fastqc*" tool. The report indicated that the data required preprocessing. *Fastp* was used to remove the variety of artifacts such as adapters, duplicated reads, and reads with small lengths, using the following parameters: "-D" for removing duplicated reads, for adapters removal *"--detect_adapter_for_pe", and "--length_required 100" for setting the length required* [53]. These modifications were made to improve the quality of reads and facilitate further analysis.

3.3 *De novo* Metagenome Assembly

After the preprocessing, the metagenome assembly of the clean reads was performed through MEGAHIT (v.2.4.3) [54] by using the KBase online server. The MEGAHIT assembly was conducted with the following *k-list 31, 59, 87, 115, 127* and a minimum contig length of *500*.

3.4 Identification of HGTs using WAAFLE

The following gene extraction count method from CoverM (8.6.1) [55] used to determine the number of reads mapping to each gene sequence. The resulting count tables for each sample were processed to create a matrix using a custom Python script. This script worked in two steps: (1) it summed up the counts of genes and (2) arranged the multi-sample counts such that each row corresponded to a unique query and columns represented counts in independent samples.

3.4.1 Extraction of Genes Involved in HGT Events

Next, sequences of genes involved in HGT events were identified and extracted from the genes predicted from the metagenome assemblies. For this, the coordinates of genes were extracted from the GFF files produced by WAAFLE [56]. Then, the FASTA of the genes was obtained from the gene catalogue using the coordinates through a custom Python script.

3.4.2 Estimation of Counts of Extracted Genes

Following the gene extraction, the count method from CoverM (8.6.1) was used to determine the number of reads mapping to each gene sequence. The resulting count tables for each sample were processed to create a matrix using a custom Python script. This script

worked in two steps: (1) it summed up the counts of genes and (2) arranged the multi-sample counts such that each row corresponded to a unique query and columns represented counts in independent samples.

3.4.3 Estimation of Rate and Frequency of HGT Events

In this step, the rates and frequencies of HGT events were calculated, within samples as well as between clade pairs. These rates of HGT events identified by WAAFLE were normalized by the assembly size. For this, the number of HGT events was obtained for each sample from the outputs and was divided by the corresponding sample's metagenome assembly size in bp and finally normalized to rates per million. The rationale behind this step was to account for the inherent variability in assembly sizes, as larger assemblies naturally have a higher likelihood of capturing more events. Thus, this normalization enabled us to remove any potential bias that could have resulted due to a larger assembly size obtained due to deeper sequencing of certain samples. This was estimated by calculating HGT events frequency between a pair of clades: A is acceptor, B is donor; the number of HGT events was observed between A and B in all samples. Then the figure was normalized to the total number of genes assembled for both acceptor and donor across all samples and normalized to 1000 genes per assembly. Likewise, the frequency of directed HGT events from a donor to an acceptor was calculated by measuring the number of transfers from a donor to an acceptor across samples and then normalized to the total number of acceptor genes assembled across all samples and then scaled to 1000 genes. The normalization of this enabled an accurate average density of transfers from the donor to the acceptor, with particular importance on the recipient clade acceptor in HGT events.

3.4.4 Extraction of ARGs

By utilizing the FASTA sequences of contigs involved in HGTs, ARG was extracted by using the Resistance Gene Identifier (RGI) online software, which is based on Comprehensive Antibiotic Resistance Database (CARD) is used to predict ARGs present in the contigs, involved in HGTs identified by WAAFLE. The analysis conducted with default parameters, ensuring high sensitivity and specificity. The RGI tool categorized ARGs based on their resistance mechanism, antibiotic classes, and associated gene families. The results of RGI included detailed annotations of each ARG, including its percentage identity, model alignment, resistance mechanism, and associated antibiotic class.

3.5 Contigs Abundance Estimation

CoverM (v0.6.1) [55] was applied to estimate the relative abundances of contigs in the metagenomes. For this, the contig identifiers obtained from the WAAFLE outputs were utilized and their corresponding FASTA sequences were extracted from their respective metagenome assemblies. The specific parameters for estimating the abundances included: --min-read-percent-identity 95, and --min-read-aligned-percent 50. The 'Relative Abundance' method from the CoverM was used to calculate the percentage of total metagenomic reads that mapped to each contig. To concatenate multiple CoverM output files into a single matrix format, a customized Python script was prepared. This script performed two major functions: (1) it summed up the abundances of contigs that belonged to the same microbial taxa and (2) arranged the data such that abundances of each sample

were represented in a single column. The first row of listed the name of the taxa that was idenfitied from WAAFLE.

3.6 Gene Annotation

HGT is conventionally linked to various molecular functions and to explore potential functional enrichments among genes involved in HGT events, eggNOG-mapper v.2.11.[57] is used for the identification of functions of genes. The extracted gene sequence files served as input. eggNOG-mapper was executed with default parameters to annotate the extracted gene sequences. This process involved mapping each gene to known orthologous groups and assigning putative functions. The output from eggNOG-mapper provided annotation files for both Control and OTC. These annotation files included information on the predicted functions of genes within each dataset, categorized according to Clusters of Orthologous Groups (COG). For this analysis, we processed the WAFFLE output and obtained the contigs that were assigned to the genes involved in HGT events. These steps collectively contributed to a comprehensive and functionally enriched representation of the gene sequences Contigs Extraction and Gene Prediction

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

In the microbiome study conducted by Falony et al., the comparison of median differences in alpha-diversity measures, the proportion of core genera, and the abundance

of specific genera was accomplished using the Wilcoxon test for categorical variables. These non-parametric tests were employed to assess statistical significance, providing a robust method to evaluate differences within and between groups without relying on strong distributional assumptions. For the comparison of contig length, N50 and largest contig distribution in each group Control and OTC, the Wilcoxon test was applied using the QUAST report. This facilitated an examination of potential differences in contig sizes between groups. In the analysis of HGT events, the Wilcoxon test was utilized in multiple scenarios. The HGT rate and transferred gene frequency were compared between the Control and OTC groups. Differences in HGTs between time points in each individual group were tested, as well as differences in HGTs between group at an individual time point. This enabled a powerful statistical comparison of HGT dynamics with consideration for different experimental conditions and factors related to time. A p-value of < 0.05 was considered to indicate statistically significant differences.

3.7.2 Comparative Analysis of α-Diversity in Control and OTC

α-diversity describes the variety and distribution of species, it comprises evenness, which shows the relative abundance of each of those species in a sample, and richness, which is the number of distinct species [58]. Because the Shannon method is well-established and frequently used, it was employed to compute the alpha diversity of the two examples, Control and OTC. The calculation of the Shannon index was done at each time point from baseline to 30 days for both groups, and the respective results were plotted to compare the trends in the alpha diversity between groups across time. Then, Wilcoxon tests was performed on the groups at each time point to produce p-values of the differences in α-diversity between the groups to analyze the observed differences statistically.

3.7.3 Full Enrichment Using Eggnog-Mapper

To identify meaningful changes in gene function between datasets, log2 fold change calculations were applied to COG categories from the annotation files. This statistical approach quantified differences in gene expression patterns over various timepoints. Bubble plots were then generated using R to visually display these differential expression patterns across the COG categories.

CHAPTER 4: RESULTS AND DISCUSSION

4.1 Data Preprocessing

The mean counts for clean reads are reduced for the Control and OTC were 16.31±6.2 M and 18.46±10.1 M, respectively. It indicated improved data quality after preprocessing. Furthermore, no significant difference was observed in the read counts in both cases.

4.2 Metagenome Assembly

Metagenomes of both groups were assembled using MEGAHIT *de novo* metagenome assembler and their quality was assessed through QUAST. The main parameters evaluated for determining assembly quality include total number of contigs, total assembly length, largest contig, and N50 length. These are discussing in detail below:

4.2.1 Total Number of Contigs in Metagenome Assemblies

The mean and std for the Control are 16.31±6.2 million base pairs (Mbp) respectively. For the OTC, the mean and std are 18.46±10 Mbp respectively. This indicates that the number of contigs in both groups was relatively consistent, with the OTC having a higher mean and std compared to the Control (Figure 4.1). However, no significant differences were observed between the groups, with p-value 0.77.

4.2.2 Total Length of Metagenome Assemblies

The total length of the metagenome assemblies for the Control was 149.93±61.47 Mbp, whereas for the OTC, it was 184.29±62.81 Mbp. This suggests that the total length of the metagenome assembly was comparatively higher for the OTC compared to the Control. However, there is significant differences were observed between the groups, with p-value of 0.016. (Figure 4.1).

4.2.3 Largest Contig Size

The largest contig size of metagenome assemblies for the Control were 516.17±154.05 Kbp, whereas for the OTC, it was 624.81± 453.66 Kbp. However, no significant differences were observed between the groups, with p-value of 0.72. (Figure 4.1).

4.2.4 N50 Length

The N50 length for the Control was 1.20±0.32 Kbp, whereas for the OTC, it was 1.35±0.57 Kbp. This suggests that the N50 is slightly higher for the OTC, compared to the Control (Figure 4.1).

However, no significant differences were observed between the groups, with p-value of 0.75. The results indicate that the metagenome assemblies for the Control and OTC were of similar quality, with no significant differences in the assembly metrics. This suggests that both groups were adequately represented in the assemblies. The assembled metagenomes of zebrafish also enabled the investigation of HGTs involved under the influence of low concentrations of antibiotics.

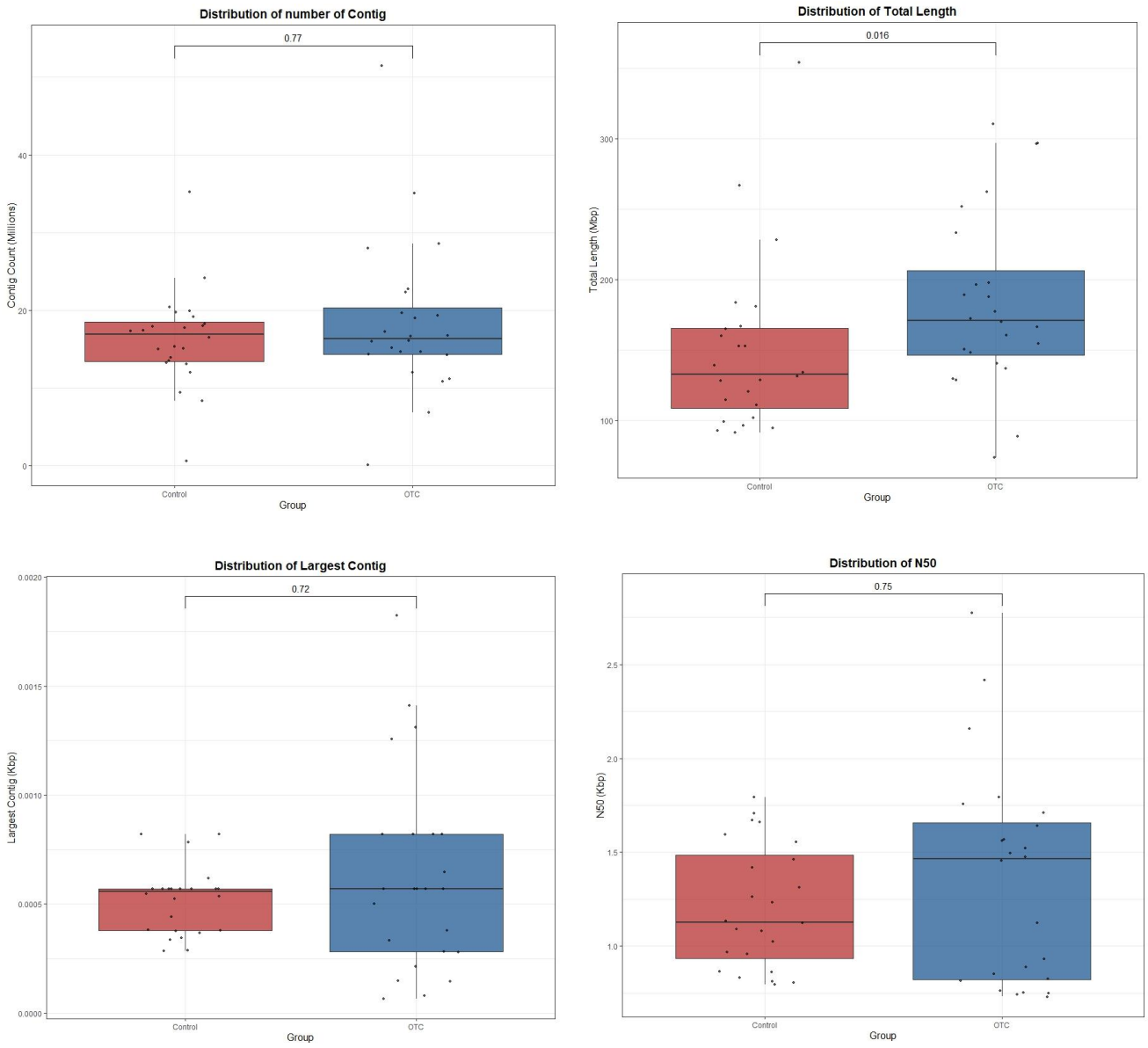

Figure 4.1: Visualization of the quality matrices of metagenome assembly

4.3 Identification and distribution HGTs in Zebrafish gut microbiome

The observed patterns of HGT events in the zebrafish gut microbiome, as depicted in Figure 4.2 shows a notable difference in Control and OTC in the zebrafish. Specifically,

Control group exhibits higher number of HGT genes at Baseline, 3, 6, 12, 18, 24 and 30 days compared to the OTC group zebrafish. However, by 9 days the number of HGT genes in the OTC group is higher than the Control group. However, no significant difference was observed as determined by the Wilcoxon rank-sum test (p-value > 0.05).

Specific families of bacteria may dominate the zebrafish gut microbiome and thus relate to the observed pattern of HGT events. For example, at baseline, OTC tend to be dominated by *Enterobacteriaceae*, while Control group will more likely to be dominated by the *Aeromonas*. By Day 3 OTC tend towards *Enterobacter*, whereas Control tends towards *Flavobacterium*. In Day 6 *Enterobacter* and *Aeromonas* again tends to be highly abundant in OTC but only *Aeromonas* in Control. This pattern is changes for Day 9 and 12, *Aeromonas* is more abundant in both groups OTC and Control. At Day 18 only *Enterobacter* is present in OTC while in Control group *Aeromonas* is again rises. For Day 24 again *Enterobacter* is more abundant in OTC, while Control group have multiple species like *Enterobacter, Curvibacter,* and *Enterobacter*. At day 30 again *Aeromonas* abundance increases for both control and OTC.

In brief, Baseline (0 day) both groups start with relatively high abundance of HGT genes. Day 3-6, in both groups there is a significant drop in HGT genes. From Day 9 to onwards both groups start to diverge. The control group maintain a relatively stable HGT gene-count, but OTC group experience further decrease. Interestingly, in the control group, the HGT gene-count shows a rebound at the 24 Day and continue to increase until the 30-day point. But the Aeromonas is more abundant in OTC group which is a dominant member in HGT due to its virulence and ability to transfer genes. This is due to the interaction of

Aeromonas with the other species like *Pseudomonas* and *Escherichia* via MGEs through HGT [59].

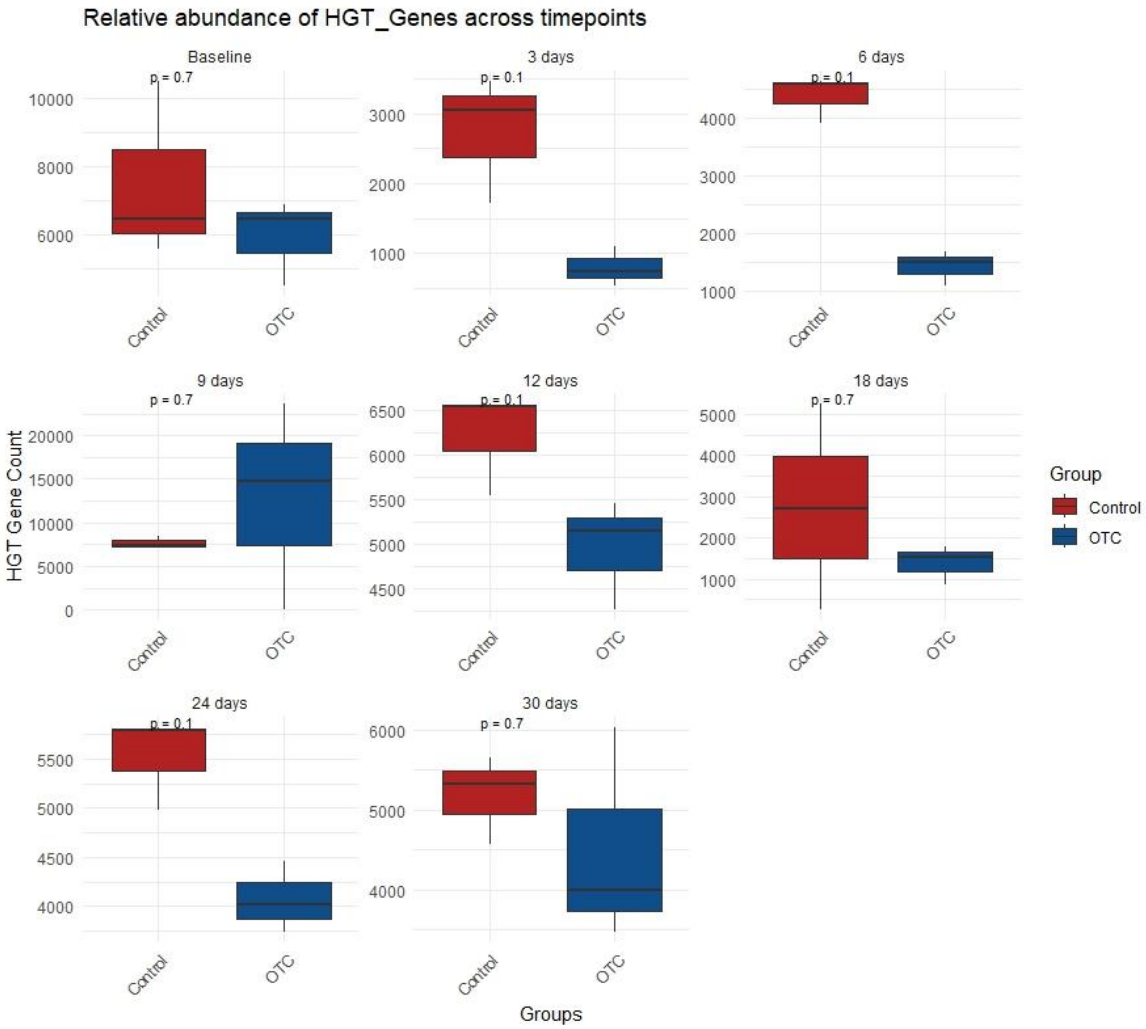

Figure 4.2: HGT Gene-count across different timepoints

4.4 α-Diversity of identified HGTs

The results of α-diversity analysis for donor and acceptor species in both Control and OTC across different timepoints reveal distinct patterns and interactions. At baseline

(0 Day), both groups exhibit similar levels of α-diversity. As time progresses, a clear divergence in α-diversity becomes evident in both Control and OTC. From Day 3 to onwards the OTC has higher α-diversity till Day 30. The OTC groups generally show a higher level of α-diversity compared to the Control group across all timepoints. The p-values associated with each timepoints indicate whether the difference in α-diversity between the groups is statistically significant.

The observed increased in α-diversity in the OTC group, coupled with the higher abundance of HGT genes at earlier timepoints, suggested that low concentrations of antibiotics within the OTC might stimulate HGT. This is consistent with previous studies that antibiotics can induce stress response in bacteria, leading to increased HGT rates as a mechanism for acquiring ARGs [60]. These results indicated that increased diversity in OTC group could be due to the HGT, which can introduce new genetic material into microbial populations, leading to the emergence of novel strains and increasing the overall diversity of the community. These findings are consistent with the hypothesis that HGT is a major force shaping microbial community structure and functions [61].

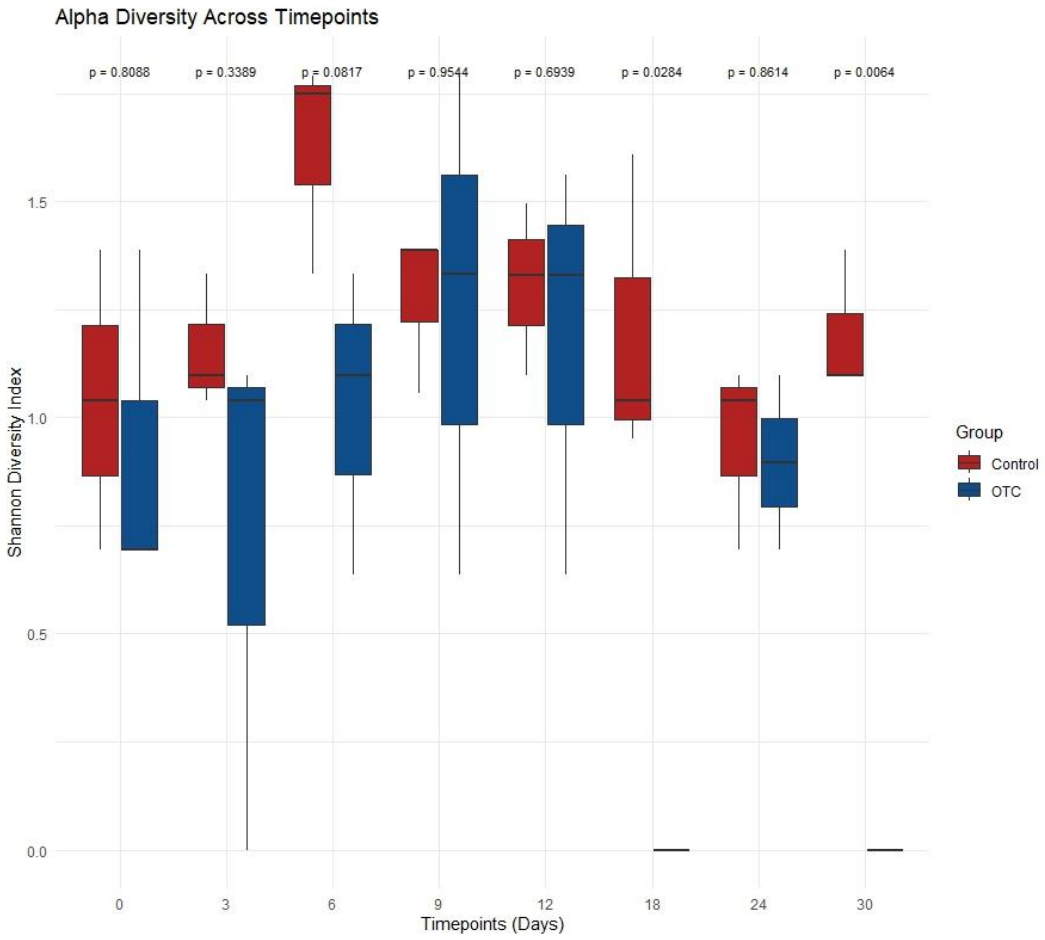

Figure 4.3: α-diversity across different timepoints

4.5 Rate and Frequency of identified HGTs

Each box plot in Figure 4.4 represents the distribution of HGT events per Mbp of assemblages within groups. At baseline, both groups exhibit a very low rate of directed HGT events. Over the time, OTC generally shows a higher rate of directed HGTs compared to the Control group across timepoints. The p-values associated with each timepoint indicate whether the difference in directed HGT rate between the groups is statistically

significant. These results demonstrated a significant impact of the OTC intervention on the rate of directed HGT, leading to a higher rate of HGT events in the OTC group compared to the Control group. These findings highlight the potential importance of HGT in shaping microbial community dynamics and the potential consequences of intervention that influence HGT rates. For non-directed HGT events, both groups exhibit a very low rate at baseline. The OTC group exhibits a notably higher rate at the Day 9. The statistical significance of the variation in non-directed HGT rate across timepoints can be determined by the p-values corresponding to each timepoint. At day 12-18 there is a significant difference is observed. Following the same trend as in the directed HGT rate, in non-directed HGT rate is also higher in OTC group as compared to the Control group across most of the timepoints indicates that the intervention of low concentration of antibiotics may promote conditions that favor the transfer of ARGs in the OTC group.

Frequency of identified HGT events in the zebrafish gut microbiome varies between OTC and Control. Frequency of HGTs was calculated for the two sample groups by dividing the HGT events by the genes. This normalization allows us to count the number of transferred genes in a given set of genes, providing a more accurate representation of HGT frequency. The results indicate that a notably difference between two groups at Day 18, the OTC group has higher frequency. The p-values (p-value < 0.05) at each timepoint indicate the statistical significance of the difference in HGT event frequency between the groups. However, a notably difference in the HGT events frequency between two points from baseline to Day 30.

The Figure 4.5 suggests that the OTC intervention has a significant role on the frequency of HGT events. The consistency higher frequency of HGT events across

timepoints in the OTC indicates that low concentrations of antibiotics may promote the favor of gene transfer. The decrease in the frequency at baseline could be because of the less or no exposure to antibiotics. Increased frequency over time indicate exposure to antibiotics increased the stability in the gut microbiome, which allows HGT events more efficient. The further increase of frequency at Day 18 indicate more exposure to antibiotics concentrations. Specifically, these results highlight the dynamics of HGT events within zebrafish gut microbiome, and significant roles of exposure of low concentrations of antibiotics.

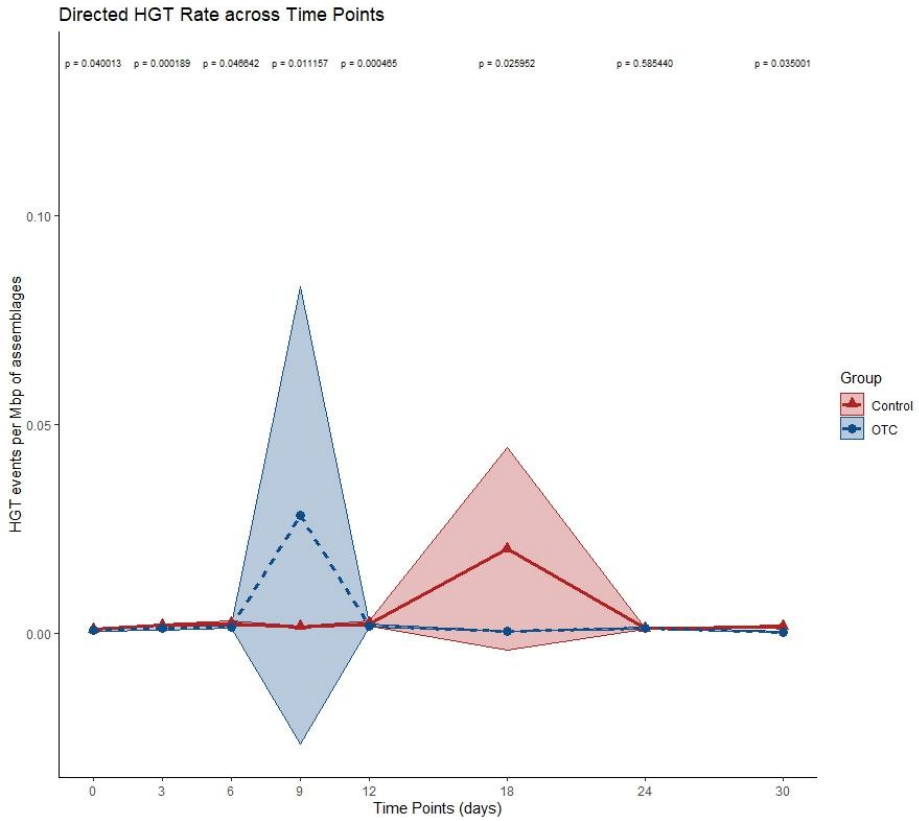

Figure 4.4: Directed HGT-rate across different timepoints

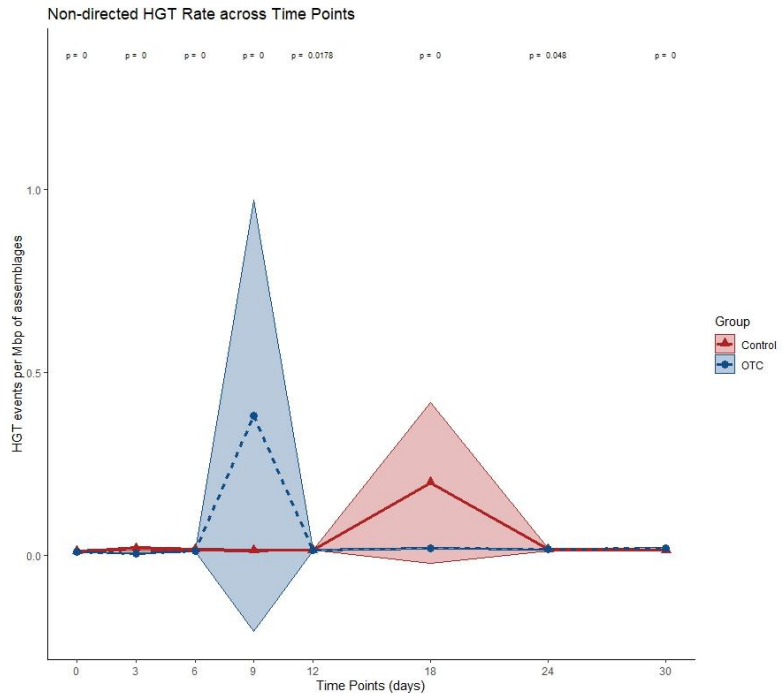

Figure 4.5: Non-directed HGT-rate across different timepoints

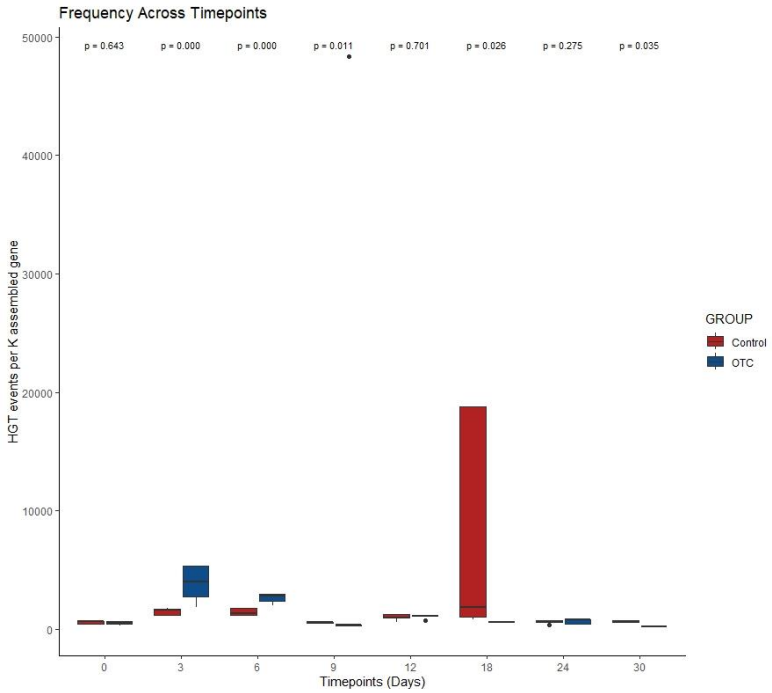

Figure 4.6: Frequency of HGT events across timepoints

4.6 Identifying Unique Driver of HGTs in Control and OTC

The results of α-diversity and frequency reveal significant differences at different timepoints between both OTC and Control groups. This difference is observed in both donor and acceptor species, the upset plots show a greater number of unique donor (Figure 4.7) and acceptor (Figure 4.8) species in OTC. The unique donor species in OTC at baseline *Caldimonas_manganoxidans* and *Vibrio_splendidus*. The unique donor species in Control at baseline are *Verminephrobacter_eiseniae* and *Variovorax_paradoxus*. The unique acceptor specie in OTC at baseline *Burkholderiales* and unique acceptor specie in Control at baseline is *Thauera_phenylacetica*. Interestingly, unique donor species in OTC donor group emerged at Day 3, 6, 9 and 12 subsequent to the baseline. The unique donor species in Control group are emerged at Day 6, 12, 18 and 24 following the baseline period.

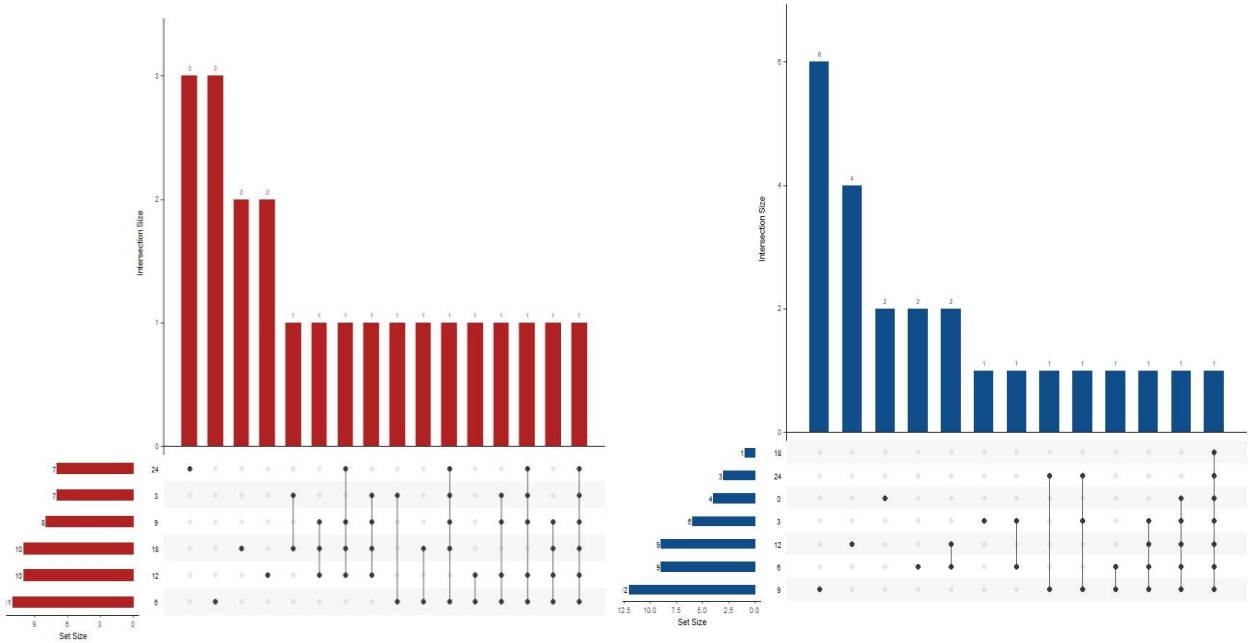

Figure 4.7: Visualization of unique and common donor species across different timepoints in Control and OTC

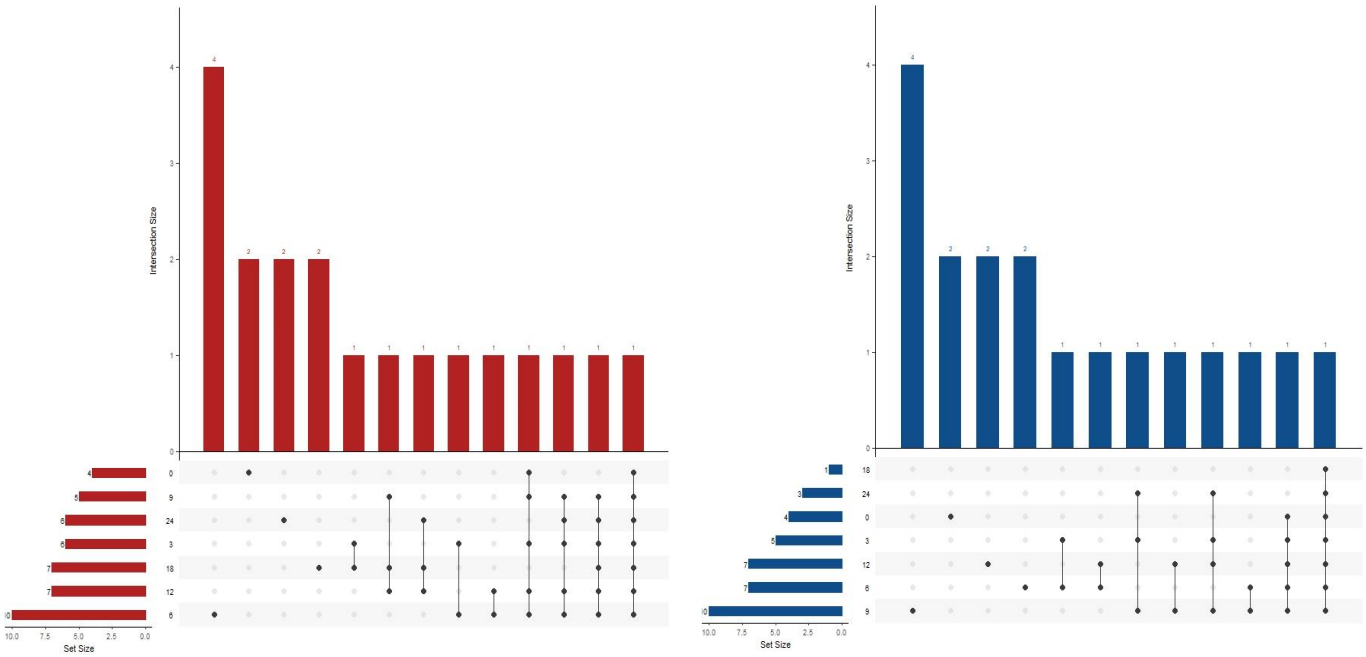

Figure 4.8: Visualization of unique and common acceptor species across different timepoints in Control and OTC

4.7 Comparative Analysis of HGT Events in Control and OTC

The donor-acceptor pair in Control, involved in HGT events (20 events), was *s-Enterobacter-clocae, s-Aeromonas-hydrophilia*, followed by *s-Aeromonas-hydrophilia, s-Vibrio-kanaloae* (15 HGT events) shown in Figure 4.9. In contrast, the donor-acceptor pair in OTC, involved in most HGT events (10 events), was *s-Enterobacter-cloacae, s-Enterobacter-sp-SST3*, followed by *s-Aeromonas-hydrophilia*, *s-Vibrio-splendidus* (8 HGT events) shown in Figure 4.10. Furthermore, the donor-acceptor pairs in Control and OTC belong to different families and genera, reflecting distinct patterns of HGT events. For example, in the Control group, *s-Enterobacter-cloacae and s-Aeromonas-hydrophila*, the pair involved in the most HGT events, belong to different families, *Enterobacteriaceae* and *Aeromonadaceae*, respectively. Similarly, the pair *s-Aeromonas-hydrophila and s-*

vibrio-kanaloae, belong to different families, *Aeromondaceae and Vibrionaceae*, respectively. In contrast, in the OTC group, the donor-acceptor pair involved in most HGT events, *s-Enterobacte-cloacae and s-Enterobacter-sp-SST3*, belong to the same family, *Enterobacteriaceae,* but different genera. Similarly, the pair *s-Aeromonas-hydrophila* and *s-Vibrio-splendidus,* belong to different families, *Aeromonadaceae and Vibrionaceae*, respectively. Bacterial interactions during HGT may be impacted by these variations in the donor-acceptor pairs, and their families, and their genera. The *Enterobacteriaceae* family is well known for contributing in the propagation of ARGs in gut microbiome [62]. The aquatic-dwelling Vibroaceae family, could haver unique ecological adaptations that affect HGTs. These differences illustrate the various manners by which they affect the dynamics of donor and acceptors pairs and the bacterial interactions that accompany along with them [63].

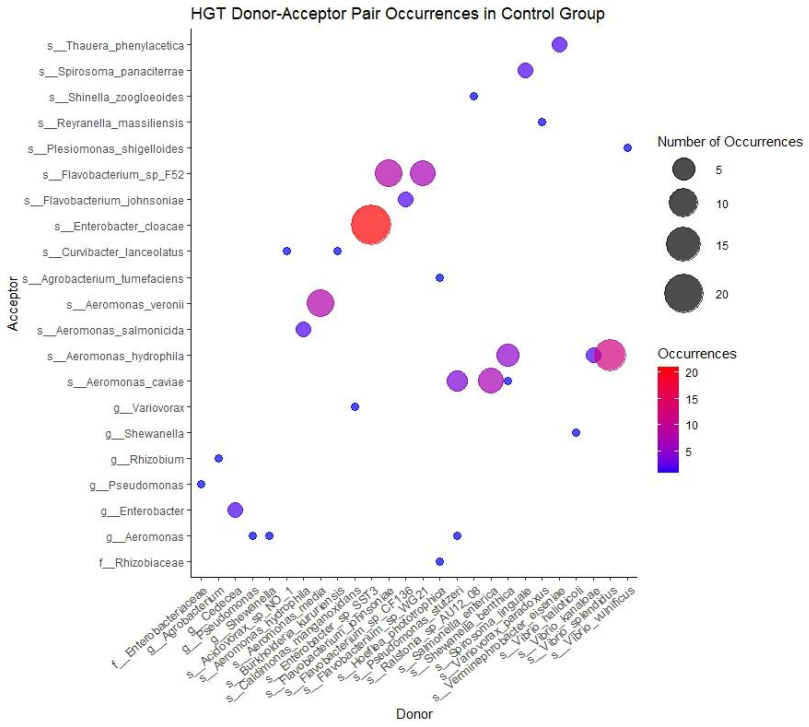

Figure 4.9: Visualization of HGT donor-acceptor pair in Control, representing the number of occurrences

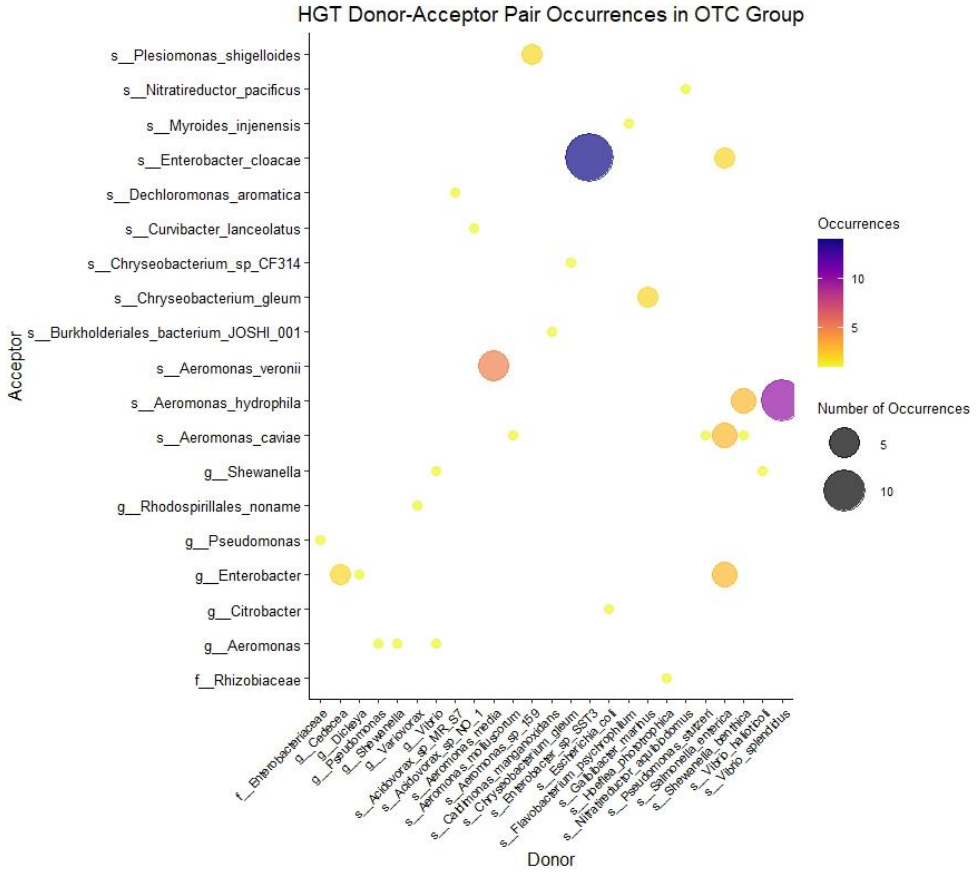

Figure 4.10: Visualization of HGT donor-acceptor pair in OTC, representing the number of occurrences

4.8 Temporal Patterns of HGTs in Zebrafish Gut Microbiomes

Understanding the gene transfer between bacteria requires more than simply counting raw numbers of gene transfers. The reason is that raw counts can sometimes be misleading if certain bacteria are very abundant or very rare in the community. Instead, these counts have been normalized to show relative abundances, which will be helpful in understanding the dynamics of gene transfer.

Figure 4.11: Visualization of temporal HGT patterns from donor to acceptor in OTC

In OTC group, the bacterium donor is mostly represented *Chryseobacterium gleum*. Over time, this bacterium starts transferring genes to other bacteria. For example, at baseline, it transfers gene to *Acidovorax sp.MR_S7* and at day 6, it transfers to *Citrobacter*, and at day 9, it transfers to *Rhodospirillales_noname*. Another important donor *Escherichia coli*. Which donates genes to *Chryseobacterium gleum* at day 9. The variability

in the gene donor and timepoints where exchanges occur underscores the notion that the gut microbial community of zebrafish is still dynamic. This means that each event of transfer, more specifically from *Chryseobacterium gleum,* is changing the gut microbial community over time in ways that can have large consequences for the health of the host. The observed HGT patterns in the OTC group suggest that low-concentrations of antibiotic exposure might have influenced the microbial community structure and dynamics, leading to changes in the patterns of gene transfer compared to the Control group.

4.9 ARG Profiling and Resistance Mechanisms

RGI analysis identified a diverse array of ARGs within the zebrafish gut microbiome across OTC group. Notably, the *catB2* gene, associated with chloramphenicol acetyltransferase protein, act by antibiotic inactivation mechanism, was identified with a percentage identity of 73.4%. Other prominent ARGs included *TxR* conferring tetracycline resistance (44.27% identity), *blaOXA-58,* a beta-lactamase responsible for cephalosporin, carbapenem and oxacillin resistance (30.28% identity), *mdsB* linked with chloramphenicol resistance by antibiotic efflux mechanism (26.05 % identity) and other *CMY-135, Ecol-AcrR-MULT* are linked with cephamycin and triclosan resistance respectively.

4.10 Gene Expression profile of Horizontal Gene Transfer in OTC:

Figure 4.12 illustrate the dynamic shifts in functional enrichment patterns across different timepoints in the OTC group, which was exposed to low concentrations of antibiotics. The size of each dot represents the abundance of genes associated with a particular metabolic pathway at each timepoint. At early timepoints (baseline and 3 days) genes related to “Defense mechanism” exhibit high abundance, suggesting a strong initial response to the

antibiotic stressor. This is consistent with previous studies showing that low concentrations can induce stress responses in bacteria, leading to upregulation of defense mechanisms [64]. As time progresses from 6-12 days, the abundance of genes involved in “Energy production and conservation”, “Carbohydrates transport and metabolism”, and “Amino acid transport and metabolism” increases. This suggests that the bacterial community is adapting to the antibiotic stress by shifting its metabolic activity to support growth and survival. At later timepoints, there is a further increase in the abundance of genes associated with cellular processes such as “Translation, ribosomal structure, and biogenesis”, “Transcription”, and “Replication, recombination, and repair”. This indicates that the bacterial community is actively growing and dividing, suggesting adaptation and recovery from the initial antibiotic stress. The Figure 4.12 reveals a dynamic interplay between different metabolic pathways over time. This suggests that the bacterial community is constantly adapting and responding to the changing environmental conditions, including the presence of low concentrations. The observed shifts in functional enrichment patterns in the OTC group suggest that low concentrations of antibiotic exposure can significantly influence the metabolic activity and functional dynamics of bacterial community. While the initial stress response involves upregulation of defense mechanisms, the community subsequently adapts by shifting towards metabolic processes that support growth and survival. These findings highlight the potential impact of low antibiotic concentrations on microbial community function and adaptation.

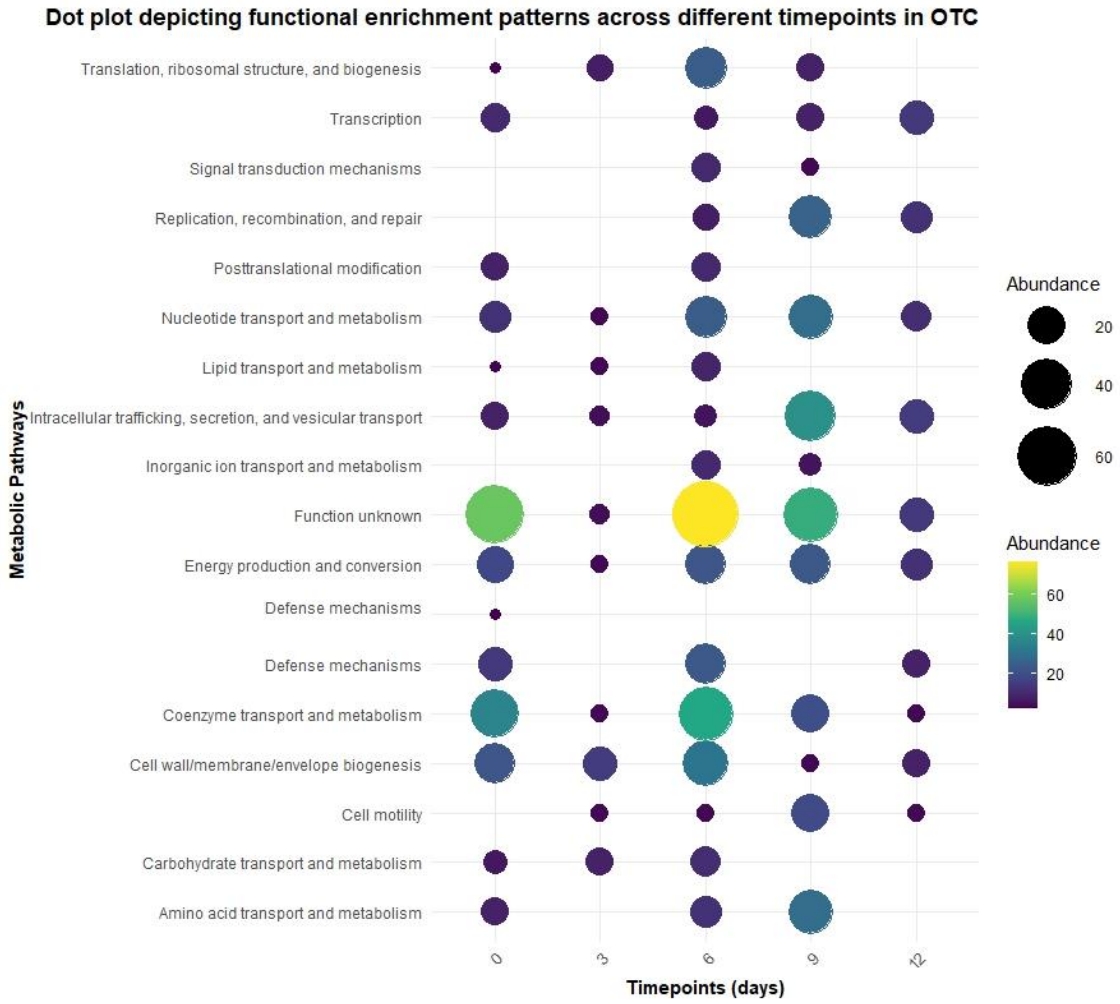

Figure 4.12: Functional enrichment patterns across different timepoints in OTC

CHAPTER 5: CONCLUSIONS

With an emphasis on the kinetics of HGT events, this study evaluated the impact of low concentrations antibiotic exposure on the gut microbiome of zebrafish. The results indicate that composition of the microbial population changes significantly after antibiotic exposure. In particular, opportunistic pathogens, such as that belong to the Proteobacteria phylum, reported increasing predominance, but beneficial bacteria, such as those from the Bacteriodetes and Firmicutes phyla, exhibited a decrease in relative abundance. A significant rise in HGT events among the remaining bacterial populations coincided with this change in microbial diversity. Analysis revealed that genes linked to the metabolic processes, virulence factors, and ARGs (*catB2, TxR, mdsB, Ecol-AcrR-MULT*) were transferred via HGT. The emergence of multi-drug resistance strains maybe facilitated by the transfer of ARGs from Proteobacteria to Firmicutes have encountered. Furthermore, HGT rendered it less difficult for opportunistic pathogens to acquire virulence factors, which boosted their propensity to colonize and disturb the host's gut microbiome. Our research also demonstrated how HGT might encourage metabolic alterations in the gut microbial community. Genes related to nutrition consumption and carbohydrate metabolism were shifted, indicating that HGT could influence the gut microbiome's overall metabolic potential. The host immune defenses, energy homeostasis and nutrition absorption may all be significantly impacted by these metabolic alterations.

These results suggest that additional research is necessary in an assortment of critical areas, to evaluate the long-term effects of low concentration antibiotic exposure on the host's health and gut microbiome, conduct long term studies. Firstly, continued

monitoring the evolution of microbial communities over time and the persistence of genes which lead to antibiotic resistance, and investigates the molecular processes that drive HGT occurrences in context to antibiotic exposure. Secondly, can identify the precise environmental variables that either encourage or prevent the HGT in the gut microbiome. Can continued by developing the probiotic strains can suppress HGT and prevent the propagation of ARGs. To establish antibiotic strategies that reduce gut microbiome disruption and minimize the chances of HGT. By describing unique gut microbiome and forecast the possible effects of antibiotic therapy, can use metagenomics and meta-transcriptomics analyses. Furthermore, encourages the prudent use of antibiotics for the benefit of human and animal health and increase the knowledge of possible hazards of exposure of low concentrations of antibiotics by establishing policies forth to reduce the number of antibiotics and genes that contribute to resistance get released into the ecosystem.

REFERENCES:

[1] A. Mullard, A. M.- Nature, and undefined 2008, 'The inside story: the human body teems with microbes. In the first of two features, Asher Mullard looks at the global efforts to catalogue this vast'microbiome'. In the', *go.gale.comA Mullard, A MandavilliNature, 2008•go.gale.com*, Accessed: Jan. 11, 2025. [Online]. Available: https://go.gale.com/ps/i.do?id=GALE%7CA183367689&sid=googleScholar&v=2.1&it=r&linkaccess=abs&issn=00280836&p=AONE&sw=w

[2] N. Cerf-Bensussan and V. Gaboriau-Routhiau, 'The immune system and the gut microbiota: Friends or foes?', *Nat Rev Immunol*, vol. 10, no. 10, pp. 735–744, 2010, doi: 10.1038/NRI2850.

[3] K. Hou *et al.*, 'Microbiota in health and diseases', *Signal Transduct Target Ther*, vol. 7, no. 1, Dec. 2022, doi: 10.1038/S41392-022-00974-4.

[4] J. F. Wardman, R. K. Bains, P. Rahfeld, and S. G. Withers, 'Carbohydrate-active enzymes (CAZymes) in the gut microbiome', *Nat Rev Microbiol*, vol. 20, no. 9, pp. 542–556, Sep. 2022, doi: 10.1038/S41579-022-00712-1.

[5] M. De Angelis *et al.*, 'Diet influences the functions of the human intestinal microbiome', *nature.comM De Angelis, I Ferrocino, FM Calabrese, F De Filippis, N Cavallo, S Siragusa, S RampelliScientific reports, 2020•nature.com*, doi: 10.1038/s41598-020-61192-y.

[6] P. Portincasa *et al.*, ‘Gut microbiota and short chain fatty acids: implications in glucose homeostasis’, *mdpi.comP Portincasa, L Bonfrate, M Vacca, M De Angelis, I Farella, E Lanza, M Khalil, DQH WangInternational journal of molecular sciences, 2022•mdpi.com*, vol. 23, no. 3, Feb. 2022, doi: 10.3390/ijms23031105.

[7] J. Montenegro *et al.*, ‘Exploring the Influence of Gut Microbiome on Energy Metabolism in Humans’, *Advances in Nutrition*, vol. 14, no. 4, pp. 840–857, Jul. 2023, doi: 10.1016/J.ADVNUT.2023.03.015.

[8] L. Crudele, R. M. Gadaleta, M. Cariello, and A. Moschetta, ‘Gut microbiota in the pathogenesis and therapeutic approaches of diabetes’, *EBioMedicine*, vol. 97, Nov. 2023, doi: 10.1016/J.EBIOM.2023.104821/ASSET/8680809A-0D8E-46B1-A794-147C00B21F72/MAIN.ASSETS/GR3.JPG.

[9] L.-Y. Zhao *et al.*, ‘Role of the gut microbiota in anticancer therapy: from molecular mechanisms to clinical applications’, *nature.comLY Zhao, JX Mei, G Yu, L Lei, WH Zhang, K Liu, XL Chen, D Kołat, K Yang, JK HuSignal Transduction and Targeted Therapy, 2023•nature.com*, vol. 8, no. 1, Dec. 2023, doi: 10.1038/s41392-023-01406-7.

[10] D. Zheng, T. Liwinski, E. E.-C. research, and undefined 2020, ‘Interaction between microbiota and immunity in health and disease’, *nature.comD Zheng, T Liwinski, E ElinavCell research, 2020•nature.com*, doi: 10.1038/s41422-020-0332-7.

[11] S. P. Wiertsema, J. van Bergenhenegouwen, J. Garssen, and L. M. J. Knippels, ‘The interplay between the gut microbiome and the immune system in the context of

infectious diseases throughout life and the role of nutrition in optimizing treatment strategies', *Nutrients*, vol. 13, no. 3, pp. 1–14, Mar. 2021, doi: 10.3390/NU13030886.

[12] R. Gao, Z. Gao, L. Huang, H. Q.-E. J. of C. Microbiology, and undefined 2017, 'Gut microbiota and colorectal cancer', *SpringerR Gao, Z Gao, L Huang, H QinEuropean Journal of Clinical Microbiology & Infectious Diseases, 2017•Springer*, vol. 36, no. 5, pp. 757–769, May 2017, doi: 10.1007/s10096-016-2881-8.

[13] M. A. Núñez-Sánchez *et al.*, 'Crohn's disease, host–microbiota interactions, and immunonutrition: Dietary strategies targeting gut microbiome as novel therapeutic approaches', *mdpi.comMA Núñez-Sánchez, S Melgar, K O'Donoghue, MA Martínez-Sánchez, VE Fernández-RuizInternational Journal of Molecular Sciences, 2022•mdpi.com*, vol. 23, no. 15, Aug. 2022, doi: 10.3390/ijms23158361.

[14] A. Fattorusso, L. Di Genova, G. B. Dell'isola, E. Mencaroni, and S. Esposito, 'Autism spectrum disorders and the gut microbiota', *mdpi.comA Fattorusso, L Di Genova, GB Dell'Isola, E Mencaroni, S EspositoNutrients, 2019•mdpi.com*, vol. 11, no. 3, Mar. 2019, doi: 10.3390/nu11030521.

[15] V. I. Reshetnyak, A. I. Burmistrov, and I. V. Maev, 'Helicobacter pylori: Commensal, symbiont or pathogen?', *World J Gastroenterol*, vol. 27, no. 7, pp. 545–560, Feb. 2021, doi: 10.3748/WJG.V27.I7.545.

[16] C. D. Davis, 'The gut microbiome and its role in obesity', *Nutr Today*, vol. 51, no. 4, pp. 167–174, 2016, doi: 10.1097/NT.0000000000000167.

[17] M. Vacca, G. Celano, F. M. Calabrese, P. Portincasa, M. Gobbetti, and M. De Angelis, 'The controversial role of human gut lachnospiraceae', *mdpi.comM Vacca, G Celano, FM Calabrese, P Portincasa, M Gobbetti, M De AngelisMicroorganisms, 2020•mdpi.com*, vol. 8, no. 4, Apr. 2020, doi: 10.3390/microorganisms8040573.

[18] D. Khanna, S. Khanna, P. Khanna, P. Kahar, and B. M. Patel, 'Obesity: A Chronic Low-Grade Inflammation and Its Markers', *Cureus*, Feb. 2022, doi: 10.7759/CUREUS.22711.

[19] J. Qin *et al.*, 'A metagenome-wide association study of gut microbiota in type 2 diabetes', *nature.comJ Qin, Y Li, Z Cai, S Li, J Zhu, F Zhang, S Liang, W Zhang, Y Guan, D Shen, Y Peng, D Zhangnature, 2012•nature.com*, vol. 490, no. 7418, pp. 55–60, Oct. 2012, doi: 10.1038/nature11450.

[20] B. Khorsand, H. Asadzadeh Aghdaei, E. Nazemalhosseini-Mojarad, B. Nadalian, B. Nadalian, and H. Houri, 'Overrepresentation of Enterobacteriaceae and Escherichia coli is the major gut microbiome signature in Crohn's disease and ulcerative colitis; a comprehensive metagenomic analysis of IBDMDB datasets', *Front Cell Infect Microbiol*, vol. 12, Oct. 2022, doi: 10.3389/FCIMB.2022.1015890/FULL.

[21] P. Gebrayel *et al.*, 'Microbiota medicine: towards clinical revolution', *J Transl Med*, vol. 20, no. 1, Dec. 2022, doi: 10.1186/S12967-022-03296-9.

[22] G. Roeselers *et al.*, 'Evidence for a core gut microbiota in the zebrafish', *academic.oup.comG Roeselers, EK Mittge, WZ Stephens, DM Parichy, CM*

Cavanaugh, K Guillemin, JF RawlsThe ISME journal, 2011•academic.oup.com, vol. 5, no. 10, pp. 1595–1608, Oct. 2011, doi: 10.1038/ismej.2011.38.

[23] J. F. Rawls, B. S. Samuel, and J. I. Gordon, 'Gnotobiotic zebrafish reveal evolutionarily conserved responses to the gut microbiota', *Proc Natl Acad Sci U S A*, vol. 101, no. 13, pp. 4596–4601, Mar. 2004, doi: 10.1073/PNAS.0400706101.

[24] E. Lucassen, C. Coomans, M. van Putten, S. de K.-C. Biology, and undefined 2016, 'Environmental 24-hr cycles are essential for health', *cell.comEA Lucassen, CP Coomans, M van Putten, SR de Kreij, JHLT van Genugten, RPM SutoriusCurrent Biology, 2016•cell.com*, Accessed: Jan. 11, 2025. [Online]. Available: https://www.cell.com/current-biology/fulltext/S0960-9822(16)30497-3?

[25] S. Brugman, 'The zebrafish as a model to study intestinal inflammation', *Dev Comp Immunol*, vol. 64, pp. 82–92, Nov. 2016, doi: 10.1016/J.DCI.2016.02.020.

[26] 'Zebrafish (Danio rerio) anatomy, illustration. This animal is a popular model organism in biological research. Its genome is fully sequenced, and its Stock Photo - Alamy'. Accessed: Jan. 16, 2025. [Online]. Available: https://www.alamy.com/zebrafish-danio-rerio-anatomy-illustration-this-animal-is-a-popular-model-organism-in-biological-research-its-genome-is-fully-sequenced-and-its-image335314280.html

[27] J. Davies, D. D.-M. and molecular biology reviews, and undefined 2010, 'Origins and evolution of antibiotic resistance', *Am Soc Microbiol*, Accessed: Jan. 11, 2025. [Online]. Available: https://journals.asm.org/doi/abs/10.1128/mmbr.00016-10

[28] E. Habjan, G. Schouten, A. S.-I. T. ERADICATE, and undefined 2024, 'Diving into drug-screening: Zebrafish Embryos as an in vivo Platform for', *research.vu.nlE Habjan, GK Schouten, A SpeerINNOVATE TO ERADICATE, 2024•research.vu.nl*, Accessed: Jan. 11, 2025. [Online]. Available: https://research.vu.nl/files/362220495/habjanevaphdthesis%20-%206707b9405220a.pdf#page=38

[29] S. Nabadda, F. Kakooza, … R. K.-J. P. H., and undefined 2021, 'Implementation of the World Health Organization global antimicrobial resistance surveillance system in Uganda, 2015-2020: mixed-methods study using', *publichealth.jmir.orgS Nabadda, F Kakooza, R Kiggundu, R Walwema, J Bazira, J Mayito, I MugerwaJMIR Public Health and Surveillance, 2021•publichealth.jmir.org*, Accessed: Jan. 11, 2025. [Online]. Available: https://publichealth.jmir.org/2021/10/e29954/1000

[30] J. O'Neill, 'Tackling drug-resistant infections globally: final report and recommendations.', 2016, Accessed: Jan. 11, 2025. [Online]. Available: https://www.cabidigitallibrary.org/doi/full/10.5555/20173071720

[31] G. Dantas, M. S.-C. opinion in microbiology, and undefined 2012, 'Context matters—the complex interplay between resistome genotypes and resistance phenotypes', *ElsevierG Dantas, MOA SommerCurrent opinion in microbiology, 2012•Elsevier*, Accessed: Jan. 11, 2025. [Online]. Available: https://www.sciencedirect.com/science/article/pii/S1369527412000926

[32] W. V. S. R. S. B. B. Sciences and undefined 2015, 'The human gut resistome', *royalsocietypublishing.orgW Van SchaikPhilosophical Transactions of the Royal Society B, 2015•royalsocietypublishing.org*, vol. 370, no. 1670, p. 20140087, 2015, doi: 10.1098/rstb.2014.0087.

[33] D. Andersson, D. H.-N. R. Microbiology, and undefined 2014, 'Microbiological effects of sublethal levels of antibiotics', *nature.comDI Andersson, D HughesNature Reviews Microbiology, 2014•nature.com*, Accessed: Jan. 11, 2025. [Online]. Available: https://www.nature.com/articles/nrmicro3270

[34] P. L. Keen and M. H. M. M. Montforts, 'Antimicrobial Resistance in the Environment', *Antimicrobial Resistance in the Environment*, Dec. 2011, doi: 10.1002/9781118156247.

[35] B. Liu, X. Zhang, X. Ding, Y. Wang, and G. Zhu, 'Regulatory mechanisms of sub-inhibitory levels antibiotics agent in bacterial virulence', *Appl Microbiol Biotechnol*, vol. 105, no. 9, pp. 3495–3505, May 2021, doi: 10.1007/S00253-021-11291-1.

[36] D. Andersson, D. H.-N. R. Microbiology, and undefined 2014, 'Microbiological effects of sublethal levels of antibiotics', *nature.comDI Andersson, D HughesNature Reviews Microbiology, 2014•nature.com*, Accessed: Jan. 13, 2025. [Online]. Available: https://www.nature.com/articles/nrmicro3270

[37] L. S. Frost, R. Leplae, A. O. Summers, and A. Toussaint, 'Mobile genetic elements: the agents of open source evolution', *Nat Rev Microbiol*, vol. 3, no. 9, pp. 722–732, Sep. 2005, doi: 10.1038/NRMICRO1235.

[38] C. Smillie, M. P. Garcillán-Barcia, M. V. Francia, E. P. C. Rocha, and F. de la Cruz, 'Mobility of plasmids', *Microbiol Mol Biol Rev*, vol. 74, no. 3, pp. 434–452, Sep. 2010, doi: 10.1128/MMBR.00020-10.

[39] M. Blokesch, 'Natural competence for transformation', *Curr Biol*, vol. 26, no. 21, pp. R1126–R1130, Nov. 2016, doi: 10.1016/J.CUB.2016.08.058.

[40] Z. Jian *et al.*, 'Antibiotic resistance genes in bacteria: Occurrence, spread, and control', *J Basic Microbiol*, vol. 61, no. 12, pp. 1049–1070, Dec. 2021, doi: 10.1002/JOBM.202100201.

[41] H. Brüssow, C. Canchaya, and W.-D. Hardt, 'Phages and the evolution of bacterial pathogens: from genomic rearrangements to lysogenic conversion', *Microbiol Mol Biol Rev*, vol. 68, no. 3, pp. 560–602, Sep. 2004, doi: 10.1128/MMBR.68.3.560-602.2004.

[42] S. R. Partridge, S. M. Kwong, N. Firth, and S. O. Jensen, 'Mobile Genetic Elements Associated with Antimicrobial Resistance', *Clin Microbiol Rev*, vol. 31, no. 4, Oct. 2018, doi: 10.1128/CMR.00088-17.

[43] A. R. Almeida, M. Alves, I. Domingues, and I. Henriques, 'The impact of antibiotic exposure in water and zebrafish gut microbiomes: A 16S rRNA gene-based metagenomic analysis', *Ecotoxicol Environ Saf*, vol. 186, Dec. 2019, doi: 10.1016/J.ECOENV.2019.109771.

[44] N. Raab-Traub and D. P. Dittmer, ‘Viral effects on the content and function of extracellular vesicles’, *Nat Rev Microbiol*, vol. 15, no. 9, pp. 559–572, Sep. 2017, doi: 10.1038/NRMICRO.2017.60.

[45] A. N. T. Nguyen, R. Gorrell, T. Kwok, T. Connallon, and M. J. McDonald, ‘Horizontal gene transfer facilitates the molecular reverse-evolution of antibiotic sensitivity in experimental populations of H. pylori’, *Nature Ecology & Evolution 2024 8:2*, vol. 8, no. 2, pp. 315–324, Jan. 2024, doi: 10.1038/s41559-023-02269-5.

[46] Y. Zhao *et al.*, ‘The NLRC4 inflammasome receptors for bacterial flagellin and type III secretion apparatus’, *nature.comY Zhao, J Yang, J Shi, YN Gong, Q Lu, H Xu, L Liu, F ShaoNature, 2011•nature.com*, 2011, doi: 10.1038/nature10510.

[47] J. Munita, C. A.-V. mechanisms of bacterial, and undefined 2016, ‘Mechanisms of antibiotic resistance’, *Wiley Online LibraryJM Munita, CA AriasVirulence mechanisms of bacterial pathogens, 2016•Wiley Online Library*, vol. 4, no. 2, Mar. 2016, doi: 10.1128/microbiolspec.VMBF-0016-2015.

[48] M. ur R. Kayani *et al.*, ‘Environmental concentrations of antibiotics alter the zebrafish gut microbiome structure and potential functions’, *Environ Pollut*, vol. 278, Jun. 2021, doi: 10.1016/J.ENVPOL.2021.116760.

[49] L. S. Frost, R. Leplae, A. O. Summers, and A. Toussaint, ‘Mobile genetic elements: the agents of open source evolution’, *Nat Rev Microbiol*, vol. 3, no. 9, pp. 722–732, Sep. 2005, doi: 10.1038/NRMICRO1235.

[50] M. ur R. Kayani, K. Yu, Y. Qiu, X. Yu, L. Chen, and L. Huang, ‘Longitudinal analysis of exposure to a low concentration of oxytetracycline on the zebrafish gut microbiome’, *Front Microbiol*, vol. 13, Sep. 2022, doi: 10.3389/FMICB.2022.985065.

[51] D. A. Benson, I. Karsch-Mizrachi, D. J. Lipman, J. Ostell, and E. W. Sayers, ‘GenBank’, *Nucleic Acids Res*, vol. 38, no. SUPPL.1, Nov. 2009, doi: 10.1093/NAR/GKP1024.

[52] ‘47. ‘Babraham Bioinformatics - FastQC A Quality... - Google Scholar’. Accessed: Jan. 13, 2025. [Online]. Available: https://scholar.google.com/scholar?hl=en&as_sdt=0%2C5&q=47.%09%E2%80%98Babraham+Bioinformatics+-+FastQC+A+Quality+Control+tool+for+High+Throughput+Sequence+Data%E2%80%99.+Accessed%3A+Mar.+31%2C+2024.+https%3A%2F%2Fwww.bioinformatics.babraham.ac.uk%2Fprojects%2Ffastqc%2F&btnG=

[53] S. Chen, Y. Zhou, Y. Chen, J. G.- Bioinformatics, and undefined 2018, ‘fastp: an ultra-fast all-in-one FASTQ preprocessor’, *academic.oup.comS Chen, Y Zhou, Y Chen, J GuBioinformatics, 2018•academic.oup.com*, vol. 34, no. 17, pp. i884–i890, Sep. 2018, doi: 10.1093/bioinformatics/bty560.

[54] D. Li *et al.*, ‘MEGAHIT v1.0: A fast and scalable metagenome assembler driven by advanced methodologies and community practices’, *Methods*, vol. 102, pp. 3–11, Jun. 2016, doi: 10.1016/J.YMETH.2016.02.020.

[55] B. Aldeguer-Riquelme, L. M. Rodriguez-R, and K. T. Konstantinidis, ‘Differences in metagenome coverage may confound abundance-based and diversity conclusions’, *bioRxiv*, p. 2024.10.10.617679, Oct. 2024, doi: 10.1101/2024.10.10.617679.

[56] T. Hsu, E. Nzabarushimana, D. Wong, C. Luo, R. B.- bioRxiv, and undefined 2023, ‘Profiling novel lateral gene transfer events in the human microbiome’, *ncbi.nlm.nih.govTY Hsu, E Nzabarushimana, D Wong, C Luo, RG Beiko, M Langille, C HuttenhowerbioRxiv, 2023•ncbi.nlm.nih.gov*, Accessed: Jan. 13, 2025. [Online]. Available: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC10441418/

[57] C. P. Cantalapiedra, A. Herṇandez-Plaza, I. Letunic, P. Bork, and J. Huerta-Cepas, ‘eggNOG-mapper v2: Functional Annotation, Orthology Assignments, and Domain Prediction at the Metagenomic Scale’, *Mol Biol Evol*, vol. 38, no. 12, pp. 5825–5829, Dec. 2021, doi: 10.1093/MOLBEV/MSAB293.

[58] A. Nadkarni *et al.*, ‘Feasibility, acceptability and cost-effectiveness of a brief, lay counsellor-delivered psychological treatment for men with alcohol dependence in primary care: an’, *Wiley Online LibraryA Nadkarni, HA Weiss, R Velleman, J McCambridge, D McDaid, AL Park, P MurthyAddiction, 2019•Wiley Online Library*, vol. 114, no. 7, pp. 1192–1203, Jul. 2019, doi: 10.1111/add.14630.

[59] J. R. Huddleston, J. M. Brokaw, J. C. Zak, and R. M. Jeter, ‘Natural transformation as a mechanism of horizontal gene transfer among environmental Aeromonas species’, *Syst Appl Microbiol*, vol. 36, no. 4, pp. 224–234, Jun. 2013, doi: 10.1016/J.SYAPM.2013.01.004.

[60] H. Ochman, J. G. Lawrence, and E. A. Grolsman, ‘Lateral gene transfer and the nature of bacterial innovation’, *Nature*, vol. 405, no. 6784, pp. 299–304, May 2000, doi: 10.1038/35012500.

[61] H. Ochman, J. G. Lawrence, and E. A. Grolsman, ‘Lateral gene transfer and the nature of bacterial innovation’, *Nature*, vol. 405, no. 6784, pp. 299–304, May 2000, doi: 10.1038/35012500.

[62] M. Sommer, C. Munck, … R. T.-K.-N. R., and undefined 2017, ‘Prediction of antibiotic resistance: time for a new preclinical paradigm?’, *nature.comMOA Sommer, C Munck, RV Toft-Kehler, DI AnderssonNature Reviews Microbiology, 2017•nature.com*, Accessed: Jan. 15, 2025. [Online]. Available: https://www.nature.com/articles/nrmicro.2017.75

[63] F. L. Thompson, T. Iida, and J. Swings, ‘Biodiversity of Vibrios’, *Microbiology and Molecular Biology Reviews*, vol. 68, no. 3, pp. 403–431, Sep. 2004, doi: 10.1128/MMBR.68.3.403-431.2004.

[64] J. Davies, ‘Origins and evolution of antibiotic resistance.’, *Microbiologia*, vol. 12, no. 1, pp. 9–16, 1996, doi: 10.1128/MMBR.00016-10.