Journal of Sustainability Science and Management
Volume 20 Number 1, January 2025: 179-191
eISSN: 2672-7226

HOSPITAL WASTEWATER MICROBIAL COMMUNITY IN RESPONSE TO CIPROFLOXACIN

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http://doi.org/10.46754/jssm.2025.01.011
Received: 19 February 2024 Revised: 8 August 2024 Accepted: 7 October 2024 Published: 15 January 2025

Abstract: Ciprofloxacin (CIP) is a broad-spectrum antibiotic effective against Gram-negative organisms in clinical and hospital settings. However, bacteria strains resistant to it have emerged in recent years. This study assesses the bacterial community in hospital wastewater treatment plants (WWTPs) and investigates the impact of CIP treatment. Next-generation sequencing targeting the 16S rRNA genes was conducted on wastewater samples (Treatment A) and samples treated with 10 mg/L CIP using different growth media: Nutrient broth (rich medium) (Treatment B) and a minimum salt medium (poor medium) (Treatment C). Decrease in Chao1, Pielou, observed OTUs, Shannon, and Simpson indices indicated reduced species richness and diversity. Microbial communities in treatments A, B, and C showed notable dissimilarities between each other. Bray-Curtis and weighted UniFrac PCoA plot explained 54.86% and 74.32% of the total variance, respectively. Analysis across samples revealed that Firmicutes exhibit the highest relative frequency of bacteria (40.62%), followed by Proteobacteria (35.19%), and Bacteroidetes (19.92%). Barnesiellaceae dominate Treatment B and Sphingobacteriaceae prevail in Treatment C at the family level. The CIP-resistant bacteria in different treatments shape the microbial community because of their adaptability to the ecosystem. Therefore, it is crucial to consider these ecological implications for environmental bioremediation and development.

Keywords: Adaptability, antibiotic resistance, bacterial community, ciprofloxacin, hospital wastewater, microbial diversity.

Introduction

Hospital wastewater contains a complex mix of substances such as patient waste, medications, and disinfectants. Ciprofloxacin (CIP), a commonly used antibiotic in healthcare is one such medication (Thai *et al*., 2023). The problem with CIP is that a large amount of it is excreted through patients' urine and ends up in hospital wastewater. This leads us to an important issue when CIP happens to be a significant source of environmental and pharmaceutical contamination. The presence of CIP in hospital wastewater is not only a concern for the environment but also impacts the microorganisms that live in it (Du *et al*., 2023). Hospital wastewater serves as a sanctuary for a variety of microorganisms. The introduction of CIP into this environment disrupts this equilibrium due to its resistance to certain microbial metabolic processes, hindering efficient degradation (Alonso-Vásquez *et al*., 2023). The current study investigates how CIP impacts these microbial communities.

The discharge of CIP-contaminated wastewater from hospitals into wastewater treatment plants (WWTPs) has raised concerns about its potential impacts on microbial communities within these treatment systems

(Fatimazahra *et al*., 2023). Bacterial communities are integral components of WWTPs and play a vital role in wastewater treatment processes by degrading organic matter and removing pollutants (Chen *et al.*, 2022; Papajová *et al*., 2022). Thus, understanding the ecological effects of CIP on these microbial communities is crucial for effective wastewater treatment and ensuring the protection of public health (Kim *et al*., 2020; Qalyoubi *et al*., 2022).

Next-generation sequencing (NGS) of the 16s rRNA gene is widely used to investigate the microbiota at any given site without the need for laborious cultivation (Gancarz *et al*., 2021; Wensel *et al*., 2022). The structure of bacterial communities can be characterised by a molecular approach using 16s rRNA, the small ribosomal subunit that is commonly used to study the taxonomic composition of microbial communities in their natural environment (Zhang *et al*., 2020). Microbiome composition of bacterial hypervariable V3-V4 region of the 16S rRNA gene from the sample was determined by direct DNA sequencing using NGS technologies that highlight the conserved genetic marker at the genus or species level (Zhang *et al*., 2020; Fadeev *et al*., 2021; Boccioni *et al*., 2022).

Kim *et al*. (2020) reported that CIP exposure changed the composition of the microbial community in aerobic activated sludge; thus, having a cascading impact on the ecosystem. CIP also disrupted the microbial community in a laboratory batch study, as reported by Miritana *et al*. (2022). A study on artificial wastewater by Dai *et al*. (2018) highlighted different microbial community structures in response to the dosage of CIP. In the sponge membrane bioreactor, the presence of CIP led to formation of different microbial community structures (Dang *et al*., 2021). A study demonstrated that a single addition of CIP can influence the structure and function of microbial communities in soil (Cui *et al*., 2014). Repeated exposure to CIP along with other treatments has been shown to alter the enzyme activity and overall microbial community structure (Han *et al*., 2019). According to Smirnova *et al*. (2023), under the action of CIP, *E. coli* retained a high rate of growth and respiration longer in LB rich media than in a minimal M9 medium. Although there is data on the effect of CIP on microbial activity and the influence of medium composition, there is a gap in the literature about the effect of direct CIP on the microbial community in hospital wastewater and different media of CIP treatments.

The objectives of this study are to investigate the influence of CIP exposure on the bacterial community structure within hospital WWTPs using 16S rRNA gene sequencing and the response of the bacterial community under different mediums of CIP treatments within the WWTPs. We hypothesise that CIP exposure changes the bacterial populations in hospital WWTPs and different CIP treatment media affects the bacterial population response.

Materials and Methods

Sampling Location and Samples Collection

Hospital Shah Alam is a secondary hospital with 385 beds, 1,435 staff, and a wastewater flow rate of 172.8 m^3/day. The sampling location at the wastewater treatment plant Hospital Shah Alam is at latitude 3.070089888215716˚N and longitude 101.48832698301037˚E. Pretreated wastewater samples that passed through the bar screen with an opening of 25 mm were collected via the grab sampling method in triplicates. The collected samples were labelled and transported to the laboratory at 4ºC in an ice box for further analysis.

Wastewater Characterisation

In-situ parameters of pretreated wastewater such as temperature, pH, pH voltage, turbidity, oxidation-reduction potential, conductivity, dissolved oxygen, total dissolved solid, and salinity were measured using a multi-parameter water quality meter (Horiba U-50, Japan). Total solid, total suspended solid, and biological oxygen demand were analysed by referring to Standard Methods for the Examination of Water and Wastewater (1998) using total solid 2540B,

total suspended solid 2540D, and biological oxygen demand 5210B methods, respectively (Clesceri *et al*., 1998).

For chemical oxygen demand analysis, the chemical oxygen demand HACH method 8000 USEPA reactor digestion method was conducted. Nitrate HACH Method 8192 cadmium reduction method and orthophosphate HACH Method 8048 USEPA phosver 3 (ascorbic acid) method were conducted for nitrate and orthophosphate analysis.

Sample Treatment

Table 1 shows the experimental set-up for the microbial community study. For Treatment A, the pretreated wastewater from sampling in Hospital Shah Alam was filtered through the 0.22 µm membrane filter (Whatman) and filters were stored at -80ºC. An amount of 0.1% (v/v) sample of wastewater was added into nutrient broth containing 10 mg/L CIP (Treatment B) and minimum salt medium with 10 mg/L CIP (Treatment C), respectively. Samples were left for five days on a shaker operating at 120 rpm at room temperature. After that, samples were filtered using a 0.22 µm membrane filter and kept at -80ºC.

Extraction of Genome DNA and PCR

Total DNA extraction from the collected samples was performed using the CTAB/SDS method. The extracted concentration of DNA and purity were assessed using 1% agarose gels. Subsequently, based on the concentration, the DNA was appropriately diluted to a final concentration of 1 ng/µL using sterile water. For PCR amplification, 16S rRNA degenerate forward primer sequence* (AGRGTTYGATYMTGGCTCAG) and 16S rRNA degenerate reverse primer sequence* (RGYTACCTTGTTACGACTT) were used along with the Phusion® High-Fidelity PCR Master Mix (New England Biolabs). The diluted genomic DNA served as the template for PCR amplification.

PCR Products Mixing and Purification

The PCR products obtained from the amplification were mixed in equidensity ratios. To confirm the presence and size of the PCR products, the mixture was subjected to electrophoresis using a 2% agarose gel. After gel electrophoresis, the PCR product mixture was purified using the Qiagen Gel Extraction Kit (Qiagen, Germany). This kit is designed to efficiently extract and purify DNA fragments from agarose gels, ensuring high-quality PCR products for subsequent analyses.

Library Construction and Sequencing

The sequencing linker was ligated to the ends of the amplified DNA fragments using DNA-binding enzymes to construct a SMRTbell library, which was carried out with SMRTbell Express Template Prep Kit 2.0. Subsequently, the DNA fragments were purified using AMPure PB magnetic beads. Following purification, the buffer-resolved fragments were screened for a specific size using the BluePippin fragment screening technique. The DNA fragment of interest was then further purified using AMPure PB magnetic beads (Beckman Coulter, United States).

The constructed library was quantified using Qubit concentration measurement (Thermo Fisher Scientific, United States) ensuring accurate determination of library concentration. The insert size of the library was

Table 1: Experimental design for microbial community study

Treatment	Description
A	Wastewater
B	Nutrient broth with 10 mg/L CIP
C	Minimum salt medium with 10 mg/L CIP

determined using the Agilent 2100 (Agilent, United States), providing information about the size distribution of the inserted DNA fragments. Finally, the library was subjected to sequence on the PacBio platform (PacBio, United States).

Bioinformatics Analysis

Paired-end reads obtained from the sequencing data were assigned to their respective samples based on unique barcodes, and the barcode and primer sequences were subsequently removed through truncation. To obtain high-quality clean tags, quality filtering on the raw tags was performed using specific filtering conditions following the QIIME (V1.7.0) quality control process. The paired-end reads were merged using FLASH (V1.2.7) to generate longer sequences for further analysis. Subsequently, denoising, species annotation, alpha diversity analysis, beta diversity analysis, and Analysis of Composition of Microbiomes (ANCOM) analysis were performed using the QIIME 2 (2020.6) bioinformatics software package with the SILVA 138 database for taxonomic assignment. The DADA2 version used in this process was QIIME 2 (2020.06) with built-in R version 4.0.2. To identify differentially abundant taxa, LEfSe analysis was conducted using the LEfSe software. Metastat was calculated using R software. *p*-values were determined through permutation tests while q-values were calculated using the Benjamini and Hochberg False Discovery Rate method. T-tests and data visualisations were performed using R software to analyse the results further and generate illustrative representations. The sequencing and processing run metrics for the samples were summarised in Table 2.

Results and Discussion

Water Quality Analysis

Table 3 summarises the results of the water quality analysis of Hospital Shah Alam. The wastewater was high in total suspended solids, chemical oxygen demand, nitrate, and orthophosphorus. This indicates the wastewater provides a suitable environment with available nutrients for bacteria growth. Comprehensive wastewater treatment is important to minimise the ecological impact of the wastewater and maintain a healthy aquatic environment (Papajova *et al*., 2022).

Alpha Diversity Analysis

Table 4 shows the alpha diversity index for treatments A, B, and C. A total of 107 observed Operational Taxonomic Units (OTUs) were found in the microbial community. Treatment A exhibited the highest number of observed OTUs with a count of 61.00 and the highest values in all alpha diversity indices, indicating a highly diverse bacterial community. Conversely, treatments B and C, which included CIP in media composition, showed reductions in the alpha diversity index. Such conditions reveal a decrease in species richness and diversity. Treatments B and C showed similar levels of species richness and diversity, suggesting a potential influence of the CIP in maintaining microbial diversity.

The incapability of some bacteria to break down CIP has major effects on the dynamics of the community and competitive interactions (Cai *et al*., 2022). Since CIP does not serve as a carbon or energy source, it hinders bacterial

Table 2: Data summaries of denoising process

Treatment	Total Reads (ccs)	Primers Removed	Filtered Reads	Denoised Reads	Non-chimeric Reads
A	14,624	13,471	13,330	5,204	5,102
B	22,123	20,531	20,371	12,813	12,144
C	14,217	13,071	12,956	6,965	6,546

Table 3: Hospital Shah Alam wastewater quality analysis

Parameters (unit)	Mean
Temperature (°C)	27.33 + 0.50
pH	7.30 + 0.07
pH voltage (pH mv)	-15 + 4
Turbidity (NTU)	247 + 24
Oxidation reduction potential (ORPmv)	4 ± 14
Conductivity (mS/cm)	0.543 ± 0.022
Dissolved oxygen (mg/L)	4.88 ± 0.58
Total dissolved solid (g/L)	0.358 ± 0.017
Salinity (ppt)	0.3 ± 0.0
Total solid (g/mL)	437 ± 114
Total suspended solid (g/mL)	93 ± 14
Biological oxygen demand (mg/L)	15.05 ± 5.14
Chemical oxygen demand (mg/L)	307 ± 40
Orthophosphate (mg/L)	12.1 ± 3.3
Nitrate (mg/L)	21.37 ± 1.79

Table 4: Alpha diversity index

Treatment	Observed OTUs	Pielou	Chao1	Shannon	Simpson
A	61	0.90	61.33	5.31	0.97
B	42	0.67	43.50	3.61	0.85
C	42	0.67	43.00	3.61	0.82

growth and survival. CIP limits bacterial growth and survival by targeting the enzymes DNA gyrase and topoisomerase IV, which are essential in DNA replication and repair (Shariati *et al*., 2022). This inhibition slowed down DNA replication, broke double-stranded DNA, and interrupted key cellular activities, which can eventually lead to bacterial cell death.

Otherwise, CIP-degrading bacteria respond adaptively; thus, increasing their chances of survival and persistence (Sridhar *et al*., 2021). Changes in metabolism, gene expression, and stress response pathways may be a part of this response, which aids the bacteria in surviving the antibiotic stress and in the presence of CIP. The competitive exclusion of the non-degrading bacteria within a microbial community, resulting the decrease in species diversity and population density. Therefore, the exposure of CIP influences the bacterial community structure within hospital WWTPs.

Beta Diversity Analysis

The heat maps based on weighted UniFrac and Bray-Curtis distances between the microbial communities are displayed in Figure 1. The deeper colours in the heat maps indicate greater differences and distances between the communities. In this study, microbial communities in treatments A, B, and C showed notable dissimilarities between each other, reflecting different levels of adaptation and tolerance towards CIP.

Adaptation increased the expression of efflux pump genes, resulting in increased efflux pump activity and decreased the intracellular concentration of antibiotics; thus, lowering their efficiency and contributing to resistance development (Yu *et al*., 2022). Papkou *et al*. (2020) supported this finding, where the overexpression of norA efflux pump activity led to an increase in the resistance to CIP in *S. aureus*. The efflux pump is important in lowering the intracellular concentration of antibiotics, which reduces their effectiveness (Jiang *et al*., 2018; Huang *et al*., 2022). Therefore, different adaptation mechanisms of efflux pumps in response to CIP influence the distances between the communities.

Figure 2 illustrates the Principal Coordinate Analysis (PCoA) plots based on Bray-Curtis and weighted UniFrac distances. The distinct clusters formed by each treatment group showed different microbial community characteristics. The Bray-Curtis PCoA plot explained 54.86% of the total variance, with PCo1 and PCo2 accounting for most of the variation. The weighted UniFrac PCoA plot explained 74.32% of the total variance, highlighting the importance of PCo1 in differentiating the communities. The distinct clustering pattern in this study indicated that growth medium and CIP play a major role in governing the composition and diversity of the bacterial community.

Nutrient broth is rich in nutrients and essential for bacterial growth (Mello *et al*., 2016). The fast adaptation of bacteria to CIP in nutrient broth led to an increase of growth rate and competitive advantage in the community due to nutrient availability. In the minimal salt medium, CIP serves as the sole carbon source for the growth of microbial species. Otherwise, the non-adapted CIP bacteria growth was suppressed and undesirable bacteria were eliminated due to the specific requirements of the microorganisms being cultured (Bonnet *et al*., 2020).

E. coli showed optimal growth and respiration in nutrient-rich media, indicating a preference for this favourable environment (Smirnova *et al*., 2023). Haikal *et al*. (2023) revealed that *Cupriavidus necator* and *Pseudomonas aeruginosa* strain HJ1012 had the maximum efficiency in acetaminophen degradation with ideal circumstances being pH 7.0, temperature range 30°C to 40°C, and a minimal salt solution. Therefore, different bacteria exhibit different medium requirements for community structure.

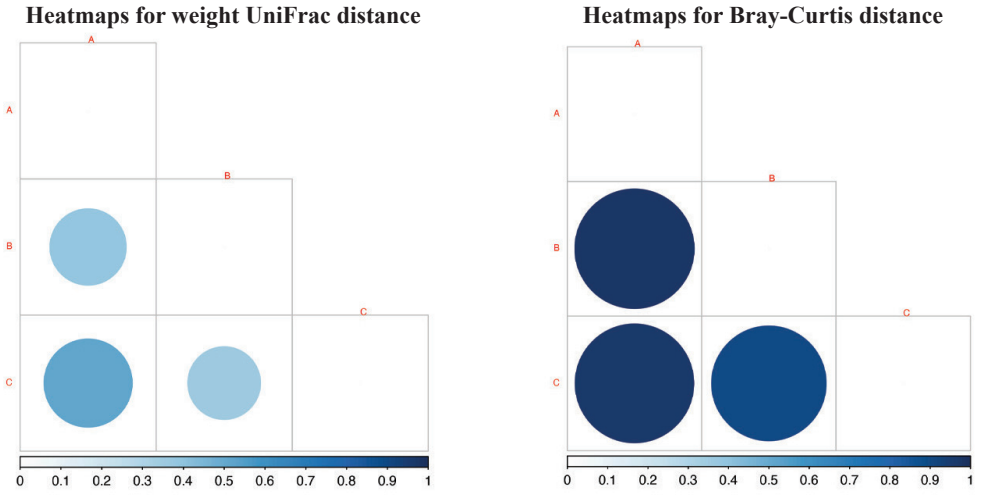
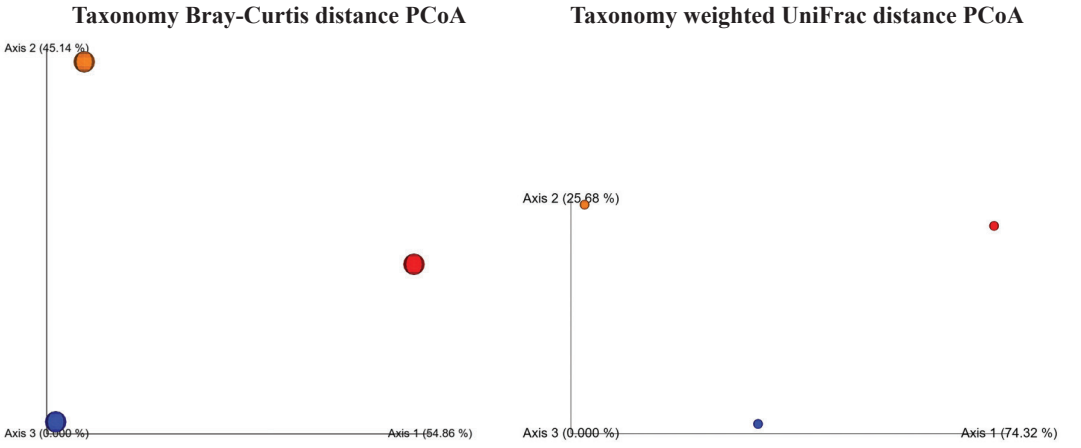


Figure 1: Heatmap for taxonomy distance matrix of (a) weighted UniFrac distance and (b) Bray-Curtis distance



(a) (b)

Figure 2: Principal coordinate analysis for taxonomy based on (a) Bray-Curtis distance and (b) weighted UniFrac distances

Note: Each circle represents the sample (A = Red, B = Blue, and C = Orange).

Sample Community Composition Analysis

Figure 3 depicts the number of shared and unique bacterial communities from treatments A, B, and C in a Venn diagram. In this analysis, each Amplicon Sequence Variant (ASV) represents a unique sequence variant identified without clustering into Operational Taxonomic Units (OTUs). Therefore, each ASV can be considered as an exclusive taxonomic unit. The Venn diagram shows that three ASVs are shared among all three treatments, indicating the presence of microorganisms that consistently exist in both wastewater and CIP-treated samples.

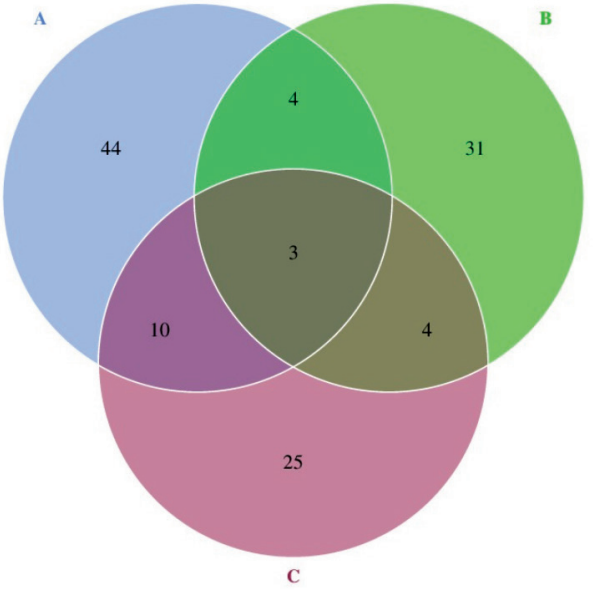


Figure 3: Venn diagram displays the number of shared and unique bacterial communities from treatments A, B, and C

This suggests a core microbial community that remains relatively stable regardless of the treatment (Shade & Handelsman, 2012).

Furthermore, the diagram showed the number of unique ASVs in each treatment. Wastewater (Treatment A) had the highest number of unique ASVs with 44 identified, followed by Treatment B and Treatment C with 31 and 25 unique ASVs, respectively. The adaptation mechanism influences the survival of bacteria in different CIP treatments. The treatment with CIP in B and C led to the development of distinct ASVs. The divergence in ASVs is due to bacterial adaptation mechanisms in response to CIP, in which only resistant bacteria survived. On the other hand, the absence of distinct ASV suggests a more homogeneous microbial community composition across treatments. Therefore, the dynamics of the microbial community resulted in the presence of core and unique ASVs in each treatment.

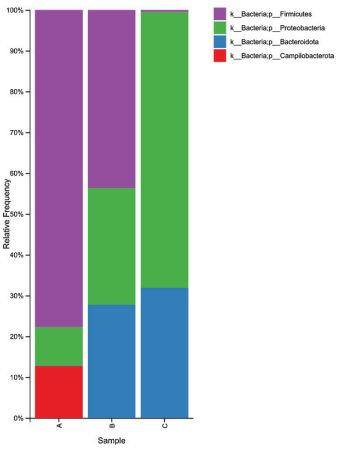
Figure 4 shows the relative frequencies of bacterial groups in treatments A, B, and C at phylum, class, and family levels. Four phyla, namely Firmicutes, Proteobacteria, Bacteroidota, and Campylobacterota were observed across all treatments. Only Firmicutes and Proteobacteria were observed in all treatments. Firmicutes had the highest relative frequency in Treatment A (77.73%) but lowest in C (0.49%) while Proteobacteria was highest in Treatment C (67.56%) but lowest in A (9.44%). Campylobacterota was only present in Treatment A (12.70%) while Bacteroidota was only present in B (27.80%) and C (31.95%).

At the class level, the dominant class in each treatment was Negativicutes in A (75.80%), Bacilli in B (43.63%), and Gammaproteobacteria in C (63.48%). Besides class Gammaproteobacteria, Treatment C also had class Alphaproteobacteria (4.08%) that consist of order Caulobacterales (2.31%) and Rhizobiales (1.77%). Another notable class was class Clostridia that contributed less than 1% in A. For analysis of community composition at the order level, the CIP and minimal salt medium changed the dominance of Bacteroidia, where Bacteroidales were dominant in Treatment B, whereas Sphinghobacteriales was dominant in Treatment C. Among Bacteroidales, family Barnesiellaceae was found to be dominant in B.

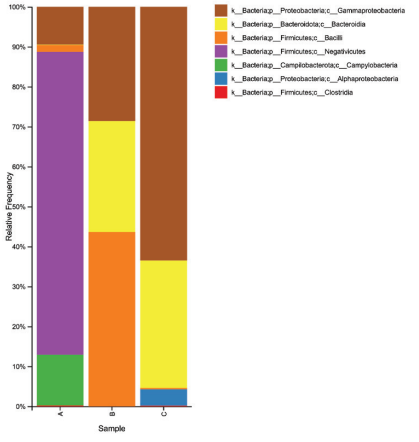
The analysis of key genera revealed distinct patterns of abundance across the different treatments. Genus Ralstonia showed a marked increase in abundance in the CIP-treated samples (Treatments B and C), suggesting its potential role in CIP degradation. Conversely, genus Comamonas was absent in Treatment B but increased significantly in Treatment C while genus Enterococcus absent in Treatment C, showed a notable rise in Treatment B. Genus Acidovorax was dominant in Treatment A, significantly represented in Treatment C, but absent in Treatment B. Similarly, genus Streptococcus was dominant in Treatment A and significantly represented in Treatment C. Genera Romboutsia and Stenotrophomonas were present in Treatments A and C only. Unique to Treatment A were genera Megamonas, Arcobacter, Zoogloea, Bacillus, and Rivicola. Treatment B had unique representation from genera Vagococcus, Bacteroides, Dysgonomonas, Erysipelatoclostridium, Porphyromonadaceae, and an uncultured genus from the family Barnesiellaceae. Finally, Treatment C was characterised by unique genera including Sphingobacterium, Bosea, Brevundimonas, Chryseobacterium, Phenylobacterium, Runella, and Fictibacillus. These findings highlight the distinct microbial communities in each treatment, emphasising the adaptation and survival mechanisms of different genera in response to CIP treatment.

The phyla in the current study have previously been reported in high abundance in municipal and clinical wastewater, except for Campylobacterota (Zhang *et al*., 2020; Shen *et al*., 2023). Poopedi *et al*. (2023) documented that the Campylobacterota was consistent with this study; however, the most predominant phyla were Bacteroidota, followed by Campylobacterota, Proteobacteria, Firmicutes, and Desulfobacteria. This finding suggests that the presence of CIP in different growth media

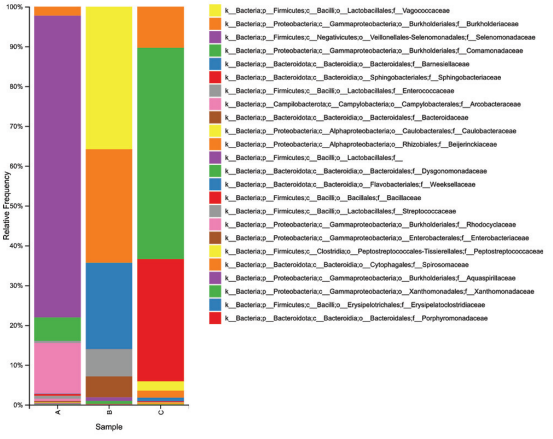
[a]


k__Bacteria;p__Firmicutes
k__Bacteria;p__Proteobacteria
k__Bacteria;p__Bacteroidota
k__Bacteria;p__Campilobacterota
Relative Frequency
Sample
A
B
C

[b]


k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria
k__Bacteria;p__Bacteroidota;c__Bacteroidia
k__Bacteria;p__Firmicutes;c__Bacilli
k__Bacteria;p__Firmicutes;c__Negativicutes
k__Bacteria;p__Campilobacterota;c__Campylobacteria
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria
k__Bacteria;p__Firmicutes;c__Clostridia
Relative Frequency
Sample
A
B
C

[c]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k__Bacteria;p__Firmicutes;c__Negativicutes;o__Veillonellales-Selenomonadales;f__Selenomonadaceae
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k__Bacteria;p__Bacteroidota;c__Bacteroidia;o__Bacteroidales;f__Porphyromonadaceae
Relative Frequency
Sample
A
B
C

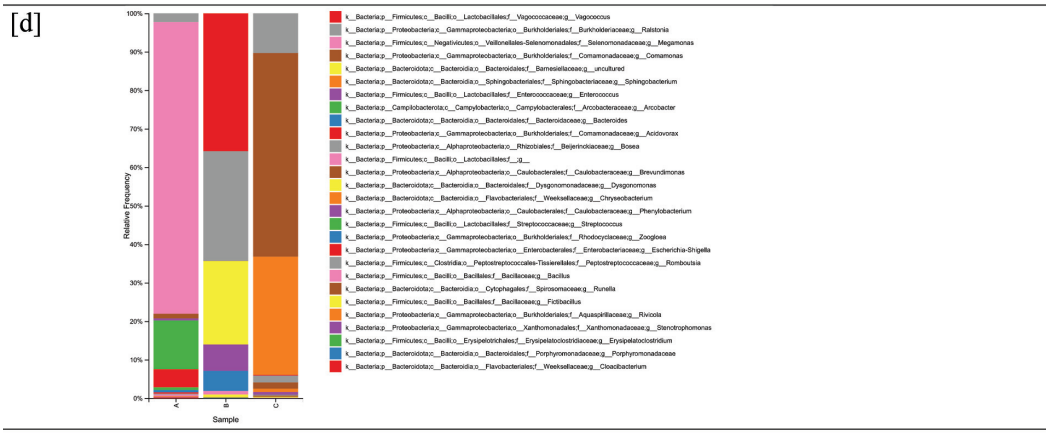

Figure 4: Bacterial profiles of wastewater samples at the [a] phylum, [b] class, [c] family, and [d] genus level. Bar plots indicate bacterial compositions of wastewater microbiome for treatments A, B, and C
Note: k = Kingdom, p = Phylum, c = Class, o = Order, f = Family, g = Genus.

was found to shape the microbial community at the family level (Ohore *et al*., 2021). In addition, it also supported the results of Alpha and Beta diversity analyses (Figures 1 and 2). Ng *et al*. (2019) had a finding consistent with this study on the survival of Bacteroidota in Treatment B that revealed the survival of gut microbiota Bacteroidetes, including Bacteroidaceae, Porphyromonadaceae, Rikenellaceae, S24-7, and Barnesiellaceae. Therefore, the presence of CIP in different growth media shapes the taxonomic composition.

Conclusions

The influence of ciprofloxacin (CIP) exposure on the bacterial community structure in hospital WWTPs was investigated. Treatment without CIP exhibited a more diverse bacterial community with higher species richness and diversity indices compared with treatments with CIP. The presence of separate clusters in the beta diversity analysis indicates a unique microbial community profile due to the adaptability and tolerance of the toxicity level of bacteria that is influenced by different media. The CIP-adapted bacteria altered the dominant structure within the microbial community. The sample community composition analysis showed the number of unique ASVs in wastewater and CIP treatment samples, indicating the capability of these groups to break down the CIP. Using metagenome binning, the phyla that showed the highest relative frequencies across the treatment is Firmicutes, followed by Proteobacteria. Campylobacterota is absent in samples treated with CIP.

The presence of CIP in various growing mediums was discovered to shape the taxonomic composition. Overall, CIP exposure changes the bacterial populations in hospital WWTPs, different CIP treatment media affect the bacterial population response. The results showed that the information on CIP-degrading bacteria obtained in this study can be used for environmental bioremediation applications. This knowledge leads to the development of tailored remediation plans and facilitates the establishment of sustainable wastewater treatment practices and potentially contributes towards achieving the United Nations Sustainable Development Goal (UN SDG) 6: Clean water and sanitation.

Acknowledgements

The authors would like to express their gratitude to the validators for their invaluable contribution in validating the questionnaire instrument.

Conflict of Interest Statement

The authors declare that they have no conflict of interest.

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