Characterization of Microbiome and Antibiotic Resistance Genes of the River Ganga

SUMMARY OF THE THESIS

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The study of the thesis discusses the bacterial diversity and the antibiotic resistance genes prevailing in the Ganga River water and sediment samples from Uttarakhand and Uttar Pradesh region of India by using the traditional culturable and unculturable (metagenomic) approach. Chapter **one** consists of the introduction part where the importance of the Ganga River, its source of pollution, and antibiotic resistance genes are discussed. Besides, the section describes the lacunae in previous investigations on bacterial diversity on various stretches of the Ganga River. This laid down the idea to further research on the bacterial diversity of the Ganga River and explore the functional attributes such as associated antibiotic resistance genes, biofilm formation activity, and profiling of xenobiotic compounds of the inhabitant bacteria. The study considered a longer stretch of the Ganga River from Uttarakhand (Rishikesh and Haridwar) and Uttar Pradesh (~ 1560 km of 2525 km) regions on water and sediment samples. Further, this section also describes the QIIME2 and PICRUSt2 pipelines used for the analysis of the metagenomic data for the microbiome and their functional annotation analysis. The study comprises four major objectives as follows are described in **chapter two** of the thesis.

- Extraction of high molecular weight quality metagenomic DNA from water/sediment samples of the river Ganga.
- Characterization of microbial diversity of river Ganga using metagenomic approaches.
- Characterization of antibiotic resistance genes of microbes present in river Ganga.
- Identifying the correlation between existing microbial diversity and antibiotic-resistant genes.

Chapter three consists of a review of the literature, where previous studies done of the Ganga River for exploring microbial diversity and antibiotic resistance genes by using both culturable and unculturable approaches have been discussed with their gaps that laid down the idea to further research to further enrich the limited information in this line of research. **Chapter four** of the thesis consisting the methodology section, where various methods used to accomplish each objective of the study were described in detail. **Chapter five** of the thesis consists of the results part covering each objective finding through culturable as well as unculturable

approaches. Chapter **six** comprised an extensive discussion of the findings and conclusions of each defined objective of the thesis. Further, it also discusses the future prospects of the present study. Chapter **seven** is the listing of the related references cited in the thesis from the Introduction to Discussion sections. Chapter **eight** has been annexed with the title page of published papers and other scientific output of work.

In the present study, 17 water and 10 sediment samples were taken from various sites of Uttarakhand (Upstream; US) and Uttar Pradesh (Downstream; DS) regions of India. A high molecular weight metagenomic DNA from water (17 samples) and sediment (a total of 10 samples or 5 pooled samples) samples were extracted to explore the bacterial diversity and antibiotic resistance genes prevailing in the Ganga River using a metagenomic approach. This metagenomic DNA was processed for Next generation sequencing (NGS)targeting V3-V4 bacterial-specific 16S rRNA hypervariable region only. Taxonomy assignment and diversity indices analysis was done by using the QIIME2 pipeline, however, pathways involved in antibiotic biosynthesis, antibiotic resistance and its related genes (ARGs), xenobiotic degradation, carbohydrate, and energy metabolism in water and sediment samples were analyzed through PICRUSt2 pipeline using KEGG and BRITE databases. Correlation and network analysis were also performed to study the correlation between the existing bacterial community and antibiotic resistance genes. Further, a portion of water and sediment samples were processed for studying physicochemical parameters such as chemical oxygen demand (COD), nitrate, sulphate, phosphate, and ammonia. A culturable approach was also used to isolate the bacterial community present in the water and sediment samples of the Ganga River from the same sampling sites, which was followed by the morphological and biochemical, and molecular (16S rRNA) characterization of the isolated bacteria. The antibiotic susceptibility test and Minimum inhibitory concentration (MIC) were also performed on these isolated bacteria. A comparative analysis between water and sediment samples was also performed for a better understanding of the data obtained through culturable as well as metagenomic approaches. A comparative analysis between the antibiotic-resistant profiling of the bacterial isolates obtained from water and sediment samples was studied.

The major findings of the present study of the thesis are summarized below.

Water samples of the Ganga River

Physicochemical analysis revealed a higher concentration of COD, nitrate, and phosphate in the downstream sites of the Ganga River water. Overall, the bacterial microbiome showed the dominance of *Proteobacteria* (42.70%), *Actinobacteria* (14.78%), *Bacteroidota* (12.65%), and *Planctomycetota* (9.57%). The US region showed a relatively high dominance of phyla such as *Proteobacteria, Actinobacteriota*, and *Planctomycetota*. Similarly, at the genus level, several common genera were observed that recapitulate the previous reports on the river water such as *Flavobacteria, Pseudomonas, Acinetobacter, Gemmatimonadetes, Verrucomicrobia, Rheinheimera, Commamonas*, and *Nitrospora*. The dominance of *Flavobacterium* and *Cyanobacteria* spp. was observed in the DS region which indicates an organic-rich environment. Moreover, *Flavobacterium* also showed antibiotic resistance for five antibiotic pathways (CAMP, Tetracycline, Quinolone, Aminoglycosides, and Phenicol). Further, a total of six phyla (*Bacteroidota, Planctomycetota, Verrucomicrobiota, Gemmatimonadota, Chloroflexi,* and *Armatimonadota)* and eleven significantly different genera (*Pseudomonas, Gemmatimonas, Sporichthyaceae, Luteolibacter, Pirellula, Novosphingobium, Arenimonas, Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium, Comamonas,* and *Schlesneria*) were significantly different in between the US and DS regions (p-value < 0.05). The comparative analysis revealed that *Bacteroidota*, *Verrucomicrobiota*, and *Gemmatimonadota* were more populated with the DS group. The prevalence of *Bacteroidetes* in the DS region seeks attention for their abundance in the heavily polluted water that may be loaded with high dissolved organic carbon (DOC) and recent fecal pollution. The prevalence of *Pseudomonadota* members (*Pseudomonas, Novosphingobium*, and *Comamonas)* in the US region (Haridwar) is noteworthy as these Gram-negative bacteria may turn into opportunistic pathogens, especially among immunocompromised individuals. The higher diversity indices in the downstream samples further emphasize that the DS regions harbor more diverse bacterial communities over the US region.

Antibiotic resistance pathways analysis revealed that the most observed antibiotic resistance was of β-lactam (33.92%), followed by CAMP (27.75%), MDR (19.17%), and vancomycin resistance (17.84%). Similarly, antibiotic biosynthesis pathways analysis revealed the dominance of biosynthesis of several antibiotics (streptomycin, monobactam, novobiocin, acarbose, validamycin biosynthesis, carbapenem biosynthesis, vancomycin, gentamicin, and tetracycline biosynthesis) pathways in the water samples of the DS region. This indicates that bacterial communities prevailing in the DS region exhibit evolved resistance systems engaged in the biosynthesis of antibiotics, self-defense, and antibiotic resistance. Correlation analysis of the antibiotic resistance with the top 50 bacteria of this investigation concluded that the majority of the bacteria showed significant resistance to tetracycline (21%) followed by phenicol (17.5%), and macrolide/CAMP (12.2%). The inhabitant bacteria showed the least significant correlation with antibiotic resistance (5.2%) towards β-lactam, MDR, and vancomycin antibiotics. Only one genus *Arenimonas* showed resistance against trimethoprim. Interestingly, antibiotic-resistant genera were more dominant in the water samples of the downstream region such as *Flavobacterium* was positively correlated with three antibiotic-resistance genes (CAMP, Tet, and Quinolone). Similarly, *Nevskia* and *Sedminibacterium* were relatively abundant in the DS region and exhibited multiple antibiotic resistance. Whereas, *Pseudomonas* (multidrug resistance), *Vicinamibacteraceae* (CAMP, aminoglycosides, and phenicol), and *Kapabacterials* (β-lactam, CAMP, and phenicol) were relatively abundant in the US region. Similarly, several bacterial genera such as *Flavobacterium*, *Pseudomonas*, *Rheinheimera*, *Kapabacteriales*, *Brevundimonas*, *and Gemmatimonas* showed a significant positive correlation with both antibiotic resistance biosynthesis and antibiotic resistance pathways. The prevalence of Gram-negative bacteria observed might be due to high exposure to antibiotics in water as Gram-positive bacteria have increased susceptibility to antibiotics.

Sediment samples of the Ganga River

Physicochemical analysis revealed a higher concentration of nitrate, sulfate, and ammonia in the downstream sites of the Ganga River sediment samples, especially in the Varanasi (VS) and Kanpur (KS) sediment samples. Overall, the study revealed that the Ganga sediment is full of Gram-negative bacteria that chiefly include the members of *Proteobacteria, Acidobacteria, Chloroflexi,* and *Bacteroidota. Proteobacteria* dominate in all five sediment samples. The prevalence of these bacteria in the Ganga River sediment indicates the huge organic load due to municipal waste and agricultural runoff. The genera-level analysis showed the prevalence of three major genera that includes *Nitrospira*, *Hydrogenophaga*, and *Thauera.* Further, Varanasi sediment also showed a higher abundance of xenobiotic degradation pathways involved in polycyclic aromatic hydrocarbon (PAH) degradation, aminobenzoate degradation, and benzoate degradation. Therefore, we hypothesize that the Varanasi region sediment is comparatively more polluted with the xenobiotic complex compounds than the other sediments studied here. This can be correlated with the level of NO^{-3} and SO_4 ²- that were higher in the VS and KS regions. Similarly, the species-level analysis uncovered several underrepresented members (*Vogesella_alkaliphila, Fluviicoccus keumensis, Lacibacter cauensis, Perlucidibaca piscinae, Leptolyngbya planctomycetes str*) that utilize various electron donors and acceptors that include complex organic compounds (polysaccharides/sugars and short-chain fatty acids) as well as metals, and other minerals. Surprisingly, the microbial diversity of the Rishikesh samples (collected from the upstream region of river Ganga) did not much deviate from the sediment microbiome of other regions and suggests that the upstream region of the Ganga is also heavily contaminated with similar hazardous toxicants and pollutants. Diversity analysis revealed high bacterial richness and evenness in the Kanpur sediment (KS) with respect to other sediment samples, which might be due to the high pollution load. Further, benzoate and aminobenzoate, and fluorobenzoate degradation pathways accounted for more than 40% of total pathways involved in xenobiotic degradation obtained in the sediment samples. Thus, the Ganga River sediments could be considered a reservoir/sink of xenobiotic, aromatic, and complex organic compounds and their bacterial degraders due to the continuous disposal of industrial and sewage effluents and wastes.

Comparative analysis of water and sediment samples of the Ganga River

While comparing the microbiome at the phyla level, six phyla (Proteobacteria, Bacteroidota, Actinobacteriota, Acidobacteriota, Gemmatimonadota, Nitrospirota, and Firmicutes) were shared between water and sediment. Similarly, a higher count of common genera (691) was observed between water and sediment samples, which hypothesize that water and sediment share a high level of bacterial diversity of common taxa both at phyla and genera level. Of the nine significantly different phyla, only two phyla (Proteobacteria and Actinobacteria) were more prevalent in the water samples. Of the nineteen significantly different genera, only two (*Pseudomonas* and

Gemmatimonas) were dominant in water samples. *Pseudomonas* showing relatively higher dominance in water indicates the contamination of the hospital as well as human waste in the river water samples of the Ganga River. ARGs level analysis showed seventy-six antibiotic resistance genes shared among water and sediment samples, of which, twenty-two ARGs showed >1% abundance. Where, β-lactam, vancomycin, multidrug resistance genes, tetracycline, and aminoglycoside constitute the top five prevalent antibiotic resistance pathways between water and sediment samples. Of the significantly different ARGs (p-value < 0.05), 32 ARGs were significantly different between water and sediment samples. Whereas, vancomycin resistance genes were more associated with sediment samples, whereas water samples were more associated with aminoglycosidase resistance genes. Vancomycin is considered the last reservoir antibiotic against several bacterial infections, therefore, the prevalence of vancomycin resistance genes in the Ganga River is a fright. The PCoA analysis revealed a higher diversity of ARGs among water samples over the sediment samples and identified them as distinguished spatial clusters. Further, the clustering of water with the sediment samples in the PCoA analysis indicates that there might be the dissemination of ARGs occurring between sediment and water or vice-versa due to low flow rate of water, seasonal effect, accumulation of extracellular DNA, and a shift in the microbial community. Network-based analysis in 14 shared genera (>1%) between water and sediment samples indicate that five genera (*Flavobacterium*, *Pseudomonas*, *Acinetobacter*, *Candidatus_divison* CL5003, and *Candidatus_division* SWB02) may be considered as the reservoir of ARGs. It chiefly includes genes of six antibiotic resistance pathways (β-lactam, vancomycin, multidrug resistance, tetracycline, aminoglycoside, and macrolide resistance pathways). Therefore, Ganga River water samples have a profusion of ARGs consisting of beta-lactam, multidrug, and tetracycline antibiotics.

Culturable method for exploring bacterial community and antibiotic resistance in water and sediment samples of the Ganga River

Overall analysis revealed that the colony-forming unit (CFU) and Most Probable Number (MPN) counts were comparatively higher in the downstream regions (Uttar Pradesh) than in the upstream regions (Uttarakhand) of the Ganga River water and sediment samples. It indicates a high bacterial load in the Ganga River water and makes it unsuitable for drinking. MPN count was also observed high in the Ganga

River water sample of the downstream (Uttar Pradesh) region (>1800 MPN/100 ML), however, the sediment samples of Kanpur and Varanasi regions showed higher MPN values (>1600 MPN/100ML), Allahabad (920 MPN/100ML), and Rishikesh sediment samples showed a negative MPN test. Morphological characterization identified 74 distinct bacterial isolates from water and sediment samples of the Ganga River. Gram-staining revealed a higher count of Gram-negative (52.70%) bacteria as compared to the Gram-positive (47.30%) bacteria. A 16S rRNA-based sequence analysis of 20 morphologically different bacteria categorized them into three different phyla (Firmicutes, Proteobacteria, and Bacteroidetes) and five different genera (*Bacillus, Pseudomonas, Enterobacteriaceae, Bacteroides, Staphylococcus,* and *Exiguobacterium*) which dominates in the Ganga River. Firmicutes harbor Gram-positive heterotrophic bacteria involved in nutrient cycling and their abundance may also be correlated with fecal pollution. Further, the prevalence of these observed genera in the Ganga River water and sediment samples hypothesizes severe domestic pollution due to the heavy disposal of sewage and agricultural waste into the river. At the species level, *Stutzerimonas stutzeri* (also known as *Pseudomonas stutzeri*), *Bacteroidetes caccae, Enterobacter hormaechei subsp. xiangfangensis strain SUE25*, and *Bacillus cereus* constitute the most dominant species observed in the present study. The abundance of such species in the Ganga River indicates the heavy contamination of fecal matter in the Ganga River. The occurrence of *Staphylococcus aureus* and *Pseudomonas aeruginosa* observed in this study further affirms that water is not drinkable and acts as a reservoir of pathogens.

Disc diffusion test revealed that out of seventy-four, seventy-two bacterial isolates showed resistance towards more than two antibiotics, thus, can be considered as multidrug resistance (MDR) bacterial isolates. >50% resistant pattern was observed for clotrimazole, bacitracin, ampicillin, polymyxin, vancomycin, and rifampicin antibiotics. The multiple antibiotic resistance (MAR) index of 72 isolates of the 74 bacteria studied was > 0.2, which indicates a high risk of antibiotic contamination, resistance potential, and prevalence of pathogenic bacteria in the surrounding environments. Whereas, Minimal inhibitory concentration (MIC) concentration of ampicillin (8.1 mg) was the highest among the bacterial isolates obtained from the Ganga River followed by vancomycin and chloramphenicol antibiotics. A higher dominance of antibiotic-resistant *Staphylococcus aureus* was also observed in the sediment samples from the Ganga River water from an anthropogenically disturbed region which is a threat and needs to be further examined. Further, a higher abundance of antibiotic-resistant bacterial genera was observed in the Ganga River water sample as compared to the sediment samples as the water sample's bacterial isolates showed high resistant pattern towards antibiotic eight different antibiotics (Ciprofloxacin (15.90%), Vancomycin (61.12%), Ampicillin (88.63%), Tetracycline (52.27%), Rifampicin (84.09%), Erythromycin (43.18%), Clotrimazole (97.72%), and Kanamycin (15.90%). Whereas, the sediment sample's bacterial isolates showed high resistant patterns towards only three antibiotics (Bacitracin (96.87%), Polymyxin (78.12%), and Chloramphenicol (34.37%). The present investigation also confirms the presence of β-lactam resistant bacteria in the Ganga River water by amplifying the *blaSHV* gene in the bacterial isolates (50%) of the Ganga River.

Conclusion of the study

The present findings provide a broader interpretation of the bacterial microbiome of the Ganga River water and sediment samples for their strong correlation with prominent antibiotic-resistance genes. The investigation concludes that the Ganga River water exhibits high bacterial richness in the DS regions over the US region of water samples. Several pathogenic bacterial genera such as *Pseudomonas, Acinetobacter, Rheinheimera, Brevundimonas,* and *Gemmatimonas* have also been observed in the Ganga River water sample in the US and DS groups which is a serious concern. The prevalence and co-occurrence of Gram-negative, chemo-organotrophic bacteria in the DS group indicate over-mineralization of the Ganga River. It confirms the consequences of agricultural run-off and industrial waste disposal in the river. These findings represent new insights into the microbiology of the Ganga River water, suggesting a potential role for bacteria in transforming C-, N-, and P-bound organic matter. Moreover, the dominance of Pseudomonadata species in the Ganga River's US region is evidence of high anthropogenic activity, primarily due to domestic wastewater disposal and sacred bathing. Additional evidence for a significant antibiotic load in the Ganga River, particularly in the DS region, comes from the prevalence of multiple antibiotic resistance genes among dominant genera. Thus, over-mineralization and routine practice of antibiotic usage also significantly altered the bacterial microbiome of the Ganga River. Further, the sediments of the Ganga River are heavily populated with diverse Gram-negative, chemolithotrophic bacteria. The prevalence of sulphur, hydrogen, and alkane/alkene oxidizers defines the broad physiological behavior of inhabitant bacteria of the Ganga River sediment. Several of these bacteria have already been reported for their role in degrading pollutants and transforming heavy metals. The functional level analysis also recapitulates the bacterial profiling obtained here. Where, the majority of the genes were observed in the degradation of benzoate, nitrotoluene, dioxin, atrazine, and styrene-like compounds. It indicates the unregulated disposal of industrial effluents and municipal sewage in the Ganga River. The findings of the study may be explored for designing better treatment strategies for the Ganga River water management. Comparative analysis between water and sediment of the Ganga River unveiled several shared and unique taxa at the level of phyla and genera. It was further observed that the sediment harbors a higher level of species richness over the water samples. Genes of antibiotic resistance pathways were also relatively more prevalent among sediment samples over the water. Vancomycin resistance genes were significantly more abundant in the sediment samples, whereas β-lactam resistance genes were equally shared in water and sediment. It indicates that the sediment harbors a reservoir of ARGs by providing a less disturbed niche to bacteria for evolving mechanisms to cope up with the present threats of antibiotics. The members of *Flavobacterium, Pseudomonas, Acinetobacter, Candidatus_divison* CL5003, and *Candidatus_division* SWB02 may be considered reservoirs for such ARGs. A culturable approach study revealed a high bacterial load in the downstream Ganga River water and sediment samples. Of them, most of the bacteria exhibited antibiotic resistance to several antibiotics. MAR index analysis of the isolated bacteria showed a high risk of antibiotic contamination and resistance potential. Further, water sample bacterial isolates have high resistant potential for antibiotics than sediment samples of the Ganga River. The prevalence of antibiotic-resistant bacterial isolates such as *Staphylococcus aureus,* and *Pseudomonas aeruginosa* observed in this study in the Ganga River is a serious concern.

The gradual decline of the Ganga River's water quality in both upstream and downstream regions highlights the need for strict implementation of policies to protect India's most sacred and valuable river. This study will assist in monitoring the water quality and develop the model to assess antibiotic resistance threat among bacteria of this holy river. Monitoring of domestic waste and agricultural runoff must be emphasized. This investigation, therefore, paves the way to study the ARGs profile among these candidates using traditional cultivation approaches along with their dissemination mechanisms. The findings can be further employed to make the policies for regulating the consumption as well as disposal of reported ARGs here. Several human-associated risk factors among inhabitant bacteria of the Ganga River need sincere efforts to monitor the quality of the most sacred river in India. More extensive research is therefore required to further establish the correlation between the inhabitant bacteria in developing antibiotic resistance mechanisms.

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