LGU Journal of
LIFE SCIENCES

LGU Society of Life Sciences

DOI: https://doi.org/10.54692/lgujls.2023.0702264

Research Article
Vol 7 Issue 2 April - June 2023

LGU J. Life. Sci
ISSN 2519-9404
eISSN 2521-0130

In Silico Comparative Metagenomic Analysis of Microbial Communities of Chromium Contaminated Sites

Rabia Sadiq[1], Nazia Kanwal[2], Yasir Rehman*[1]

1 - Department of Life Sciences, School of Science, University of Management and Technology (UMT), Lahore, Pakistan.
2 - Department of Biological Sciences, The Superior University, Lahore, Pakistan.
Corresponding Author's Email: Yasir.rehman@umt.edu.pk

ABSTRACT: Chromium is one of the highly toxic and carcinogenic heavy metals. Due to increased anthropogenic activities, high concentration of chromium is found in many areas. Many microorganisms have the ability to detoxify chromium. Metagenomics allow us to comprehensively study microbial communities present at different sites without culturing them. The objective of this study was to analyze the abundance of microbial groups in different environments contaminated with chromium. For this purpose, chromium contaminated soil, anaerobic sludge and reactor samples were chosen. 16S rRNA data of these samples was retrieved from NCBI SRA database. The sequences were analyzed by Mothur software accessed via Galaxy server, and were classified using SILVA database. Venn diagram, phylogenetic tree, heatmap, relative abundance graphs and Krona pie charts were generated. Statistical analysis was also performed in the form of AMOVA and HOMOVA tests. According to results of our study, *Proteobacteria*, *Leucobacter*, *Actinomycetales*, *Actinobacteria*, *Arthrobacter*, *Rhizobiales*, *Sphingomonas*, *Bradyrizobium* and *Nucardioidaceae* were present in all the samples. *Firmicutes*, *Planctomycetes*, *Verrucomicrobia* and *Bacteroidetes* were more abundant in chromium contaminated samples as compared to control samples. The results were also found to be statistically significant. The above-mentioned bacteria can be targeted and studied to discover their roles in bioremediation of chromium contaminated sites.

Keyword: Chromium; bioremediation; metagenomics; bacteria; microbial community analysis

INTRODUCTION

Heavy metals are of great interest in today’s era (Joseph et al., 2019). Despite of their toxic properties, they are still being used widely in different industries, putting the environment at risk (Esmaeili and Beni, 2108). Until now, 53 elements have been identified as heavy metals as they have densities greater than or equitant to 5g cm^{-3} and atomic weight more than 20. Due to increased industrial effluents, highly urbanized societies and over-population, many hazardous materials in the environment are increasing, causing serious health problems (Li et al., 2019). Chromium (Cr) is one of the naturally existing heavy metal having a molecular weight of 51.1 a.m.u and density of 7.19 g cm^{-3}. It is 17th most abundant element on Earth and is highly reactive to oxygen presence in air, and hence various oxidation states have been reported ranging from 0 to +6 (Oliveira, 2012). Among all other, Cr (III) trivalent and Cr(VI) hexavalent are considered as most stable forms of Cr in the environment. Different industrial processes like burning of coal and oil, drilling of oil wells, metal refining, leather industries, stainless steel production, and chemical dye production use Cr (Coetzee et al., 2020). Cr contamination via air emissions and industrial effluents reach the local population and affects them (Welling et al, 2015). Cr is considered as Group I carcinogen by the International Agency for Research on Cancer (Ray, 2016). Exposure to high concentration of Cr can have detrimental health effects on human beings as it may affect the working of different organs such as lungs, kidney, liver and brain, and can results in different types of cancers as well. Studies have also shown that long term exposure to Cr can cause muscular and neurological alteration, Alzheimer’s disease, Parkinson’s disease, muscular dystrophy and even cancer (Pushkar et al., 2021).

Cr affects the microbes in various ways and can alter the composition of microbial communities (Pei et al., 2018). However, many microbial species are known to exhibit resistance against Cr and can detoxify it. Different microbial remediation techniques (bioremediation) such as biosorption, bioaccumulation, biotransformation, and bioleaching have been employed to remove Cr and other heavy metals from industrial wastewaters (Fernández et al., 2018).

Metagenomics is an approach which allow us to analyze directly the extracted genetic materials from sites and decipher the information of uncultivated microbial communities present in them. It can be a good tool to trace out the microbes and the genes involved in bioremediation (Datta et al., 2020). Next Generation Sequencing technologies (NGS) have made possible the enormous data output from metagenomics samples. This gives a very comprehensive picture of the microbial communities, both culturable and non-culturable. *In silico* metagenomic analysis plays a crucial role in unraveling the intricate complexities of microbial communities and their functions within diverse ecosystems. By harnessing the power of computational tools and algorithms, this approach allows researchers to study the genetic content of entire microbial populations without the need for culturing individual species. Such metagenomics analysis can provide insights into the diversity, composition, and functional potential of microbial communities, shedding light on their roles in nutrient cycling, disease development, and environmental processes (Mitra et al., 2015). Such *in silico* studies can serve as a powerful tool for advancing our knowledge of microbial ecology and can pave the way for the development of targeted interventions and strategies for harnessing the potential of these complex microbial communities (Llorens-Marès et al., 2015).

The objective of the present study was to compare microbial communities of different sites contaminated with Cr in order to find out the bacteria common in all those sites. Such bacteria might have a role to play in Cr detoxification. For this purpose, 16S rRNA gene sequences of metagenomes of Cr affected microbial communities were downloaded from internet databases and were analyzed comparatively.

Methodology

Sample selection

Soil, anaerobic sludge and bioreactor sites contaminated with Cr were selected for this study. Sequence data published on National Center for Biotechnology Information (NCBI) Short Read Archive (SRA) was downloaded with accession numbers. A total of 24 samples were randomly selected. Out of these 24 samples, 9 samples were of soil, 4 were of reactor, 5 samples were of anaerobic sludge and 6 were selected of control soil (Table 1)

Table 1. List of samples retrieved from NCBI SRA

No	Sample	SRA	No. of spots	Published
1	Soil	SRR12524814	89,977	2020-12-01
2	Soil	SRR12524811	90,490	2020-12-01
3	Soil	SRR12524812	85,982	2020-12-01
4	Soil	SRR12524829	72,900	2020-12-01
5	Soil	SRR12524822	92,943	2020-12-01
6	Soil	SRR12524821	90,379	2020-12-01
7	Soil	SRR12524813	87,836	2020-12-01
8	Soil	SRR12524805	97,779	2020-12-01
9	Soil	SRR12524803	83,440	2020-12-01
10	Reactor	DRR218029	21,232	2020-09-03
11	Reactor	DRR218030	33,806	2020-09-03
12	Reactor	DRR218031	25,714	2020-09-03
13	Reactor	DRR218032	27,722	2020-09-03
14	Anaerobic Sludge	SRR10015225	48,497	2019-08-26
15	Anaerobic Sludge	SRR10015226	51,373	2019-08-26
16	Anaerobic Sludge	SRR10015227	48,540	2019-08-26
17	Anaerobic Sludge	SRR10015228	44,026	2019-08-26
18	Anaerobic Sludge	SRR10015229	47,111	2019-08-26
19	Control	SRR16005226	54,660	2021-09-22
20	Control	SRR16005227	41,830	2021-09-22
21	Control	SRR16005228	43,288	2021-09-22
22	Control	SRR16005233	51,433	2021-09-22
23	Control	SRR16005234	47,857	2021-09-22
24	Control	SRR16005235	62,514	2021-09-22

All the samples had been sequenced through Illumina MiSeq paired end sequencing technology. The data selected was comparable to each other according to 16S rRNA gene region sequenced (V3-V4), base numbers, number of spots, etc.

Processing of sequences:

The processing of the sequences and the analysis was performed in Mothur software (Schloss et al., 2009) through Galaxy server (https://usegalaxy.org/). All the collected and downloaded data and the reference files [such as Silva.v4.fasta for alignment, Trainset9_032012.pds.fasta and Trainset9_032012.pds.tax for classification obtained from Mothur Miseq SOP website (https://mothur.org/wiki/miseq_sop/)] were uploaded on the Galaxy server. Contigs were made by aligning both reverse and forward files of the data. The data was cleaned by eliminating all unnecessary sequences and by reducing the number of ambiguous reads by using commands such as screen.seqs and unique.seqs

Sequence Alignment

Sequences were aligned to the SILVA reference file of 16S rRNA dataset named “Silva.v4.fasta” (Quast et. al., 2012). It improves the clustering of the sequences. Any read which did not overlap the V4 region of 16S rRNA gene was removed. The next step was to merge near-identical sequences together to further reduce the spread of the data. Sequences that differed by 1 in every 100 bases were likely to represent sequencing errors, not true biological variation, and therefore these were combined to further reduce the data.

Taxonomy classification

After the alignment of the sequences, next was to assign taxonomy to the sequences. For this *classify.otu* command was run using RDP reference dataset *Trainset9032012.pds.fasta* (Cole et al., 2014). *Remove.lineage* command was used to remove the undesirables such as the sequences belonging to chloroplast, mitochondria, unknown groups and eukaryota. After this *Summary.seq* was run to summarize the quality of sequences.

OTU clustering

For this, *Cluster.split* command was used which assigned the sequences to OTUs and to split large matrices of the data to reduce further computation. To find out how many sequences are in each OTU from each group, *make.shared* command was used. Then *classify.otu* command was used to learn about the taxonomy for each of OTUs. For this step, level 0.03 was considered which actually means 97% similarity threshold or specie level. *Count.group* was performed to find out the number of sequences in each sample.

Diversity Analysis

Diversity analysis was performed in two ways, alpha and beta diversity analysis. In alpha diversity analysis, *summary. single* command was used to calculate the diversity indices of the Cr-contaminated sites. For beta diversity analysis, heatmap tool was used which is a graphical representation in which shades of red and black are used to depict the extent of similarities and dissimilarities between the samples. *Venn diagram* was used to determine species richness and diversity. Phylogenetic tree was made to find out how different sites are grouping with each other based on their sequence similarities.

Krona pie chart

It was used to visualize the proportions of all the bacteria present in the samples. For this purpose, *input file* was selected as *"Taxonomy-to-krona"*. In first krona pie chart, all the samples were plotted together to see the diversity of all the bacteria present in the samples. Krona pie chart of each sample was also made to visualize diversity of bacteria in each sample.

Relative Abundance

To further find out the relative abundance of the bacteria present in control and Cr-contaminated sites visually, relative abundance bar graph was generated by using biom (biological observation matrix) format which is used to represent OTUs. The results were visualized and analyzed in Phinch tool https://www.phinch.org/.

Statistical Analysis

To assess whether the diversity of bacteria present in control and different Cr contaminated sites have statistically significance differences, AMOVA test was performed. An alternative way to observe whether the spread of data differs between groups is by comparing the homogeneity of molecular variance (HOMOVA). It is molecular based statistical test. Parsimony analysis was also performed to compare communities with similar structure. All the statistical analysis was performed in Mothur software through Galaxy server, as mentioned above.

RESULTS

Cleaning, alignment and classification of sequences

A total number of sequences in the start were 1355509. The shortest sequence had a chain length of 36 base pairs and contained three polymer repetition. The longest sequence had 601 base pairs. The number of sequences decreased to 821612 after *screen.seqs*. The total number of sequences declined further as we removed unwanted, ambiguous and duplicated sequences. The total number of sequences gradually declined to 356249 in the end, with the longest sequence having a chain length of 636 base pairs. On alignment with the reference database silva.v4.fasta, most of the sequences aligned starting at base pair location 638. The analysis was further conducted by clustering the samples into operational taxonomy units (OTUs) and classifying them.

Diversity Analysis

Alpha diversity analysis

The command *summary.single* showed different diversity metrics that indicated the species diversity and richness in the samples (Table 2).

Table 2: Diversity indices of the control and chromium contaminated samples

Sample	Chao	Simpson	Shannon
ChromiumAS1	3945.778	0.592248	1.841371
ChromiumAS2	2953.97	0.213452	3.036724
ChromiumAS3	961.2419	0.278226	3.126684
ChromiumAS4	839.8261	0.033787	4.356874
ChromiumAS5	7672.648	0.014806	5.572186
ChromiumS1	48263.94	0.00203	7.74438
ChromiumS2	5309.645	0.002695	6.82159
ChromiumS3	42452.19	0.002428	7.539418
ChromiumS4	38661.15	0.005417	7.305137
ChromiumS5	51454.69	0.001781	7.841346
ChromiumS6	43433.49	0.005209	6.962666
ChromiumS7	39666.46	0.005297	7.55263
ChromiumS8	49367.84	0.003491	7.606628
ChromiumS9	49650.59	0.001852	7.822735
Control01	5707.387	0.036701	5.5797
Control02	4952.492	0.059163	5.285571
Control03	5505.077	0.041767	5.465372
Control04	6047.503	0.025453	5.767816
Control05	8176.775	0.022387	6.058845
Control06	10200.18	0.016929	6.131441

Shannon index indicated the richness and evenness and according to results, in anaerobic sludge site highest value was 5.57219 of sample ChromiumAS5 and lowest value was of sample ChromiumAS1. In soil site, highest value was of ChromiumS5 sample and lowest value was of ChromiumS2 sample. Chao indicated the total richness in each sample, the results showed that in anaerobic sludge site the sample ChromiumAS1 had the highest value and ChromiumAS4 showed the lowest value. While in soil site, ChromiumS5 was the sample having highest value and ChromiumS2 showed the lowest value. Simpson showed the diversity of the bacteria, and in anaerobic sludge the sample ChromiumAS3 had the highest diversity and ChromiumAS5 had the lowest. While in soil, ChromiumS4 sample showed highest diversity and ChromiumS5 showed the lowest value.

Beta diversity analysis

In diversity analysis, different tests were used to find the similarities and differences between the samples. In Heatmap analysis, shades of red and black colors showed that whether the samples were more similar or dissimilar (Fig. 1).

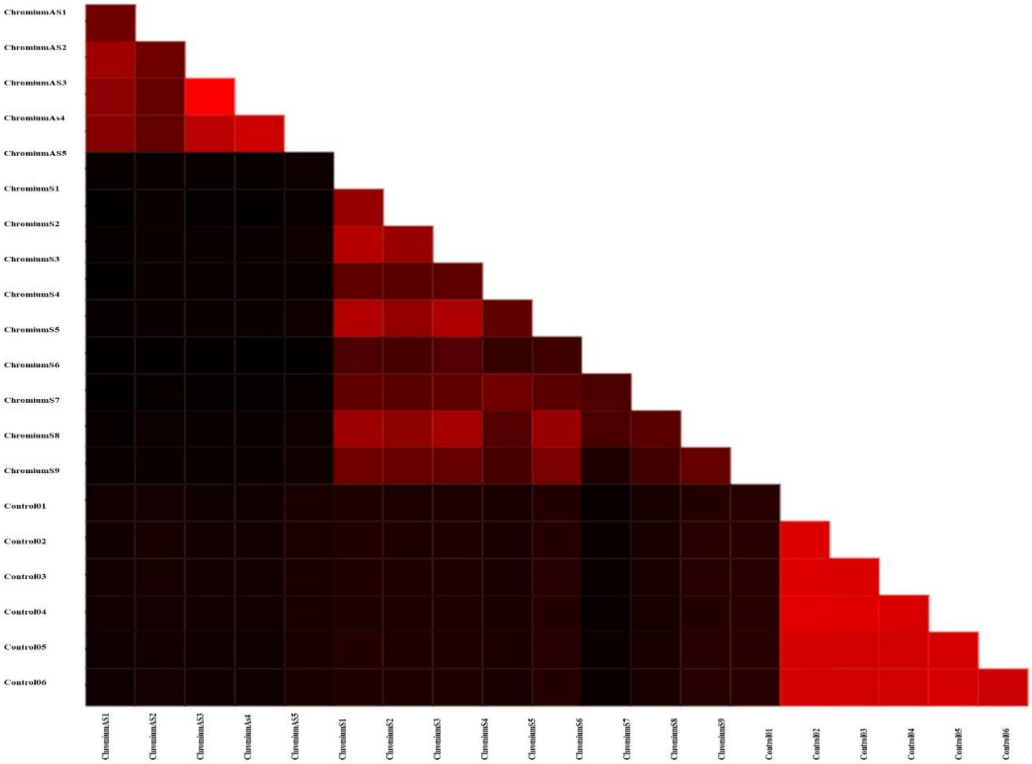

Fig. 1. Heatmap of the control and chromium contaminated samples

Brighter red shades indicated high similarities between the samples whereas darker shades indicated higher differences between the samples.

According to results, samples of anaerobic sludge showed higher dissimilarities as compared to the samples of soil. Where Cr-contaminated soil samples showed dissimilarities when compared to the control samples. Venn diagrams were created based on a four-way analysis of samples to determine the number of sequences common in different samples (Figure 2). The similarity among bacterial diversity was represented by nine different Venn diagrams each comprising of 4 random samples. The overlapping circular areas in each Venn diagram indicated the number of OTUs common in those samples. 129 OTUs were found to be common in ChromiumAS5 and ChromiumAS3 samples, and 48 OTUs were common in ChromiumS6 and ChromiumS9 samples. No common OUT was found among ChromiumS6, ChromiumAS3 and ChromiumAS5 samples (Figure 2). Similarly, 220 OTUs were found common in Control01 and Control04 samples. Overlapping of Control04, Control01 and ChromiumAS3 7 common OTUs. When Control04, ChromiumS6 and ChromiumAS3 samples were compared, no common OTUs were found (Fig. 2).

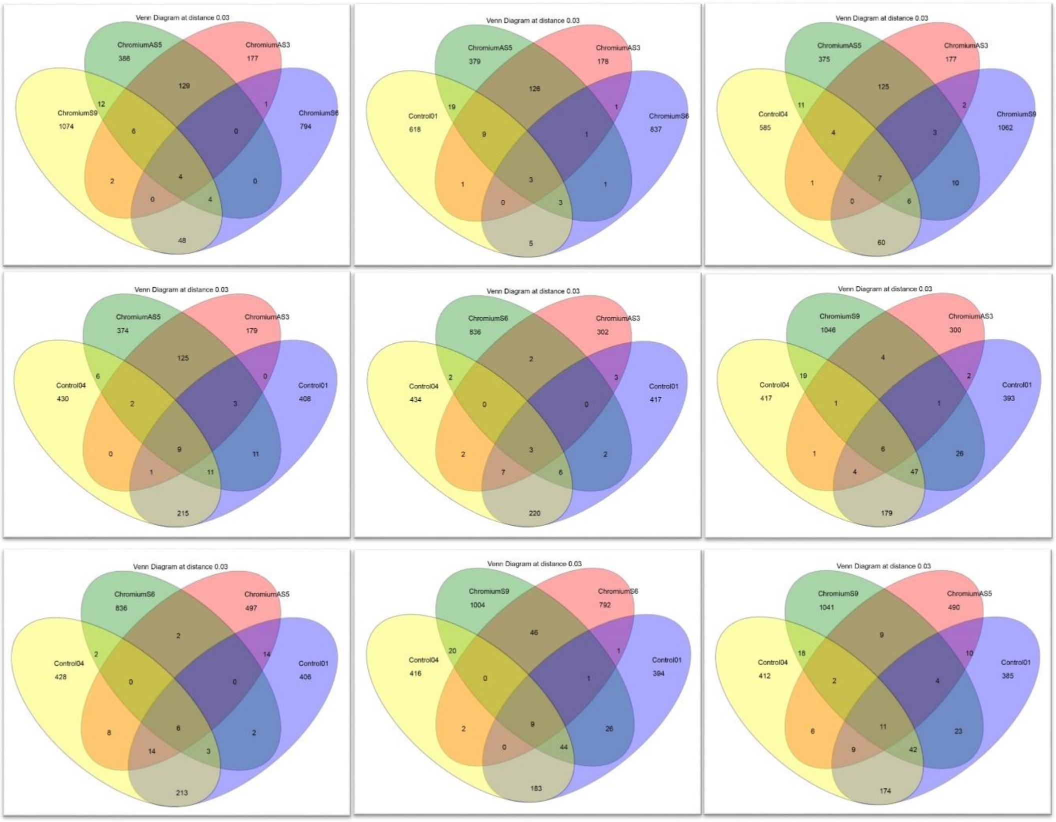

Fig. 2. Selected Venn diagrams showing common and exclusive number of sequences in different samples

A phylogenetic tree was generated to determine how the samples group with each other based on the similarity of the sequences (Fig. 3).

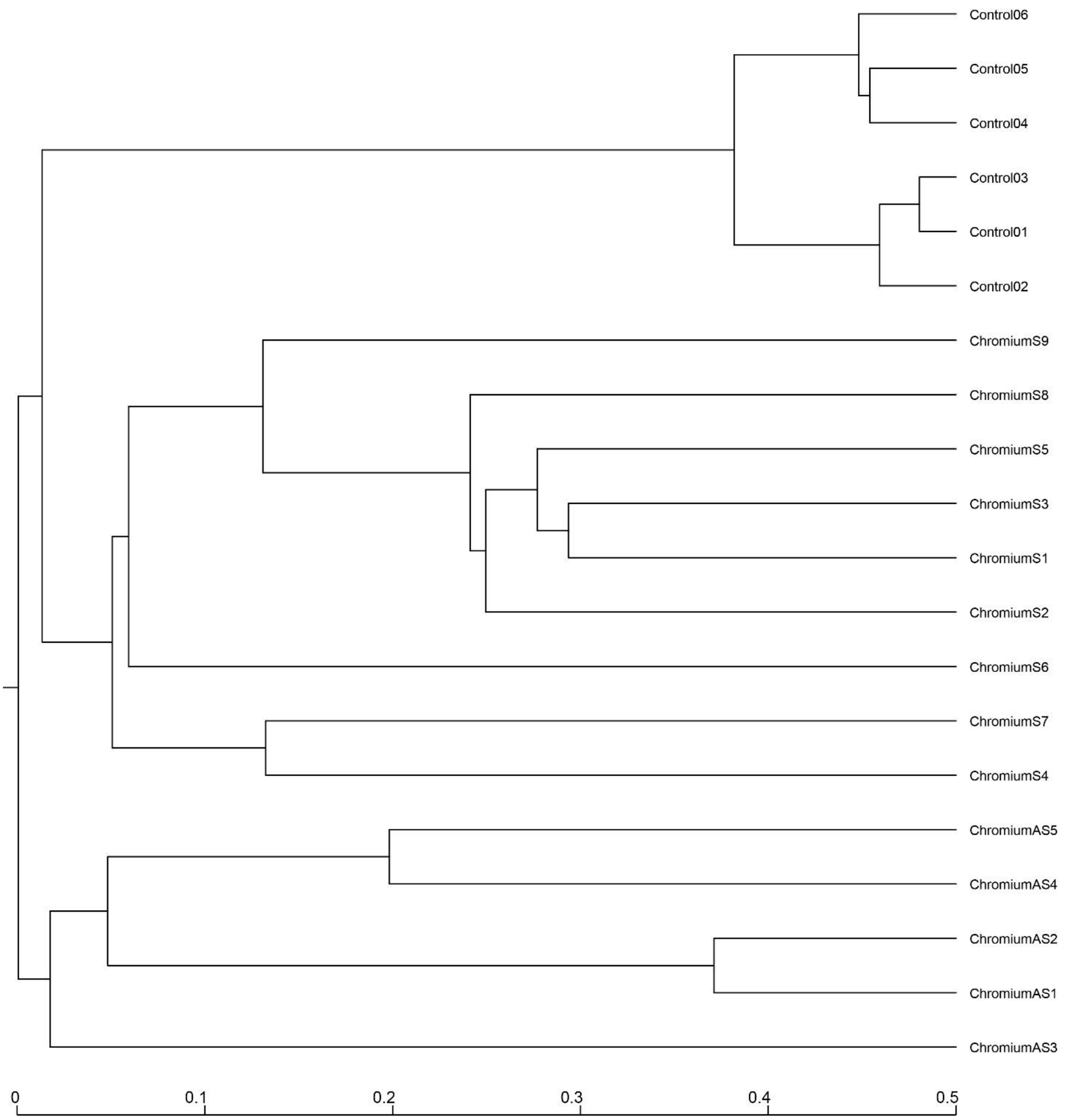

Fig. 3. Neighbour-joining Phylogenetic showed grouping of the samples based on their sequences similarities

The samples ChromiumS1, ChromiumS5, ChromiumS3 and ChromiumS8 showed more similarities with each other as compared to other samples.

Krona pie chart Analysis

Krona pie chart was used to visualize the microbial composition of the samples (Fig. 4).

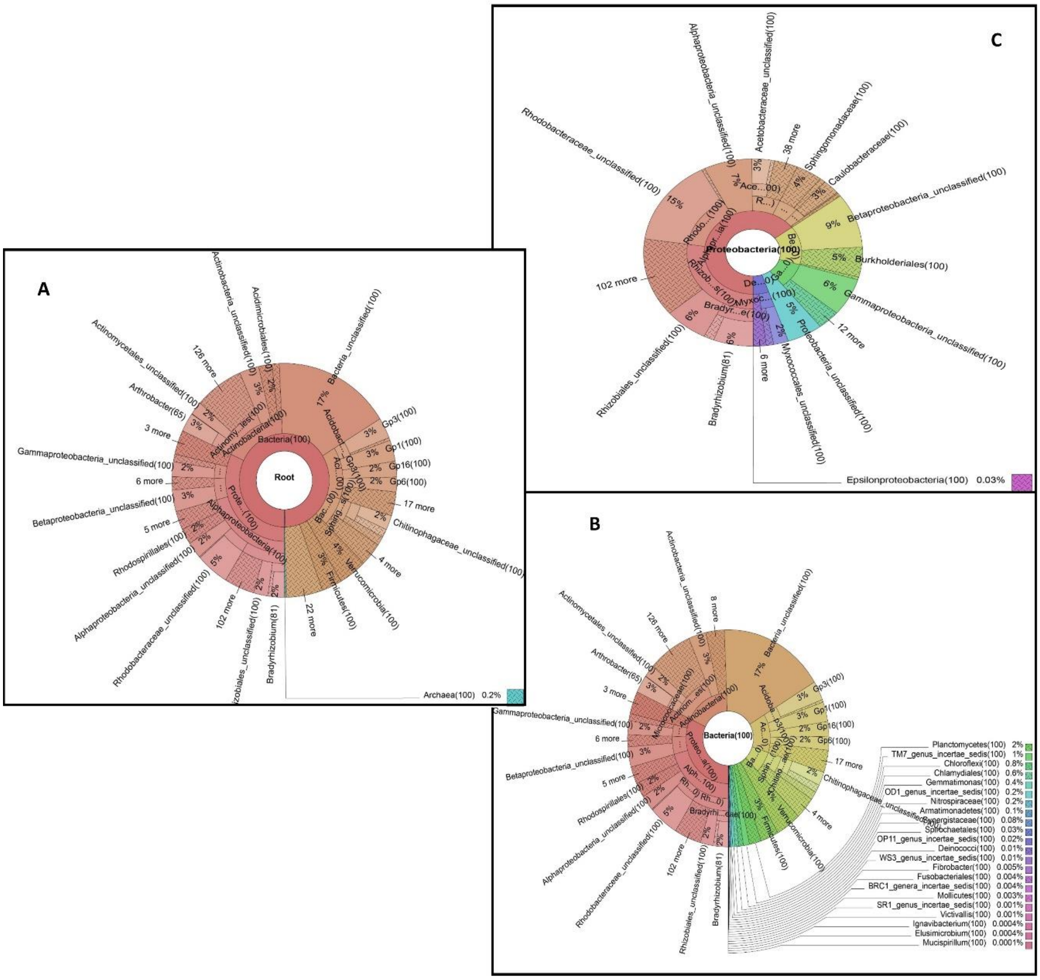

Fig. 4. Krona pie charts showing proportions of different microorganisms in the samples. **A:** the percentages of all the microorganisms, **B:** the percentage of different bacterial groups, **C:** the percentage of different groups of proteobacteria

In krona pie chart, total number of sequences were 703092 out of which 701597 sequences (99.8%) were classified as bacterial sequences, and 1495 sequences (0.2%) were classified as archaeal sequences. In the bacterial domain, 224779 (32%) classified sequences belonged to *Proteobacteria* (7% unclassified sequences of Alphaproteobacteria, 9% unclassified sequences of Betaproteobacteria, 6% unclassified sequences of Gammaproteobacteria, 1% unclassified sequences of Deltaproteobacteria). Unclassified sequences of *Acidobacteria* were 24%, *Actinobacteria* were 3%, and *Bacteriodetes* were 2%. Acidimicrobiales classified sequences were 2%. *Arthrobacter* classified sequences were of 3%. Unclassified *Rhodobacteraceae* sequences were 5% while classified *Planctomycetaceae* sequences were 2%.

Relative abundance

Relative abundance was determined by using biom file and was visualized in Phinch software (Fig. 5).

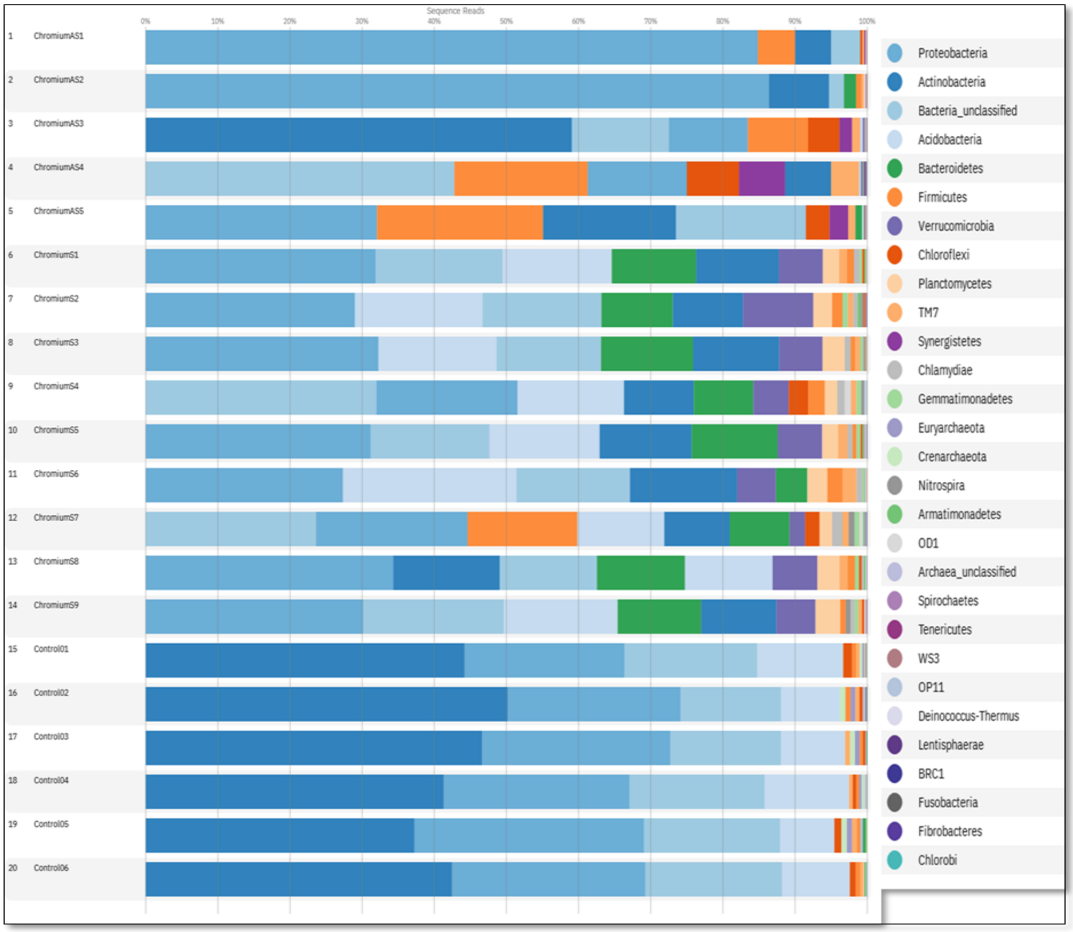

Fig. 5. Relative abundance of different bacterial groups in the control and chromium contaminated samples

TableAccording to it, different Cr (anaerobic sludge, control and soil) samples differed by the percentages of bacteria present in them. Figure 5 represents the percentage and relative abundance of major bacterial taxa found in the samples.

The *Actinobacteria* were present in all the samples but were most abundant in ChromiumAS3 and control samples. *Proteobacteria* were present in all samples but were most abundant in ChromiumAS2 sample. *Acidobacteria* were present in all soil and control samples but were not detected in anaerobic sludge samples. *Firmicutes* were also found in all the samples but were most abundant in the sample ChromiumS7. Another bacteria *Verrucomicrobia* was present in samples of anaerobic sludge and soil but was found in very less numbers in control samples. *Planctomycetes* bacteria was abundant in soil samples but it was either absent or in very few numbers in anaerobic sludge and control samples. High abundance of *Planctomycetes* was found in ChromiumS9 samples. *Chloroflexi* was one of the bacteria which were present in all samples, but in some samples it was found in very numbers while in other samples it was found in large proportions. In anaerobic sludge ChromiumAS4 sample, *Chloroflexi* was abundant while the least amount of this bacteria was found in soil samples (ChromiumS3 and ChromiumS6). In some samples the presence of *Bacteriodetes* was abundant while in others these bacteria were found in very low numbers. In soil sample ChromiumS3, *Bacteriodetes* was found in abundance.

Firmicutes, Planctomycetes, Verrucomicrobia and *Bacteroidetes* were present in very low numbers in control, while were abundant in all Cr-contaminated samples. Relative abundance of *Acidobacteria* and *unclassified Bacteria* in control and Cr contaminated sites were almost similar.

Statistical analysis

Statistic tests AMOVA, HOMOVA and Parsimony were performed. When AMOVA was applied on all the samples, and when comparing samples of any two sites at a time, every time the p-value obtained was <0.001, indicating that the difference between the samples was statistically significant.

In HOMOVA, the results showed that the p-value was less than 0.01 which determined it as significant. It showed that all the samples were different to each other. Parsimony was a general test for comparing communities with similar structures. It could only specify the probability of groups having the same structure but did not indicate the level of similarity.

DISCUSSION

Exposure to high concentrations of Cr can alter the physiological and biological functioning of living organisms (Urbano et al., 2012). Metagenomics is an advance technique by which we can extract total DNA of microbial communities directly from the sample without culturing the microbes. In order to determine microbial diversity of Cr contaminated sites in this comparative metagenomic study, 16S rRNA sequence data was used and analyzed through Galaxy server (Kozich et al., 2013). 16S rRNA sequence data was chosen as it is both highly conserved and has variable regions as well, and huge reference databases are available. All these samples had been sequenced via Illumina sequencing technology. Next generation sequences (NGS) techniques are very strong and effective tools to find out comprehensive information of complex prokaryotic communities.

In this analysis, Silva reference file was used for alignment of the contigs of 16S rRNA genes, whereas RDP based database file was used for the purpose of classification of OTUs. Diversity analysis was performed in two ways: *alpha diversity* (number of species coexisting within a local site) and *beta diversity* (the magnitude of similarity in species composition among different sites) (Zhou et al., 2020). In heatmap analysis, the brighter shade of red color showed more similarities between samples while the darker shade showed more dissimilarities. According to this graphical representation, number of bacteria which are more dissimilar were abundant among the samples, while a few samples showed similarities of bacteria. Neighbour joining Phylogenetic tree was made to find out similarities and dissimilarities among the samples. According to this analysis, the samples that grouped together were more similar e.g., ChromiumAS3, ChromiumAS1 and ChromiumAS2 were more similar to each other and dissimilar to the other samples and control. Diversity indices such as Chao, Simpson and Shannon were also determined. Chao indicates the total richness of microbial communities, Shannon indicates the richness and evenness while Simpson shows the diversity. To further analyze the data, relative abundance graph was generated and visualized in Phinch software. It was found that although the relative abundance of bacteria varied from sample to sample, but similarities were also present.

In one of the previous studies about Cr contaminated soil, Actinobacteria and Proteobacteria (Alphaproteobacteria and Gammaproteobacteria) were found to be the important phyla showing resistant to Cr toxicity at different contamination levels. *Lactobacillus*, *Pseudomonas*, *Nitrospira*, *Clostridium*, *Bacillus*, and *Escherichia* were found to be the dominant genera in active mining areas (Pradhan et al., 2020). While according to another study *Ochrobactrum* sp. and *Microbacterium* sp. were rich in abundance (Kao et al., 2021). According to our results of krona pie chart, *Proteobacteria* (Alphaproteobacteria, Betaproteobacteria, Gammaproteobacteria) were abundant in all samples of Cr contaminated sites and less in control. Most of the studies showed *Pseudomonas* is one of the best potential microbe for the bioremediation of Cr. As Arisah et. al. (2021) stated that *Pseudomonas* showed high efficiency of Cr (VI) removal, up to 85%. Another study by Gong et. al. (2020) investigated Cr removal efficiency of *Pseudomonas* sp. and according to this study the bacteria was able to resist high concentrations of Cr. Under the optimal conditions, the removal rate of Cr was 94.26% in contaminated soil. One of the research by Sousa et. al. (2023) evaluated bioremediation of Cr by *Rhodobacter* sp. According to the results of this study, under optimal conditions this bacteria showed complete removal of hexavalent Cr. Rajyalaxmi et al. (2019) reported the potential of the photosynthetic bacterium *Rhodobacter* to remediate hexavalent Cr. According to this study, *Rhodobacter* sp. showed reduction of Cr(VI) up to 35 µM on 8th day of its incubation under anaerobic light conditions. Some researchers have also reported the presence of Rhizobiales in Cr contaminated soil (Araujo et al., 2023). Relative abundance bar graph in our study showed that samples of Cr contaminated soil had higher abundance of Proteobacteria, Acidobacteria and Bacteroidetes, while Actinobacteria, Verrucomicrobia, Planctomycetes, Chlamydiae, Gemmatimonadetes, Nitrospira, Firmicutes and Armatimonadetes were found in less abundance.

According to one of the studies about microbes present in heavy metal contaminated anaerobic sludge, Proteobacteria, Firmicutes, Bacteroidetes, and Thermotogae were observed in high percentage as compared to other species of bacteria (Lim et al., 2017). In our study, anaerobic sludge sites showed that Proteobacteria and Actinobacteria sp. were highly abundant. while Bacteroidetes, Firmicutes, Acidobacteria, Verrucomicrobia, Terenicutes and Planctomycetes were present in lower proportions.

CONCLUSION

According to this study, *Chloroflexi, Firmicutes, Planctomycetes, Gemmatimonadetes, Verrucomicrobia* and *Bacteroidetes* were present in less numbers in control, while more in all Cr-contaminated samples. *Actinobacteria* were abundant in control while less in all other groups. Relative abundance of *Acidobacteria* and *Proteobacteria* in control and other Cr contaminated sites was almost similar. Bacteria which are abundant in Cr contaminated sites might have some role to play in detoxification of Cr. There is also strong need to focus more on functional genes of these bacteria related to Cr detoxification in order to find out better Cr remediation solutions.

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