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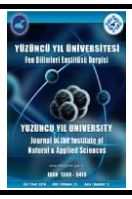

Research Article

A Comparative Study on Microbial Air Quality of Radiology and Patient Waiting Rooms of a Full-Equipped Hospital in Aydın Province

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Abstract: Every year many patients die due to hospital-acquired infections. These infections also put the lives of healthcare workers at risk. Additionally, hospitals are one of the main reservoirs of antimicrobial resistance. In this study, the effect of X-rays on species diversity and functions and antimicrobial resistance were observed. Metagenome analysis was performed on air samples taken from the x-ray room (radiation exposure) and the waiting room (non-radiation area) to examine whether the radiation affected the species present in the air. The diversity of microorganisms was analysed based on phylum, genus, and species levels. Functional profiling and resistance screening were also performed. X-ray radiation was found to have a major effect at the phylum level. It was observed that Proteobacteria species almost dominated the microbiome in the x-ray room (99%) while Actinobacteria species dominated the microbiome in the waiting room (84%). No significant differences were observed between the two areas in functional profiling. A total of thirty-eight functions were observed, twenty-four of which were overlapping. Antimicrobial resistance was not as diverse as expected. Only beta-lactam, penicillin, tetracycline, and lincomycin resistance genes were present (2364 reads belonging to four different genes). According to the results, it was observed that X-rays affected the air microbiome, as expected. A decrease in the number of microorganisms was expected, but it was also observed that the dominant microorganism types changed. On the other hand, no significant difference was found in terms of functional profiling and no significant antimicrobial resistance was observed.

Aydın İlindeki Tam Teşekküllü Bir Hastanenin Radyoloji ve Hasta Bekleme Odalarının Mikrobiyal Hava Kalitesinin Karşılaştırmalı Olarak İncelenmesi

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Öz: Her yıl çok sayıda hasta hastane kaynaklı enfeksiyonlar nedeniyle hayatını kaybetmektedir. Bu enfeksiyonlar sağlık çalışanlarının hayatını da riske atmaktadır. Ayrıca hastaneler, antimikrobiyal direncin ana rezervuarlarından biridir. Çalışmada X-ışınlarının tür çeşitliliği ve fonksiyonları üzerine etkisi ve antimikrobiyal direnç gözlemlenmiştir. Röntgen odasından (radyasyona maruz kalan) ve bekleme odasından (radyasyon olmayan alan) alınan hava örneklerine radyasyonun havada bulunan türleri etkileyip etkilemediğini görmek için metagenom analizi yapıldı. Mikroorganizmaların çeşitliliği şube, cins ve tür seviyelerinde analiz edildi. Ayrıca fonksiyonel profilleme ve direnç taraması

Anahtar Kelimeler
Hava kalitesi,
Hava mikrobiyomu,
Metagenom,
Röntgen odası

yapıldı. X-ışını radyasyonunun şube düzeyinde büyük bir etkisi olduğu bulundu. Röntgen odasında Proteobacteria türleri mikrobiyomu neredeyse domine ettiği (%99), bekleme odasında Actinobacteria mikrobiyomu domine ettiği görüldü (%84). Fonksiyonel profillemede iki alan arasında önemli bir fark görülmedi. Yirmi dördü örtüşen toplam otuz sekiz fonksiyon gözlemlendi. Antimikrobiyal direnç beklenilen kadar çeşitli değildi. Sadece beta-laktam, penisilin, tetrasiklin ve lincomycin direnç genleri mevcuttu (dört farklı gene ait 2364 okuma). Çıkan sonuçlara göre, beklenti doğrultusunda, X-ışınlarının hava mikrobiyomunu etkilediği gözlemlendi. Mikroorganizma sayısında azalma bekleniyordu fakat baskın mikroorganizma türlerinin değiştiği de gözlemlendi. Fonksiyonel profilleme açısından önemli bir fark bulunamamış ve önemli bir antimikrobiyal direnç görülmemiştir.

1. Introduction

Many factors are shaping microbial communities around us (O'Hara et al., 2017). These communities can harbour pathogens with serious effects on our health. The development of technologies like Next Generation Sequencing and metagenomic analysis gives us opportunities to analyze and understand these microbial communities (Roux et al., 2016). From homes to offices, humans spend most of their time in indoor environments. There are many studies related to the indoor microbiome (Lax et al., 2014; Lax & Gilbert, 2015; Lax et al., 2017). Indoor Air Quality (IAQ) is important to protect humans since they are directly affected by the air quality of the indoor environment (Leung & Chan, 2006). For hospitals, IAQ is more important because hospital-acquired infections (HAI) are considered one of the main reasons for patient deaths (Lax & Gilbert, 2015). Hospital air represents a reservoir for Hospital-acquired infections for both patients and healthcare workers (Alrazeeni & Al Sufi, 2014). Therefore, many studies focused on identifying hospital-associated pathogens and their transmission routes (Marinella et al., 1997; Bures et al., 2000; Akinyemi et al., 2009; Wiener-Well et al., 2011). Fewer studies were conducted on indoor air microbiome and their transmission routes (Li et al., 2021).

Antibiotics are used for the treatment of bacterial infections and to prevent bacterial infections (Adedeji, 2016). However, over time bacteria develop resistance mechanisms to these drugs once seen as miracle substances. Extensive antibiotic applications caused a crisis worldwide (Ventola, 2015). In addition to soil and water-borne resistant bacteria, air-borne resistant bacteria are also becoming a problem (Li et al., 2021). Several researchers focused their studies on airborne resistance (Li et al., 2018; Xie et al., 2018).

Many industries use UV, ionizing, and gamma radiation to inhibit microbial growth (Farkas & Mohácsi-Farkas, 2011; Kumar et al., 2012). Ionizing radiation is used in the food industry to prevent microbial spoilage (Farkas & Mohácsi-Farkas, 2011). X-ray radiation does not produce radioactive waste and can pass 30-40 cm thick objects (Oner & Wall, 2013). Studies of the effects of X-ray radiation go back over a century (Clark & Boruff, 1929). There are also studies on X-ray applications for foodborne pathogens like *E.coli* O157H7, *Listeria monocytogenes*, *Salmonella entrica*, and *Shigella flexneri* (Mahmoud, 2009, 2010 and 2012).

Due to the high rates of hospital-acquired and antimicrobial-resistant infections, the need to characterize hospital microbiomes increased. Radiology is one of the busiest departments in hospitals. There are always people coming in and out. Likewise, the waiting room in front of the X-ray room becomes more crowded as both patients and their relatives arrive. The workload of the X-ray room is between 350-400 patients per day on average. The number of patients who had X-rays taken during the time we sampled was eighty. This greatly increases the diversity of microorganisms. In this study, a metagenomic-based method was applied to identify microorganisms, analyze the functions of these microorganisms, profile the virulence factors and resistance genes in hospital indoor air, and compare the X-ray room with the waiting room air microbiome.

2. Material and Methods

2.1. Sample collection

Air samples were taken with the Merck MAS100 Air Sampler device. For each sampling, 50 liters of air were drawn at once from two different locations; one sample from the X-ray room (50 L) and one sample from the patient waiting room (50 L) which is considered to be a non-radiation area. Sampling was made between 10 am and 12 pm when the patient density was highest. Airborne particles smaller than 3 µm were directed into buffered peptone water (Merck, pH:7.4) using a modified air sampler (Whon et al., 2012).

2.2. Metagenome analysis

For metagenome analysis samples were sent to Eurofins Genomics (Germany) company. From both samples, taxonomic, functional profiling, and resistance screening were made.

DNA isolation, PCR, and sequencing were made by Eurofins Genomics company. DNA isolation of the samples was performed using GeneMark Bacterial Genomic DNA isolation kit. V3 (5′-CCTACGGGNGGCWGCAG-3′) -V4 (5′-GACTACHVGGGTATCTAATCC-3′) primer region was used for PCR of the samples (Klindworth et al., 2013). Illumina NovaSeq platform was used for sequencing (IlluminaTM, Inc., San Diego, CA, United States). The software used for analyzing metagenomic data is given in Table 1.

Table 1. Name, version, and description of relevant programs

Software	Reference	Description
Bowtie 2.2.9	Langmead et al., 2009	A short-read aligner with high memory efficiency. The Burrows-Wheeler transform algorithm serves as its foundation.
Kraken 0.10.5	Wood & Salzberg, 2014	Kraken is a program that assigns taxonomic names to metagenomic DNA sequences.
Krona 2.5	Ondov et al., 2011	By using zoomable pie charts, Krona enables the exploration of hierarchical data.
Picard 1.131	Anonymus, 2018	A Java-based command-line tool for parsing SAM/BAM files.
R 2.15.3	R team, 2010	R is a programming language and environment for statistical computing.
SAMTools 0.1.18	Li et al., 2009	SAMtools offers many tools for working with alignments in the SAM format.
Trimmomatic 0.33	Lohse et al., 2012	Trimmomatic performs a variety of useful trimming tasks for Illumina paired-end and single-end data.
Bamtools 2.3.0	Barnett et al., 2011	A compact yet potent set of command-line utility applications called BamTools are available for modifying and searching BAM files for information.
Sambamba 0.6.3	Tarasov et al., 2015	A high-performance, modern, dependable, and quick program (and library), Sambamba is used to work with SAM and BAM files.

Low-quality reads (a read is a small section of DNA) were removed before further processing. If the average quality is less than 15, bases were extracted. For the following phases, only pairs (forward and reverse read) were employed.

Non-host readings were then subjected to a taxonomic profiling technique following the removal of host sequence reads. Kraken (Wood & Salzberg, 2014) was used as a reference database for taxonomic profiling. Each read was divided into overlapping k-mers by Kraken to classify the reads. The genomes that make up each k-mer were mapped to their lowest common ancestor (LCA) in a reference database.

Read counts of samples detected at Phylum, Genus, and Species levels were collected and normalized by using the Vegan package in R to compare species richness analysis (Oksanen et al., 2007). Normalized read counts facilitate better correlations with different sample sizes.

Non-host sequence reads were mapped against the integrated reference catalogue (IGC) using Bowtie (Langmead et al., 2009) with default parameters. The Kyoto Encyclopedia of Genes and Genomes (KEGG) functional annotations were used to aggregate IGC-associated data (Ogata et al., 1999). Using molecular-level data, particularly from sizable molecular datasets generated by high-throughput technologies, KEGG is a database resource for understanding high-level functions and utility of a biological system, such as the cell, the organism, and the ecosystem.

Using Bowtie (Langmead et al., 2009) with the default parameters, non-host sequence reads were mapped against the microbial virulence database (MvirDB) (Zhou et al., 2007). MvirDB is a collection of genes with traits associated with virulence, such as transcription factors, pathogenicity islands, resistance proteins, and antibiotic resistance.

3. Results and Discussion

In the X-ray room, there were 33.952.806 sequence reads in total with 95.6% high-quality reads. In the waiting room, there were 34.785.046 sequence reads in total with 94% high-quality reads.

A classification tree was created for each read by chopping down the taxonomy and only keeping the taxa (including ancestors) connected to the k-mers in that read. The read was classified using the path from the root to the leaf with the highest sum of weights after each node was given a weight based on the number of k-mers mapped to the node. Although high-quality reads have a high percentage, classification rates were low (51% for X-ray room, 30% for waiting room).

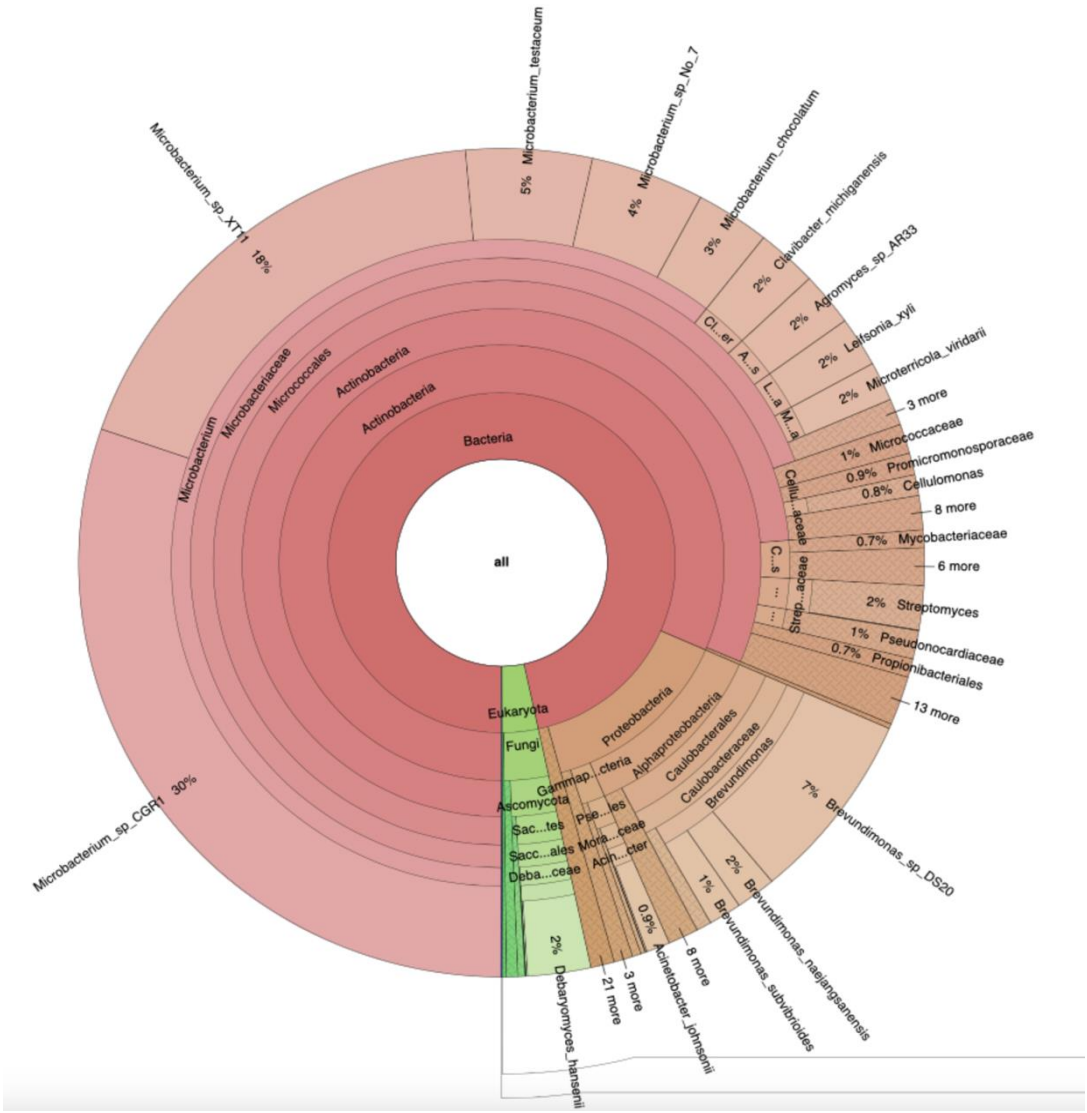

Figure 1. An illustration of an interactive plot created by Krona.

Most of the classified reads belong to the Bacteria kingdom (99.47% in the X-ray room, 75% in the waiting room). There were also reads assigned to Viruses, Fungi, and ambiguous reads. Results from Kraken (Wood & Salzberg, 2014) were used to generate plots using Krona (Ondov et al., 2011).

At the levels of the Phylum (Figure 2), Genus (Figure 3), and Species (Figure 4), abundance was calculated as the percentage of OTU-assigned readings from different taxonomic levels.

A diversity index is a quantitative measure that reflects how many different species are in a dataset and simultaneously takes into account how evenly the basic individuals are distributed among those types. Simpson diversity index value ranges from 0 to 1. Smaller values indicate diverse environments. Shannon diversity index, Inverse Simpson diversity, and Alpha diversity have values greater than zero. A higher score indicates more diversity. SpeciesNo refers to the complete number of species found in samples. The distribution of individuals across species is referred to as evenness. The index gets closer to zero when communities with few species become less even. Six diversity indices were inferred based on the number of species found in each sample (Figure 5).

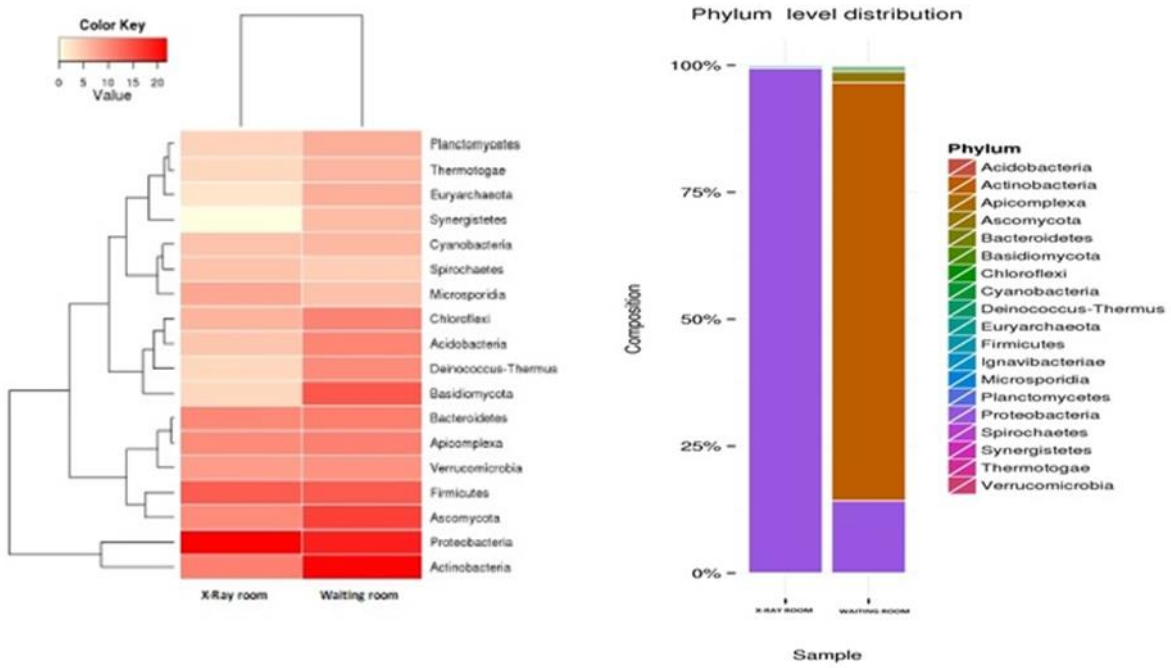

Figure 2. The taxonomic abundance and its relationship across the samples were shown via a heat map and a bar plot. The link between the species (left) and the samples (top) was displayed in dendrograms created by performing hierarchical clustering from the abundance levels. For clarity, the abundance levels (amount of reads linked to each taxon) were logarithmically converted to base 2. Taxa level: Phylum.

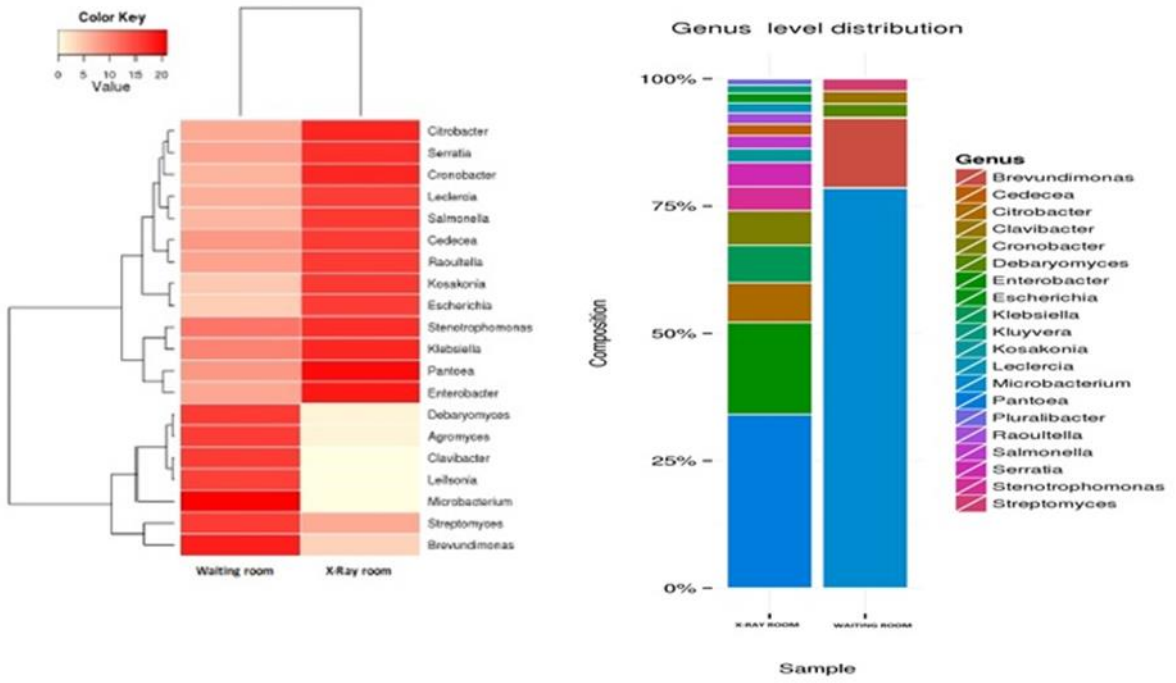

Figure 3. The taxonomic abundance and its relationship across the samples were shown via a heat map and a bar plot. The link between the species (left) and the samples (top) was displayed in dendrograms created by performing hierarchical clustering from the abundance levels. For clarity, the abundance levels (amount of reads linked to each taxon) were logarithmically converted to base 2. Taxa level: Genus.

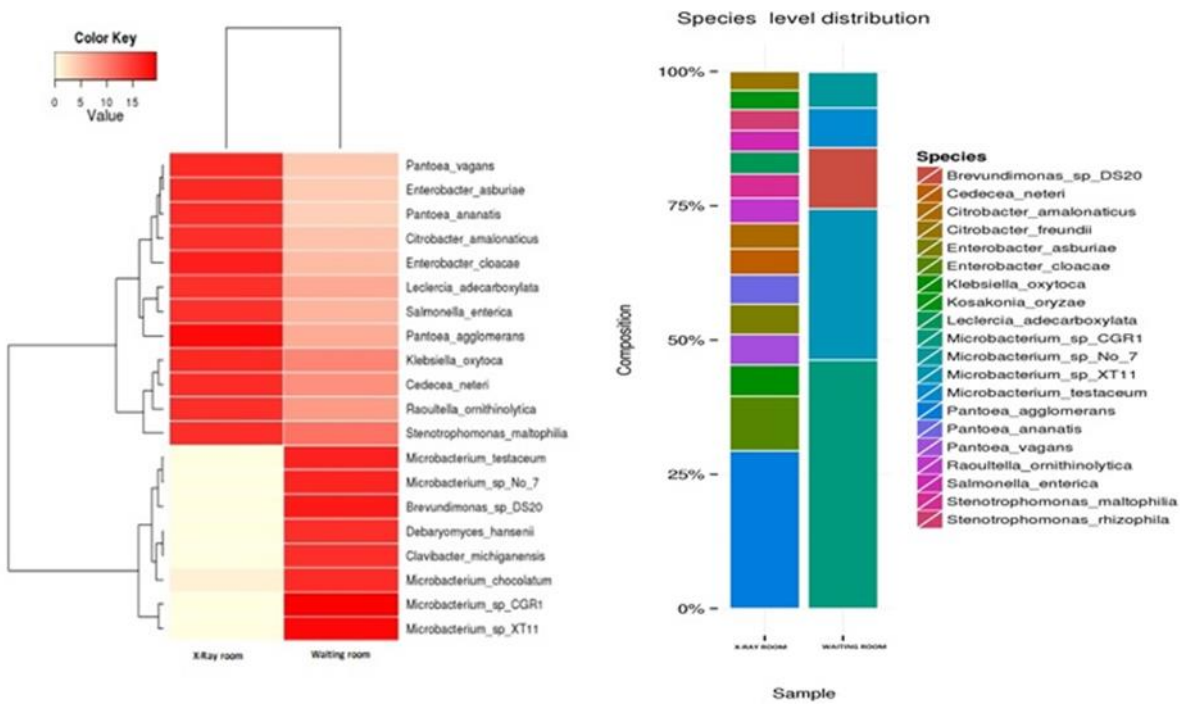

Figure 4. The taxonomic abundance and its relationship across the samples were shown via a heat map and a bar plot. The link between the species (left) and the samples (top) was displayed in dendrograms created by performing hierarchical clustering from the abundance levels. For clarity, the abundance levels (amount of reads linked to each taxon) were logarithmically converted to base 2. Taxa level: Species.

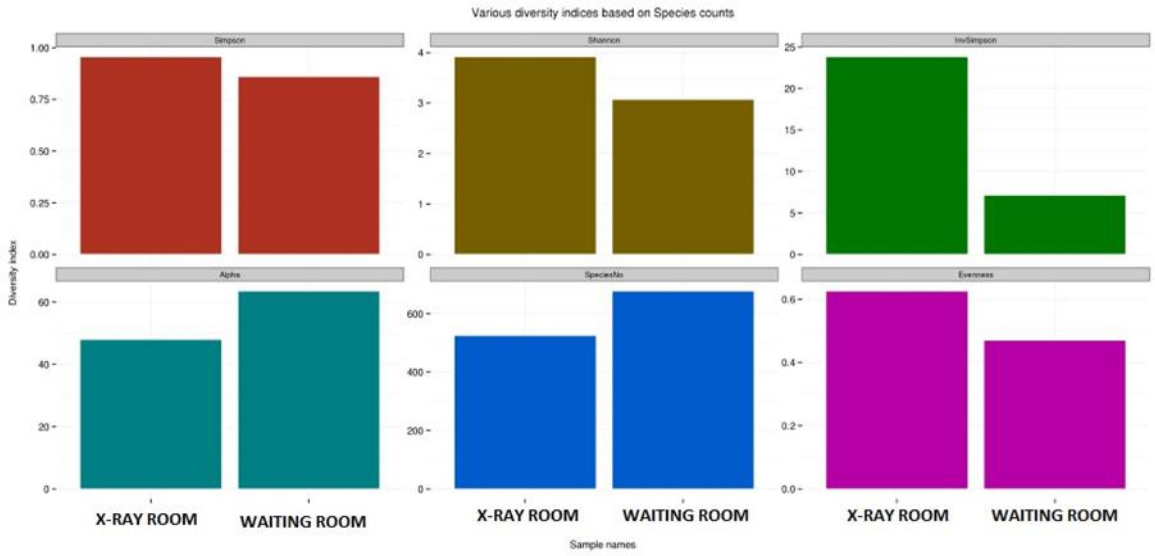

Figure 5. Diversity indices are calculated based on species counts discovered in each sample.

Simpson index values were 0.96 in the X-Ray room and 0.86 in the waiting room. Shanon's index values were 3.92 for the X-ray room and 3.07 for the waiting room. Inverse Simpson index values were 23.85 for the X-ray room and 7.18 for the waiting room. Alpha index values were 48.08 for the X-ray room and 63.6 for the waiting room. There were 526 species in the X-Ray room and 678 species in the waiting room. Evenness values were 0.63 for the X-ray room and 0.47 for the waiting room. Species Diversity is simply the number and relative abundance of species found in a given biological organization. According to the Simpson index and Alpha index, the waiting room was more diverse, while the Shannon index, Inverse Simpson index, and Evenness told us the X-ray room was more diverse. Also, SpeciesNo suggests waiting room had more species than the X-ray room.

In the waiting room, viruses account for 0.04% (1088 reads) of all diversity, while in the X-ray room, they account for 0.001% (58 reads) of all diversity. Most of the viruses were bacteriophage species. Some animal viruses were also present like cyprinid, equid, and caviid herpesviruses, and plant virus-like Pothos latent virus. Human endogenous retrovirus K was found in the waiting room and constitutes only 0.0001% (3 reads) of all viruses. Human endogenous retrovirus K is related to cancer (Curty et al., 2020). In normal cells, their expression is inhibited, but in tumor cells, their expression is increased (Bannert et al., 2018).

In the X-ray room, almost all of the identified taxa (99.47 %) belong to the Bacteria kingdom, and in the waiting room, the majority of taxa (74.92 %) belong to the Bacteria kingdom. In the X-ray room, only 0.46 % of the reads cannot be assigned to any kingdom while in the waiting room, 22.85 % of the reads can not be assigned to any kingdom. In the waiting room, Actinobacteria was dominant but almost all species found in the X-ray room belong to Proteobacteria. Phylum Proteobacteria contains several human pathogens. Members of Phylum Actinobacteria are commonly found in soil and water. They are economically important for agriculture and forestry.

At the genus level in the X-ray room, *Pantoea* was most abundant. Generally, *Pantoea* species were not considered harmful but some species can be opportunistic pathogens (Delétoile et al., 2009; Yılmaz et al., 2015). In addition to *Pantoea*, *Enterobacter* and *Cedecea* genera were abundant. Genus *Cedecea* is an extremely rare bacteria that originated from the Centers for Disease Control (CDC) (Grimont et al., 1981). Members of the genus *Cedecea* can cause a wide spectrum of acute infections in immunocompromised hosts (Dalamaga & Vrioni, 2011; Ahmad et al., 2021). Furthermore, multidrug-resistant *Cedecea* prevalence is becoming more concerning because of their wide distribution in the environment. In the waiting room, the bacterial profile was different from than X-ray room. Members of the genus *Microbacterium* and *Brevidomonas* were abundant whereas *Cedecea*, *Pantoea*, and *Enterobacter* species were less frequent. Species of *Microbacterium*, *Brevidomonas*, and *Debaromyces* genus can be susceptible to radiation because they were near or less than detectable in the X-ray room. In Oshoma et al. (2010) studied microbial air quality of X-Ray rooms in Nigeria using cultural methods.

They investigated both indoor and outdoor environments of the X-Ray rooms for five days. They found the microbial load between 2.00 cfu/m^3 and 41.5 cfu/m^3. According to their study *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *E.coli* were abundant. Our study showed different results. This is a normal outcome since we used a stronger method. Girma & Lamore (2022) studied indoor air bacterial profiles in a university hospital in Ethiopia using cultural methods. They conducted their study in ten different wards and found a bacterial load between 280-6369 cfu/m^3. They isolated *Staphylococcus aureus*, *E.coli*, *Klebsiella* spp., *Bacillus* spp., *Proteus* spp., and *Streptococcus* spp. They did not include an X-ray room in their study. This differs from our study. Also, they used cultural methods which also differs from our study.

Fungal species in both rooms mostly belong to Ascomycota. The difference is in the waiting room Basidiomycota was abundant after Ascomycota while in X-ray room, Microsporidia was abundant after Ascomycota. Although there were fewer fungi in the X-ray room (2383 reads) than in the waiting room (87.118 reads); the X-ray room had more fungi species. Both rooms contain various animal and plant pathogens. *Debaryomyces hansenii*, the most abundant fungi species in the waiting room, is an invasive yeast species also known as *Candida famata*. It can cause problems in immunocompromised patients (Karapetsa et al., 2019). On the other hand, *Trichosporon asahii*, found in the waiting room causes skin infections and can be dangerous in immunocompromised patients (Yayla et al., 2018).

A review written by Ilyas et al. (2019) states Hospital Acquired Infection risk has increased in X-ray rooms due to the increasing number of patients. They also discussed how patients and healthcare workers can be exposed to pathogens in X-Ray rooms. In our study, the workload of the X-ray room was between 350-400 patients per day. This is a relatively high number and supports the mentioned review.

There were 2.803.505 reads in the X-ray room associated with IGC while the waiting room had 58.966 reads. The reads were further filtered to only include those that could be paired together and be uniquely positioned. Reads of high quality were processed and reported.

The accompanying table and figure (Table 2, Figure 6) summarize the makeup of various functional categories for each sample.

Table 2. Composition of top 20 functional categories for all samples (%)

Function	X-Ray Room	Waiting Room
Unknown	27.60	50.35
Carbohydrate Metabolism	11.37	5.13
Nucleotide Metabolism	8.63	3.52
Energy Metabolism	6.01	3.50
Amino Acid Metabolism	6.87	2.49
Genetic Information Processing	3.53	5.61
Translation	6.75	2.30
Membrane Transport	4.82	2.57
Cellular Processes and Signaling	3.87	3.47
Poorly Characterized	2.66	4.54
Metabolism	2.55	4.43
Replication and Repair	3.63	2.65
Folding, Sorting, and Degradation	3.27	2.34
Enzyme Families	2.01	1.60
Transcription	2.45	0.92
Glycan Biosynthesis and Metabolism	0.92	1.02
Lipid Metabolism	0.66	0.91
Metabolism of Cofactors and Vitamins	0.72	0.80
Signal Transduction	0.53	0.72
Metabolism of Terpenoids and Polyketides	0.29	0.37

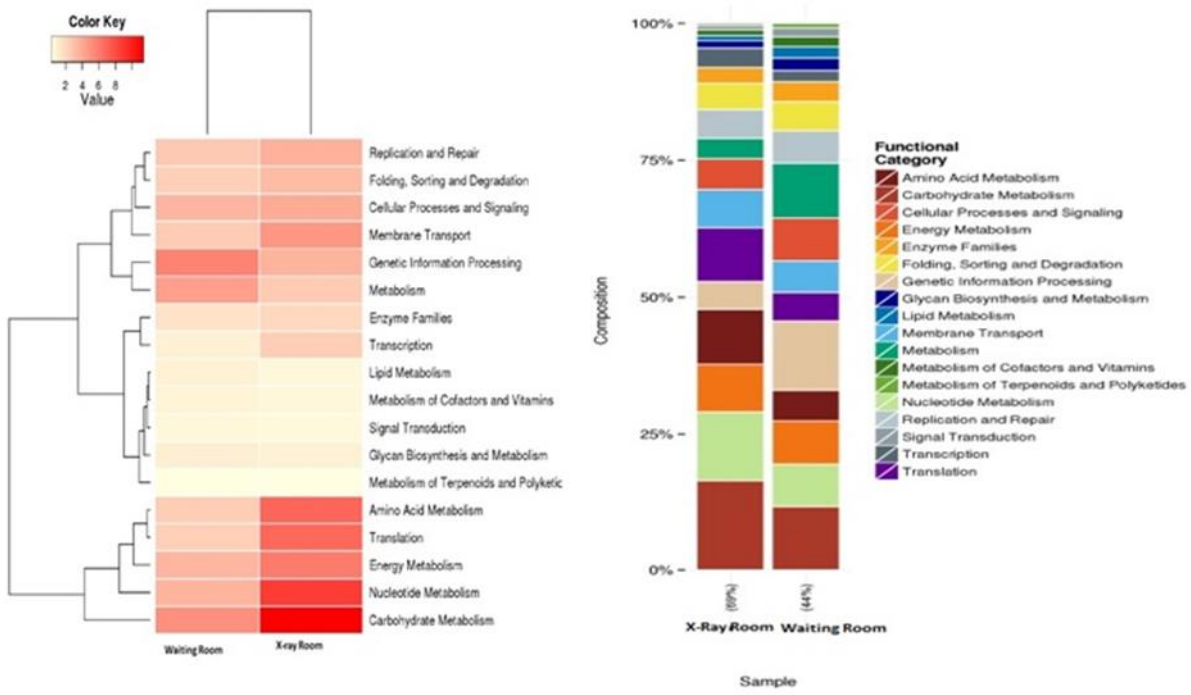

Figure 6. Heat map displaying the relative frequency of the most prevalent functional categories in the samples and their correlations. The relationships between the various functional categories (left) and the samples (top) were displayed in dendrograms created by generating hierarchical clustering from the frequencies. Bar graph displaying the proportion of genes discovered in the most prevalent functional categories across all samples.

Only a small percentage (0.18 %) of the reads in the waiting room were mapped. This, again, makes the comparison harder. Furthermore, 50 % of them remained unknown after the profiling. Unknown and poorly characterized functions are not included in our evaluation of the results.

Our analysis detected 38 different functions with 24 overlapping functions, with the top functions being carbohydrate, nucleotide, and energy metabolism.

In the X-ray room, carbohydrate metabolism had the highest reads among other functions. This fits our expectations. On the other hand, the replication and repair functions were lower than our expectations. Under constant X-rays, we expected increased repair functions. O'Hara et al. (2017) conducted a metagenomic study on ambulances. They found cofactors, prosthetic groups, and electron carriers as top functions followed by secondary metabolites biosynthesis function. In our study, these two functions belong to the lowest function groups. It is possible to X-Rays cause this difference but there is not enough data to reach a solid conclusion.

For the waiting room, considering low mapping, functions were almost equally distributed with genetic information processing taking the lead and followed by metabolism functions.

The distribution of reads on various virulence factors for each sample is given in Table 3 and Figure 7.

Table 3. Distribution of virulence factors for all samples (number of reads)

Virulence Factor	X-ray room	Waiting room
Antibiotic resistance	2364	26
Differential gene regulation	286	0
Pathogenicity island	10936	162
Protein toxin	6	32
Virulence protein	8480	478

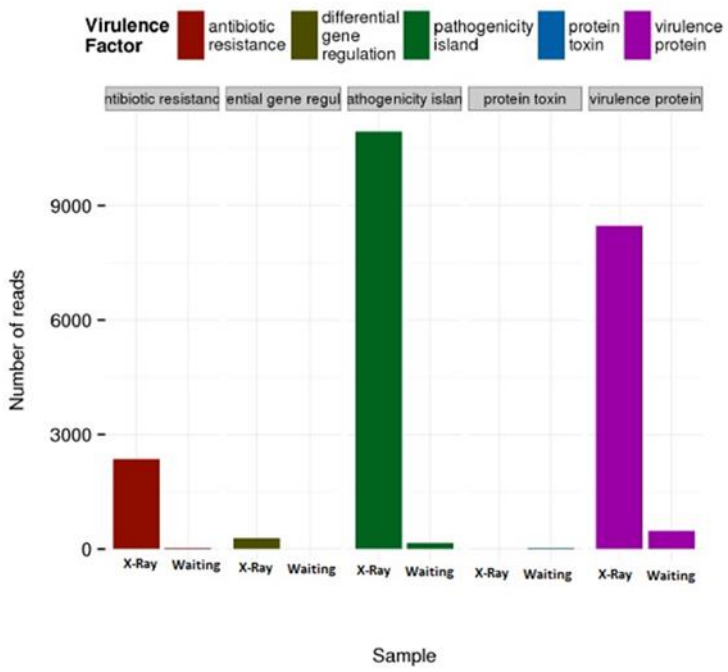

Figure 7. Sample-based read distribution of different virulence factors.

Most reads belong to pathogenicity islands and virulence proteins. There weren't many antimicrobial resistance (AMR) reads but from 2364 reads multi-resistance beta-lactam, penicillin, lincomycin, and tetracycline resistance genes were found. During the sampling time, airborne antimicrobial resistance was low. It is possible the presence of radiation shifted antibiotic resistance genes related to the radiation-related genes. It is also possible antimicrobial resistance genes could be frequent in the surface samples instead of air samples.

We expected methicillin-resistant *Staphlococcus aureus* (MRSA) but *Staphlococcus aureus* has only 76 reads. It is approximately equal to %0.0002 of all reads. Considering this it is normal not to find MRSA. O'Hara et al. (2017) also found similar results from the surfaces of ambulances.

4. Conclusion

Although there are several methods available for identification in a sequenced sample, no methods have perfect accuracy. Both rooms had almost equal reads but 30 % of reads in the waiting room were classified. This makes the comparison somewhat harder. In the X-ray room, this ratio was almost 50-50. This indicates large part of the data remains unknown. This is also true for functional profiling and resistance screening. Classified data was inferred and interpretations were made based on these classified data. It should also be noted that drawing air samples on buffered peptone water and doing the metagenomic analysis wasn't a routine technique.

In previous studies there was not much about air metagenome in hospitals, let alone in X-ray rooms. The sampling technique used was a modified technique from Whon et al. (2012) They collected viral particles with a 1µm filter. We used a 3 µm filter to collect fungal particles as well as bacterial and viral particles.

In our study, we want to see the effects of X-rays on airborne bacteria. Although problems were encountered with waiting room data, the results were satisfying. X-Rays have a major effect on phylum. They also affect the species diversity though more data were needed to make a solid conclusion. For functional analysis, results were within the expectations except for repair functions. For antimicrobial resistance fewer resistance genes were found than expected but this can be because our samples were airborne.

This study will be a foundation for further studies. Today's technology, software, and databases allow us to dig deeper into the microbiome. In the future transcriptomic, proteomic, and metabolomic studies can be made. With more robust studies on interactions between microbiota, residents can be

understood and solutions can be offered for HAI, AMR, and other pathogen-related problems in hospitals.

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