INSTITUTO POLITÉCNICO DE LISBOA

ESCOLA SUPERIOR DE TECNOLOGIA DA SAÚDE DE LISBOA

CHARACTERIZATION OF MICROBIOLOGICAL CONTAMINATION OF A CLINICAL PATHOLOGY SERVICE AT A CENTRAL HOSPITAL IN LISBON

STUDENT: RAQUEL MARIA VALÉRIO LAMPREIA LOURENÇO

PROJECT ADVISOR: PhD. CARLA VIEGAS – ESTeSL-IPL

Master's Degree in Clinical Laboratory Technologies

Lisboa, 2020

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“*Thus, the task is not so much to see what no one yet has seen, but to think what nobody yet has thought about which everybody sees*”

- Arthur Schopenhauer

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Abstract

Indoor air quality (IAQ) has been a target of great interest for the scientific community in recent years because of strong evidences that poor air quality has proven harmful effects on its occupants, affecting health, comfort and productivity. This situation becomes alarming in places where the health of its occupants is impaired, such as in hospitals. IAQ proves to be extremely important in the hospital environment due to the aerial spread of bacteria, potentiating nosocomial infections. There is a lack of studies on this topic in Portugal.

Fungi dispersed through the atmosphere are susceptible to causing diseases, when they come into contact with skin and mucous membranes, by direct contact or inhalation. The contamination of fungi in health facilities has shown several infections, caused by several species of *Aspergillus*, *Cladosporium* and *Penicilium*.

The most prevalent bacteria in indoor environments are gram-positive bacteria (*Micrococcus* and *Staphylococcus*), although gram-negative bacteria might also be detected.

This work aims to characterize the biological contamination in ventilation grids of a Clinical Pathology Service of a Central Hospital in Lisbon. Samples were made with swabs in ventilation grids, in all sections within the Clinical Pathology service and, plates for Electrostatic Dust Cloths (EDC) were placed for 15 days.

Total bacteria presented the highest prevalence in both matrixes, whereas gram- bacteria presented the lowest. Swabs presented a higher prevalence (27.6%) for fungal burden while EDC shown its high prevalence in DG18 (91.9%). *Chrysonilia sitophila* presented the highest prevalence in swabs for both medias (MEA 52.50%, DG18 57.39%), followed by *Penicillium* spp. in MEA (18.43%) and *Cladosporium* spp. in DG18 (23.56%). For EDC, *C. sitophila* and *Mucor* spp. were the most prevalent in MEA (both with 44.52%), whereas *Cladosporium* spp. was the most prevalent in DG18 (45.98%). Concerning *Aspergillus* genera on swabs, section *Flavi* was the one with the highest prevalence (58.02%), whereas, for EDC, section *Versicolores* was the only section observed (100%). Section *Fumigati* was detected in 10 swabs and in 7 EDC samples and section *Versicolores* was detected in one EDC sample by molecular tools. These results were confirmed through the PCR technique.

The obtained results revealed a potential threat to public and occupational health, allowing the clinical staff to raise awareness of the problem.

Keywords

Electrostatic Dust Cloths, Surface swabs, Fungi, Bacteria, Aspergillus

// Resumo

A qualidade do ar interior (QAI) tem sido alvo de grande interesse por parte da comunidade científica nos últimos anos por existirem evidências claras de que uma má qualidade do ar tem comprovado efeitos nefastos nos seus ocupantes, afetando a saúde, o conforto e produtividade. Esta situação torna-se alarmante em locais onde a saúde dos seus ocupantes é debilitada, como em hospitais. A QAI revela-se extremamente importante no ambiente hospitalar devido à disseminação aérea de bactérias potenciar infeções nosocomiais. Estudos nesta temática são raros em Portugal.

Os fungos dispersos na atmosfera são suscetíveis de provocar doenças, quando entram em contacto com a pele e muscosas, por contacto direto quer por inalação. A contaminação de fungos em serviços de saúde apresenta várias infeções, causadas por espécies de *Aspergillus*, *Cladosporium* e *Penicilium*.

As bactérias mais prevalentes em ambientes fechados são bactérias gram-positivas (*Micrococcus* e *Staphylococcus*), embora bactérias gram-negativas também possam ser detetadas.

Este trabalho tem como objetivo caracterizar a contaminação biológica em grelhas de ar de um Serviço de Patologia Clínica de um Hospital Central de Lisboa. Foram efetuadas colheitas com zaragatoas nas grelhas de ventilação em todas as secções do serviço de Patologia Clínica e colocadas placas para EDC durante 15 dias.

As bactérias totais apresentaram a maior prevalência em ambas as matrizes em relação às gram-negativas. Zaragatoas apresentaram maior prevalência (27,6%) de carga fúngica enquanto os EDC tiveram no DG18 (91.9%). *Chrysonilia sitophila* apresentou maior prevalência nas zaragatoas para ambos os meios (MEA 52,20%, DG18 57,39%), seguida de *Penicillium* spp. em MEA (18,43%) e *Cladosporium* spp. em DG18 (23,56%). Para os EDC, *C. sitophila* e *Mucor* spp. foram os mais prevalentes em MEA (ambos com 44,52%), e o *Cladosporium* spp. no DG18 (45,98%). Relativamente aos gêneros *Aspergillus* em zaragatoa, a secção *Flavi* foi o que apresentou maior prevalência (58,02%), enquanto, para os EDC, a secção *Versicolores* foi a única observada (100%). A secção *Fumigati* foi detetada em 10 zaragatoas e 7 amostras de EDC e a secção *Versicolores* foi detetada em apenas uma amostra de EDC. Estes resultados foram confirmados através da técnica de PCR.

Os resultados obtidos, revelaram uma potencial ameaça à saúde pública e ocupacional, permitindo uma maior sensibilização para esta temática.

Palavras-chave

Electrostatic Dust Cloths, Zaragatoas, Fungos, Bactérias, Aspergillus

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Acronyms List

ABPA Allergic Bronchopulmonary Aspergillosis
CCOHS Canadian Center for Occupational Health and Safety
CFU Colony Forming Unit
DG18 Agar Dicloran Glicerol
DMSO Dimethyl sulfoxide
EDC Electrostatic Dust Cloths
HAI Healthcare Associated Infections
HVAC Heating, Ventilating and Air Conditioning
IA Invasive aspergillosis
IAQ Indoor Air Quality
INSA Instituto Nacional de Saúde Dr. Ricardo Jorge
ITS Internal Transcribed Spacer
MEA Malt Extract Agar
mL milliliter
MRSA Methicillin-Resistant Staphylococcus Aureus
NaCl Sodium Chloride
NI Nosocomial Infection
PCR Polymerase Chain Reaction
qPCR quantitative Polymerase Chain Reaction
RPM Rotations Per Minute
SBS Sick Building Syndrome
TSA Tryptic Soy Agar
VRBA Violet Red Bile Agar
WHO World Health Organization
µL Microliter

1. Introduction

Several studies recognize ambient air as a source of propagation of microorganisms. Although the majority of hospital infections are related to diagnostic and therapeutic methods (endogenous origin), these infections can be transmitted by air and must be considered. Particles, ventilation rate, occupation and degree of activity performed by people (patients and health professionals) occupying this physical space are some of the main factors of the level of indoor air contamination (1).

Bioaerosols are viable and non-viable aerosolized biological particles present in the atmosphere of natural or anthropogenic origin, accounting for about 5-34% of indoor environmental pollution. (2,3) Bioaerosols produced routinely in hospitals can be originated from several processes, namely through breathing, sneezing, coughing, medication and surgery (4). In this context, the most important source of atmospheric pathogens occurs when pathogenic microorganisms are transferred from an infected individual to a susceptible individual by air.

The predominant mechanism that allows the presence of microorganisms in an indoor environment is the production of aerosols mainly through sneezing or coughing, and their subsequent loss of water which allows them to shrink in size, increasing their ability to remain in the air (4,5). Many airborne organisms are commensal and inhabit the surface of the skin and can be transported to the surrounding areas, enhancing the cross-contamination phenomenon (6).

In both, developing and developed countries, a substantial risk factor for human health are the problems of Indoor Air Quality (IAQ). However, given the different pollution sources and, climatic, geographic, housing and socioeconomic conditions in different parts of the globe, the exposure to indoor pollution and correspondent health effects vary (7).

The biggest constraint in applying a solution is based on the complexity of the process due to the surrounding’s several variables such as the weather, building’s location, Heating, Ventilation and Air Conditioning (HVAC) equipment (8) and, the existence of sources of pollution that may be originated from inside or from outside and that infiltrate the building through ventilation systems. There are also other factors to take into account, such as thermal comfort (temperature and humidity), odors, psychosocial factors and the individual's own organism are variables that complicate the equation, affecting occupants in different ways and intensities (9,10).

In today's society, it is essential to ensure a good management of IAQ in buildings, a mission that does not prove to be easy, not only due to the large number and different types of interior spaces, but also due to the complex relationship between IAQ and the materials, activities, cleaning, building design, user behavior, population density in large buildings and with internal dynamics. Koistinem et al. state that the management of IAQ has shown, sometimes very complicated, yet critical to providing a greater well-being to its occupants/users (11).

Hospitals and other healthcare buildings are examples of a particularly complex environment that must ensure a healthy IAQ. The existence of chemical, physical, biological and / or psychosocial factors, characteristic of this type of environment, may compromise the health of not only patients, but also health professionals. Studies show that hospitalization and medical procedures are designed to promote the health not only of patients but also of health professionals and that exposure to a deficient IAQ is a cause of morbidity and mortality, being the cause not only of several symptoms and diseases, as well as Healthcare Associated Infections (HAI) (12).

Fungi spread through the atmosphere are likely to cause disease in humans when in contact with skin and mucous membranes, either by direct contact or after inhalation. The first reports on the importance of the hospital environment as a source of transmission of infectious agents were associated with aerial contamination by spores of *Aspergillus* spp. (13).

Currently available data demonstrate that several other microorganisms can be aerosolized. In fact, MRSA (*Methicillin-Resistant Staphylococcus Aureus*), *Pseudomonas aeruginosa* and *Mycobacterium tuberculosis* are among the species described as responsible for hospital outbreaks related to environmental contamination (14,15) and according to studies in indoor air conducted in Europe, gram-positive cocci, namely the *Micrococcus* and *Staphylococcus* species, are the bacteria that most occur in the interior, although gram-negatives are also present (16).

Another recent study, focused on the detection of bacteria and fungi in hospital air, determined that the predominant genera of aerobic bacteria are *Staphylococcus* (50%), *Micrococcus* (15-20%), *Corynebacterium* (5-20%) and *Bacillus* (5 -15%), while the predominant fungi are *Cladosporium* (30%), *Penicillium* (20-25%), *Aspergillus* (15-20%) and *Alternaria* (10-20%) genus (17).

1.1 Relevance of the Topic

Considering the aforementioned facts, the choice of this dissertation topic is of great importance in the study of quantification and identification of microorganisms in the ventilation grids through swabs and EDC in a hospital environment. Although there are several studies related to air conditioner filters, studies have not been found related to ventilation grids that often go unnoticed during cleaning and maintenance in different hospital environments.

1.2 Main Objective

The main objective was the identification of microorganisms present in the ventilation grids through surface swabs and EDC in a service of a hospital environment.

1.3 Specific Objectives

It is intended to identify the different types of microorganisms present in the aforementioned ventilation grids together with the present microbial load through direct contact (surface swabs) and through air particles collected with EDC. Another objective is to identify microorganisms that may be a threat to public health.

1.4 Type of Study

The study carried out has a cross-sectional component, in which it was intended to describe the environmental and biological phenomena of microbial contamination (bacteria and fungi) in a hospital environment, in a given period of time and to explore possible associations between variables.

The variables of this study are the two types of sampling methods, the usage of four culture media, size of the studied areas, amount of EDC and Swabs collected and the number of attendees per location sample.

2. State of the Art

2.1 Indoor Air Quality (IAQ)

According the World Health Organization (WHO), an acceptable IAQ is defined as: "*the physical and chemical nature of the indoor air that is breathed by the occupants of a building and that produces complete well-being, mental, physical and social, and cannot cause absenteeism, illnesses or weaknesses.*" (18).

The indoor atmosphere represents an ecological unit with an impact on public health. Indoor air, like outdoor air, presents, in addition to chemical pollutants, a complex range of microorganisms, endotoxins, mycotoxins and volatile organic compounds potentially adverse to health (3,19).

Fungi, bacteria and viruses, part of biological pollutants, are key requirements for the IAQ definition. Although, IAQ is a much more complex concept which includes physical factors (ambient temperature, humidity), contaminant and pollutant chemicals, and mechanical factors (ventilation) of the indoor air. The combination of these factors will restrain the comfort and health conditions of all occupants in a closed environment (20–22).

2.2 Parameters influencing the IAQ

The characterization of IAQ should take into account the parameters of thermal comfort, chemical parameters and microbiological parameters, both in an isolated perspective and in the interaction between them.

Temperature is a factor that mainly conditions the comfort of occupants in a building and microbial growth. This is usually a point of contention among the occupants when it comes to an HVAC system, as it depends on the individual susceptibility of each one (23).

Temperature, despite not having a limit value defined in the legislation on IAQ, plays a very important role with regard to the parameters of thermal comfort and microbiology, since both influence microbial growth (23)

Like temperature, relative humidity is a physical parameter that influences microbial growth. According to the Canadian Center for Occupational Health and Safety (CCOHS), when values are outside of what is considered acceptable, it can cause discomfort in occupants, such as dryness of mucous membranes (eyes, throat and nose) for low relative humidity and breathing problems and allergic reactions when the value of this parameter is high (due to the increase in the development of microbiological matter in the air) (24).

2.2.1 Microbiological parameters

Microbiological contamination comprises hundreds of species of bacteria and fungi that can proliferate when conditions of temperature, humidity and nutrients are suitable for their growth. According to Lingle (2008), it is a matter of time before microbiological growth begins on materials and surfaces that have moisture, the same stress that humidity is even the limiting factor in microbiological growth, since the remaining predominant factors (temperature and nutrients) are normally checked.

2.3 IAQ in hospitals

The hospital environment, especially the affection for providing medical and health care to the community, constitutes a particular environment. Although hospitalization and medical procedures are designed to promote health, there are several risk factors within the hospital, whether chemical, physical, biological and/or psychosocial, capable of inadvertently causing harmful effects on health, not only on patients but also of health professionals and assistants. These are factors that subject the hospital community to an increased risk compared to other community environments and the requirements for good IAQ are much higher in hospitals than in other buildings (12).

Hospital employees are exposed to many chemical agents from anesthetic, disinfectant and sterilizing gases in addition to microbiological pollutants, which reinforces the importance of a good IAQ that is known to be associated with a sense of well-being and comfort, a factor preponderant for concentration since many of the tasks performed in hospitals are very demanding and any mistake can have serious consequences. Patients are the group of occupants most likely to get an infection because they have the weakest immune system (25).

The three major groups of daily occupants in a hospital are patients, health professionals and visitors, all of whom have different immune systems and who therefore also have different susceptibilities to contaminated air. In a Hospital there are several types of services and existing spaces, such as waiting rooms, operating rooms, intensive care units, recovery rooms, pharmacies, radiology rooms, laboratories, emergency services, among others. Each unit has its functions and the day-to-day life of each one can be very different from the others (26).

In developed countries, the organisms most often associated with infections are the members of the usual flora of humans, especially the skin, upper respiratory and gastrointestinal tracts, and saprophytic organisms in the hospital environment. The most common are the coagulase negative *Staphylococcus*, *Staphylococcus aureus*, *Enterococcus sp*, *Clostridium difficile*, *Escherichia coli*, *Klebsiella pneumoniae*, and *Proteus sp*. In developing countries, infections caused by *Mycobacterium tuberculosis* and *Salmonella* are still common, originating from employees and visitors (27).

2.3.1 Health Care Associated Infections

In the context of criticality in hospital areas, it is pertinent to address the HAIs, since the transmission of infections within the hospital shows a relationship close to a reduced IAQ.

WHO defines Nosocomial Infection (NI), also called Healthcare Associated Infections (HAIs), as (28) "*an infection acquired in the hospital, by a patient who was admitted for a reason other than the infection. (…) Infection that occurs in a patient admitted to a hospital or other health institution, which was not present or in incubation at the time of admission. Included are infections acquired in the hospital that are detected after discharge, as well as occupational infections in health professionals.*".

HAIs occur worldwide, affecting both developing and developed countries. This type of infection is among the main causes of death and increased morbidity in hospitalized patients, especially those who have a more vulnerable immune system. (28)Although there is a great innovation in health and hospital care, HAIs continue to develop among hospitalized patients, and may also affect the employees who work there. (28) This type of infection is the most common complication in hospitalized patients and mainly affects the urinary tract, the surgical site, the respiratory tract and the bloodstream (29).

2.3.2 Sick Building Syndrome

Under favorable conditions, bacteria and fungi in the atmosphere are able to grow and propagate on a variety of building materials and surfaces, causing indoor pollution. Many bacteria and fungi have developed defense mechanisms that allow them to survive in the aerosolized state. Some bacterial genera form bacterial spores that can remain viable for years and are resistant to environmental stresses such as heat and cold (30). Several studies have been conducted on the survival of aerosolized bacteria. In this context, the bacteria have different types of coatings: Gram Positive coated with an outer layer of peptidoglycan and Gram Negative coated with an outer layer of lipopolysaccharides. Indoor air studies have shown that Gram-positive bacteria predominate indoors, although Gram-negative bacteria are also present (16).

The Sick Building Syndrome (SBS) is a term used to describe situations in which the occupant's manifest symptoms that seem to be related to the stay inside a given building, having no specific cause. The occupants can present symptoms, related to a certain area of the building or to the whole (31).

The characteristic symptoms of SBS are irritation of the mucous membranes, nose and throat, dry skin, headaches and fatigue. However, the referred symptoms can be common to many diseases. The factor that incorporates it in the SBS is the temporal relationship with work or the occupation of a given building. Thus, most symptoms disappear after the occupants leave the building (32,33). The symptoms associated with SBS are subjective, that is, there may be individuals with many symptoms and others who do not manifest any type of symptoms, when present inside the same building. This subjectivity is motivated by the different sensitivities of the occupants to the conditions of the internal environment (34). The consequences arising from the SBS can affect job satisfaction, stress and productivity of workers (35).

According to WHO (2009), the age groups that manifest more symptoms associated with the internal environment are: children, the elderly and the chronically ill.

IAQ related legislation

To date, no specific standards have been established for air quality in hospitals in most countries, including Portugal (36,37), there is only a decree law implemented in Portugal but for air conditioning systems, which in this work it will not be referenced, since it will only determine the microbiological presence in the ventilation grids, and not in HVAC systems.

The typical contamination sources in health care environments are related with clinical staff, circulating patients, ventilation systems, including air conditioning systems, and outdoor air entering indoors (38–42). The modern ventilation systems have better and better included filtering systems and may reduce the microbiological contamination of indoor air. Although, if these systems don't have an appropriate maintenance, they can jeopardize the IAQ by getting contaminated and become a secondary source of microbial contamination (42,43). Under these current circumstances, it is of utmost importance to implement monitoring and control measures of the microbiological load (41) in several countries adopting more and more the legislation regarding IAQ.

To comply with the changes imposed in Decree-Law No. 118/2013, of August 20, Ordinance No. 353-A/2013, of December 4 was published, which determines the minimum values of fresh air flow per space, as well as the protection thresholds and the reference conditions for indoor air pollutants in new commercial and service buildings, subject to major and existing intervention and the respective assessment methodology.

The protection thresholds for physicochemical pollutants and the reference conditions for the microbiological parameters to be considered are those presented in the following Tables.

	Matrix	Units	Reference Conditions
Bacteria	Air	CFU.m^{-3}	Total bacteria concentration is lower than the concentration outside and should not be higher than 350 CFU. m^{-3}
Legionella spp	Water	CFU.L^{-1}	Concentration less than 100 CFU.L^{-1}, except in the case of cooling tower tanks research in which a concentration of less than 1000 CFU.L^{-1}
Fungi	Air	CFU.m^{-3}	Fungal concentration inside is lower than that detected outside

Table 2.1 - Reference conditions for microbiological pollutants according to Ordinance No. 353-A/2013.

When compared with the previous laws (Decrees-Law No.: 78/2006; 79/2006 and 80/2006, of April 4), this ordinance defines more restricted reference conditions at the level of microbiological pollutants. While the previous laws stipulated a concentration of biological agent (bacteria, fungi) of 500 CFU/m^3, the current law states that the concentration varies according to the type of biological agent (bacteria, fungi) to be investigated in the indoor air.

However, decree 353-A/2013 of December 4, only states that the concentration of viable bacteria (commensal, opportunistic and pathogenic pathogens) found in environmental harvests must be lower than the concentration abroad, plus 350 CFU/m^3 and not takes into account aspects addressed with the potential danger to the public health of the biological agent (bacteria). Bearing in mind that this is not a specific legislation for hospitals, this legislation encompasses all areas as equal, which should not be applicable, as each area must have the type of environment according to its needs.

Species		Specific compliance conditions
Common species (excluding toxin producers)	*Cladosporium* spp	Mixture of species: concentration less than or equal to 500 CFU.m^{-3}
	Penicillium spp	
	Aspergilus spp	
	Alternaria spp	
	Eurotium spp	
	Paecilomyces spp	
	Wallemia spp	
Rare species	*Acremonium* spp	Each species: concentration less than 50 CFU.m^{-3} Mixtures of species: concentration less than 150 CFU.m^{-3}
	Chrysonilia spp	
	Tricothecium spp	
	Curvularia spp	
	Nigrospora spp	
Pathogenic species	*Chryptococcus neofromans*	Absence of any and all species
	Histoplasma capsulatum	
	Blastomyces dermatitidis	
	Coccidioides immitis	
Toxigenic species	*Staphybotrys chartarum*	Each species: concentration below 12 CFU.m^{-3} (several colonies per plate)
	Aspergillus versicolor	
	Aspergillus flavus	
	Aspergillus ochraceus	
	Aspergillus terreus	
	Aspergillus fumigatus	
	Fusarium moniliforme	
	Fusarium culmorum	
	Trichoderma víride	

Table 2.2 - Specific conditions for checking the conformity of fungi based on the danger of different species according to Ordinance No. 353-A/2013.

Regarding fungi, the specific conditions of compliance established by Decree 353-A/2013 depend on the species found in indoor air samples compared to the concentration of fungi, indicating which species are more pathogenic and less pathogenic for people in their respective study areas (Table 2.2).

2.4 Microorganisms present in hospital environment

The biological agents relevant to human health are quite heterogeneous, ranging from pollens and plant spores (mostly from abroad), to bacteria, fungi, algae and protozoa emitted inside or outside. Exposure to microbial contaminants is clinically linked to

respiratory symptoms, allergies, asthma and immunological reactions (7), and can be fatal to individuals whose immune system is weakened. In 2019 it was investigated, that fungi are among the most important and least understood indoor air pollutants, being their frequent presence in urban environments (44).

The main source of microorganisms in indoor environments is generally outside air, and the occupants and their activities also contribute to an increase in microbiological contamination. The microbiological concentrations outside can vary with the season and the time of day, consequently influencing the concentrations recorded inside, and the highest concentrations can be recorded in the summer (45).

In a building there are several factors that enhance the growth of the microbial population, such as high levels of humidity, reduced ventilation, availability of nutrients, adequate temperature for their development, the existence of sources of indoor contamination (HVAC systems, construction materials and decoration, water infiltrations, occupants), as well as air intakes (46).

2.4.1 Bacteria

Bacteria are prokaryotic beings, with diameters between 0.25 and 8 μm (47,48), which can be found in isolation or in colonies. Among the groups of bacteria, the mesophilic stand out for having an optimum growth interval between 20°C and 49°C (49), very close to terrestrial temperatures. Bacteria of human origin are Gram positive and generally do not pose a risk to human health. Gram-negative bacteria, such as *Pseudomonas* spp., *Enterobactereaceas* and *Legionella pneumophila*, are generally rare in indoor environments and can be potentially pathogenic to humans. Bacteria like *Legionella* can be the source of infections that result in pneumonia - Legionnaire's disease. The proliferation of this bacterium is generally associated with systems that have water in their constitution, serving as amplifiers for this microorganisms (8).

The main source of bacteria inside buildings is the occupants, with most of the interior bacteria coming from the skin and the human respiratory tract. The bacteria that usually dominate indoor environments are Gram Positive, which are human diners and usually do not pose a serious health hazard. Gram Negative bacteria, such as *Pseudomonas* spp., *Enterobacteteaceas* and *Legionella pneumophila* are, in general, pathogenic to humans and rare in indoor environments (8).

The concentration of total viable bacteria in indoor environments varies between 10^1 and 10^3 CFU/m^3. These levels probably represent the degree of occupation of the building and the effectiveness of the ventilation systems (7).

Exposure to bacteria can trigger the following symptoms in the occupants: infections, irritation of the mucous membranes, hypersensitivity pneumonia and immunological reactions. Respiratory infections can originate from the upper or lower respiratory tract. These diseases are mostly mild, which makes infected people often ignore the disease. However, respiratory diseases are an important cause of morbidity and mortality worldwide (50).

2.5.4 Fungi

Fungi are heterotrophic organisms and have an aerodynamic diameter between 1 and 30 μm. There are two morphological types of fungi: filamentous fungi and yeast-like fungi (yeasts) (47,48). Among the species of fungi, *Alternaria* spp., *Cladosporium* spp., *Aspergillus* spp., *Fusarium* spp., *Stachybotrys chartarum* and *Cryptococcus neoformans* stand out, which are related to allergenic reactions. Some of these fungi produce metabolites, such as mycotoxins, which can cause allergic reactions in the host (45).

The ideal temperature for the development of fungi varies according to genus and species, in a range between 18ºC and 32ºC (51).

The proliferation of fungi in indoor environments, in addition to presenting a danger to the health of the occupants, is also an indicator that the conditions of relative humidity can provide the proliferation of other biological agents (52).

The existence of fungi in an indoor environment is easily detected by occupants through odor. Exposure to these biological agents can cause the following symptoms: headache, difficulty breathing, skin irritation, allergic reactions and worsening asthma.

Allergens produced by fungi

There are many fungi, namely those of the genera *Alternaria* spp., *Aspergillus* spp. and *Cladosporium* spp. that produce important type I allergens, of which the following stand out: Cha h I (*Cladosporium herbarum*), Alt a I and Alt a II (*Alternaria alternata*) and Asp f I and Asp f II (*Aspergillus fumigatus*) (53).

Mycotoxins

Some fungi produce mycotoxins, as by-products of their metabolism. These metabolites are generally of low molecular weight and their chemical structures and physical properties are quite diverse. Many types of mycotoxins are cytotoxic, being that they cause the rupture of structures, such as cell membranes and interfere with protein synthesis, RNA and DNA (54,55).

The mycotoxins of greatest importance for public health are: aflotoxins, ochratoxins, trichothecenes, zearalenone and fumonisins. In humans, mycotoxins can cause outbreaks of acute infections and in more severe cases, cancer. The WHO has classified aflotoxins as carcinogenic and ochratoxins and fumonisins as possible carcinogens. Trichothecenes and zearalenone have been designated as non-carcinogenic. Over time and with the advancement of research, the importance of mycotoxins for human health is increasingly recognized. Some fungi of the genus *Stachybotrys* spp., considered non-pathogenic, have been extensively studied, due to the high toxicity of the produced toxins. These toxins have been associated with cases of infant mortality (56,57).

The production of mycotoxins depends on several environmental factors, such as humidity, temperature and availability of oxygen. However, once produced, mycotoxins are stable and are not affected by varying environmental conditions (58).

2.5 Critical sections of *Aspergillus*

2.5.1 *Aspergillus* genus

The *Aspergillus* genus belongs to the phylum *Ascomycota* of the Fungi kingdom, *Eurotiales* order and to the *Thichonomacea* family. Those belonging to the *Aspergillus* genus are characterized by being filamentous organisms. In the anamorphic phase, that is, asexual, reproduction is carried out through asexual spores, called conidia. (59)

This genus is considered quite diverse, being made up of 344 species (36) having a great economic and also social impact. The species belonging to this genus can be found all over the world and in diverse habitats.

Many species of *Aspergillus* are used in biotechnology for the production of various metabolites such as antibiotics, organic acids, enzymes, drugs, or as fermenters in the food industry (60).

They commonly contaminate food and some produce mycotoxins. Rarely does inhalation of conidia by healthy (immunocompetent) individuals cause any adverse effect, since conidia are eliminated relatively effectively by the mechanisms of human innate immunity. However, some species are often described as pathogenic to humans and animals, being responsible for a wide spectrum of fungal infections, namely: (36,59–61).

- Allergic pulmonary aspergillosis (develops essentially in susceptible individuals who are frequently exposed to high concentrations of conidia)
- Aspergilloma (growth of a fungal mass inside the lung cavities resulting from sequelae in patients with previous tuberculosis)
- Invasive aspergillosis (spread to different organs, usually fatal in immunocompromised patients)

The pathologies associated with the *Aspergillus* genus (particularly, with *Aspergillus fumigatus*) mostly affect the lungs, although they can also occur in other organs (62,63).

2.5.2 Identification of species of the genus *Aspergillus*

The correct identification of *Aspergillus* spp. it is a very important factor, and the most recent discoveries have shown that, unfortunately, many strains continue to be identified incorrectly. This happens because its identification is made through the observation of phenotypic characteristics (observation of the colony morphology and microscopy identification), with species of *Aspergillus* morphologically identical to each other, sometimes indistinct morphologically, but when analyzed at the molecular level it turns out to be different species (the so-called cryptic species, all belonging to the same species section). On the other hand, strains isolated from human or animal tissues sometimes tend to have a more restricted sporulation and may show differences in micromorphology, such as conidiophores being more branched, elongated or conidia varying greatly in terms of size and shape. This variation makes it poorly identified, leading to the description of "new" species of *Aspergillus* of clinical origin, including *Aspergillus fumigatus*. Through several studies it was realized that through molecular biology and through phylogeny all species are synonymous with *Aspergillus fumigatus*, having recently been revised the taxonomy of *Aspergillus fumigatus* species and the like, through molecular, morphological and physiological parameters (60,62,64).

2.5.3 Cryptic sections and species

According to recent molecular methodologies it has been found that the genus *Aspergillus* is divided into eight subgroups that, in turn, are subdivided into several sections that include a wide variety of closely related (cryptic) species (36). The most clinically relevant sections are *Fumigati*, *Flavi*, *Terrei*, *Usti*, *Nigri* and *Nidulantes*. Most of the studies use the sequencing of the Internal Transcribed Spacer (ITS) region of the ribosomal DNA that allows the identification of the *Aspergillus* species at the section level. It is through the sequencing of these genes that it is possible to identify cryptic species within the different sections of *Aspergillus*. Molecular studies have revealed that there are numerous cryptic species in different sections of the *Aspergillus* genus (36,62).

When we only do the morphological identification or through the sequencing of the ITS region, we speak only at the section level, we can only designate the species identified by *sensu lato* and only after sequencing these genes can we identify the species as a *sensu stricto* or as one of its species cryptic.

2.5.4 Used antifungals

There are currently three main families of antifungals used in the clinical practice of treating invasive fungal infections (65).

Polyenes (represented by amphotericin B and nystatin); azoles (consisting of itraconazole, fluconazole, voriconazole, posaconazole, ketoconazole and miconazole); and echinocandins, are represented by caspofungin, micafungin and anidulafungin (65,66).

Over the past few years there has been an increase in new antifungals, providing doctors with a greater variety in treatments. The increasing use of antifungals has caused selective pressure on the different species/strains of fungi, leading to the emergence of resistance: sensitive species/strains are being replaced by resistant strains, thus changing the epidemiology of fungal infections, leading to the development of secondary resistance in several species/strains (66).

The performance of susceptibility tests to certain antifungals, makes it possible to detect antifungal resistance earlier and in this way it becomes easier to determine the best treatment for a given fungus (66).

2.5.5 Pathologies associated with infection by *Aspergillus* spp.

In general, the pathologies caused by *Aspergillus* are three: allergic bronchopulmonary aspergillosis, aspergilloma and invasive aspergillosis. In all pathologies, the organ primarily involved is the lung and Aspergillus fumigatus is the dominant species (59).

Allergic Bronchopulmonary Aspergillosis (ABPA) is the result of the response to airway hypersensitivity to colonization by *Aspergillus* spp., occurring in people with asthma and cystic fibrosis it is an indolent disease, that is, without pain, but it is potentially progressive. The pathological physiology of this disease is complex, with inflammation of sites resulting from ineffective spore removal, which in turn leads to increased mucus production, airway hyper-reactivity, bronchiectasis, recurrent cough and wheezing. Most of the time, a bronchial allergic reaction (asthma) develops after inhaling *Aspergillus* spp. (59,67).

The most familiar localized infection caused by *Aspergillus* spp. normally, aspergilloma develops in patients with structurally abnormal lungs, with pre-existing cavities, for example, in old cavities caused by tuberculosis. Aspergilloma represents a non-invasive form of pulmonary aspergillosis, forming a ball-like mass, consisting of an intricate mesh of hyphae normally of *Aspergillus* spp. (59,68).

At last but not least, Invasive aspergillosis (IA) can be defined as an opportunistic infection that has become a major cause of concern due to the high rates of morbidity and mortality in patients with hematological malignancies, with an average mortality rate of 50% in patients with leukemia. It is also one of the main causes of death in transplant patients (bone marrow transplantation). This poor prognosis is, in part, due to difficulties in obtaining an early diagnosis resulting in a harmful delay in the initiation of appropriate antifungal treatment. As this is a fast-progressing infection (1 to 2 weeks from onset to death), doctors sometimes start treating the patient empirically rather than waiting for the diagnosis to be made. The main etiologic agent of IA is *Aspergillus fumigatus* (in 90% of cases), however, it is not the only pathogen within this genus (62,64,69–71).

2.5.6 Nosocomial infections

Invasive fungal infections acquired in the hospital, especially aspergillosis, have become an increasing problem in immunocompromised patients (72). Exposure to contaminated bio aerosols is problematic, particularly in hospital environments, where under conditions

of adequate temperature and humidity, increased sporulation and conidia present in the air and surfaces may occur. Several studies have shown that the highest percentage of nosocomial infections is caused by fungi (72) According to some authors, the values allowed for the presence of fungi in intensive care units cannot be higher than 300 CFU/m^3 in indoor air, in the operating rooms the number of CFU/m^3 allowed is zero, whereas in treatment room, the allowed value is 50 CFU/m^3, and in the other hospital units the allowed value is 200 CFU/m^3 (73).

It is assumed that the hospital environment is the main source of contamination for patients who end up developing IA and the elimination of *Aspergillus* spp. spores. of this environment has been shown to be important for reducing the incidence of IA acquired in hospitals (70).

The detection of *Aspergillus fumigatus* in hospital units with high-risk patients is an important way of preventing nosocomial IA. According to the AIHA (American Industrial Hygiene Association) guide, the confirmed presence of *Stachybotrys chartarum*, *Aspergillus versicolor*, *Aspergillus flavus*, *Aspergillus fumigatus* and *Fusarium moniliforme* in a hospital environment requires making decisions that lead to the implementation of corrective and preventive measures (70).

2.6 Exposure evaluation methods (active and passive)

There are two main methods used to sample the air quality: the active air sampling and the passive air sampling.

The active air sampling is commonly associated with pumped sampling. This means that with this type of microbial air samplers, the air is continuously forced to pass over a Petri dish that is used as an artificial media. After collecting these samples over a short period of time, the Petri dishes are taken from the air samplers and incubated. This incubation method will allow microorganisms to grow. The active samplers are widely used when having low microbial load (74).

Instead of using machines as in active air sampling, passive air sampling commonly uses sedimentation plates or contact plates exposed to air. However, the use of EDC instead of sedimentation or contact plates is also common. Swabs are also used regularly not only in places where it is impracticable to use EDC, or any type of plates, but also to take samples directly from certain surfaces (74,75).

When comparing both methods (active and passive) it can be seen that an important difference between them is the exposure time to the air samples. While the active methods can collect the contamination from short periods of time commonly up to several minutes, the passive methods collect contamination for longer periods that can take larger periods until weeks or even several months. The methods' cost is another important difference where the passive samplers might be cheaper (usage of EDC and swabs) when compared with used active samplers (such as impingers and impaction machines) (76–78).

2.6.1 Advantages of passive methods

Over the past few years, it has been seen a higher adoption of passive sampling methods to overcome the known limitations when compared with active methods (76–78).

The passive sampling measures the harmful part of the airborne population that falls on a critical surface, thereby, according some authors, this makes this passive sampling offering a valid risk assessment (15,79). Since it allows a direct measurement of microorganisms settling on surfaces, this passive measurement is better than

volumetric (active) measurements when trying to predict the likelihood of the contamination rate at a surgical site (79–82).

Other keys advantages are the simplicity, low cost and lightweight of this method when compared with active sampling. The passive one also doesn't need any trained personnel to collect the samples while the active sampling require some training and knowledge for the sampling pumps and flow meters usage. Looking into passive samples, workers can work uninterruptedly (83). When comparing the performance in terms of sensitivity and reproducibility, several passive samplers are being able to provide comparable performance with the existing active samplers. Moreover, these characteristics are of added value since accurate air contamination measurements are imperative when assessing exposure for health effects (84).

3. Methodology

This Chapter aims to show the whole process carried out since the collection of samples, the location of the collections and all their processing.

3.1 Location of the samples

The samples were collected at the Clinical Pathology Service at a Hospital in Lisbon during July of 2019 in the following sections: Microbiology, Mycobacteria, Immunology, Hematology, Immunoserology and Core Labs and, Collection Room of emergency area, Triage area, Bedroom, lunch area, bathroom, office and also in all Routine Collection Rooms.

A total amount of 30 swabs were collected throughout the sections in the same day. The EDCs, 16 in total, were placed in the same day at 1.5 meters height in the sections for 15 days. In some these studied sections, due to the absence of ventilation grids, swabs' samples were not collected. The reason, in some other sections, for not collecting EDC samples, was the lack of a surface at 1.5 meters of height.

Location	No. of ventilation grid swab samples	No. of EDC samples
Collection Room – emergency area	2	1
Core Lab	7	4
Microbiology Lab	6	2
Collection Room no. 8	1	0
Collection Room no. 4	1	0
Collection Room no. 3	1	0
Collection Room no. 5	1	0
Collection Room no. 6	1	0
Collection Room no. 7	1	0
Triage Area	1	1
Mycobacteria Lab	2	1
Office	1	1
Hematology Lab	2	1
Immunology Lab	2	1
Worker's Bedroom	0	1
Lunch Area	0	1
Immunoserology Lab – PCR room	1	1
Worker's Bathroom	0	1

Table 3.1 – Samples performed in each sampling location from the Clinical Pathology Service

3.2 Materials

The material used for collecting the needed samples for this project is of easy access for everyone.

- Electrostatic precipitators – electrostatic dust cloths (EDC). The EDC used for this project are the common Swiffer that can be easily found in any nearby supermarket. They were used together with a Petri dish. EDC were put inside the Petri dishes in order to collect the particles being expelled by the ventilation girds.

Figure 3.1 - Example of an EDC being used inside a Petri dish

- Swabs – swabs were the second type of material used for collecting the needed samples for this project by rubbing them directly on the ventilation grids.

Figure 3.2 – Swabs used for collecting the microbiological load

Figure 3.3 – Example of the swab's collecting in a ventilation grid

3.3 Sampling and characterization of bioburden

The plates were incubated at 27ºC and examined for fungal contamination densities (colony forming units, CFU/m²) after 5-7 days. The fungal isolates were identified by macroscopic and microscopic morphology, using adhesive tape and lactophenol blue (85). When colony overgrowth was observed due to rapidly growing fungi (*Mucorales*, *Chrysonilia sitophila* and *Trichoderma* spp.), making colony counting impossible, a quantitative cut of 500 isolates (CFU) was applied (86).

As for the bacteria evaluation, TSA and VRBA plates were incubated at 30ºC and 35ºC for 7 days, respectively. Bacterial densities (colony forming units (CFU) per 1 m² of swab area) were determined in the two different culture media.

The microbial content on the surfaces of the ventilation grids was collected using 10 by 10 cm square swabs. Each swab was washed with 10 mL of 0.1% Tween™ 80 saline (NaCL 0.9%) by 30 minutes at 250 rpm on an orbital laboratory shaker (76).

The EDC had an exhibition area of 94.2 cm^2 and were placed 1.5 m high for 15 days. After 15 days, they were weighed and washed with 20 mL of 0.05% Tween™ 80 saline (0.9% NaCL) for 60 minutes at 250 rpm on an orbital laboratory shaker (Edmund Buhler SM-30, Hechingen, Germany) (77).

3.4 Inoculation

As for the bioburden bacteria evaluation, the suspensions were inoculated on both triptych soy agar (TSA) supplemented with 0.2% nystatin and, violet red bile agar (VRBA). TSA is a non-selective culture media, where different bacteria grow. This media is rich in tryptone and peptone, a source of carbohydrates, proteins and lipids for the development of various microorganisms, more specifically bacteria. The TSA supplemented with Nystatin is widely used for environmental monitoring preventing fungal growth, being then a specific media for bacterial growth (87).

In the other hand, VRBA is used for counting coliforms in foods and dairy products. The group of coliforms (group of bacteria that are indicators of contamination) includes facultative aerobic and anaerobic bacilli and, non-spore forming Gram-negative bacilli. Coliforms ferment lactose and form acid and gas at 35°C within 48 hours. Members of the *Enterobacteriaceae* family include the majority of the group. Coliform colonies lower the pH of the media, subsequently causing a red coloration of them (Neutral Red Dye) and the precipitation of bile salts (88).

150 µL of the resulting swab suspensions were inoculated into malt extract agar (MEA) supplemented with chloramphenicol (0.05%), and in a media based on dichloran glycerol agar (DG18) supplemented with chloramphenicol (0.01%).

MEA is a malted barley extract soluble in clarified water. This extract is a useful ingredient in culture media developed for the spread of yeasts and molds. This ingredient is suitable for yeasts and molds because it contains a high concentration of carbohydrates, particularly maltose. The approximate percentage of reducing sugars is 60–63%. Malt extract is generally used in culture media in concentrations of 10 to 100 grams per liter (89).

The DG18 agar is recommended for counting and isolating yeasts and molds from dry and semi-dry foods, including fruits, spices, cereals, nuts, meat and fish. The highly selective composition of this media allows counting the growth of the fungus. About 0.01% of chloramphenicol is added, which is an antibiotic used to prevent bacterial growth, as this means without chloramphenicol is specific for dehydrated products with water activity less than or equal to 0.95%. For products with water activity greater than 0.95%, chloramphenicol should be added. The reduction in water activity in this media is achieved by adding approximately 18% glycerol, this is very important, as many yeasts and molds actually require low water activity to improve the growth and development of the colonies. The media also contains the antifungal agent dichloride, which restricts the spread of mucoraceous fungi and restricts the colony size of other genera, making colony counting an easier task (90).

3.5 Molecular detection of critical sections of *Aspergillus*

The molecular identification of the *Circumdati*, *Flavi*, *Fumigati* and *Versicolores* sections of *Aspergillus* were carried out through real-time PCR (qPCR) resorting to the automation system (CFX Connect Real-Time PCR Detection System – Bio-Rad), following the procedures already published (91). Using positive DNA amplification controls, the reactions were obtained. These reactions include 1 iQ Supermix (Bio-Rad), with 0.5 µM from each primer (Table 3.2) and, 0.375 µM of TaqMan probe in a total of 20 µL of volume.

From reference strains belonging to the culture collection of the Reference Unit for Fungal Infections, Department of Infectious Diseases of the National Institute of Health Dr. Ricardo Jorge (INSA), DNA was obtained for each amplified gene and a non-

template control and a positive control were used. These strains were sequenced for internal transcribed spacer (ITS), tubulin B and calmodulin (92).

***Aspergillus* Sections Targeted**	**Sequences**	**Reference**
Flavi		
Forward Primer	5’-GTCCAAGCAACAGGCCAAGT-3’	
Reverse Primer	5’-TCGTGCATGTTGGTGATGGT-3’	(93)
Probe	5’-TGTCTTGATCGGCGCCCG-3’	
Fumigati		
Forward Primer	5’-CGCGTCCGGTCCTCG-3’	
Reverse Primer	5’-TTAGAAAAATAAAGTTGGGTGTCGG-3’	(94)
Probe	5’-TGTCACCTGCTCTGTAGGCCCG-3’	
Circumdati		
Forward Primer	5’-CGGGTCTAATGCAGCTCCAA-3’	
Reverse Primer	5’-CGGGCACCAATCCTTTCA-3’	(95)
Probe	5’-CGTCAATAAGCGCTTTT-3’	
Versicolores		
Forward Primer	5’-CGGCGGGGAGCCCT-3’	
Reverse Primer	5’-CCATTGTTGAAAGTTTTGACTGATcTTA-3’	(96)
Probe	5’-AGACTGCATCACTCTCAGGCATGAAGTTCAG-3’	

Table 3.2 – Sequence of primers and TaqMan probes used for real-time PCR

4. Results

4.1 Bacteria

The presented results come from the quantification of the colonies grown in the media. More precisely, TSA to quantify the number of total bacteria and VRBA to identify Gram negative bacteria.

The graphs in this section are divided by sampling method, EDC and swabs but each of them contains the information of all the analyzed areas of the hospital.

In the below Figure 4.1, it is presented a graph with the results of the bacteria growth when using swabs to collect the samples. It is possible to verify that the highest prevalence is 99.99% of total bacteria (TSA) and only 0.01% belong to Gram Negatives (VRBA).

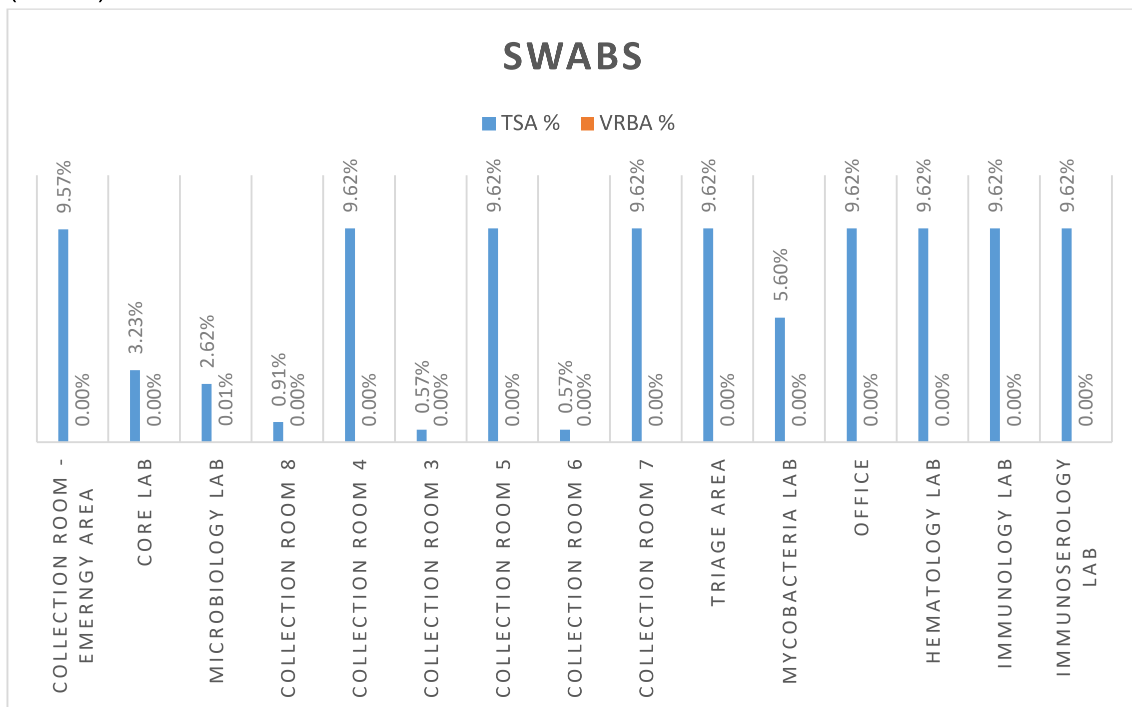

Figure 4.1 - Bacteria growth of swabs' samples collected in all the studied areas of the hospital

Comparing the different sampling sites in Swabs, it can be said that in the TSA media, the emergency collection rooms, the office, hematology, immunology and immunoserology laboratories as well as the routine collection rooms 4, 5 and 7 were the ones that showed the highest bacterial load. In contrast, routine collection rooms 3, 6 and 8 had the lowest total bacterial counts.

In the VRBA media, only gram-negative bacteria were detected in the Microbiology laboratory, which represents a residual value when compared to the total bacterial value found in the TSA media.

Next, it will be shown the graph with the bacteria growth when resorting to EDC in Figure 4.2 for all the studied areas in the hospital. The prevalence is 100% for total bacteria (TSA), with no Gram-negative bacteria (VRBA).

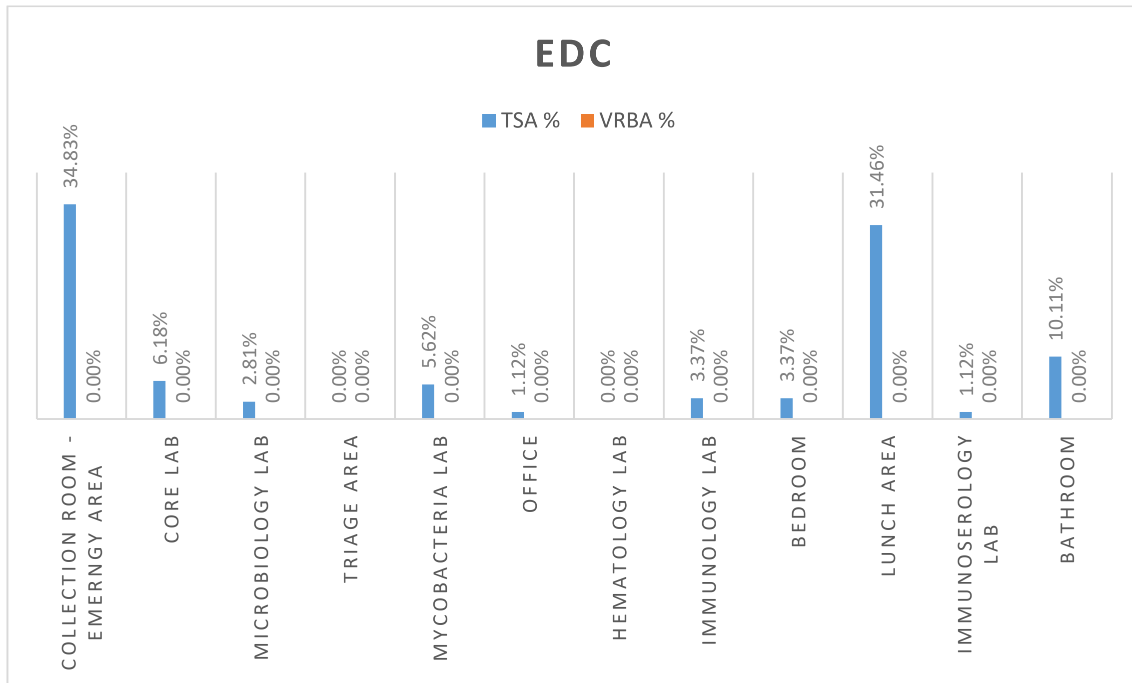

Figure 4.2 - Bacteria growth of EDC' samples collected in all the studied areas of the hospital

In the EDC' sampling sites, in the TSA media, the rooms that showed the highest prevalence of total bacteria were the emergency collection room and the lunch area, respectively.

4.2 Fungi

The presented results in this section were obtained through quantification and the identification and of growth colonies in the Screening environment. The identification was made based on the morphological characteristics (macro and micromorphological). The culture media used were MEA and DG18 for both Swabs and EDC.

4.2.1 Swabs

In this Collection Room from the Emergency Area (Figure 4.3), two samples were collected with swabs from two ventilation grids, placed in two culture media, MEA and DG18.

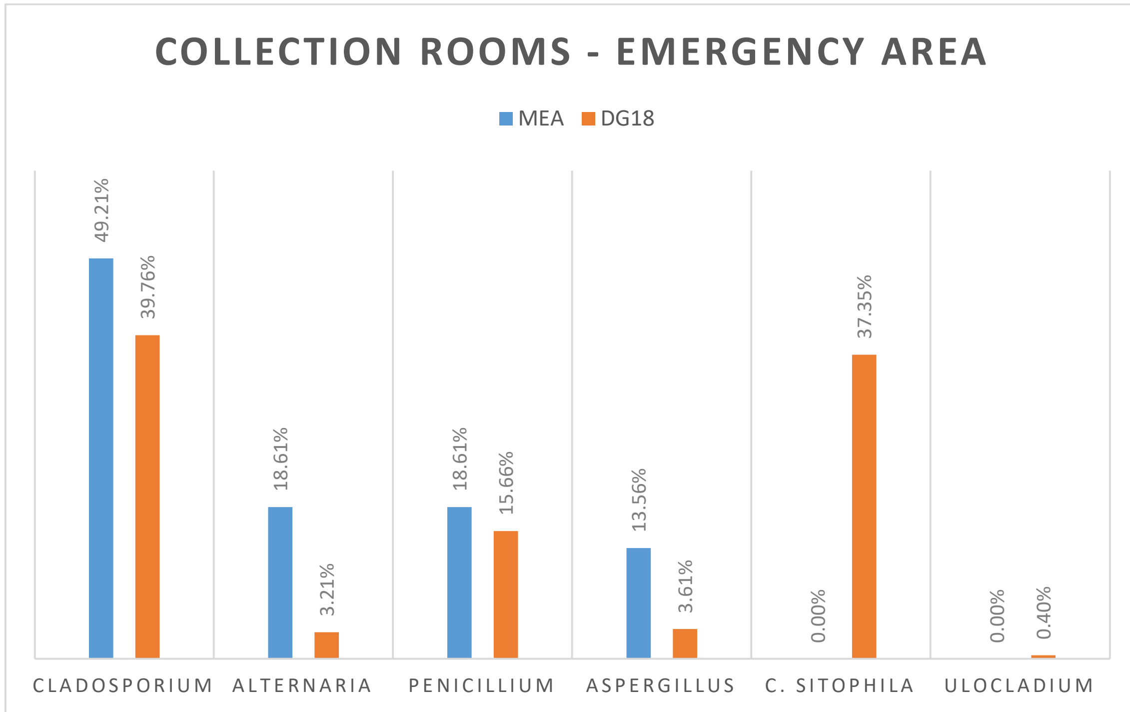

Figure 4.3 - Fungi growth of swabs' samples collected in the collection room of the emergency area

By observing the above Figure 4.3 it can be been that six different species were found in the DG18 media and four different species in the MEA media. The most prevalent species in the DG18 media were *C. sitophila*, *Cladosporium*, and *Penicillium*, while in the MEA media the most prevalent species were *Alternaria*, *Cladosporium* and *Penicillium*.

In the Core Lab (Figure 4.4), seven samples were collected with swabs. Comparing both culture media, by observing the below Figure 4.4 it can be seen that the most prevalent species were *C. sitophila*, followed by *Cladosporium*, *Mucor* and *Penicillium*.

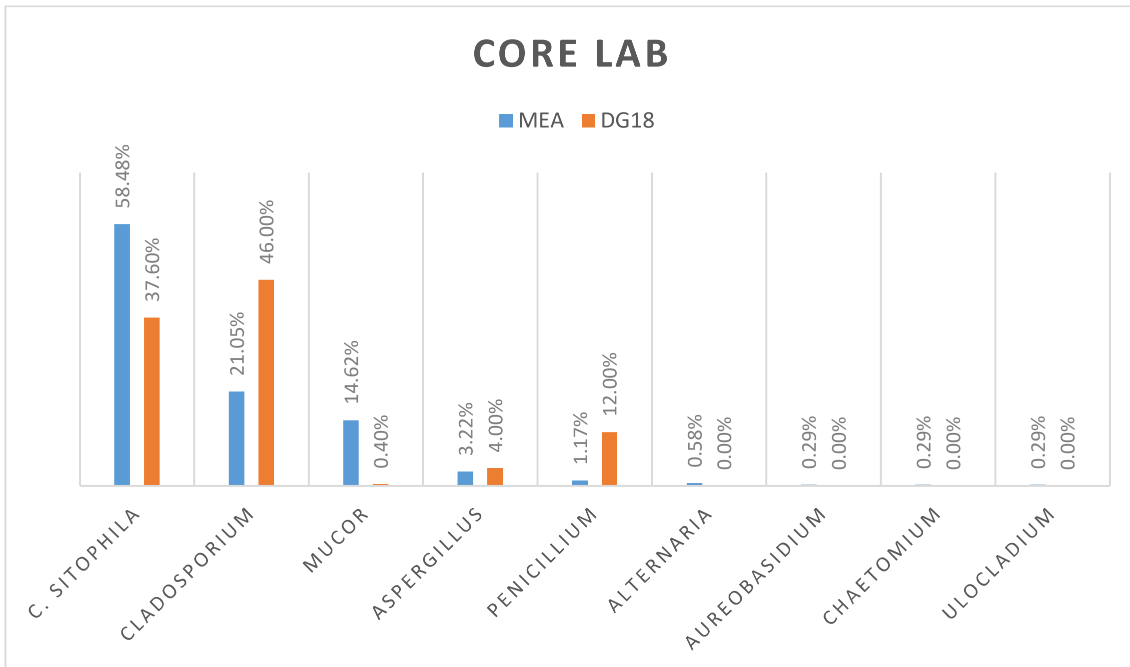

Figure 4.4 - Fungi growth of swabs' samples collected in the Core Lab

This is the place with the greatest number of workers, being 24 hours, a day receiving biological products to be processed. Since *C. sitophila* is the most commonly found fungus, it makes perfect sense, as it is a fungus that spreads very quickly (97).

The next hospital section was the Microbiology Lab where six samples were collected with swabs (Figure 4.5).

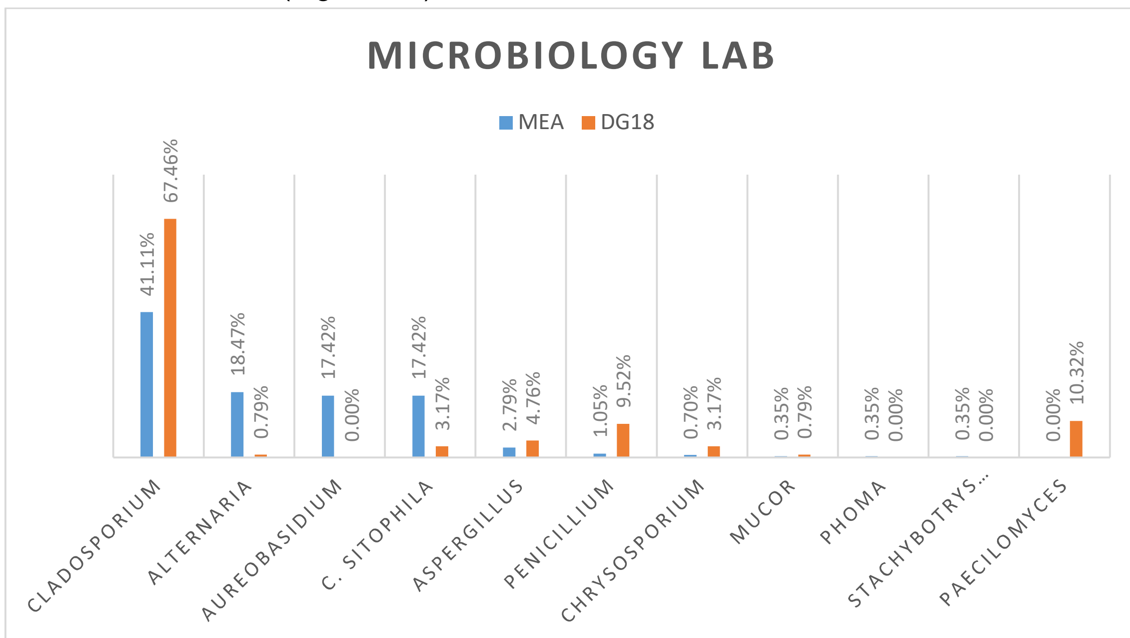

Figure 4.5 - Fungi growth of swabs' samples collected in the Microbiology Lab

The most prevalent species was *Cladosporium* spp. as it is, once again, a fast-spreading fungus and one of the most common in the environment.

From the six Collection Rooms analyzed, the bellow Figure 4.6 presents all the information. To collect the samples, one swab per room was used.

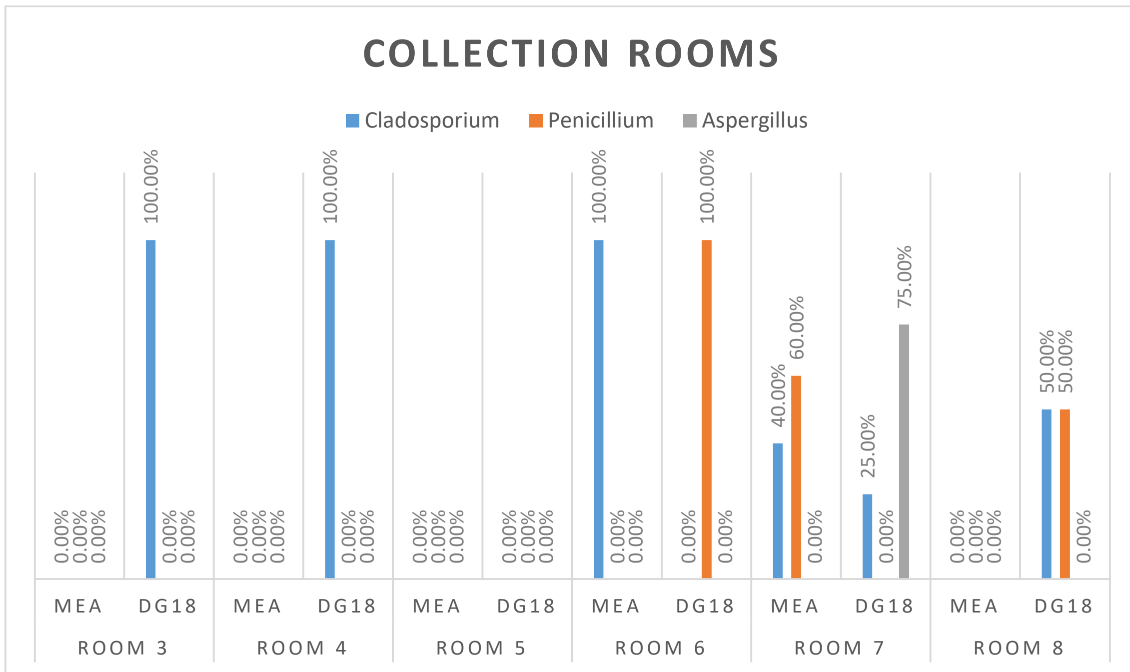

Figure 4.6 - Fungi growth of swabs' samples collected in the Collection Rooms

In the Triage area (Figure 4.7), only one swab was collected. As this room was submitted to a renewal process, where some structural works has been done, few number of species were found. The most prevalent ones were *Cladosporium* spp. and *Penicillium* spp.

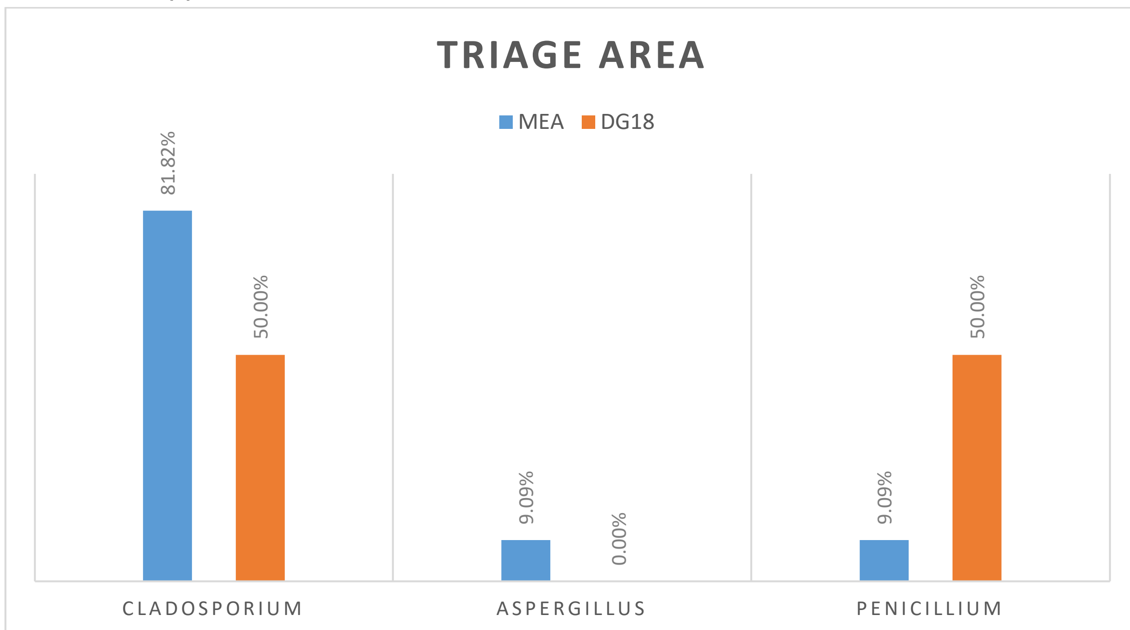

Figure 4.7 - Fungi growth of swabs' samples collected in the Triage Area

A large number of bacteria were also found, which is not qualitatively valued in this case. The fact that two of the most common fungi have been found in the environment, may mean that, due to the high number of people using this room, and because it is an area where the doors are constantly open, it means that the circulation of dust are constant, there will soon be a greater number of *Cladosporium* spp. and *Penicillium* spp. in the ventilation grids.

In the following Mycobacteria Lab (Figure 4.8), two swabs were collected from the ventilation grids.

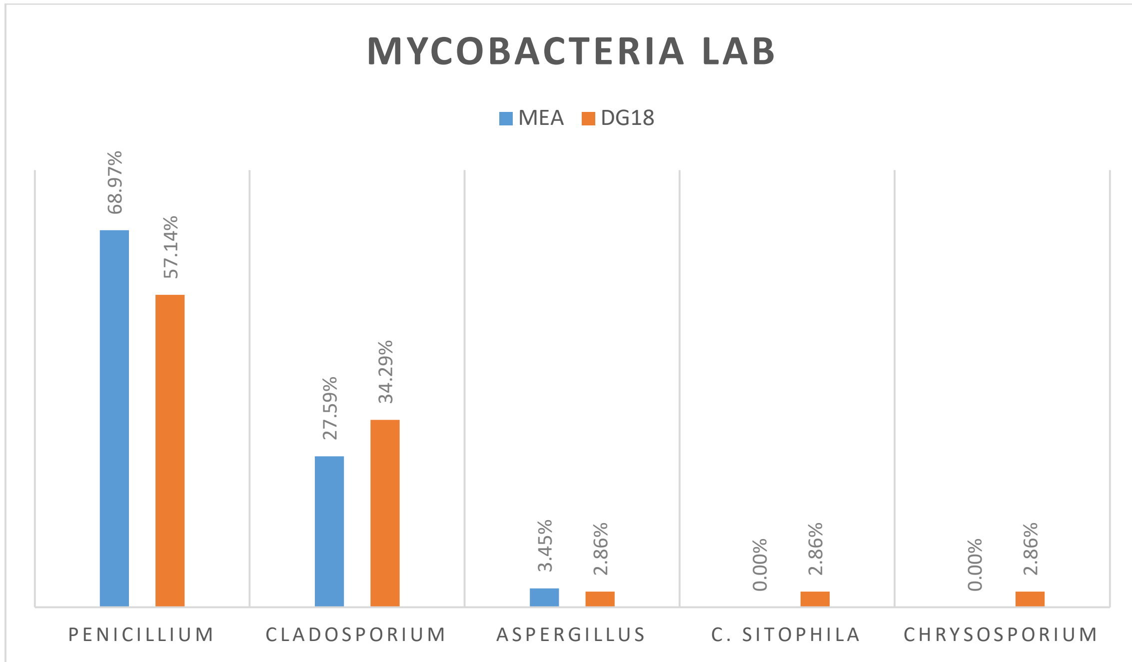

Figure 4.8 - Fungi growth of swabs' samples collected in the Mycobacteria Lab

The species found are the most common in the environment, *Cladosporium* and *Penicillium*.

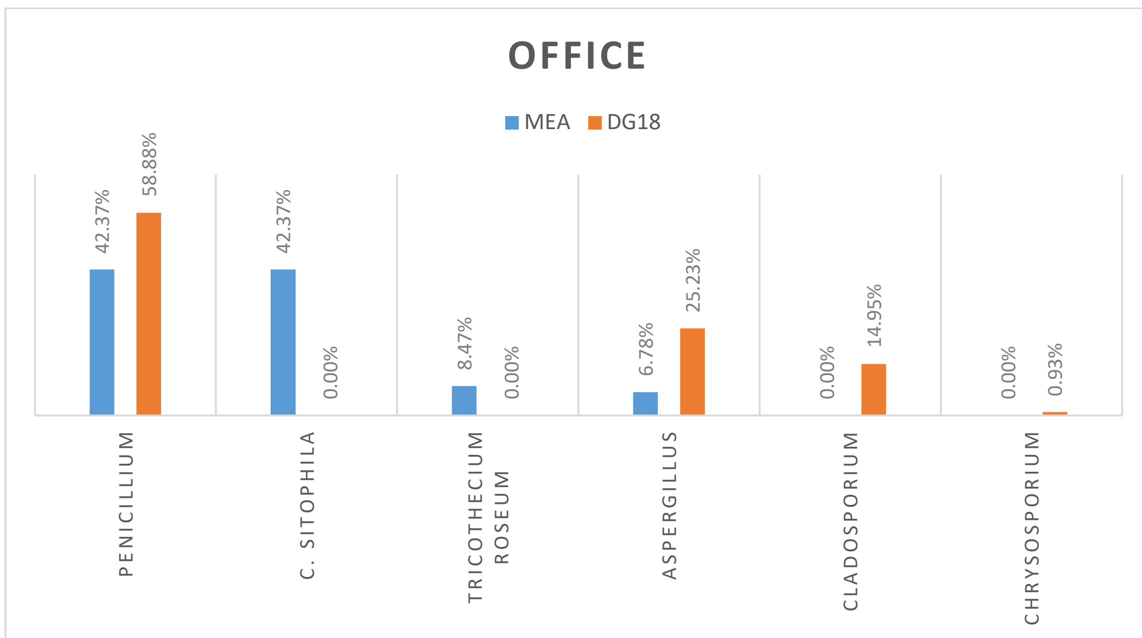

Figure 4.9 - Fungi growth of swabs' samples collected in the Office

Only one swab was collected in the Office (Figure 4.9). The most prevalent species were *Penicillium* in both media, and *C. sitophila* in MEA. *Penicillium* spp., as one of the most common fungi of the "mold", was found in a large percentage in this room, due to the amount of dossiers and older material kept on the shelves. This room also does not have good ventilation, due to the fact that the only ventilation grid is inactive

and does not have an auxiliary ventilation system (such as Air Conditioning), only open windows, for air circulation, which makes more dust entering this room and accumulating.

The Hematology Lab is a room for pathological results validation and slide viewing. In this room, two swabs were collected from the ventilation grid (Figure 4.10).

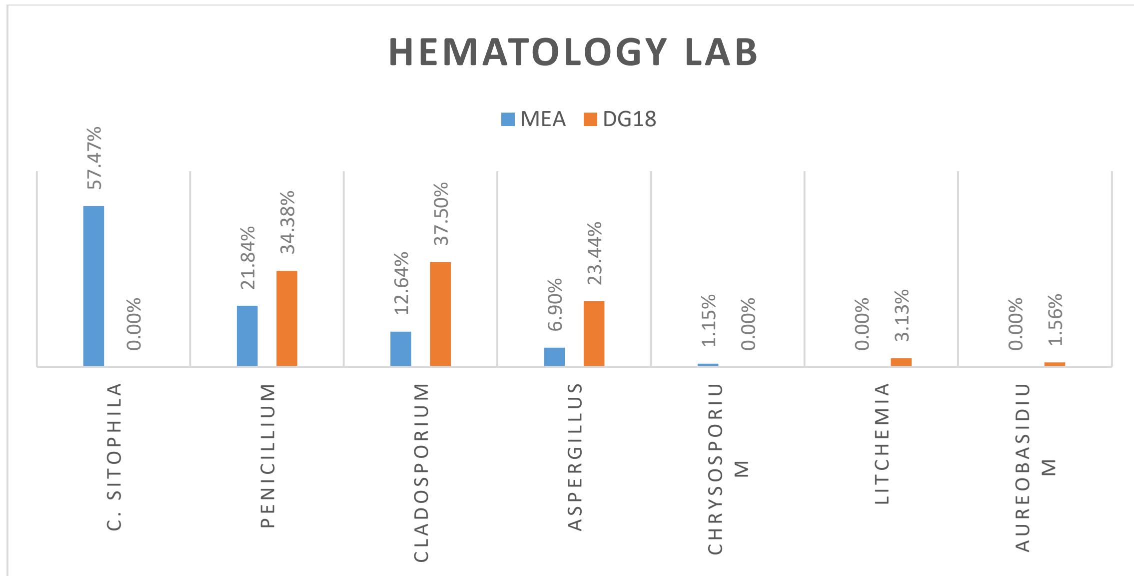

Figure 4.10 - Fungi growth of swabs' samples collected in the Hematology Lab

The most common species in both media was *Penicillium*, followed by *C. sitophila* in the MEA media and *Cladosporium* in the DG18 media.

In the Immunology Lab (Figure 4.11), similar to the previous Hematology Lab (Figure 4.10), also two swabs were used to collect fungi from the ventilation grid.

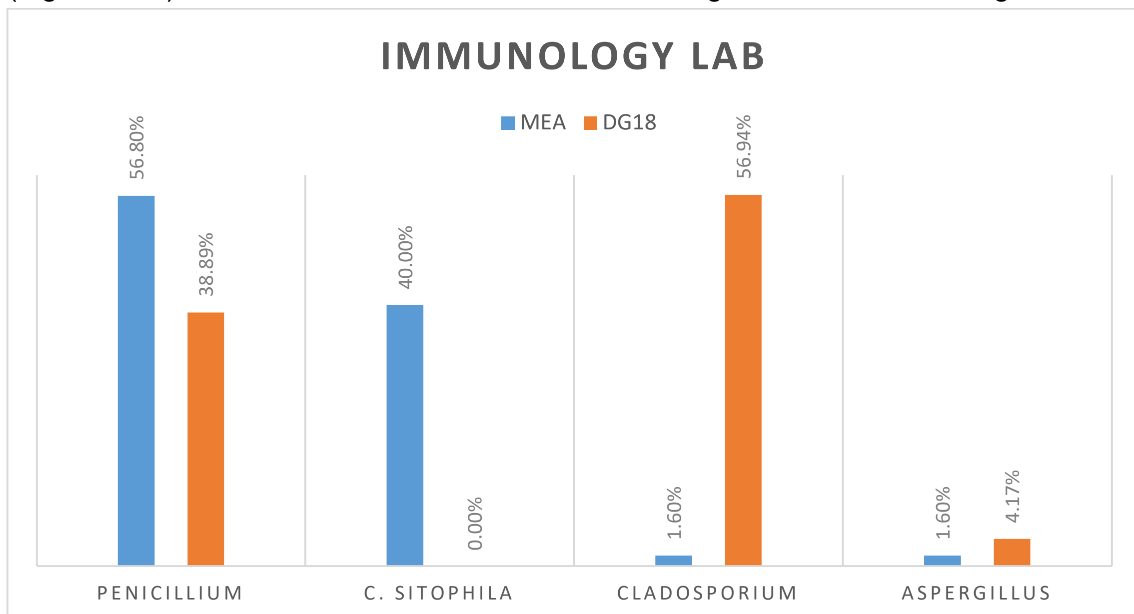

Figure 4.11 - Fungi growth of swabs' samples collected in the Immunology Lab

The most prevalent species in both media was *Penicillium*, followed by *Cladosporium* in the DG18 media and *C. sitophila* in the MEA media.

The most common species found in the Immunoserology Lab (Figure 4.12) were *Cladosporium* in both media and only *C. sitophila* was identified in the MEA media with a large percentage.

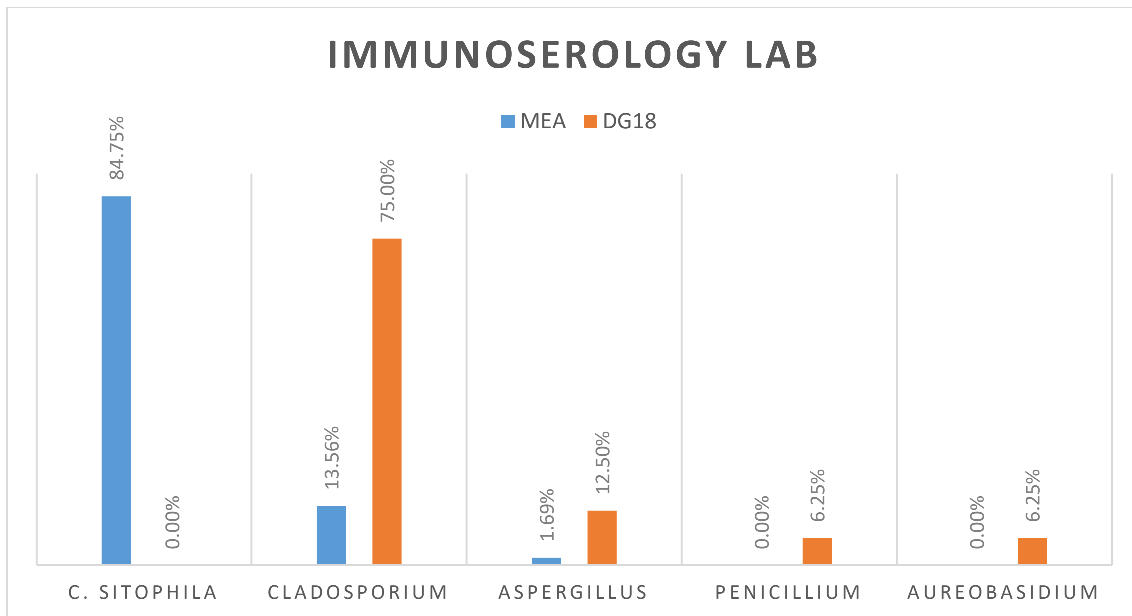

Figure 4.12 - Fungi growth of swabs' samples collected in the Immunoserology Lab

In this room, one swab was collected from the ventilation grid. It is a room that is only used a few days a week, and only by one or two people at a time, so only a few number of different species has been identified.

In the areas of bedroom, lunch area and toilet, swabs were not collected due to the lack of ventilation grids.

4.2.2 EDC

In the Collection Room from the Emergency Area (Figure 4.13), an EDC was placed and the growth of four different species was identified, the most significant of which were *C. sitophila*, *Cladosporium* and *Penicillium*.

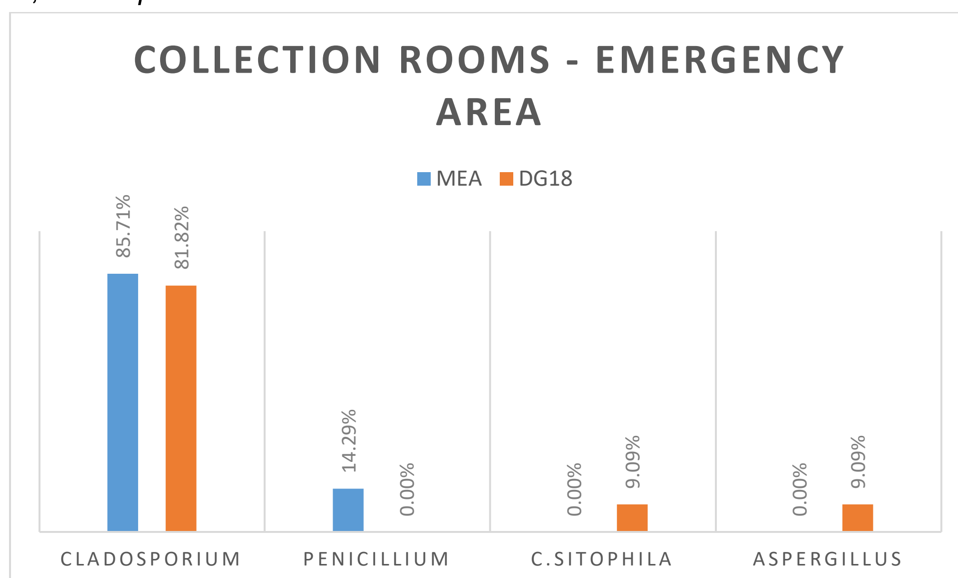

Figure 4.13 - Fungi growth of EDC' samples collected in the Collection room from the emergency area

In the core lab, as it is a very large section, four EDC were placed.

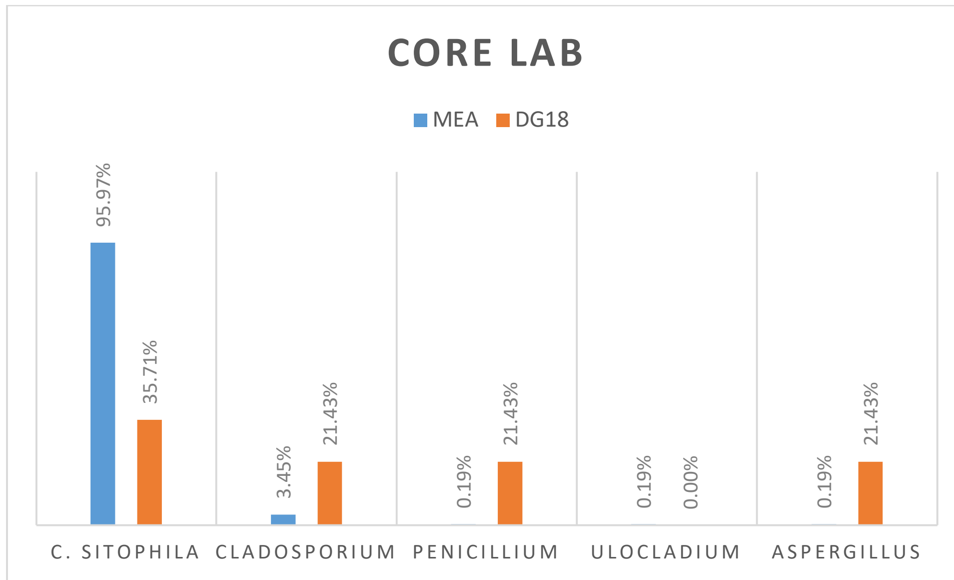

Figure 4.14 - Fungi growth of EDC' samples collected in the Core Lab

Five different species of fungi grew (Figure 4.14), the most relevant of which were *C. sitophila* which grew significantly in MEA media.

In the Microbiology section, two EDC were placed, where four different fungi were identified, the most prevalent being *Cladosporium* spp., which grew in both media (Figure 4.15).

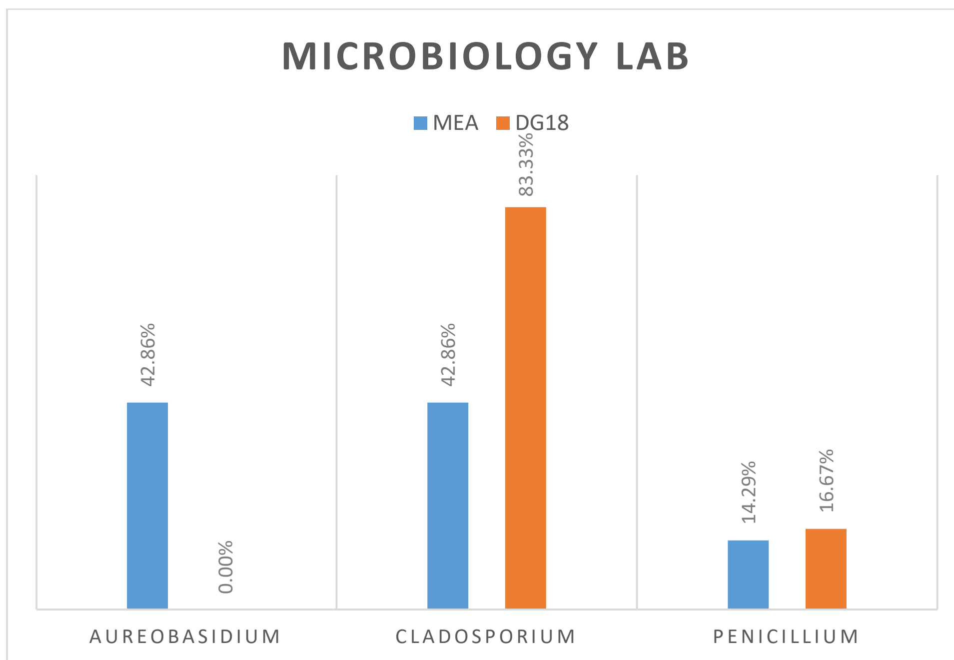

Figure 4.15 - Fungi growth of EDC' samples collected in the Microbiology Lab

As previously mentioned, the Triage area was renewed recently. Only one EDC was placed in this room, and only one fungus, *Cladosporium* spp., was detected as it can be seen in the below Figure 4.16.

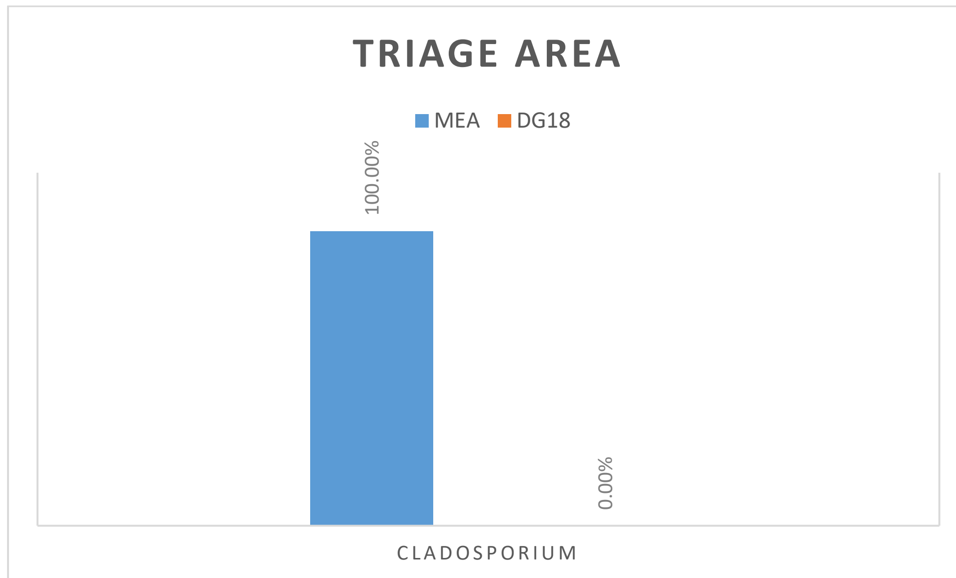

Figure 4.16 - Fungi growth of EDC' samples collected in the Triage area

In the Mycobacteria Lab, only one EDC was placed. There was growth of two types of fungi, *Cladosporium* spp. and *Penicillium* spp. (Figure 4.17).

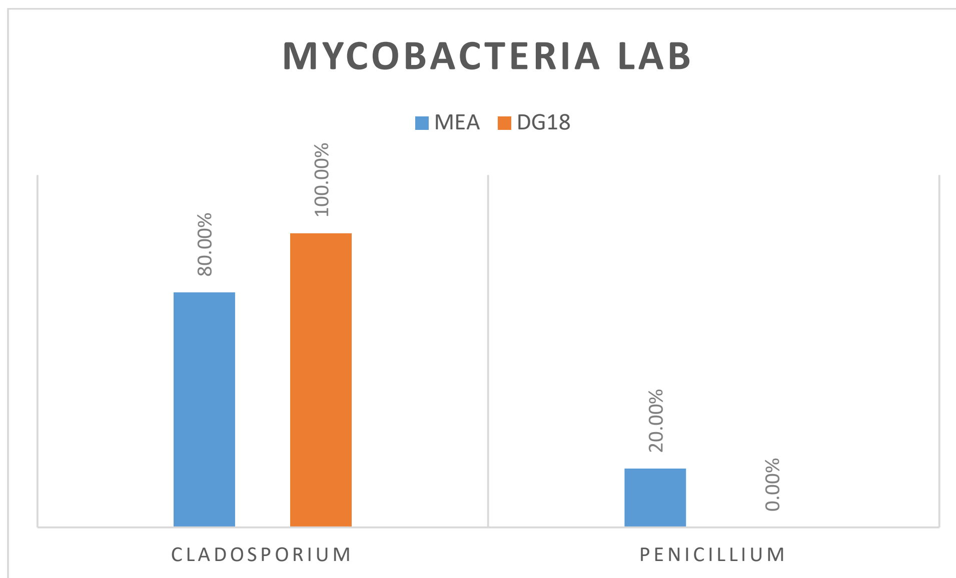

Figure 4.17 - Fungi growth of EDC' samples collected in the Mycobacteria Lab

Cladosporium spp. grew in both MEA and DG18 media but *Penicillium* spp. only grew in MEA media.

An EDC was also placed in the Office and three different species of fungi were found (Figure 4.18).

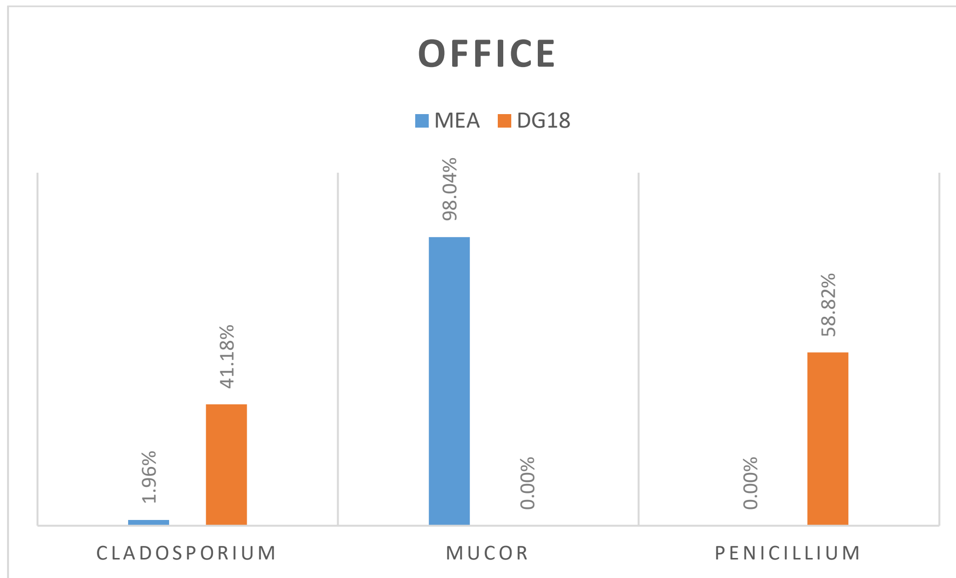

Figure 4.18 - Fungi growth of EDC' samples collected in the Office

An EDC was placed in the Hematology Lab where three different fungi grew (Figure 4.19).

- *Penicillium* spp. grew only in DG18 media;
- *Cladosporium* spp. was found in MEA;

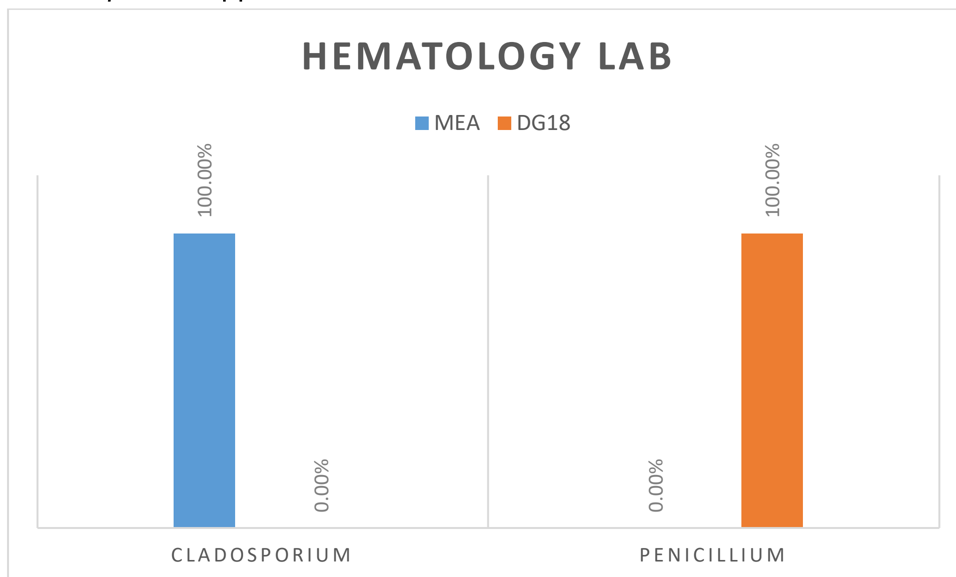

Figure 4.19 - Fungi growth of EDC' samples collected in the Hematology lab

Only one EDC was placed in the Immunology Lab. Three types of fungi were found (Figure 4.20): Chrysosporium spp., *Cladosporium* spp. and *Penicillium* spp. which grew only in MEA and DG18 media, the most prevalent being *Cladosporium* spp.

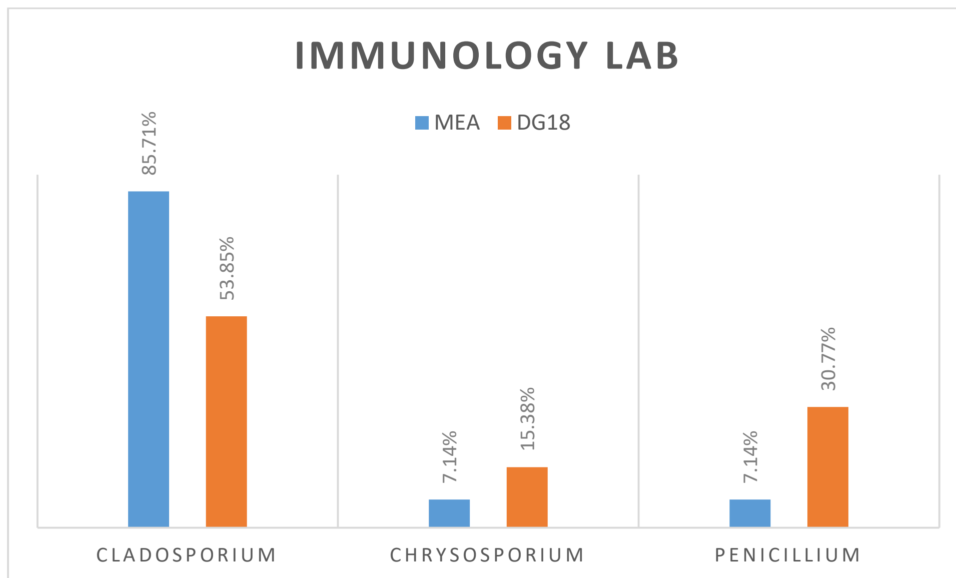

Figure 4.20 - Fungi growth of EDC' samples collected in the Immunology lab

An EDC was placed in the Bedroom, but 2 distinct species were found as is can be seen in the below in Figure 4.21.

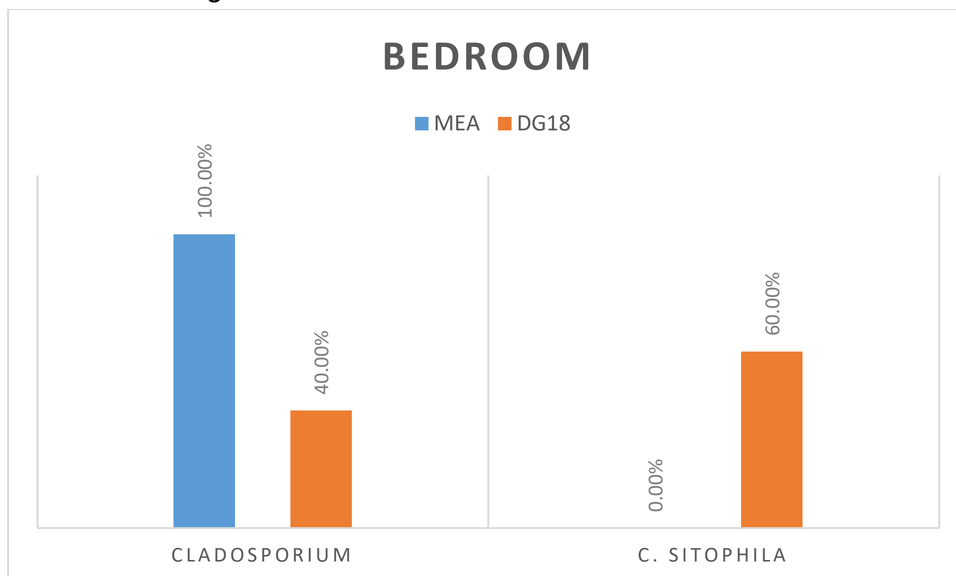

Figure 4.21 - Fungi growth of EDC' samples collected in the Bedroom

The most prevalent species found were *Cladosporium* in both media and also *C. sitophila* that only grew in DG18.

In the Lunch area, only one EDC was placed but, probably, due to the high number of people using this area, five different species of fungi were found as it can be seen in the below Figure 4.22.

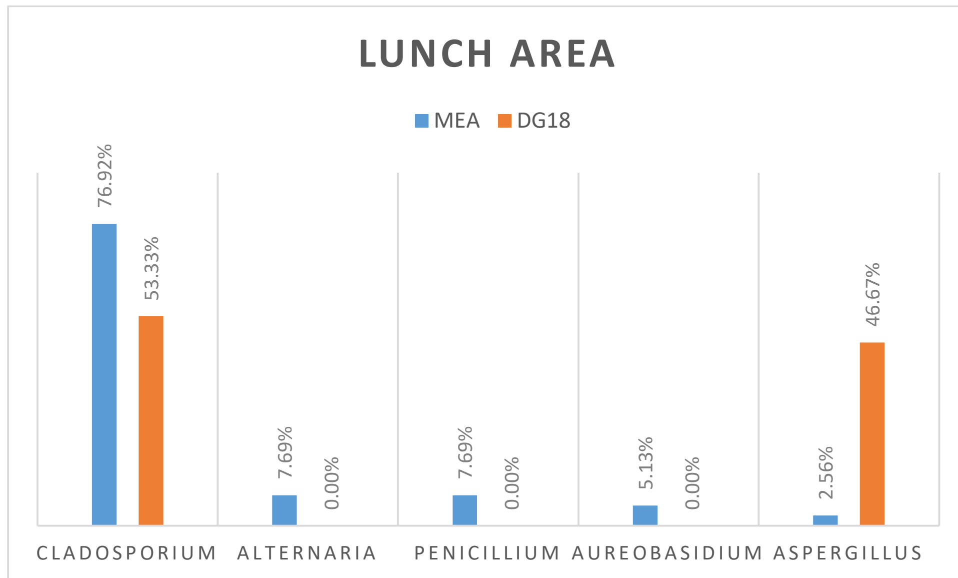

Figure 4.22 - Fungi growth of EDC' samples collected in the Lunch area

The most prevalent were *Cladosporium* spp., which grew in MEA and DG18 media.

Next, in both Immunoserology Lab (Figure 4.23) and Bathroom (Figure 4.24), only one EDC was used per location to collect the needed samples.

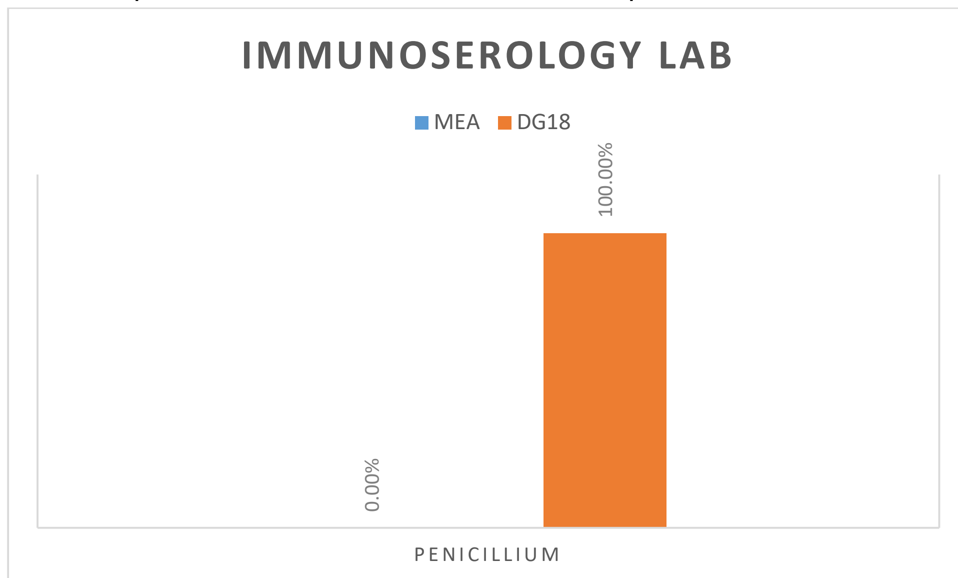

Figure 4.23 - Fungi growth of EDC' samples collected in the Immunoserology Lab

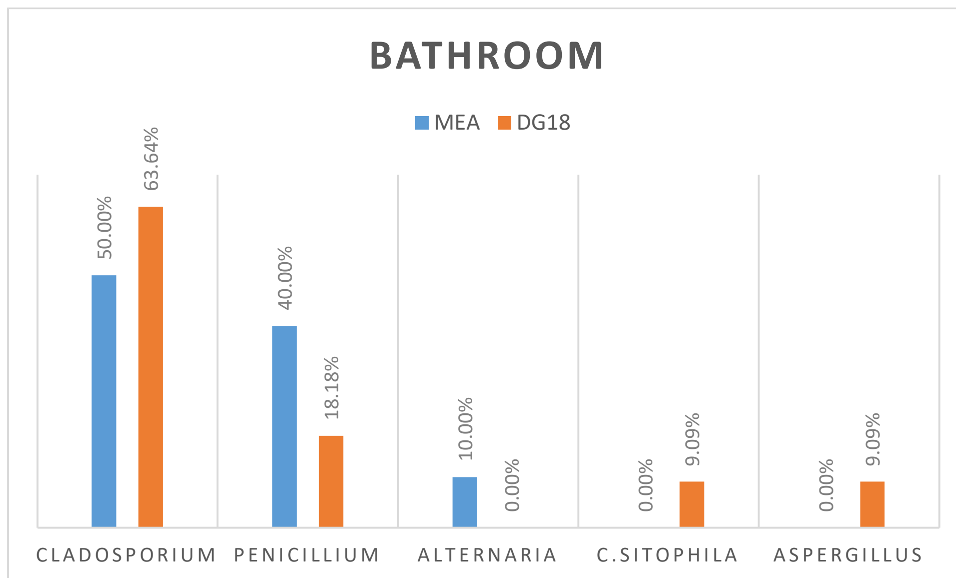

Figure 4.24 - Fungi growth of EDC' samples collected in the Bathroom

In the Immunoserology lab (Figure 4.23) there was growth of *Penicillium* spp. only in DG18.

In the Bathroom (Figure 4.24), there was growth of five different species being the most prevalent *Cladosporium* and *Penicillium* which grew in DG18 and MEA media.

Below, it can be seen an aggregated graph of the most prevalent fungal species found in as hospital sections combined for swabs and EDC in both MEA and DG18 medias.

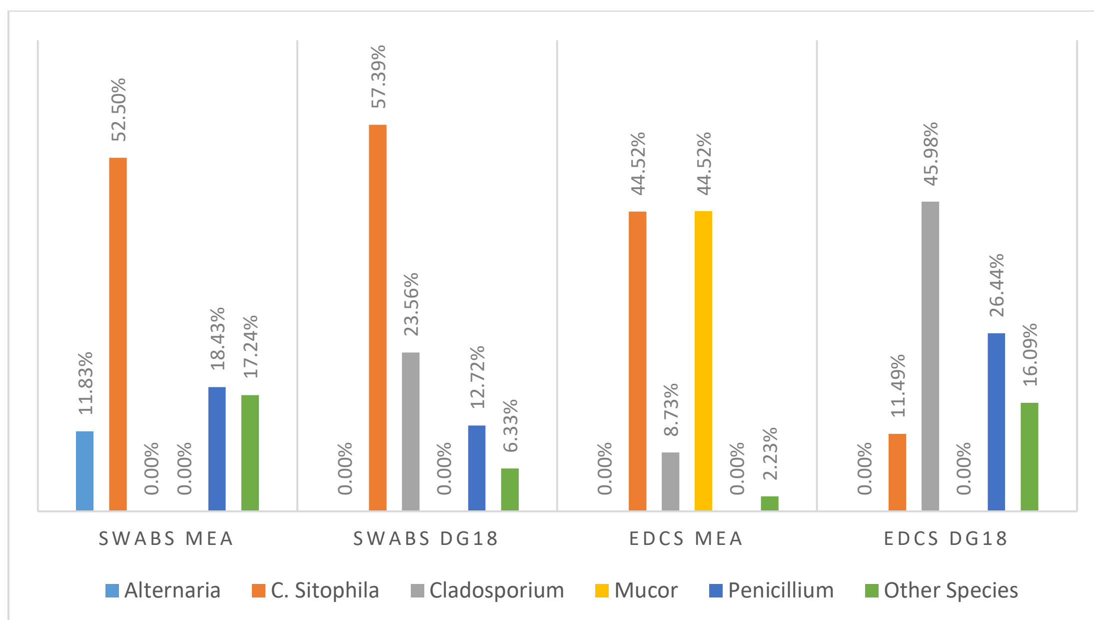

Figure 4.25 - Overall results of EDC and swabs samples in both MEA and DG18 medias

In the swabs, the most prevalent species was *Chysonilia sitophila*, which presented 52.50% in the MEA media and 57.39% in the DG18 media, followed by *Penicillium* spp. in the MEA media with 18.43% and finally *Cladosporium* spp. in DG18 with 23.56%.

In EDCs, *Cladosporium* spp. showed the highest prevalence in the DG18 media with 45.98% while in the MEA media the most prevalent fungi were *C. sitophila* and *Mucor* spp. with 44.52% in both.

4.2.3 *Aspergillus* sections

Among the *Aspergillus* sections, in the swabs the *Flavi* section was the one with the highest prevalence in the MEA media with 58.02% and the *Nigri* section showed the highest prevalence in the DG18 media with 55.41%.

Swabs						
Aspergillus sections	**MEA**			**DG18**		
	CFU	**CFU m²**	**%**	**CFU**	**CFU m²**	**%**
Flavi	47		58.02%	14		18.92%
Nigri	28		34.57%	41		55.41%
Versicolores	3		3.70%	2		2.70%
Circumdati	3		3.70%	14		18.92%
Aspergilli	0		0.00%	3		4.05%
Total	81		100.00%	74		100.00%
EDC						
Versicolores	2	212.31	100.00%	6	636.94	75.00%
Circumdati	0	0.00	0.00%	2	212.31	25.00%
Total	2	212.31	100.00%	8	849.26	100.00%

Table 4.1 - Aspergillus sections found in both sampling methods in MEA and DG18 media

In the EDC samples, the *Versicolores* section had a higher prevalence in both media (100% MEA and 75% in DG18), however, the section *Circumdati* was found in DG18 media.

4.2.4 qPCR

From the four investigated *Aspergillus* sections (described in Section 3.5), only two were found resorting to the qPCR technique. From the next table 4.2 it can be seen the following:

- *Aspergillus* section *Fumigati* was:
  - detected in 10 out of 15 Swabs corresponding to 66.67% using qPCR;
  - detected in 7 out of 12 EDC corresponding to 58.33% using qPCR;
  - not detected for culture-based methods.
- *Aspergillus* section *Versicolores* was:
  - detected in 1 out of 12 EDC corresponding to 8.33% using qPCR;
  - not detected in any Swabs using qPCR;
  - detected in 3 out of 12 EDC corresponding to 25% using culture-based methods;
  - detected in 4 out of 15 Swabs corresponding to 26.67% using culture-based methods;

Aspergillus Section Detected	Sample Type	Location	CFU/CFU m^2 (MEA/DG18)	C_q
Aspergillus* section *Fumigati	**Swabs**	Core Lab	0/0	36.08
		Collection room No. 8	0/0	39.36
		Collection room No. 5	0/0	35.94
		Collection room No. 6	0/0	35.23
		Collection room No. 7	0/0	39.25
		Triage area	0/0	36.57
		Mycobacteria Lab	0/0	37.85
		Hematology Lab	0/0	36.23
		Immunology Lab	0/0	35.56
		Immunoserology Lab - PCR room	0/0	38.85
	EDC	Collection room - emergency area	0/0	35.93
		Microbiology Lab	0/0	37.82
		Hematology Lab	0/0	37.77
		Immunology Lab	0/0	38.66
		Workers´ bedroom	0/0	35.34
		Canteen	0/0	36.82
		Immunoserology Lab - PCR room	0/0	39.71
Aspergillus* Section *Versicolores	**Swabs**	Collection room - emergency area	0.5/0	-
		Core Lab	0/0.14	-
		Microbiology Lab	0.17/0.17	-
		Hematology Lab	0.5/0	-
	EDC	Core Lab	26.54/0	-
		Workers´ bedroom	0/0	32.79
		Canteen	106.16/530.79	-
		Workers´ bathroom	0/106.16	-

Table 4.2 - Molecular detection results in Swabs and EDC

5. Discussion

This work had as main objective the identification and characterization of bioburden in a hospital environment, more specifically in the Clinical Pathology service of a hospital in the Lisbon region.

It is necessary to consider the requirements for a good IAQ in a hospital, which must be more demanding than in other buildings. In addition to being a very particular environment, with a wide range of elements that can disturb the health, comfort and productivity of health professionals, it is also a place with patients who have the weakest immune system, being more vulnerable to a deficient IAQ**.** (98)

The fact that a health unit is a very particular environment, where there is a plurality of activities, conditions and occupations, which characterize the different services, makes the IAQ assessment to be carried out individually. Thus, each service has specific requirements and it is necessary to adapt a good IAQ to the objective of each activity, providing an optimization of the productivity of its workers, as well as the well-being and comfort of all occupants (41).

It should be mentioned that the assessment of IAQ proved to be a complex process that relates different variables and, for this aspect, can lead us to serious errors in the significance of the results. That is, occasional events or a limitation of measurements can influence one or more variables, compromising the final results, namely in shorter campaigns (98). In order to overcome this problem, two sampling methods were used to make the evaluation as representative as possible, but still limited in terms of the specificity of the hospital environment. When comparing the two sampling methods, active and passive, an important difference is noted, which is the needed time of exposure to air samples. While active methods can collect contamination for short periods of time, usually up to minutes, passive methods collect contamination for longer periods such as weeks or months. The cost of the methods is also relevant since the passive methods are cheaper when compared to the active methods.

Regarding the characterization of bacteria, two different medias were used. In order to be able to account for the growth of total bacteria, the TSA media was used, and to be able to identify gram-negative bacteria, which are generally more harmful to public health, the VRBA media was used (87,88).

The high concentration of bacteria inside the spaces, may be related to the high permanence of people (health professionals, patients and companions), since according to (99), humans are the main source of indoor air contamination. Occupants of the space constantly release bacteria from hair, skin, nostrils and the oral cavity (100). This helps to sustain the obtained results where some hospital sections had a high count of total bacteria, because they are more used than other sections in the hospital, that showed a much lower count of total bacteria, since they are substantially less used. The authors in (101), point this as a clear indicator of different variations in terms of bacteria load per area. Other possibilities could be related with temperature, humidity or even malfunctioning or insufficient ventilation of each room as mentioned in (102).

From the results, it was observed an expected higher prevalence of total bacteria when compared to the number of gram-negative bacteria. These were expected results mainly due to the difference between the used media. The TSA media was used to determine the total bacterial load (bacteria gram-positive and negative) in which the samples were placed at a temperature of 30ºC. In VRBA media, a more selective media, the samples were placed at 35ºC (77), which is the approximate temperature of the human body. With the latter, it is expected that not many colonies would grow because

they do not exist or simply because existing gram-negative bacteria are not viable, meaning that they won't be pathogenic to humans. It would be concerning and harmful to health if there was a critical growth in this environment (103). Also, according to (104), the absence or low value of gram-negative bacteria can also be explained by the fragility of their cell walls that don't tolerate dehydration caused by air conditioning (76).

Regarding the fungal load, two different medias were also used, MEA and DG18.

In the swabs the highest prevalence was found in the MEA media (27.6%), which is in line with the literature, since the swabs were collected from the ventilation grids, where there is a large percentage of moisture and water, therefore the fungi that growth in this media are not xerophilic (105).

In the EDC, on the other hand, the highest prevalence is in the DG18 media (91.9%), because as the samplings were made in the air, in places with low humidity, the fungi that growth in this environment are xerophilic, that is, they do not need great conditions for its growth and it is controlled by the size of the colonies, making counting easier and more accurate (105).

The routine collection and screening rooms obtained exceptionally low results in relation to the fungal load. These rooms, although with the presence of several patients and workers, are rooms that had recently been built, so the presence of fungi is lower in relation to older rooms. Recently built rooms tend to avoid the SBS due to the new material being used. Although many people attend those rooms, with a proper maintenance cleaning and maintenance, the SBS can be avoided suppressing the possible growth of fungi. (32,34,35).

The mycobacteria room would be an area where many fungi were expected to grow, due to the type of biological products processed in that room, but that was not what was revealed. One of the possible reasons for not having many fungi detected in this room is due to the sample processing and all the techniques are done in a laminar flow chamber. The usage of laminar flow chamber helps to extract of all microorganisms present making the working environment safer (106).

The remaining rooms obtained expected results due to the high fungal load since they are rooms where there is a high number of workers, patients and biological products to be processed. In the case of the office and the lunch area, despite having no biological products or patients, these are areas with a large affluence of workers, day in day out. Besides the number of people attending to the lunch area, another factor that sustains the expected high fungal load result is the fact the food itself can also produce fungi thus, helping increasing the fungal load in this specific area (107).

Regarding fungi, the different number of species in each sample was of 16 species in the MEA and DG18 medias for swabs and in the EDC 9 different species were obtained in the MEA media and 7 in the DG18 media.

Mucor sp., one of the most prevalent fungi in EDC of MEA, may cause serious, though rare, infections in immunocompromised patients in hospitals. The infection caused by *Mucor*, called Mucormycosis is caused by a group of molds called mucormycetes. The most common genera that cause this infection in humans are *Rhizopus* and *Mucor* species, however there are also other species. Mucormycosis infection can affect almost any part of the body from lung infections caused by inhaling spores, skin infections, the entry of spores into the skin and, gastrointestinal infections when *Mucor* is present in food (108).

The *Cladosporium* genus was the most prevalent species found in both environments because it comprises a large number of dematiaceous fungi with worldwide distribution and which are among the most common environmental fungi. They are frequently isolated as contaminants however, some species are pathogenic and

toxigenic for humans, being associated with superficial infections of the skin and soft tissues and include disseminated sepsis with high mortality. *Cladosporium* spp. are aero-allergens and cause severe allergic diseases of the respiratory tract, as well as intrabronchial lesions (109).

Chrysonilia sitophila, is considered a contaminant without pathogenicity, but it can have implications for the onset of asthmatic diseases (occupational asthma) (110). It can be a contaminant when spread in laboratories and hospitals, because it can grow very easily and thanks to spore multiplication it can easily contaminate many surfaces (111) and workspaces used for various microbiological procedures.

Fungi of the genus *Penicillium* spp., are found in soil, decaying vegetation or wood, dry foods, spices, dry cereals, fresh fruits and vegetables, growing in building materials in damaged water environments, as well as in indoor air and house dust. They are mesophilic fungi that need to grow at a temperature between 5-37°C (optimal, 20-30°C) (105).Some species of the genus *Penicillium* are mycotoxin producers. Ecological and biological factors react with each species, varying the importance of these toxic compounds (112). In this study it was not possible to identify which section of *Penicillium* was found in each sample.

Aspergillus is a genus composed of more than 180 anamorphic species and is divided into several sections, such as *Flavi*, *Circumdati*, *Nigri*, *Fumigati*, *Versicolores*, *Restricti*, *Cervini*, *Nidulantes*, *Flavípedes*, *Usti*, *Terrei*, *Candidi*, *Cremei*, *Sparsi* and *Wentii* (113).

The fungi found in swabs (*Flavi* in MEA and *Nigri* in DG18) and EDC (*Versicolores* and *Circumdati*) are fungi capable of metabolizing and synthesizing various organic compounds. One of the most well-known organic compounds are mycotoxins, produced under favorable conditions such as humidity and temperature, which can be harmful to human health (39,72).

Of all the *Aspergillus* sections found, the most worrying is *Flavi*, as it is a cosmopolitan fungus which produces a mycotoxin (secondary metabolites) called aflatoxins. Aflatoxins B1 is considered capable of causing cancer and is generally the main aflatoxin produced by toxigenic strains (114). It is a fungus of great medical importance with regard to opportunistic infections such as aspergillosis (colonization of the airways and respiratory tract) and respiratory allergies (115).

Molecular biology techniques have been widely used, as they allow species identification in a few hours or days.

The identification of fungi is done with the aid of several laboratory techniques, through classical microbiology (sowing and macro and microscopic identification). However, these techniques are considered time-consuming and require specialized professionals (116). Therefore, the polymerase chain reaction (PCR) technique has been widely accepted as an important tool for the diagnosis of fungi and bacteria (117).

In this study, the molecular tools were very efficient in detecting the *Aspergillus* section *Fumigati*, while for the *Aspergillus* section *Versicolores*, the culture-based methods were more valuable. It was possible to investigate the *Aspergillus* sections using the qPCR technique in swab samples from the ventilation grids and in the EDC. Only two sections were detected. *Aspergillus* section *Fumigati* that was not possible to identify through culture media and the *Aspergillus* section *Versicolores* that was only found in the culture media.

Molecular tools have several advantages over conventional methods, such as greater typing and discrimination power, greater speed, good detection limit, greater selectivity, specificity, potential for automation and the possibility of working with bacteria and fungi that are not cultivable in regular culture medias (118). One of the main

obstacles to its implementation in the laboratory routine is the inability of the method to differentiate between living and dead cells and because it is an expensive technique.

According to recent studies, the reason for not having determined *Aspergillus* section *Fumigati* in culture media is tied with the fact that the fungus is no longer alive, so it has no growth capacity in culture media. Since it was found only in PCR, it means that the fungus is present but is not viable, meaning that it is not harmful to public health (119,120).

In relation to *Aspergillus* section *Versicolores*, it was detected in seven locations through culture and in one location using the PCR technique. As previously reported (76,121,122), the fact that it wasn't possible to identify *Aspergillus* section *Versicolores* using the qPCR technique in most locations can be explained due to the samples contain complex environmental substances interfering with the inhibition of PCR amplification.

6. Conclusion

Overall, the two sampling methods used in this study - swabs and EDC - revealed a more complete feature of bioburden, allowing us to obtain information on the exposure of workers and patients. In addition, culture-based methods and molecular tools used in parallel should be used to perform an accurate characterization of fungal contamination.

Other studies still need to be done, in other areas of the hospital and at another time of the year to be able to compare and reflect on the results and allow to establish a better comparison.

The comparison between the sampling sites regarding microbial contamination allowed us to prioritize an intervention in which biocides (to eliminate fungi / bacteria) should be applied at each sampling site. However, it was verified between the analyzed fungal contamination that a positive correlation was applied between the two sampling methods.

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Appendixes

Appendix 1

Scientific Article associated to this study – published article

Article

Bioburden Assessment by Passive Methods on a Clinical Pathology Service in One Central Hospital from Lisbon: What Can it Tell Us Regarding Patients and Staff Exposure?

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Abstract: The assessment and control of microbial contamination in health care facilities is presently a mandatory and vital part of strategies to prevent and control hospital-acquired infections. This study aims to assess the bioburden with two passive sampling methods (30 ventilations grids swabs and 16 electrostatic dust collectors (EDCs)) at Clinical Pathology Services. The fungal burden was characterized through molecular tools, antifungal resistance, and the mycotoxins and cytotoxicity profile. Total bacteria presented the highest prevalence in both matrixes, whereas Gram-bacteria presented the lowest. Swabs presented a higher prevalence (27.6%) for fungal burden. *Chrysonilia sitophila* presented the highest prevalence in swabs, whereas for EDCs, *C. sitophila* and *Mucor* sp. were the most prevalent. Concerning *Aspergillus* genera on swabs, section *Flavi* was the one with the highest prevalence (58.02%), whereas, for EDCs, section *Versicolores* was the only section observed (100%). *Aspergillus* section *Fumigati* was detected in 10 swabs and 7 EDC samples and *Aspergillus* section *Versicolores* was detected in one EDC sample. Fungal growth on azole-supplemented media was observed in eight EDC samples. No mycotoxins were detected in any of the samples. A low cytotoxic effect was observed in two sites upon incubation of collected samples with A549 and SK cells and in two other sites upon incubation of collected samples with SK cells only. A medium cytotoxic effect was observed with one EDC sample upon incubation with A549 cells. This study reinforces the need of determination of the azole resistance profile for fungal species and allowed a preliminary risk characterization regarding the cytotoxicity. An intervention including the use of a ultraviolet with wavelength between 200 nm and 280 nm (UVC)—emitting device and an increased maintenance and cleaning of the central heating, ventilation, and air conditioning (HVAC) systems should be ensured to promote the reduction of microbial contamination.

Keywords: bioburden; passive methods; *Aspergillus*; azole resistance profile; cytotoxicity analysis

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1. Introduction

Hospital facilities are indoor environments with a high risk of cross infection between their patients and staff. Studies performed in European hospitals [1] reported that nosocomial infections add significantly to morbidity and mortality rates and that many of these infections are transmitted by airborne pathogens [2]. Several everyday activities from health care facilities can boost bio-aerosols dispersion [3–6], leading to infectious disease transmission. As such, the assessment and control of microbial contamination in health care facilities is currently a mandatory and vital part of the strategies to prevent and control hospital-acquired infections [7,8]. Aiming at addressing this challenging assessment, several studies have already reported the upmost importance of applying passive sampling methods [9–13]. These methods allow characterization of the contamination from a larger period of time (weeks to several months) [14].

The presence of distinct species of airborne microorganisms in clinical environments may concur for a wide range of negative health effects, both in patients, a potentially more vulnerable population, and in clinical staff, long-term exposed to resident bioburdens during work tasks. The toxicity and pathogenicity for humans of microorganisms varies greatly among genus and among species within the same genus. The emergence worldwide of drug-resistant human pathogenic microbial species, such as Methicillin-resistant *Staphylococcus aureus* (MRSA) and fungal species, such as *Candida* sp. and *Aspergillus fumigatus*, and the increasing reports of therapeutic failure against fungal infections [15–17] has revealed the need of surveillance of both bacterial and fungal resistance in health care organizations [18,19].

Although it is not currently well known to which degree the exposure to toxigenic airborne microbiota may contribute to actual adverse effects on human health, it is accepted, for instance, that fungi development in buildings is unhealthy and must be prevented [20]. Bacteria and fungi can promote allergic and infectious responses, and also inflammatory responses via inhalation of endotoxin or ß-glucans [21,22]. Additionally, it has been suggested by previous studies that modification of immune responses may be induced by long-term exposure to toxigenic fungi, leading to the development of neurological symptoms, fever, cough, and fatigue [23].

Regarding fungi, *Aspergillus fumigatus* is one of the most studied species in clinical environments due to their high prevalence as contaminants in clinical material, while being responsible for invasive fungal infections among immunocompromised patients [24]. Their great toxicity is due to the production of specific mycotoxins, such as aflatoxin B1, ochratoxin A, and gliotoxin. Indeed, in a review by Viegas et al. from 2018 [25], it was possible to conclude the relevance to monitor mycotoxins presence in clinical environments.

There are several published guidelines regarding ventilation of health care facilities [26–29]. However, the majority focuses on specialist facilities, such as isolation rooms, operating theatres, and bronchoscopy wards, where the risk of infection is well characterized. In contrast, guidelines regarding the ventilation of general wards, patient rooms, intensive care wards, or even other specific areas are much scarcer and often dedicated to clinical cases and not to the indoor environment. The same gap was found in microbial contamination studies, as indicated in scientific databases [13,14]. Furthermore, almost all studies focused on patients' health, disregarding clinical and laboratory health of staff [14]. However, the staff can transport and disseminate microbial contamination through all hospital facilities, thus affecting patients [13].

Several studies were performed aiming at testing and recommending new technologies to ensure air cleaning [30–33]. However, efforts should prioritize the exposure assessment to better characterize and control the risk. This will allow the selection of the best and more suitable technologies.

To the best of our knowledge, no former studies were performed in a Clinical Pathology Service of a central hospital, applying passive methods, such as a sampling approach. This study aims to assess the bioburden using two passive sampling methods (ventilations grids and electrostatic dust collectors). The fungal burden was also characterized through molecular tools for the toxigenic species, antifungal resistance, and the mycotoxins and cytotoxicity profile.

2. Materials and Methods

2.1. Indoor Environment Assessed and Sampling Locations

The present study was performed in June, 2019 in a Clinical Pathology Service from one central hospital with 802 total number of hospital beds, comprising all hospital services. The hospital was located in the Lisbon district. It was part of an enlarged exploratory study with financial support to establish protocols for assessing occupational exposure to bioburdens in clinical environments. The Clinical Pathology Service assessed included laboratory facilities, in which diagnosis of disease was performed based on body fluids (biological samples). In this area, only hospital staff were allowed in, with the exception of the collection rooms.

A walkthrough survey and checklist were used in order to prioritize the more critical workplaces/areas regarding bioburdens, as previously published [13]. The assessed areas were equipped with central heating, ventilation, and air conditioning (HVAC) systems, with air handing units and a ductwork system. Filtered outdoor air was supplied through diffusers, coupled with grids (ventilation grids) in the ceiling/above the door, and evacuated by corner outlets at the floor level. Regarding the cleaning procedures, floors and surfaces were cleaned daily after laboratory service and working hours and were performed by an external company.

The sampling procedures were focused in specific areas, namely, one collection room in the emergency area, the core laboratory, the microbiology laboratory, six collection rooms, the triage area, the mycobacteria lab, one office, the hematology lab, the immunology room, the worker's bedroom, bathroom, and canteen, and the immunoserology laboratory. In each area. two different types of samples were collected: swab samples in the ventilation grid and electrostatic dust collector (EDC) samples (Table 1). All the samples were collected in the same period of time, during a normal working day.

Table 1. Samples collected in each location of the Clinical Pathology Service.

Location	Number of Ventilation Grid Swab Samples	Number of EDC Samples
Collection room—emergency area	2	1
Core lab	7	4
Microbiology lab	6	2
Collection room No. 8	1	
Collection room No. 4	1	
Collection room No. 3	1	
Collection room No. 5	1	
Collection room No. 6	1	
Collection room No. 7	1	
Triage area	1	1
Mycobacteria lab	2	1
Office	1	1
Hematology lab	2	1
Immunology room	2	1
Workers' bedroom		1
Canteen		1
Immunoserology lab—PCR room	1	1
Workers' bathroom		1

2.2. Sampling and Characterization of the Viable Bioburden

Passive sampling methods were applied to determine the contamination levels of a greater period of time and also to avoid any kind of disruption of the clinical activities. Ventilation grids (with 3 dm^2 of area) samples (a total of 30) were collected by swabbing the grids. Swabs were subject to microbial extraction following a specific protocol for extraction [13] and plated onto the selected media, namely malt extract agar (MEA) supplemented with chloramphenicol (0.05%) and dichloran glycerol agar (DG-18) were used for fungi; tryptic soy agar (TSA) supplemented with cycloheximide (0.1%) was used to assess the bacterial load, and violet red bile agar (VRBA) to assess Gram negative bacteria.

Each EDC (a total of 16) had a surface expose area of 0.00942 m^2 and dust was allowed to settle down for 15 days before analysis. Settled dust collected by the EDC was weighted after sampling to allow mass determination of the collected dust. EDC samples were subject to extraction and bioburden, characterized by culture-based methods, as previously performed [14]. EDC extracts were seeded onto the same culture media applied for the swabs.

EDC samples were screened for antifungal resistance on Sabouraud dextrose agar (SDA) media, supplemented with 4 mg/L itraconazole (ITRA), 1 mg/L voriconazole (VORI), or 0.5 mg/L posaconazole (POSA), with non-supplemented SDA as the control as performed previously [13,14].

2.3. Molecular Detection of Aspergillus Sections

Molecular identification of the different fungal species/strains was obtained by real-time PCR (qPCR), using the automation system (CFX)-Connect PCR System (Bio-Rad). Reactions were performed, using positive controls of DNA amplification, as indicated previously [13]. Reactions included 1× iQ Supermix (Bio-Rad), 0.5 μM of each primer (Table 2), and 0.375 μM of TaqMan probe in a total volume of 20 μL. For each gene amplified, a non-template control and a positive control, consisting of DNA obtained from a reference, belonged to the culture collection of the Reference Unit for Parasitic and Fungal Infections, Department of Infectious Diseases of National Institute of Health Dr. Ricardo Jorge. These strains have been sequenced for Internal transcribed spacer (ITS), B-tubulin, and Calmodulin.

Table 2. Sequence of primers and TaqMan probes used for real-time PCR.

Fungal Species/Sections Targeted	Sequences	Reference
Flavi **(Toxigenic Strains)**		
Forward Primer	5′–GTCCAAGCAACAGGCCAAGT–3′	
Reverse Primer	5′–TCGTGCATGTTGGTGATGGT–3′	[34]
Probe	5′–TGTCTTGATCGGCGCCCG–3′	
Fumigati		
Forward Primer	5′–CGCGTCCGGTCCTCG–3′	
Reverse Primer	5′–TTAGAAAAATAAAGTTGGGTGTCGG–3′	[35]
Probe	5′–TGTCACCTGCTCTGTAGGCCCG–3′	
Circumdati		
Forward Primer	5′–CGGGTCTAATGCAGCTCCAA–3′	
Reverse Primer	5′–CGGGCACCAATCCTTTCA–3′	[12]
Probe	5′–CGTCAATAAGCGCTTTT–3′	
Versicolores		
Forward Primer Reverse Primer	5′–CGGCGGGGAGCCCT–3′	
	5′–CCATTGTTGAAAGTTTTGACTGATCTTA–3′	
Probe	5′–AGACTGCATCACTCTCAGGCATGAAGTTCAG–3′	[36]

2.4. Mycotoxins Analysis

EDC samples (0.05 g) were extracted by shaking 2.5 mL of mixture of acetonitrile (ACN) with: water (H_2O): acetic acid (AcOH) (79:20:1) for 90 min. After centrifugation (5 min, 5000 rpm), each sample (2 mL) was evaporated under nitrogen and reconstituted in a mobile phase (0.2 mL; A:B 7:3). Mycotoxins detection was developed through the use of a high-performance liquid chromatograph (HPLC) Nexera (Shimadzu, Tokyo, Japan), with a mass detector 5500 QTrap (Sciex, Foster City, CA, USA) (Table 3). Mycotoxins were separated on a chromatographic column Gemini C18 (150 × 4.6 mm, 5 µm) (Phenomenex, Torrance, CA, USA). The flow rate was 1 mL/min and the injection volume was 5 µL. Mobile phases were: a methanol/water/acetic acid 10/89/1 (v/v/v) and B methanol/water/acetic acid 97/2/1 (v/v/v) (both phases contained 5 mmol/L ammonium acetate) with the following gradient: 0% B up to 2 min, 50% B from 2 to 5 min, 100% B from 5 to 14 min, 100% B up to 18 min, then 0% B to 22.5 min. The Tandem Mass Spectrometry MS/MS was performed in scheduled multiple reaction monitoring (sMRM) mode in both in negative and positive polarities in one chromatographic run per sample. The Characterization of Electrospray Ionization (ESI)-source parameters were as follows: curtain gas 30 psi, collision gas medium, ionspray voltage 4500 V (negative polarity) and 5500 V (positive polarity), temperature 550 °C, ion source gas1 80 psi, ion source gas2 80 psi.

Table 3. Detection and quantification values for each mycotoxin (ng/g).

Mycotoxins	Limit of Detection (LOD)	Limit of Quantification (LOQ)
15-Acetyldeoxynivalenol	2.8	9.4
3-Acetyldeoxynivalenol	5.1	1.9
Aflatoxin B1	0.3	0.9
Aflatoxin B2	0.2	0.8
Aflatoxin G1	0.6	2.1
Aflatoxin G2	0.7	2.2
Aflatoxin M1	0.3	0.9
α-Zearalanol	1.0	3.3
α-Zearalenol	1.3	4.5
β-Zearalanol	1.1	3.8
β-Zearalenol	1.7	5.6
Deepoxydeoxynivalenol	6.2	20.7
Deoxynivalenol	3.5	11.7
Diacetoxyscirpenol	1.6	5.4
DON-3-Glucosid	3.2	10.6
Fumonisin B1	12.2	40.8
Fumonisin B2	7.5	25.0
Fumonisin B3	8.4	27.9
Fusarenon-X	7.7	25.6
Gliotoxin	4.2	13.9
Griseofulvin	0.8	2.7
HT-2 Toxin	1.6	5.2
Mevinolin	1.1	3.7
Moniliformin	2.7	8.9
Monoacetoxyscirpenol	3.4	11.4
Mycophenolic acid	2.5	8.2
Neosolaniol	3.1	10.3
Nivalenol	4.3	14.5
Ochratoxin A	0.5	1.6
Ochratoxin B	1.7	5.7
Patulin	8.3	27.6
Roquefortine C	4.1	13.6
Sterigmatocystin	0.7	2.2
T-2 Tetraol	8.0	26.7
T-2 Toxin	0.7	2.4
T-2 Triol	2.5	8.4
Zearalanone	1.3	4.3
Zearalenon	0.9	3.0

2.5. Cell Culture and MTT Assay

Cell viability of was determined after incubation of a swine kidney monolayer (SK) and adenocarcinomic human alveolar basal epithelial (A549) cell lines with EDC samples. Cells were maintained in tissue culture flasks (TPP) medium (minimum essential medium (MEM) with Earle's salts, Sigma-Aldrich, St. Louis, MO, USA), supplemented with antibiotics (Penicillin, Streptomycin (Sigma-Aldrich, St. Louis, MO, USA)) and fetal bovine serum (Sigma-Aldrich, St. Louis, MO, USA) at 37 °C in a humidified atmosphere with 5% CO_2. For the assay, cells were harvested with trypsin/ Ethylenediaminetetraacetic acid solution (EDTA) (1:10, v:v) in phosphate buffered saline (PBS) and plated in flat bottom 96-microtiter plates (TPP) in 100 μL culture medium at densities of about 2.5×10^5 cells/mL.

Colorimetric assay for assessing cell metabolic activity (MTT) assay was used to assess cellular viability after incubation with EDC samples through cleavage of MTT tetrazolium salt (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide). The formation of formazan crystals was measured by spectrophotometrical absorbance of the samples using the ELISA microplate reader (ELISA LEDETECT 96, biomed Dr. Wieser GmbH) at a wavelength of 510 nm.Data analysis was performed with MikroWin 2013SC software (Labsis, Neunkirchen-Seelscheid, Germany). The threshold toxicity level was defined as the lowest concentration of the extract that causes a drop in sample absorption to values <50% of cell division activity, as evaluated by the dilution method.

2.6. Statistical Analysis

The data were analyzed in statistical software, SPSS 22.0 for Windows. The results were considered significant at the 5% significance level. The Shapiro–Wilk test was used to test the normality of the data. Frequency analysis (n; %), and the calculation of the minimum and maximum medians were performed for the qualitative and quantitative data, respectively. The Spearman correlation coefficient was used to study the relationship between fungal and bacterial contamination and cytotoxicity.

3. Results

3.1. Bioburden Characterization

Total bacteria (TSA) presented the highest prevalence in both matrixes (99.99% Swabs; 100% EDC), whereas Gram-Bacteria (VRBA) presented the lowest (0.01% Swabs; 0% EDC) (Table 4).

Table 4. Bioburden distribution on swabs and EDC.

Bacterial Contamination	
Swabs	
	Mean (SD) CFU
TSA	2.3×10^2 (2.4×10^2)
VRBA	3.3×10^{-2} (17.9×10^{-2})
EDC	
	Mean (SD) CFU m^{-2}
TSA	7.2×10^2 (9.6×10^2)
VRBA	0.0×10^2 (0.0×10^2)
Fungal contamination	
Swabs	
	Mean (SD) CFU
MEA	2.4×10^2 (3.2×10^2)
DG18	0.6×10^2 (1.4×10^2)
EDC	
	Mean (SD) CFU m^{-2}
MEA	0.7×10^2 (0.06×10^2)
DG18	1.7×10^2 (0.05×10^2)

Concerning the fungal burden, the highest prevalence for swabs was observed on MEA (27.6%), whereas EDC presented the highest prevalence on DG18 (91.9%) (Table 4).

Concerning the number of different fungal species in each matrix, swabs presented 16 different species in both media, whereas EDC presented nine different species in MEA media and seven in DG18 media.

Chrysonilia sitophila presented the highest prevalence in swabs in both media (52.50% MEA; 60.93% DG18), followed by *Penicillium* sp. in MEA (18.43%) and *Cladosporium* sp. in DG18 (25.02%) (Figure 1). In EDC, *Cladosporium* sp. presented the highest prevalence in DG18 media (45.98%), whereas, on MEA, the highest prevalence belonged to *C. sitophila* and *Mucor* sp. (44.52%) (Figure 1).

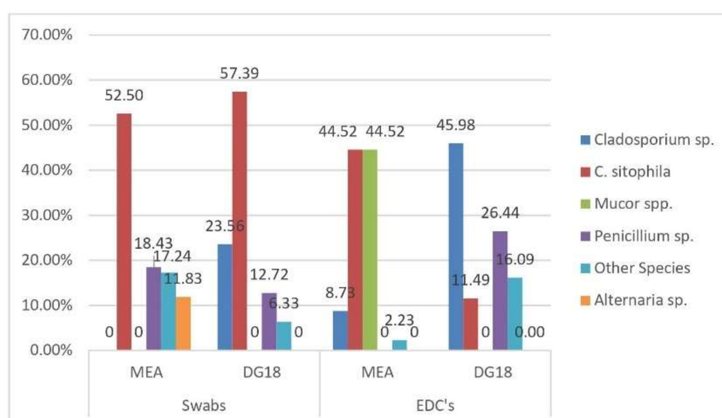

Figure 1. Fungal distribution on both environmental matrices.

Among *Aspergillus* genera, section *Flavi* was the one with the highest prevalence on MEA (58.02%) and *Nigri* section on DG18 (55.41%). Referring to the EDC samples, section *Versicolores* was the section that presented the highest prevalence in both media (100% MEA; 75% DG18). However, the *Aspergillus* section *Circumdati* (25%) was also found in DG18 (Table 5).

Table 5. *Aspergillus* section distribution on swabs and EDC.

Swabs					
	MEA			**DG18**	
Sections	**CFU**	**%**	**Sections**	**CFU**	**%**
Flavi	47	58.02	*Nigri*	41	55.41
Nigri	28	34.57	*Circumdati*	14	18.92
Versicolores	3	3.70	*Flavi*	14	18.92
Circumdati	3	3.70	*Aspergilli*	3	4.05
			Versicolores	2	2.70
Total	81	100.00	Total	74	100.00
EDC					
	MEA			**DG18**	
Sections	**CFU m^{-2}**	**%**	**Sections**	**CFU m^{-2}**	**%**
Versicolores	212.31	100.00	*Versicolores*	636.94	75.00
			Circumdati	212.31	25.00
Total	212.31	100.00	Total	849.26	100.00

Fungal growth on azole-supplemented media was observed in eight EDC samples on ITRA, 10 samples on VORI, and three samples on POSA. The most frequent species were: *C. sitophila* on SAB (97.51%), *Cladosporium* sp. on ITRA (27.27%) and VORI (68.00%), and *Penicillium* sp. on POSA (66.67%) (Table 6). *Cladosporium* sp. was able to grow in more than one azole in two EDC samples, namely from "Core lab" (ITRA+VORI) and from "Microbiology" (ITRA+VORI+POSA).

Table 6. Fungal azole resistance distribution.

	EDC							
	SAB		ITRA		VORI		POSA	
Species	CFU m^{-2}	%	CFU m^{-2}	%	CFU m^{-2}	%	CFU m^{-2}	%
Acremonium sp.			106.16	4.55				
Alternaria sp.			106.16	4.55				
Aureobasidium sp.	212.31	0.08						
C. sitophila	266,029.72	97.51	530.79	22.73	530.79	20.00		
Chrysonilia sp.	212.31	0.08						
Chrysosporium sp.	318.47	0.12	530.79	22.73				
Aspergillus section *Circumdati*	212.31	0.08						
Cladosporium sp.	4140.13	1.52	636.94	27.27	1804.67	68.00	106.1571	33.33
Fusarium verticilloides	318.47	0.12						
Penicillium sp.	1380.04	0.51	424.63	18.18	318.47	12.00	212.31	66.67
TOTAL	272,823.78	100.00	2335.46	100.00	2653.93	100.00	318.47	100.00

Among the four *Aspergillus* sections investigated by qPCR in swab samples from ventilation grids and in the EDC, only two were detected. *Aspergillus* section *Fumigati* was detected in 10 swab samples (10 out of 15; 66.67%) and 7 EDC samples (7 out of 12; 58.33%) (Table 7). Interestingly, *Fumigati* was not detected by culture-based methods in any of the samples (EDC and ventilation grid swabs).

Table 7. Molecular detection results from the EDC and ventilation grids swab samples.

Aspergillus Section Detected	Sample Type	Location	CFU/CFU m^{-2} (MEA/DG18)	C_q
Aspergillus section *Fumigati*	Ventilation grids swabs	Core lab	0/0	36.08
		Collection room No. 8	0/0	39.36
		Collection room No. 5	0/0	35.94
		Collection room No. 6	0/0	35.23
		Collection room No. 7	0/0	39.25
		Triage area	0/0	36.57
		Mycobacteria lab	0/0	37.85
		Hematology lab	0/0	36.23
		Immunology lab	0/0	35.56
		Immunoserology lab—PCR room	0/0	35.85
	EDC	Collection room—emergency area	0/0	35.93
		Microbiology lab	0/0	37.82
		Hematology lab	0/0	37.77
		Immunology lab	0/0	38.66
		Workers' bedroom	0/0	35.34
		Canteen	0/0	36.82
		Immunoserology lab—PCR room	0/0	39.71

Table 7. *Cont.*

Aspergillus Section Detected	Sample Type	Location	CFU/CFU m^{-2} (MEA/DG18)	C_q
Aspergillus section *Versicolores*	Ventilation grids swabs	Collection room—emergency area	0.5/0	-
		Core lab	0/0.14	-
		Microbiology lab	0.17/0.17	-
		Hematology lab	0.5/0	-
	EDC	Core lab	26.54/0	-
		Workers' bedroom	0/0	32.79
		Canteen	106.16/530.79	-
		Workers' bathroom	0/106.16	-

The *Aspergillus* section *Versicolores* was detected in one EDC sample by qPCR (1 out of 12; 8.33%) (Table 7); although it was detected in more samples by culture-based methods (3 out of 12 EDC samples; 25%, and 4 out of 15 swab samples; 26.67%), none of which corresponded to the sample detected at the molecular level (the workers' bedroom EDC sample).

3.2. Cytotoxicity Analysis

EDC samples were submitted to a cytotoxicity evaluation in two distinct cell lines A549 and SK cells via application of the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay. The MTT assay assesses cell viability by means of reducing the yellow tetrazolium salt to insoluble formazan. A semi-quantitative scale for cytotoxicity grading was adopted: low cytotoxic effect (+) for percentage of extinction values ranging from 80% to 90%, medium cytotoxic effect (++) for values ranging from 60% to 79%, and a high effect (+++) for values below 60%. The absence of cytotoxicity was determined when the extinction values were at ≥90. A low cytotoxic effect was observed in two locals with A549 and SK cells ("Collection room-emergency area" and "Core lab") and in other two locals with SK cells only ("Immunoserology lab-PCR room" and "Worker's bathroom"). The medium cytotoxic effect was observed with one EDC sample from "Canteen" on A549 cells. Although isolated analysis of two EDC samples from "Core lab" also revealed a medium cytotoxic effect on SK or A549 cells, in average, and "Core lab" presented a low cytotoxicity effect in vitro (Figure 2).

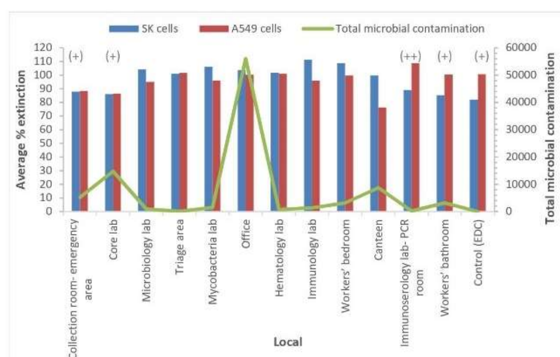

Figure 2. Cumulative microbial contamination (TSA, VRBA, MEA, and DG18) and cell viability (average percentage of extinction, MTT assay) in EDC samples. (+), low cytotoxic effect; (++), medium cytotoxic effect.

Regarding mycotoxins analysis, in all the samples analyzed, there were no mycotoxins detected.

3.3. Comparison and Correlation Analysis

Comparing the various collection sites from the swabs, it was found that the room-emergency area and office sites were the ones with the highest fungi counts on MEA (CFU) and on DG18 (CFU), whereas triage areas, offices, hematology labs, immunology rooms, and immunoserology lab-PCR rooms were the ones with the highest total bacteria counts (TSA (CFU)). Concerning Gram-bacteria (VRBA (CFU)), none of the sites revealed their presence (Figure 3). In EDC, all sites had identical counts of both fungi and bacteria, except for the office, which had higher counts of fungi on MEA (Figure 4).

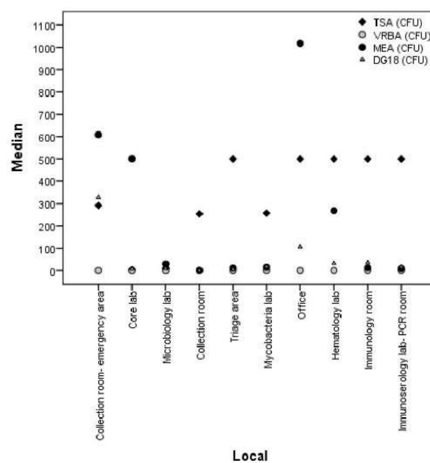

Figure 3. Comparison of fungal and bacterial load between collection sites in swabs.

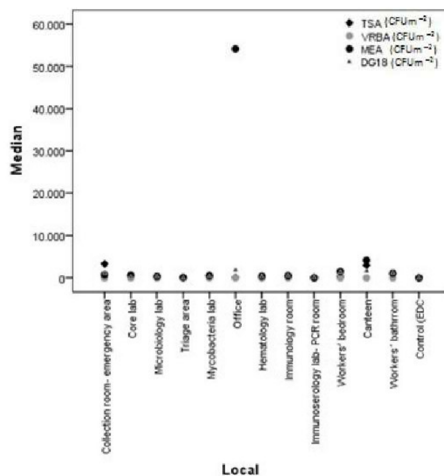

Figure 4. Comparison of fungal and bacterial load between collection sites in EDC.

The following correlations were detected on swabs: (i) total bacteria (TSA) counts on swabs (CFU) and TSA counts on EDC (CFU m^{-2}) ($r_s = -0.719$, $p = 0.006$), which meant that higher TSA counts (CFU) on swabs were related to lower TSA counts (CFU m^{-2}) on EDC; (ii) TSA counts on swabs (CFU) and A549 cells ($r_s = 0.610$, $p = 0.027$), revealing that higher TSA counts (CFU) on swabs were related to higher A549 cell viability, thus, lower cytotoxicity; (iii) Fungi counts on MEA (CFU) on swabs and fungi counts on DG18 (CFU) on swabs ($r_s = 0.628$, $p < 0.0001$), revealing that higher MEA counts on swabs (CFU) were related to higher DG18 counts (CFU) on swabs; (iv) MEA counts (CFU) on swabs and MEA counts (CFU m^{-2}) on EDC ($r_s = 0.742$, $p = 0.004$) and with DG18 counts (CFU m^{-2}) on EDC ($r_s = 0.645$, $p = 0.017$), indicating that higher MEA counts (CFU) on swabs were related with higher MEA counts (CFU m^{-2}) on EDC and higher DG18 counts (CFU m^{-2}) on EDCs (Table 8).

Table 8. Study of the relationship between microbial contamination and cell viability (MTT assay) (Spearman correlation coefficient results).

		Swabs		EDC			Cell Viability	
		MEA (CFU)	**DG18 (CFU)**	**TSA (CFU m^{-2})**	**MEA (CFU m^{-2})**	**DG18 (CFU m^{-2})**	**SK Cells (% Extinction)**	**A549 Cells (% Extinction)**
Swabs	TSA (CFU)	−0.022	0.231	−0.719 **	−0.196	−0.006	0.158	0.610 *
	MEA (CFU)		0.628 **	0.502	0.742 **	0.645 *	−0.071	−0.484
	DG18 (CFU)			−0.003	0.350	0.383	0.196	−0.047
EDC	TSA (CFU m^{-2})				0.509 *	0.490 *	−0.172	−0.729**
	MEA (CFU m^{-2})					0.861 **	0.033	−0.462
	DG18 (CFU m^{-2})						0.232	−0.344
Cell viability	SK cells							0.127

** Correlation is significant at the 0.01 level (2-tailed). * Correlation is significant at the 0.05 level (2-tailed).

The following correlations were detected on EDC: (i) TSA counts on EDC (CFU m^{-2}), MEA counts (CFU m^{-2}), and DG18 counts (CFU m^{-2}), both in EDC ($r_s = 0.509$, $p = 0.037$ and $r_s = 0.490$, $p = 0.046$, respectively), which means that higher TSA counts (CFU m^{-2}) on EDC were related to higher MEA counts (CFU m^{-2}) and higher DG18 counts (CFU m^{-2}) on EDC; (ii) TSA counts (CFU m^{-2}) on EDC and A549 cells ($r_s = -0.729$, $p = 0.001$), revealing that higher TSA counts (CFU m^{-2}) on EDC were related to lower A549 cell viability, thus, higher cytotoxicity; (iii) MEA counts (CFU m^{-2}) and DG18 counts (CFU m^{-2}) on EDC ($r_s = 0.861$, $p < 0.0001$), indicating that higher MEA counts (CFU m^{-2}) on EDC were related to higher DG18 counts (CFU m^{-2}) on EDC (Table 8).

4. Discussion

The sampling approach applied on this study allows us to recognize the advantages of using more than one sampling method [13]. Indeed, different counts and biodiversity (swabs: 16 different species in both media, EDC: 9 different species in MEA, and 7 in DG18) were observed in each environmental sample, allowing us to obtain a more accurate bioburden characterization. Additionally, using different agar media allowed bacteriota discrimination between total bacteria and Gram-bacteria. Regarding fungi, this approach has allowed the detection of a wider diversity concerning the *Aspergillus* genera, as well as the identification of different resistant profiles within the identified fungi. As such, the combination of these factors has promoted differentiation of the fungal species detected. Thus, it will be possible to introduce targeted cleaning procedures in specific surfaces, such as walls and floors, consistent with the fact that hospital surfaces might be a source for dissemination of microorganisms transported to the hands of patients or staff [37]. Indeed, hospital environments could be a source of outbreaks of resistant organisms [38], and experts suggest indirect transmission through the environment, besides direct person-to-person transmission [39].

The efficacy of biocides in eliminating microbial contaminants within healthcare facilities has been studied in the last years [40–44]. The widespread and increasing use of products containing low concentrations of biocidal agents or presenting low activity raises the concern of microorganisms' selection through development of acquired resistance and selection of less susceptible microorganisms following exposure to biocidals. Although this phenomenon is mainly described for bacteria, the same principle applies to fungal development of resistance to antifungal agents used in the environment and cross-resistance to antifungal drugs. The rational of using biocidals in healthcare facilities should, therefore, be supported by the evidence on their added value and targeted to specific applications, for which the risks have been established. For that reason, the characterization of a microbial resistance profile in the healthcare facilities' environment is of upmost importance.

Therefore, more than one sampling approach and more than one culture media should be the trend to be applied by exposure assessors (industrial hygienists or Indoor Air Quality assessors) when analyzing/sampling health care facilities [13].

Molecular tools were very efficient in detecting *Aspergillus* section *Fumigati*, whereas, for section *Versicolores*, the culture-based methods were more valuable. These differences highlight the importance of using culture-based methods for assessing the viable component of bioburden [13], combined with real-time PCR assay for targeting the indicators of harmful fungal contamination [45]. Indeed, culture-based methods can reveal less abundant fungi in a specific occupational environment when compared with more refined molecular tools (e.g., high-throughput sequencing) that provide a more exhaustive diversity profile [46]. However, although in less counts, the presence of some *Aspergillus* sections (opportunistic pathogens) may represent a health risk for patients and workers who are exposed on a daily basis [46]. Of note is the increased detection of *Aspergillus* section *Fumigati*, responsible by 80% of the cases of invasive aspergillosis, the most common invasive fungal infection [47].

Concerning the fact that mycotoxins null results, this can be due to several aspects, including the fact that the fungi are not stimulated to produce mycotoxins due to environmental reasons. However, since toxigenic species were found in the samples collected, this situation can change if some environmental variable changes. Therefore, the fact that no mycotoxins were measured does not allow discarding the need of future campaigns that will also monitor mycotoxins. Indeed, previous reports have already detected mycotoxins in health care centers, particularly in air and HVAC filter samples [13], justifying this need.

No *Aspergillus* sections were observed in azole screening media. Nevertheless, the *Circumdati* section was observed in Sabouraud. The burden of *Aspergillus* species in azole supplemented media might be underestimated due to presence of fast-growing species, such as *C. sitophila* on ITRA and VORI, and requires further investigation. In fact, molecular tools revealed the presence of other *Aspergillus* sections (*Fumigati* and *Versicolores*) in EDC not detected by culture. The fact that two samples (from Core lab and Microbiology) showed the same fungal species (*Cladosporium* and *Penicillium*) in two different azole media might suggest the development of multi-resistance to azoles, although this requires confirmation by minimum inhibitory concentration (MIC) determination by a reference method, such as The European Committee on Antimicrobial Susceptibility Testing (EUCAST). Previous studies from our group performed in health care centers also revealed (in settled dust samples) the co-occurrence in the same sample of *Penicillium* growth in ITRA and VORI [48]. Another study on indoor air quality also describes fungal growth in the three azoles for *Penicillium* and *Cladosporium* [13] and raises the question of the need of determination of the azole resistance profile for fungal species that might represent some health risk.

EDC sampling allowed a preliminary risk characterization regarding the cytotoxicity in human alveolar basal epithelial cells as a model for in vitro inhalation toxicology [49,50] and in swine kidney monolayer cells as a model for renal in vitro toxicology [51] through incubation of cell lines with airborne substances present in the sampled places. According to the defined threshold levels for cytotoxicity, airborne substances with a potential medium cytotoxic effect were recovered from three EDC samples from locals with some of the highest bacterial (TSA counts) and/or fungal (MEA)

contamination levels: "canteen" and "core lab". However, no cytotoxic effect was observed in the "office", where the highest microbial contamination was found. Statistical analysis revealed no relation between fungal contamination and cytotoxicity and contradictory results, regarding the role of bacterial contamination on cytotoxicity, depending on the sampling method. While bacterial contamination (TSA) on EDC seems to negatively affect A549 cell viability, the contrary was observed with swab sampling. These results suggest that other factors besides microbial contamination may be associated with in vitro cytotoxicity, namely, the presence of specific toxigenic species and/or particulate matter and/or other irritant agents in the collected samples. It is noteworthy that toxigenic *Aspergillus* species were detected in EDC samples collected in canteen (*Aspergillus* sections *Fumigati* and *Versicolores*) and in core lab (*Aspergillus* section *Versicolores*). However, such a statement may not provide grounds for a definite conclusion about the compared species of fungi that display a more cytotoxic effect than others.

The comparison between sampling sites regarding microbial contamination allowed us to prioritize intervention regarding which biocidals (for fungi/bacteria elimination) should be applied in each sampling site. However, it was found among the bioburden analyzed that a positive correlation between both sampling methods was applied. Thus, an intervention aiming at air cleaning with ultraviolet germicidal irradiation, such as ultraviolet with wavelength between 200 nm and 280 nm (UVC) [30] should be ensured, covering both fungi and bacteria elimination in the Clinical Pathology Service. Additionally, HVAC systems (including ventilation grids) should be maintained and properly preserved, since their efficacy in reducing microbial contamination has already been demonstrated [52,53].

5. Conclusions

Overall, it was possible to conclude that the two sampling methods used in this study—swabs and EDCs—unveiled a more complete characterization of the bioburden, permitting us to obtain information regarding workers and patient's potential exposure. Additionally, culture-based methods and molecular tools used in parallel should be used in order to perform an accurate characterization of the fungal contamination. This study also reinforces the need of analyzing the azole resistance profile for fungal species that might represent some health risk. EDC sampling allowed a preliminary risk characterization regarding the cytotoxicity present in the sampled places. Still, further studies are needed for a definite conclusion about the fungal species that display more cytotoxic effects when compared to others.

An intervention including the use of a UVC-emitting device in the Clinical Pathology Service and an increased maintenance and cleaning of the HVAC systems should be ensured to promote the reduction of microbial contamination.

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