UNIVERSIDADE DE LISBOA
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Microbiological contamination in Portuguese firefighters headquarters

BIANCA ROSÁRIO GOMES

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Dissertação orientada por:
Professora Doutora Carla Viegas (IPL-ESTeSL–H&TRC)
Professora Doutora Maria Teresa Rebelo (FCUL)

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Resumo:

Em ambientes ocupacionais, os trabalhadores podem estar expostos a uma diversidade de agentes microbiológicos. De facto, a exposição a microrganismos e os seus metabolitos pode levar a vários efeitos adversos na saúde, variando desde condições agudas até alguns tipos específicos de cancro. Mesmo em pequenas doses, a exposição pode representar um risco para a saúde dos trabalhadores. Além disso, o uso frequente de antibióticos, contribuiu para o surgimento de resistência antimicrobiana. *Aspergillus* secção *Fumigati* é uma das estirpes mais comuns e resistentes identificadas em contextos ambientais em Portugal. É o principal transmissor da doença invasiva, nomeadamente a aspergilose. Assim, em determinados ambientes ocupacionais é expectável aexposição a contaminação fúngica com potenciais efeitos sobre a saúde.

Actualmente existem alguns estudos relativos à exposição ocupacional a microrganismos em diferentes contextos em Portugal, com destaque para as unidades de saúde. No entanto, pouco se sabe sobre a exposição de bombeiros no ambiente de trabalho. A presença de agentes patogénicos em ambulâncias de bombeiros já foi sugerida por Viegas e membros da equipa (2021b), salientando a lacuna de conhecimento existente quanto à eficácia dos procedimentos de limpeza. Devido à natureza do local de trabalho e às tarefas realizadas, os bombeiros podem estar expostos a diversos perigos. Na verdade, a exposição microbiológica neste ambiente ocupacional tem sido negligenciada. Assim, este estudo pretendeu caracterizar a contaminação microbiológica em quartéis de bombeiros em Lisboa. A detecção do vírus *SARS-CoV-2* e a avaliação do perfil de resistência fúngica foram também realizados. Foram avaliados onze quartéis de bombeiros. A campanha de amostragem compreendeu um total de 760 amostras de ar (método de impactação) colhidas para meios de cultura. Com o intuito de aumentar a seletividade, dois meios de cultura foram utilizados para o crescimento de fungos: malte extract agar (MEA) suplementado com cloranfenicol (0,05%) e agar diclorano-glicerol (DG18), enquanto agar triptíco de soja (TSA) suplementado com nistatina (0,2%) e agar de bile vermelho violeta (VRBA) foram utilizados para pesquisa das bactérias. A amostragem foi também realizada através de métodos passivos, sendo recolhidos 82 coletores electrostáticos de pó (EDC), 102 zaragatoas de superfície do pavimento, 90 filtros de aspirador (1 por cada local de amostragem e agrupado por quartel), 67 etiquetas de identificação dos bombeiros, 14 esfregonas e 25 panos utilizados na rotina de limpeza de cada quartel. Em relação à avaliação de *SARS-CoV-2*, foram colhidas 23 amostras de ar interior (método do impinger) e 21 zaragatoas foram recolhidas em áreas de maior uso.

A avaliação microbiológica abordou métodos dependentes de cultura e independentes. Em relação aos métodos de amostragem ativa, as amostras foram analisadas através de microbiologia clássica (MEA; DG18; TSA; VRBA). Após a extração, as amostras de métodos de amostragem passivos foram analisadas utilizando os mesmos meios de cultura. Para a realização da avaliação de resistência a azóis, seguindo as instruções EUCAST (2019), os extratos das amostras foram inoculados em meio agar sabouraud dextrose (SDA) suplementado com azóis Itraconazole (ITR), Voriconazole (VOR) e Posaconazole (POS)). Além disso, isolados de *Aspergillus* secção *Fumigati* que cresceram nos meios (MEA; DG18; SDA; ITR; VOR; POS), foram analisados pelo método de avaliação de resistência a azóis seguindo as diretrizes EUCAST (2019). A detecção molecular de *Aspergillus* secção *Fumigati* e *Nidulantes* foi realizada por qPCR. No que se refere ao *SARS-CoV-2*, o RNA viral foi extraído das amostras e a detecção foi realizada por meio de RT-QPCR.

Em geral, de todas as matrizes os géneros predominantes foram *Cladosporium* (59,17% MEA; 73,43% DG18) e *Penicillium* (17,85% MEA; 14,83% DG18). Em relação à prevalência do género *Aspergillus* (1,52% MEA; 2,20% DG18), o mesmo foi mais frequentemente identificado em amostras de ar de Andersen six-stage (1,52% DG18) quando comparado com Millipore (0,78%). O maior valor em amostras de métodos passivos usando DG18, foi identificado em esfregonas (15,63% DG18) seguido

de panos de limpeza (7,08%). No MEA, o género foi mais frequentemente observado em filtros (3,37%), seguido de panos de limpeza (1,67%). Entre *Aspergillus* sp., oito secções foram referidas em DG18 sendo as secções *Fumigati* (33,01%) e *Nidulantes* (29,38%) as mais dominantes, enquanto no MEA as mais prevalentes foram *Nidulantes* (57,31%) e *Circumdati* (32,49%). Em relação à carga de *Aspergillus* sp. em Andersen six-stage, *Nidulantes* foi a secção mais frequente em MEA (45,45%). Apesar da distribuição em todos os estágios, *Nidulantes* teve a maior prevalência (44,85%) no estágio 4 (2,1 µm). Em DG18, a secção mais frequente foi *Candidi* (30,57%), sendo predominante no 4º estágio (64,20%). Os métodos de amostragem passivos apresentaram o maior número de secções em EDC (seis) (34,29% *Circumdati*; 29,75% *Aspergilli*; 13,73% *Flavi*; 10,78% *Candidi*; 9,16% *Restricti*; 2,28% *Nidulantes*), enquanto em DG18 foi nos filtros (44,76% *Fumigati*; 20,95% *Aspergilli*; 15,24% *Circumdati*; 12,39% *Nidulantes*; 5,71% *Flavi*; 0,95% *Candidi*;).

Em relação à carga microbiológica em ambientes interiores, de acordo com os critérios científicos para avaliação da exposição ocupacional (I / O ≤1), dos 11 FFH amostrados, 5 (45,45%) ultrapassaram o valor estipulado no que se refere à carga fúngica. A mesma tendência foi obtida para bactérias, com 7 FFH (63,63%) fora do limite estipulado. Ao utilizar o valor quantitativo sugerido pela Organização Mundial de Saúde (OMS) (valor máximo de 150 UFC.m^{-3}), 7 FFH (63,63%) ficaram acima do limite da carga bacteriana. No caso dos fungos, 5 FFH (45,45%) não respeitaram o critério. Ao aplicar os limites impostos pela legislação portuguesa para a Qualidade do Ar Interior (Portaria no353-A, 2013) a carga de espécies toxigénicas de *Aspergillus* identificada ultrapassou o limite de 12 UFC.m^{-3} na maior parte dos FFH (9 de 11), tendo sido identificadas as espécies indicadoras de contaminação fúngica prejudicial como *Aspergillus* secção *Fumigati* e, ainda outras secções como *Flavi*, *Nidulantes* e *Circumdati*.

A avaliação do perfil de resistência aos azóis revelou *Cladosporium* sp., *C. sitophila* e *Penicillium* sp. como os fungos mais recorrentes identificados em ITR, VOR e POS. O género *Aspergillus* foi também detectado em ITR e VOR (0,03% respectivamente), com a secção *Fumigati* a ser a única isolada (100%) em VOR em amostras de EDC e filtros. Entre os isolados de *Aspergillus* secção *Fumigati* o método de *screening* evidenciou seis isolados observados em ITR e VOR.

As ferramentas moleculares permitiram a detecção de *Aspergillus* secção *Fumigati* em quase todas as amostras de métodos de amostragem passivos (67,8% EDC; 66,6% Filtros; 56% panos de limpeza; 50% esfregonas, 5,97% etiquetas de identificação), enquanto a secção *Nidulantes* foi detectada em uma amostra de pó (9,09%).

No geral, o protocolo contendo uma abordagem múltipla, no que concerne aos métodos de amostragem e análise, permitiu uma caracterização microbiana mais ampla. Amostras de ar obtidas por Andersen six-stage revelaram uma carga fúngica mais diversificada. *Aspergillus* secção *Nidulantes* e *Candidi* observadas no 4º estágio, que corresponde aos brônquios secundários do sistema respiratório humano, podem sugerir um potencial fator de risco para as doenças respiratórias. Entre os métodos passivos, a diversidade fúngica obtida sugere o EDC como uma matriz adequada para avaliação da exposição. Verificaram-se resultados diferentes utilizando diferentes meios de cultura e complementando a microbiologia clássica com as ferramentas moleculares utilizadas.

A ausência de limites de exposição ocupacional para os agentes microbiológicos em Portugal levou à utilização de orientações científicas gerais. Os níveis de bactérias podem ser representativos de espécies não patogénicas, pertencendo à flora cutânea. No que diz respeito às bactérias gram-negativas, outro possível perigo está associado à capacidade de produção de endotoxinas e aos efeitos adversos na saúde associados à exposição. A carga fúngica interna ultrapassou o limite estipulado pela OMS na maioria dos quartéis (5 de 11), e a presença de espécies toxigénicas, potencialmente produtoras de micotoxinas enfatizam a necessidade de medidas corretivas. Falta de ventilação natural, estruturas danificadas e aparecimento de infiltrações nas paredes e tetos podem ser alguns dos fatores que

influenciam a contaminação fúngica. Assim sendo, a contaminação fúngica obtida neste local era a esperada ao considerar as condições físicas observadas nos FFH.

O crescimento de *Aspergillus* secção *Fumigati* em ITR e VOR destaca o surgimento de resistência antifúngica. Testes adicionais de sensibilidade aos antifúngicos devem ser preconizados. A detecção molecular das secções *Fumigati* e *Nidulantes* em diferentes matrizes sugere as ferramentas moleculares como uma abordagem adequada para superar as limitações dos métodos dependentes de cultura. Em relação à avaliação de *SARS-CoV-2*, nenhuma deteção foi obtida, sugerindo a eficácia dos procedimentos de limpeza no controlo viral.

Globalmente, este estudo corrobora a generalização da contaminação microbiana nos ambientes analisados, sendo o primeiro a caracterizar os quartéis de bombeiros em Lisboa. Estas instalações são, na verdade, um ambiente ocupacional a ter em consideração no que diz respeito à contaminação microbiológica. De facto, mais pesquisa é necessária de forma a implementar medidas adequadas para reduzir o risco dos trabalhadores e minimizar a exposição.

Palavras – Chave: Exposição Ocupacional; Contaminação microbiológica; *Aspergillus fumigatus*; Perfil de resistência fúngica

Abstract:

In occupational environments, employees might be exposed to a diversity of microbiological agents. However, less is known about firefighter's exposure in the work environment. Thus, this study aims to characterize the microbiological contamination in firefighter's headquarters from Lisbon, Portugal. *SARS-CoV-2* and fungal resistance profile assessment were also performed.

Eleven firefighters headquarters (FFH) were assessed and the sampling campaign comprised active and passive sampling methods.

The microbiological assessment covered culture-dependent and culture-independent methods. Additionally, to perform the azole resistance screening the samples extracts were inoculated in Sabouraud dextrose agar (SDA) azole supplemented media (Itraconazole (ITR), Voriconazole (VOR) and Posaconazole (POS)).

In general, from all the matrices the predominant genera were *Cladosporium* and *Penicillium.* Regarding microbiological load indoors, in accordance with the scientific criteria for occupational exposure assessment (I /O ≤1), from the 11 FFH sampled, 5 (45.45%) surpassed the stipulated value in what concerns fungal levels. The same trend was reported in 7 FFH (63.63%) for bacteria. Moreover, when using the World Health Organization (WHO) suggested indoor guideline (maximum value of 150 $CFU.m^{-3}$), the same FFH were above the stipulated limits for fungal and bacterial load respectively.

Overall, the use of a multi-approach protocol for sampling and analyses allowed a broader microbial characterization. Also, fungal growth in azole supplemented media suggests the presence of multidrug resistance. Thus, further antifungal susceptibility tests should be preconized.

The molecular detection of sections *Fumigati* and *Nidulantes* in additional matrices suggests molecular tools as a suitable approach overcoming culture-dependent methods limitations. Regarding *SARS-CoV-2* assessment, no detection was obtained which suggest the effectiveness of cleaning in viral control.

In short, these facilities are an occupational environment to have into consideration regarding the microbiological contamination. In fact, more research is needed in order to implement proper measures to reduce workers risk and minimize the exposure.

Key-words: Occupational Exposure; Microbiological contamination; *Aspergillus fumigatus*; Fungal resistant profile

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List of abbreviations

ACGIH American Conference of Governmental Industrial Hygienist
AFs Aflatoxins
AME Monomethyl ether
AOH Alternariol
CDC Centers for disease control and prevention
CFUs Colony-forming units
DG18 Dichloran-glycerol agar
DON Deoxynivalenol
EDC Electrostatic dust collectors
EPS Extracellular polysaccharides
EUCAST European Committee on Antibiotic Susceptibility Testing
FFH Firefighters headquarter
FMs Fumonisins
GC-MS Gas chromatography - mass spectrometry
HIV Human immunodeficiency virus
HPLC-FLC High performance liquid chromatography with fluorescence detection
HVAC Heating ventilation air conditioning
ITR Itraconazole
LAL Limulus amebocyte lysate
MALDI-TOF Matrix-assisted laser desorption/ionization time-of-flight
MEA Malt extract agar
MIC Minimum inhibitory concentration
MRSA Methicillin resistant Staphylococcus aureus
NGS Next generation sequencing
NOEL No observed effects level
OTA Ochratoxin A
OTs Ochratoxins
PBP Penicillin-binding protein
PCR Polymerase chain reaction
PFGE Pulse Field Gel Electrophoresis
POS Posaconazole
PPE Personal protective equipment
qPCR Quantitative polymerase chain reaction
RFLP Restriction Fragment Length Polymorphism Analysis
RT-PCR Reverse transcriptase - polymerase chain reaction
SARS Severe acute respiratory syndrome
SDA Sabouraud dextrose agar
SOP Standard operating procedure
TEN Tentoxine
TRC Trichothecenes
TSA Tryptic soy agar
VOCs Volative organic compounds
VOR Voriconazole
VRBA Violet red bile agar
WHO World Health Organization
ZEN Zearalenon

Part I - Theoretical framework

Chapter I: Evaluation of occupational exposure to microbiological agents

1. Microbiological agents

Microorganisms covers a diverse group (Burrell, 1991). According to the definition described by *Artigo 2 (Directiva* 2000/54/EC), microorganisms are any microbiological entity, cellular or non-cellular capable of reproducing or transferring genetic material. A similar definition is described for biological agent in Durando and collaborators (2019), encompassing genetically modified microorganism, or even cell culture, referring to their singular capacity of causing health adverse effects.

They can be evaluated by their effects on human health, bacteria, fungi and virus can trigger human infectious (Sastry & Bhat, 2016). Their metabolites, endotoxins, mycotoxins, ß(1.3)-glucans and others can also promote health hazards (Cox *et al.*, 2020).

Microorganisms and their components can be present in a diversity of places, possibly turning airborne, being easily transported due to their small proportions (Cox *et al.*, 2020), making part of bioaerosols (USEPA, 1995, Ki-Hyun *et al.*, 2017), also designated organic dust (Douwes *et al.*, 2003). The organism itself or their parts may enter the human nasal cavity, possibly penetrating the lung (Burrell, 1991). Indeed, asthma and inflammation of respiratory routes have been reported after bioburden exposure (Ki-Hyun *et al.*, 2017).

1.1 Fungi

Fungi are eukaryotic ubiquitous microorganism able to proliferate in a variety of environments (Aleksic *et al.*, 2017). There is about 1.5 million species of fungi, and six phyla of which Ascomycota, Basidiomycota, Glomeromycota, Blastocladiomycota, Chytridiomycota e Neocallimastigomycota (Watkinson *et al.*, 2016).

Composed by a plasma membrane, nuclei, endoplasmic reticulum, Golgi apparatus, vacuoles and in majority by mitochondria they own a rigid cell wall composed by chitin (Sastry & Bhat, 2016), a distinct feature (Watkinson *et al.*, 2016), (1→3)-β-glucans, mannose proteins and sterol (Sastry & Bhat, 2016,Green, 2017). In addition to chitin some species might have chitosan, a polymer composed by deacetylate sugar (Watkinson *et al.*, 2016). Also present in cell wall and periphery of fungal structures are extracellular polysaccharides (EPS). On the cell membrane there is ergosterol (Green, 2017), the main component modulating the permeability and fluidity (Watkinson *et al.*, 2016). Normally both EPS and ergosterol are used as markers for fungal exposure (Mensah-Attipoe & Taubel, 2017).

Fungal anatomy can vary from unicellular yeasts to filamentous hyphae, being monomorphic or dimorphic presenting both forms (Viegas, 2010). Able to produce sexual spores or asexual conidia, the concentration produced can vary depending on biotic and abiotic conditions (Green, 2017). Some species are less selective and capable of growing on different surfaces depending of environmental conditions (Aleksic *et al.*, 2017). Some can even produce spores, being easily released into the air (Ramos *et al.*, 2016). Exposure to spores, conidia, hyphae, chlamydospores and other submicron fragments can occur during work performance through aerosolization. Indeed, there is some evidence pointing out Basidiomycota and Ascomycota as the most common phyla reported in occupational environments (Green, 2017).

The phylum Basidiomycota which includes species such as rust, yeast, smuts, or mushroom fungi (James *et al.*, 2006), contemplates a diversity of ecological roles, capable of producing basidiospores, supporting forest ecology through decomposition or even establishing insect symbioses. In contrast they can cause adverse effects, leading to potential lethal infections on humans (Watkinson *et al.*, 2016).

Ascomycota, the largest phylum (James *et al.*, 2006), is characterized by yeasts, filamentous, mycorrhizal, saprotrophs and pathogenic species. Some might have asexual reproduction, forming

conidia, the asexual spores. Although most ascomycetes have sexual phases, producing ascospores (Watkinson *et al.*, 2016). The phylum covers the dimorphic fungi, some with clinical relevance (Viegas, 2010). After inhalation, spores are converted into a budding yeast (Green, 2017) possibly due to temperature, nutrients and CO_2 levels (Viegas, 2010).

Spores work as a fungal transmission route through the environment. The release of such structures can be promoted by airflow, electrostatics and cavitation (Watkinson *et al.*, 2016). In workplace, spores exposure is in general from monomorphic fungi, being the most predominant fungal particles. In contrast, exposure to dimorphic fungal pathogens may have health consequences especially in immunocompromised hosts, causing mycoses (Green, 2017).

Associated to fungi secondary metabolism, a unique fungal species can produce a variety of different mycotoxins in response to environmental stress, such metabolite is able to resist to a range of temperatures and boiling processes (Viegas *et al.*, 2018a). Until now mycotoxins, capable of inducing toxicity in humans health (Watkinson *et al.*, 2016), are established as non-viral biological occupational carcinogens (Douwes *et al.*, 2003). Aflatoxins(AFs), a well-known human carcinogenic, is splitted in four main groups of which B_1,B_2,G_1 e G_2, being AFB1 the most relevant due to the capacity of causing chronic illness (Watkinson *et al.*, 2016). Currently many are identified, being the most common AFs, Trichothecenes (TRC), Ochratoxins (OTs), Fumonisins (FMs) and Zearalenone (ZEN) (Pereira *et al.*, 2012). Even though exposure to mycotoxins is associated with respiratory occupational risk, less is known about their dose-response (Dutkiewicz *et al.*, 2011). The main route of exposure is through ingestion of contaminated food (Ki-Hyun *et al.*, 2017). However, there is some evidence supporting inhalation as a greater route, since mycotoxins can be found in airborne dust, spores and fragments of microbial growth (Huttunen & Korkalainen, 2017). In occupational environments, exposure can occur through dermal contact and inhalation (Viegas *et al.*, 2018a). Some examples of mycotoxin producers, are A*spergillus flavus* (AFs production), *Aspergillus fumigatus* (gliotoxin producer), or even *Aspergillus nidulans* (aspyridones producer) showing moderated cytotoxic activity (Watkinson *et al.*, 2016). Recently *Aspergillus niger*, placed in *Aspergillus section Nigri* has been associated with mycotoxins production capacity, as an example FMs B_2, B_4, and B_6, and OTA (Frisvad *et al.*, 2018). Other genera might also produce the same metabolite such as *Penicillium, Fusarium* (Ramos *et al.*, 2016) and *Cladosporium* (Dutkiewicz *et al.*, 2011). In addition, they can even produce allergens, volatile organic compounds (VOCs) and enzymatic proteins (Ki-Hyun *et al.*, 2017).

Aspergillus species, conidia chain producers (Sastry & Bhat, 2016), are commonly found in a diversity of environments (Viegas *et al.*, 2020a). Among filamentous fungi, *Aspergillus* sp. are described as having a major role in opportunistic infections (Pinto *et al.*, 2018). The Genera identification through phenotypic characteristics can involve a series of limitations, due to the existence of cryptic species (Francisco, 2017), exhibiting an almost identical morphology (Pinto *et al.*, 2018). However, when analyzed at the molecular level, the species distinction is verified (Francisco, 2017). The internal transcribed space (ITS), B-tubulin and Calmodulin are the main regions used to discern between them (Pinto *et al.*, 2018).

As reported by Francisco (2017), through molecular, morphological and physiological parameters, the taxonomy of *Aspergillus fumigatus* species was reviewed. Based on molecular tools, the *Aspergillus* genus was divided into eight subgroups, which are separate into several sections, including a variety of cryptic species (Francisco, 2017). To sum up, the genus is divided into 22 sections, being *Aspergilli, Fumigati, Circumdati, Terrei, Nidulantes, Ornati, Warcupi, Candidi, Restricti, Usti* and *Flavi* the clinical relevant ones (Viegas *et al.*, 2020a). Since virulence and resistance to certain antifungals can vary between cryptic species, the correct identification is crucial, that can be accomplished by sequencing the ITS region of the ribosomal DNA identifying the species at the section level. To discern between cryptic species within the same section an additional sequencing of B-tubulin

and Calmodulin can be performed, due to the interspecific variability of these regions relatively conserved between species (Francisco, 2017).

Special attention relies on *Aspergillus* section *Fumigati*, an opportunistic pathogen often associated with the ability to cause adverse respiratory effects. The small conidia and hydrophobicity contribute to his presence on air for an extended period, and viability even in drought conditions (Viegas *et al.*, 2020a). Indeed, fungal exposure can occur from indoor and outdoor environments (Bush *et al.*, 2006).

Currently, there is a concern on the excessive use of antifungal substances such as azoles, to combat fungal infection, resulting in the emergence of antifungal resistance (Sabino, 2017) .Mostly related to fungal single mutations (Pinto *et al.*, 2018). Some known resistant fungal genera are *Candida* and *Aspergillus* (Viegas *et al.*, 2020a). The resistance is associated with several changes such as activation of alternative pathways, enhance of the antifungal targets, changes in the cell wall and others (Sabino, 2017). According to Pinto and team, since the nineties azole resistance in *Aspergillus* sp. became more frequent, now extending to the six continents (Pinto *et al.*, 2018). A current example is the mutation in the CYP51A gene *(*TR43/L98H) in *A. fumigatus.* Additionally, other mutations on the same azole target were already reported such as TR46/Y121F/T289A, leading to the resistance of all medical azoles (Sabino, 2017).

1.2 Bacteria

There is a diversity of bacterial species present in the environment, including the species that make part of the human natural flora, the opportunistic and pathogenic (Barnes, 2017). This unicellular microorganism (Rogers, 2011), included in the prokaryotic group, lack some specific organelles like nucleus, endoplasmic reticulum, mitochondria or golgi apparatus (Barnes, 2017).

The simplicity of bacteria metabolic capabilities and small size contributes to a better adaptation capacity, prospering in almost any environment, being the smallest living creature. Binary fission is the most common reproduction process used. However, bacteria have developed other paths for genetic recombination even between species, incorporating new genes, leading to a better adaptation to new environmental conditions. This process can also be the starting point for antibiotic resistance, an emerging concern in medical treatment for bacterial infection (Rogers, 2011). Unlike eukaryotes, genetic diversity comes from horizontal gene transfer methods (Barnes, 2017)38, and genetic information is restricted to a circular chromosome (Rogers, 2011). Still, similar to the eukaryotic group, they use DNA as genetic material and ribosomes for protein production (Barnes, 2017).

They can be characterized by three basic forms which are rods (bacillus), spherical (coccus) and spiral, beyond the shape they can also have filamentous appendages, bifid protrusions, and cellular membrane protrusions (Barnes, 2017). In addition, bacteria optimal growth relies on environmental conditions and the nutritional requirements can vary drastically from species to species (Rogers, 2011).

Some bacteria of the Firmicutes phylum can produce endospores, a non-vegetative structure capable to resist to a diversity of environment conditions. Other species might form a capsule preventing dehydration and protection from phagocytosis, the association between capsule formation and virulence is reported in literature (Rogers, 2011). Furthermore, aggregates of bacteria involved in a carbohydrate matrix capable of adhere to surfaces are commonly designated biofilms, composed by polysaccharides, nucleic acids and proteins (Barnes, 2017). Such structure can group different species (Rogers, 2011), contributing to nutrient acquisition, motility and virulence of the microorganism (Barnes, 2017), promoting bacterial survival (Mai-Prochnow *et al.*, 2016). The aggregates can contribute to antimicrobial resistance (Mai-Prochnow *et al.*, 2016) and induce severe infections on humans (Rogers, 2011).

Two primary morphological groups can be distinguished by differences in the cell wall (Douwes *et al.*, 2003). A thick wall containing a high amount of peptidoglycan composed by amino acids and sugar polymers, characterize gram-positive species (Mai-Prochnow *et al.*, 2016, Barnes, 2017), in general being the larger group present (Ramos *et al.*, 2016). The long-chain polymer namely peptidoglycan is composed by two sugars designated N-acetylglucosamine and N-acetyl muramic acid connected by peptide bridges giving rigid stability to the bacteria cell wall (Rogers, 2011). Some of gram-positive genus are *Bacillus*, *Staphylococcus* and *Streptococcus* (Rogers, 2011). A multiple layer cell wall distinguishes gram-negative, having a membrane on top of a thin peptidoglycan layer (Siegel *et al.*, 2016), harbouring several pores and appendices (Mai-Prochnow *et al.*, 2016). Phospholipids make up the outer membrane, having phosphate and lipopolysaccharidesalso designated endotoxins (Rogers, 2011). Constituted by a core- and a O-specific- polysaccharide chain and a lipid component namely Lipid A (Münch & Sahl, 2015, Ki-Hyun *et al.*, 2017), makes of the peptidoglycan layer a barrier against possible harmful chemicals, enabling bacteria survival in harsh environmental conditions (Münch & Sahl, 2015). Some of gram-negative genus are *Enterobacter*, *Escherichia* and *Salmonella* (Rogers, 2011).

Nowadays the challenge relies on the emergence of antimicrobial-resistant bacteria being promoted by the uncontrolled use of antibiotics (Ribeiro *et al.*, 2018). Antimicrobial agents overused and deficient hygiene control practices enables resistant strains dissemination, being acquired by horizontal gene transfer (Sastry & Bhat, 2016). Also, since bacteria integrate part of bioaerosols, prolonged exposure, especially in workplaces can contribute to health risks (Ribeiro *et al.*, 2018). In short, bacteria can develop resistance by a diversity of mechanisms. One case is *Staphylococcus aureus*, possessing several virulence factors, being the most pathogenic specie among the genera able to develop drug resistance. A modification in penicillin-binding protein (PBP) to PBP-2a, seems to be the mechanism promoting the resistance, allowing a minor affinity for β-lactam antibiotics, as an example the methicillin resistant *Staphylococcus aureus* (MRSA) (Sastry & Bhat, 2016).

Due to the important role of cell wall in prokaryotic integrity maintenance and other functions such as virulence expression, its biosynthetic pathway is usually the target of several classes of antimicrobials (Münch & Sahl, 2015).

As health concern, special attention relies on the risk groups such as, children, ill patients and elderly people, being susceptible to a hard to treat infection due to antibiotic resistance (Rogers, 2011). Exposure to such microorganisms can occur at the hospitals and patients possibly obtain the microorganisms as transient flora from the environment (Sastry & Bhat, 2016), developing health problems contributing to a long-term incapacity (Dutkiewicz *et al.*, 2011).

1.3 Viruses

Several features differentiate virus from bacteria and other prokaryotes, devoid of cell wall, proper cellular organization or organelles (Gallasch *et al.*, 2020). Being an acellular microorganism able to replicate inside living cells and naturally dependent on the host cell machinery to reproduce (Croston, 2017). They use the energy as well as other metabolic functions from the host cells (Rogers, 2011). Capable of infecting all forms of life, they are considered the most abundant biological particles (Gallasch *et al.*, 2020).

Virions are characterized as an independent viral particle that is not already infected a living cell. Consisting of two parts, one with the genetic material within a protective proteinaceous coat called capsid (Croston, 2017), enabling sites for recognitions and attachment to the host cell (Rogers, 2011). The capsid is composed by proteins namely capsomeres which can be surrounded by a lipid layer, such structure can have both viral and host cells membranes. Despite the wide variety of morphologies, the majority is characterized by a helical or icosahedral structure, as example the rhinoviruses, an

icosahedral virus capable of causing common cold (Croston, 2017). In addition, the size and shape of viruses relies on the quantity and arrangement of proteins and nucleic acids, varying between 20 up to 400 nanometers, regarding the size, there are two main shapes namely rods or filaments (Rogers, 2011).

Virus can be classified by their genotype and method of replication, the genetic material is either double or single – stranded DNA or RNA (Rogers, 2011,Croston, 2017). Some viral species can have both at different times during life cycle, is the case of HIV virus (Croston, 2017). Examples of DNA viruses are herpesvirus and papillomavirus, while RNA viruses are orthomyxovirus, retrovirus or coronavirus (Rogers, 2011). Replication type relies on viral genome, in case of RNA the replication occurs primarily in the cell cytoplasm, while for DNA virus the process occurs in the cell nucleus. Once in contact with the host cell, the viral genome is released, the replication begins and the viral components are produced being liberated from the host cell (Croston, 2017). The pathogenicity depends on the nature of the virus and the host (Gallasch *et al.*, 2020). In some conditions the pathogenic microorganism can even go dormant for an indeterminate time subsequently being activated under favorable condition, and integrated in the hosts cells DNA. The mechanism of infection can result in lysogeny and malignant transformation leading to cancers or viral infections (Rogers, 2011).

Influenza virus, in which viral exposure may occur through inhalation of viral airborne particles (Croston, 2017), is one example of how viral microorganisms can be transmitted through the respiratory route due to aerosolization (Rogers, 2011). Starting the infection cycle on the respiratory tract, eventually entering the bloodstream, reaching other areas (Rogers, 2011). Similar route of exposure can occur in occupational environments, is the case of viral hepatitis especially in healthcare workers and patients (Croston, 2017).

Due to the adaptability, virus can develop new strains, consequently infecting other hosts, is the case of the viral species from the Coronaviridae family, originated from an animal reservoir and inducing the SARS, a pathogenic disease, in humans (Gallasch *et al.*, 2020).

Nowadays, an actual problem relies on the worldwide pandemic caused by the new *SARS-CoV-2* coronavirus, commonly designated by the disease COVID-19, being the seventh coronavirus known to infect humans followed by SARS-CoV, HKU1, NL63, OC43 and 229E (Andersen *et al.*, 2020). When accessing aerosol suspension of *SARS-CoV-2* results have shown a stability of the infectivity level over short distances, being resilient in aerosol form. Thus, aerosol transmission seems to be a crucial pathway (Fears *et al.*, 2020). In addition contact with contaminated surfaces (Pena *et al.*, 2021) and salivary droplets from an infected person (Gallasch *et al.*, 2020), being a source of infection to others (Lai *et al.*, 2020).

2. Health effects of microbiological agents and their metabolites

Associated with occupational exposure to total organic dust, respiratory symptoms and lung impairment are possibly the most commonly studied effects, ranging from acute conditions (Douwes *et al.*, 2003) to inflammation of airway (Ki-Hyun *et al.*, 2017),increasing the risk of lung cancer (Dutkiewicz *et al.*, 2011). Bioburden exposure can be associated with the develop of some specific cancers, such as lung, pancreatic or even liver cancers (Ki-Hyun *et al.*, 2017) The severity of health problems relies on the duration of exposure, concentration and individual health (Ramos *et al.*, 2016, Ki-Hyun *et al.*, 2017, Viegas *et al.*, 2018a). Therefore, there is an increasing tendency of health effects caused by airborne microorganisms, once in the respiratory system they can cause human disorders (Dutkiewicz *et al.*, 2011), having allergic, irritant or toxic effects (Oppliger, 2014). One example is the allergic disease Hypersensitivity Pneumonitis resulting from allergen inhalation of bacteria, fungi, vegetable, or animal origin (Dutkiewicz *et al.*, 2011).

2.1 Fungi

The ability of fungi to cause human disease relies on the capacity to grow at 37ºC, the diversity of nitrogen and carbon sources used and the adaptability to the host (Watkinson *et al.*, 2016).

Exposure to fungi and fungal spores through inhalation may trigger several adverse health effects (Douwes *et al.*, 2003). Is the case of dimorphic fungal conidia able to originate a symptomatic pulmonary infection (Green, 2017). The opportunistic *Aspergillus* section *Fumigati*, is often associated with adverse effects on respiratory tract (Viegas *et al.*, 2021a). Contributing to the invasive disease namely aspergillosis, being Cardiac, Ocular and Cerebral or the most common, the Pulmonary Aspergillosis (Sastry & Bhat, 2016). It can also induce allergic sensitization, lung disease and infectious mycosis (Douwes *et al.*, 2003).

Allergic symptoms may arise from spores exposure causing an overactive immune response, leading to hypersensitivity contributing to asthma, eczema or rhinitis (Watkinson *et al.*, 2016). Likewise, direct contact with fungal spores when having transcutaneous injuries can lead to subcutaneous infections, with some professions being susceptible to this type of transmission (farmers, gardeners and laborers). Although unusual there is some reports, as an example the occupational infection Sporotrichosis caused by *Sporothrix schenckii* going from painless to a chronic cutaneous lesion (Green, 2017).

When it comes to mycotoxins, the main concern is associated with the carcinogenic, teratogenic, immunotoxic and nephrotoxic effects that they may cause (Assunção *et al.*, 2018). One mycotoxin can lead to multiple reactions, after inhalation some reported effects are membrane irritation, acute or chronic liver damage and endocrine effects. In addition, inhalation of AFB_1 possibly contributes to the appearance of cancer, and OTA has been related to renal failure and respiratory distress (Viegas *et al.*, 2018a). Despite the lack of knowledge related to FMs on humans health, there is some information about the hepatotoxic, immunosuppressive and embryotoxic effects obtained by experimental animal system (Frisvad *et al.*, 2018).

A study conducted by Viegas and team (2018b) in a fresh bread company reported Deoxynivalenol (DON), belonging to TRC, as the mycotoxin with the highest values present in settled dust sample. The same results were obtained when analyzing biological samples of workers, highlighting the occupational exposure. Consequently, DON is associated to retarded growth, hepatotoxic and immunotoxic effects being able to modulate various physiological processes such as cell development and apoptosis (Viegas *et al.*, 2018a). Also, the toxicity of some emerging mycotoxins, such as Alternariol toxins (AOH), their monomethyl ether (AME) or Tentoxine (TEN), need to be classified.AOH and AME are characterized by their in vitro genotoxic effect, affecting topoisomerases I and II (Braun *et al.*, 2020). Also pre-term births and late abortions were reported in farm women after exposure (Douwes *et al.*, 2003).

2.2 Bacteria

Bacteria can trigger infectious diseases, as example the Legionnaires disease caused by *Legionella* sp. (Barnes, 2017). With symptoms ranging from febrile illness (Pontiac fever), to a severe pneumonia (Ki-Hyun *et al.*, 2017). On the other hand, *S.aureus* can lead to localized pyogenic to systemic infections on human body (Sastry & Bhat, 2016). Also, *Mycobacterium tuberculosis* inhalation can cause Tuberculosis (Ki-Hyun *et al.*, 2017).

Occupational exposure to bacteria and its metabolites may lead to the rarely febrile disease namely Brucellosis, causes by *Brucella* sp. generally acquired by exposure to contaminated animal products. Besides, exposure to endotoxins, characterized as the most biologically active pro-inflammatory constituent of organic dust, were associated with asthma-like symptoms possibly

developing atopic or non-atopic asthma (Basinas *et al.*, 2017), and systemic effects (Cyprowski *et al.*, 2016). When submitted to high levels can lead to impairment of lung function, or toxic Pneumonitis (Dutkiewicz *et al.*, 2011).

Peptidoglycan impact on humans health is still less understood when compared to endotoxins (Cyprowski *et al.*, 2016), derived mainly from gram-positive but also from some gram-negative bacteria (Poole *et al.*, 2010), the exposure may result in respiratory effects (Sastry & Bhat, 2016). It seems that bacterial peptidoglycans play a crucial role in the pathogenesis of complex infection, increasing the biological activity of endotoxins, even in small doses, such active compounds can influence humans health (Cyprowski *et al.*, 2016).

Also, subcutaneous and cutaneous infections may occur through direct contact, is the case of a common skin infection, caused by *Streptococcus* sp. and *Staphylococcus aureus*, often acquired by industries workers during work activities, (Sastry & Bhat, 2016). Shivering, fever, blood leukocytosis and influenza-like symptoms were some of the clinical effects reported by experiments after endotoxin inhalation and peptidoglycans seems to cause similar results (Douwes *et al.*, 2003). Furtheremore, microbial VOCs are known to be responsible for eye and upper-airway irritation, based on exposure studies in humans and animals (Huttunen & Korkalainen, 2017).

2.3 Viruses

More than 100 viral types are the main cause of common cold, among them rhinovirus, human coronavirus, and adenoviruses, being transmitted through airborne droplets or direct contact (Ki-Hyun *et al.*, 2017). As example, herpes simplex virus types I and II are highly contagious herpes viruses, capable of causing visual impairment or cervical cancer respectively. On the other hand, papilloma virus can induce malignant cancers in the genital tract. Retrovirus such as the human immunodeficiency virus (HIV) affects the immune system, gradually decreasing host defenses. Besides, in orthomyxoviridae there is four genera of influenza virus known for their pandemic outbreaks character (Rogers, 2011).

Finally, coronavirus a crucial agent of gastrointestinal disease is also capable of inducing SARS (Rogers, 2011). Exposure to *SARS-CoV-2* can range from mild clinical symptoms such as, fever, dry cough or sore throat to a more critical situation (Gallasch *et al.*, 2020). In short clinical symptoms can vary from asymptomatic carriers to pneumonia with several degrees of severity (Lai *et al.*, 2020). More recently, there is some evidence pointing out the possible occurrence of COVID 19 associated pulmonary aspergillosis in hospitalized patients on the intensive care unit, reporting an association of 19 to 33% (Ghelfenstein-Ferreira *et al.*, 2020).

2.4 β -D-Glucans

β-D-glucans are immunologically-active glucose polymer, naturally present in the cell wall (Dutkiewicz *et al.*, 2011), of fungi and bacteria, characterized by different molecular weight and branching degree (Douwes *et al.*, 2003). The majority characterized as stable molecules, insoluble in water, with a β-D-linked linear backbone (Basinas *et al.*, 2017). The linkage with proteins, chitin, lipids and the (1→6)-β-side-branches contributes to fungi cell wall structuring, characteristics such as primary structure, degree of branching, and solubility are crucial to designate glucans biological activity. Although with some contradictory results, some studies associate (1 → 3)-β-D-Glucan exposure to the airways inflammation and irritation (Basinas *et al.*, 2017).

Inflammatory responses and atopy were associated with β-glucans airborne exposure (Douwes *et al.*, 2003, 2005). Indicating the possible contribution of β-glucans to pulmonary diseases (Dutkiewicz *et al.*, 2011). Simultaneously there is some evidencing pointing out an increase of T helper 1 cell after β-D-glucans exposure (Douwes, 2005) and a disregulation of cellular signaling leading to the

malfunction of the barrier. Despite all the reported effects the mechanism of action remains poorly understood (Du *et al.*, 2015).

Ergosterol and muramic acid seem to have impact on human health, possibly contributing to the appearance of asthma (Basinas *et al.*, 2017). Despite the gap of knowledge, evidence points out ergosterol as a marker of respiratory disease (Davidson *et al.*, 2018). In similarity, muramic acid has been already associated to inflammatory symptoms in exposed workers (Poole *et al.*, 2010).

3. Standards and recommendations for occupational exposure assessment

In occupational environments, workers can be exposed to a variety of microbiological agents (Durando *et al.*, 2019). However, due to the extensive diversity of potential health effects together with bioburden variability, standards formulation on health risk assessment remains difficult to practice (Ki-Hyun *et al.*, 2017). In part conditioned by individual susceptibility, varying the health effects that microorganisms may cause (Viegas, 2010). Thus, there is a gap in human dose-response, associated to microbiological agents exposure (Ki-Hyun *et al.*, 2017). In some situations, a worst case approach is performed, being the sampling period selected taking into consideration the occurrence of higher exposure (Viegas, 2010). Allowing the results comparation of a low cost exposure assessment and the limit values generally defined by the country (Viegas *et al.*, 2016).

In general, the guidelines available in literature are referring to indoor air quality, not taking into account the health effects that microorganism may cause on workers (Ki-Hyun *et al.*, 2017). Summarizing, when it comes to indoor air quality, the Portuguese legislation establishes general limit values for microbiological contamination. Concerning the *Portaria no353-A* (2013),the fungal load indoor should be lower comparatively to the outdoor value. In other words, the ratio between Indoor and Outdoor should be lower 1. Concerning total bacteria, indoors should not exceed the outdoor load by 350 colony-forming units (CFUs).m^{-3}. In case of a higher load, a second criteria must be applied, for bacteria ([Indoor] + 350 UFC).m^{-3} > [outdoor] and [CO_2] < 1800 mg).m^{-3} and ratio Gram-Negative/ Total bacteria ≤0.5) (Viegas *et al.*, 2019a). For fungi, if the first quantitative requirement is not fulfilled and there is no visible fungal growth, an evaluation for some specific species described in *Portaria nº353-A* (2013) , should be performed.

The *Directiva 2000/54/EC* was transposed for the national law by the *Decreto Lei nº 84/97* that is currently the main Portuguese legislation related to workers protection against biological risks, it comprises the list of biological agents, microorganisms classification, hygiene measures and confinement levels. The *Directiva 2000/54/EC* transposition covers the information presented on *Diretivas do conselho nº 90/679 / CEE -26 Novembro, 93/88 / CEE-12 Outubro,* and the *Directiva 95/30/CE da Comissão de 30 de Junho,* refering the protection of the safety and health of workers from the risks associated with exposure to biological agents at work (Decreto-Lei 84/97, 1997). Regarding the national legislation, includes also the *Portaria nº405/98 - 11 Julho,* which approves the classification list of biological agents for the purpose of preventing occupational risk, and the *Portaria 1036/98 - 15 Dezembro* which has altered the previously mentioned classified list, approved by the Administrative rule (Portaria 405/98, 1998). However, this list only considers the infectious potential neglecting the toxigenic potential of each microorganism (Madsen *et al.*, 2020,Viegas *et al.*, 2021a).

Regarding occupational exposure assessment, there is a lack of European standards and legal limits are scarse in Portugal (Viegas *et al.*, 2016, 2017a, 2019a). Thus, some scientific proposed limits are often used as guidance (Cox *et al.*, 2020), such as the ratios between Indoor/Outdoor (Luksamijarulkul & Pipitsangjan, 2015) that suggests an acceptable indoor environment when the results are below 1 (Rao *et al.*, 1996, Cox *et al.*, 2020). Indeed, the relative standards enable results comparation from different studies, as long as the methods used indoor are identical to those used in the outdoor (Rao *et al.*, 1996). For quantitative analysis, there is also a stipulated limit for maximum indoor load (150

CFU.m^{-3}) proposed by WHO (2009), followed by a qualitative assessment, identifying species as indicators of harmful fungal contamination (Viegas *et al.*, 2015a), such *Aspergillus* sections *Fumigati, Nidulantes, Flavi e Circumdati* (Viegas *et al.*, 2019b).

Despite the lack of well-defined values for dose-response to microbiological agents during work shifts, there is a wide list of recommendations for workers who are or may be exposed to biohazards, (Burnett *et al.*, 2009, Rim & Lim, 2014).

Chapter II: Field sampling and microbial characterization

1. Sampling strategy

When defining the sampling strategy for occupational exposure assessment, the exposure assessor needs to take into consideration all the factors that could compromise the physical sampling efficiency. Additionally the type of biological particles and particular components needs to be taken into consideration (Reponen, 2017).

In several sectors, during work activities, workers can be exposed to organic dust from different origins containing fungi, bacteria, their components and secondary metabolites such as mycotoxins. However, guidance for exposure limits in the occupational environments by majority only includes an estimation of the concentration of cultivable microorganism and occasionally endotoxin concentration (Viegas *et al.*, 2018a).

Exposure levels can range between tasks even at the same industry, also different exposure can occur over time. Indeed, there are some evidencing point out fungal seasonality (Rao *et al.*, 1996, Viegas *et al.*, 2017a, Cox *et al.*, 2020) Thus, it is important to consider each task individually (Viegas *et al.*, 2018a). Since there is no standard protocol available, the methodology performed must be adapted to the sampling goal (Reponen, 2017). Also, identifiying workers behaviors regarding the use of protective equipment as well as workplace peculiarities that may influence the exposure, contributes to a more reliable exposure assessment (Viegas *et al.*, 2018a, 2019a).

Bioburden assessment relies on the collection of samples onto solid or liquid media, following microscopic, biochemical, microbiological, or molecular biological analysis (Douwes *et al.*, 2003). The samples are collected in each area of interest, and an outdoor sample is also collected for reference (Viegas *et al.*, 2019a).

Qualitative and quantitative methods can be used to acess microbial exposure, visual and olfatory inspection of molds characterize the qualitative criteria. Quantitative methods includes sampling and analysis, with the main goal of checking and quantifying the presence of microorganisms, identifying the cause and efficacy of control measures, assessing human exposure (Reponen, 2017). Also supplemental information about location conditions such as ventilation or air conditioning, activities during the procedure, temperature and relative humidity contribute to a better description of the surrounding environment (Cox *et al.*, 2020). Although the Portuguese legislation relies only in active air sampling and culture-based methods for bioburden assessment, a multi-approach regarding sampling methods and assays should be implied to better characterize the microbiological composition even though there is no regulation mentioning (Viegas *et al.*, 2019a).

An integrated approach, comprising active and passive sampling, culture-based methods and molecular tools enables a broader characterization of fungal diversity (Reponen, 2017),since species composition and concentration is affected by several factors, causing sudden variations (Viegas, 2010). In fact, qPCR targetting section *Fumigati* was used to overcome culture-based methods limitations in several studies (Eduard *et al.*, 2012, Viegas *et al.*, 2015a, Mbareche *et al.*, 2019, Madsen *et al.*, 2020,Viegas *et al.*, 2020c).

Based on sampling campaign experience in critical settings as well as culture-based methods limitations, Viegas and team members (2017a) define some crucial steps for sampling strategy in order to accomplish a suitable exposure assessment to *Aspergillus* sp. burden. Some of the identified criteria were: having the most critical scenario assessment, selecting periods of greater exposure during work activities, using culture-base methods combined with molecular tools, defining the appropriate indicators of fungal contamination (assuming at least *Aspergillus* sections *Flavi, Fumigati, Circumdati* and *Nidulantes*) as well as screening the azole-resistant strains followed by microdilution methods to analyzed antifungal susceptibility. Finally, results should be compared with revelant guidelines, enabling risk characterization (Viegas *et al.*, 2017a). As example two approaches can be used for the same purpose, by comparing the results with the lowest observed effect level or a perspective more

related to the dose-effect (Figure 2.1). More recently in 2021, the same team provided an review of diverse studies regarding occupational exposure to *Aspergillus* section *Fumigati* in Portugal,highlighting in the occupational environments assessed, the emergence of azole-resistance and the sampling protocols used. Overall, the work evidence some guidance for the assessment of fungi in general and in particularly for *Aspergillus* section *Fumigati* (Viegas *et al.*, 2021a).

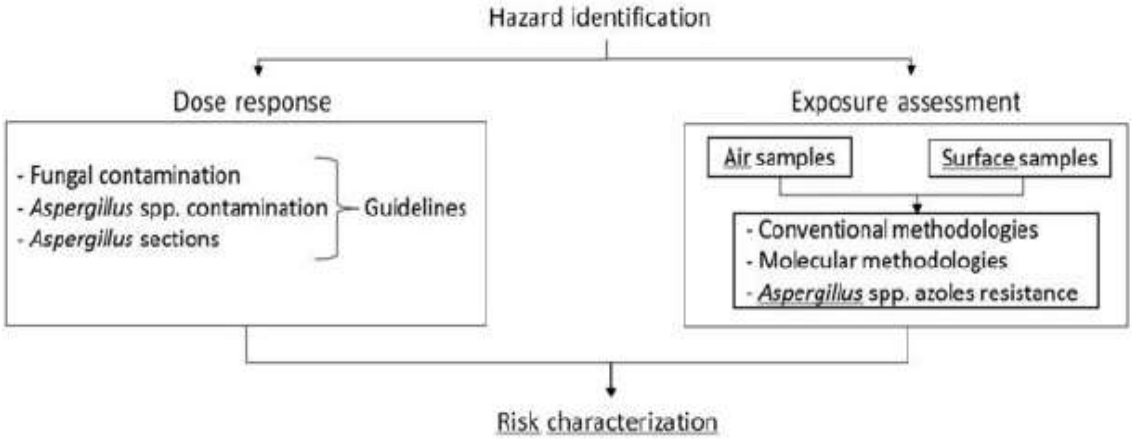


Figure 2.1 - *Aspergillus* sp. risk characterization proposal. Adapted from Viegas *et al.* (2017a).

2. Sampling methods

In general, microbial determination procedures can be performed by direct source evaluation techniques, such as surface and dust sampling or air sampling (Reponen, 2017). As an example, the frequent use of impactors and impingers for the assessment of airborne viruses, besides surface swabs (Pena *et al.*, 2021). For each sampling campaign a certain equipment is required. The volume of air sampled, the number of places to visit, and if the case, the type of culture medium and incubation conditions need to be defined before the study (Cox *et al.*, 2020).

2.1 Active sampling methods

For bioaerosols assessment, methods used can range from impingers (Viegas *et al.*, 2019a), impactors (Ramos *et al.*, 2016-, Viegas *et al.*, 2019a), filters (Wang *et al.*, 2019), electrostatic precipitation (Mainelis *et al.*, 1999) and others. Such devices promote particle separation from the air stream, and some can be directly inoculated onto a pre-selected medium or solid filters (Douwes *et al.*, 2003, Ki-Hyun *et al.*, 2017).Such procedure can be done by several ways since physical features of airborne particles and physical parameters of the sampler determine the removal efficiency of the bioburden. Nevertheless, inertial impaction is the major used mechanism for sampling in bioaerosols samplers, allowing the biological particles collected to remain undamaged (Nevalainen *et al.*, 1992). During sampling, microbial stress must be minimal, since maintaining nucleic acid integrity of microbes is crucial when applying molecular tools (Cox *et al.*, 2020) as well when cultivation is needed.

When providing the appropriate conditions, the organism must be able to form colonies or to be otherwise identified. Also, during the procedure, the samples must be collected in sealed containers, properly labelled. After collection, specimens should go to the laboratories, with the appropriate transport media if necessary and in the suitable temperatures (Sastry & Bhat, 2016).

2.1.1 Impactors

Methods such as impaction can be inertial or centrifugal (Viegas *et al.*, 2017b). Impactors promote particle separation through an air stream and utilize particle inertia to force deposition on a collection media (Santos, 2020). Through airflow streamlines, the air is forced to pass through a set of curves while smaller particles go through the streamline. bigger particles, are incapable of passing through the 90º curve, falling out of the air flow (Ki-Hyun *et al.*, 2017). Finally inertia leads to the impaction of the particles onto a surface, usually solid or semi-solid (Nevalainen *et al.*, 1992). As example the Millipore air Tester (Viegas *et al.*, 2019a). The number of stages can vary from one, namely Air-O-Cell, to a multiple stage, is the case of the cascade impactor Andersen 6-stage (Reponen, 2017), in which, by varying the pore size in the 6 sections, simulates the human respiratory system (Viegas *et al.*, 2021b) (Table 2.1).

Table 2.1- Size and stage variation of Andersen six-stage sampler adapted from Viegas *et al.* (2021b) and Andersen (1958).

Stage	Cut-off size (µm)	Correspondence with the respiratory system
1	7.0	Nose and mouth
2	4.7	Pharynx
3	3.3	Trachea and primary bronchi
4	2.1	Secondary bronchi
5	1.1	Terminal bronchi
6	0.65	Alveoli

Centrifugal impactors use centrifugal forces to collect bigger particles, incapable of being collected through air streamlines. Thus, the particles are collected onto agar plate, tape, filter or glass slide (Reponen, 2017).

2.1.2 Impingers

Impingers collect the airborne by transmitting an air flow through nozzles, being collected into a chamber containing a liquid medium (Ki-Hyun *et al.*, 2017). The size diameter of the collected particles can vary by changing the air flow rate or distance between nozzles (Ki-Hyun *et al.*, 2017). Impingers normally work with water-based suspensions, as example phosphate buffer or peptone water, in some cases glycerol and mineral oil can be used (Reponen, 2017). Liquid impingers operates mainly through inertial forces, ranging from a pre-separating unit to a multi-stage (Nevalainen *et al.*, 1992). After sampling, samples can be analyzed though culture-dependent and independent methods (Franchitti *et al.*, 2020).

2.1.3 Electrostatic precipitators and others

Electrostatic precipitators have also been used for bioaerosol sampling, working through an electrostatic field, charging particles in an air stream, forcing them to the surface which has an opposite charge. Currently there are some modifications of electrostatic samplers available, as example, a water based condensation system with laminar-flow for the collection of nanoscale viruses (Reponen, 2017).

Another method is air filtration, using different filters composed by certain constitution and porosity as the air passes through it consequently, airborne particles with different particle sizes are trapped in the porous material (Franchitti *et al.*, 2020, Santos, 2020). The process works through impactation, interception and diffusion of the collected particles with the filter material and electrostatic forces. The selected filter relies on the analytical method used subsequently, as example the capillary pore filters used for culturing bioaerosols, gelatin membrane filters for viral detection, black polycarbonate filters for epifluorescence microscopy analysis or even PVC- filters for simultaneous analysis. In addition, multi-layers filters, such as Teflon, are capable of sampling a variety of particle sizes (Reponen, 2017).

2.2 Passive sampling methods

Trough passive methods it is possible to access contamination levels from a large period of time, such low-priced techniques enables (Ribeiro & Faria, 2017) to characterize occupational exposure, since workers pass most of their time in the same location (Viegas *et al.*, 2021a). For surface assessment, samples can be collected by swabbing indoor sites using a square stencil (Viegas *et al.*, 2019a). Another passive sampling method is the EDC. Consisting of an electrostatic propylene cloth placed on a sterile petri dish, which is kept open during sampling (Viegas *et al.*, 2020b). The particles will be retained on the cloth, allowing an efficient collection of dust present in the air (Liebers *et al.*, 2012, Viegas *et al.*, 2020b). Surface samples can be obtained through coated microscope slides, or by swiping the area of interest with a cotton swab. Additionally, through vacuuming it is possible to obtain large samples and analyzing multiple agents. Both methods are assumed to be representative of a long-term exposure (Viegas *et al.*, 2017b). Gravitational sampling can be performed allowing the collection of particles onto agar plates by gravitational forces also, dust and material collection can be carried out for a wider sampling campaign (Ki-Hyun *et al.*, 2017).

3. Assays

3.1 Culture media selection and microorganism's inoculation

When performing culture methods there is the need of picking the right culture media. They can be distinguished by their selectivity, as example basic elements like water and nutrients characterize an non-selective media (Bonnet *et al.*, 2020), such as Agar enabling the grow of a wide range of microorganisms (Ribeiro & Faria, 2017). Additionally, some growth factors such as nucleic acids, amino acids, vitamins, blood and derivates can be add, benefiting the spread of specific species characterizing an enriched medium (Bonnet *et al.*, 2020). As example the bile salts in MacConkey agar, limiting microbial growth (Ribeiro & Faria, 2017), allowing to differentiate lactose fermenting organisms by the color of the colony (Sastry & Bhat, 2016).

Isolation of desired microbiota can also be accomplished by the use of antibiotics and antiseptics (Bonnet *et al.*, 2020). For instance the use of different culture media provides a broader characterization of microbial contamination, as the use of DG18 in addition to MEA permits an wider characterization of *Aspergillus* contamination due to size restriction of fungal colonies with higher growth rates (Viegas *et al.*, 2021a).Other media types are also available such as anaerobic, synthetic or even transport media, often associated with the transport of sensitive microorganisms, maintaining the viability (Sastry & Bhat, 2016).

Through solid culture media it is possible to isolate different colonies by morphological characteristics, enabling identification. In contrast to culture broths, that despite the greater availability of nutrients does not allow species identification since they are all mixed in the liquid (Bonnet *et al.*, 2020).

In what concerns inoculation, generally, streak culture is a routine method to isolate microorganisms from a diversified culture, briefly, after medium selection, the sample is smeared onto the surface of the culture plate using a sterile wire. The inoculum is spread all over the plate in parallel lines, finally the plates are incubated at a certain temperature associated with microorganism optimal growth. All the procedure should be performed in a biosafety cabinet, decreasing the risk of contamination. Other culture techniques can be applied depending on research purpose (Sastry & Bhat, 2016).

When the goal is microorganisms preservation some procedures such as a short-term preservation is performed by adding a cryoprotectant substance, glycerol or sterile distilled water, and storing in the freezer at -20ºC. A long-term preservation can be achieved by the same steps incubating the sample at lower temperatures (Sastry & Bhat, 2016), as example -80ºC (Francisco, 2017).

3.2. Microorganisms identification and characterization

3.2.1 Culture-Dependent methods

Culture methods are performed for a wide range of purposes such as microorganism isolation, identification and to access the antimicrobial susceptibility (Sastry & Bhat, 2016). It allows to gatter information about microbial viability and what has increased infection potential (Viegas *et al.*, 2020c). Which is crucial to estimate health risk, since viability can restrain microorganisms cytotoxic and inflammatory potential (Ki-Hyun *et al.*, 2017, Madsen *et al.*, 2020). In fact, the inflammatory potential of a microorganism can vary depending on its bioaerosol composition (Madsen *et al.*, 2020).

The Portuguese legislation relies only in culturable methods from air sampling to access the indoor air quality (Viegas *et al.*, 2019a).This approach permits the microorganisms quantification and identification. Enabling to study infectious organisms, (Viegas *et al.*, 2019b) and the comparison with legal and scientific criteria (Viegas *et al.*, 2017a). Indeed, the proposed thresholds of bioaerosol concentrations are generally taken from the results associated with culture methods (Ki-Hyun *et al.*, 2017). Despite the advantages, the exact microbial contamination may not be obtained due to the need of selecting the specific culture media and culture conditions (Viegas *et al.*, 2015a, 2020c). During incubation, fast growing fungal species may benefit (Mensah-Attipoe & Taubel, 2017)32, leading to an overgrowth subsequently inhibiting fastidious species due to chemical competition (Viegas, *et al.*, 2015a). Therefore, only certain microorganisms will be able to grow and the total microbial exposure can be underestimated, perhaps overestimating the most tolerant species (Viegas *et al.*, 2019a). Furthermore, associated with culturable microorganisms counting, microbial components and dead microorganisms are not detected, even though they can have allergenic properties (Douwes *et al.*, 2003). Due to associated limitations other independent methods have been developed (Mensah-Attipoe & Taubel, 2017).

3.2.2 Culture-Independent based methods

Through an instrument-based technique it is possible to obtain microorganism identification, or even have a continuous monitoring of the microbial growth (Sastry & Bhat, 2016). Indeed, molecular tools are crucial to select specific strains normally associated to pathogenic bioburden (Viegas *et al.*, 2019a), and DNA extraction from an environmental sample is the first step required for subsequent analysis (Mensah-Attipoe & Taubel, 2017).

Polymerase chain reaction (PCR) based assays have improved detection of microorganisms (Basinas *et al.*, 2017). In comparison with the conventional culture methods, PCR is more sensitive and specific (Sastry & Bhat, 2016). For this molecular tool, primers and probes are necessary, generally

designed for a specific genus, genera or single species Mensah-Attipoe & Taubel (2017). According to Mensah-Attipoe & Taubel (2017), certain regions, such as 18S ribosomal RNA gene can be used for primers and probes design due to the conservated region between individuals belonging to the same species.

Nowadays, there was some modification on the PCR procedure such as the develop of reverse transcriptase PCR (RT-PCR), making it possible to amplifying RNA, by adding the reverse transcriptase enzyme, enabling the detection of RNA viruses (Sastry & Bhat, 2016). Also, DNA quantification can be performed by real-time quantitative PCR (qPCR) (Mensah-Attipoe & Taubel, 2017) the most used PCR technique (Ribeiro *et al.*, 2017).The data are monitored by software for each cycle, producing a graph of quantitative amplification, allowing the identification and quantification of the amplified fragments (Ribeiro *et al.*, 2017). As example, qPCR gives information related to the occurrence and levels of the main indoor fungi. Also, there is some literature pointing out the priorization of this molecular procedure for virus assessment in environmental samples (Mensah-Attipoe & Taubel, 2017). More recently, fungi detection and quantification can be accomplished by next generation sequencing (NGS) more precisely amplicon sequencing, capable of analyzing large database (Mensah-Attipoe & Taubel, 2017).

Bio-molecular methods allows to assess microorganism concentrations in a higher order when compared to culture-dependent methods. Consequently, it is possible to gatter more information about bioaerosol composition, identifying even non viable microorganisms. In contrast to culture-dependent methods only restricted to species capable of creating colonies (Franchitti *et al.*, 2020).

Some limitations are associated to the cost and weak standardization mainly because of bioinformatic analysis (Anedda *et al.*, 2019).The need of choosing the most suitable regions to amplify to obtain accurate information about the microbial content (Mbareche *et al.*, 2017). The constrains associated to an inefficient DNA extraction (Viegas *et al.*, 2020c), or even amplification errors, as example the incomplete amplification of DNA fragments, leading to other recombinations, and biases in diversity results (Mbareche *et al.*, 2017).

In short, some of the challenges faced when applying such method emphasize the need for a better standardization for nucleic acid recovery, the choice of genetic markers used and data interpretation (Mbareche *et al.*, 2017).Despite the restrictions such tool opens future molecular perspectives, becoming a good resource if more standardized, including processing steps for quality control purpose, contributing to a better comprehension of the microbial exposome (Gangneux *et al.*, 2020).

3.3 Antimicrobial resistance assessment

Microorganisms can develop resistance to antimicrobial agents, such resistance can be intrinsic or acquired, for antimicrobial susceptibility determination there are some defined methods (Sastry & Bhat, 2016).

3.3.1 Antimicrobial classes

Several factors underlie the choice of the drug to be tested, its usefulness in therapies, microorganism properties, toxicity and others. Taking into account these factors, there are first and second-line drugs, the name being associated with the order of use respectively(Sastry & Bhat, 2016).

Based on mode of action antifungal drugs can be divided in azoles, affecting ergosterol synthesis, essential for the synthesis of one of the main constituents of fungal membrane (Francisco, 2017). Polyenes, acting on fungal membrane influencing spores formation leading to osmotic desregulation, fluoropyrimidines and achinocandins inhibiting DNA and β-D-glucan synthesis respectively (Sabino, 2017). In general, the agents used are mainly focused on the cell envelope (Odds *et al.*, 2003).

Antifungal agents namely triazole compounds included in the azoles group (Odds *et al.*, 2003), have been successfully used for antifungal theraphy of *Aspergillus* section *Fumigati* sensu stricto (Viegas *et al.*, 2021a). In fact, Itraconazole (ITR), Voriconazole (VOR) and Posaconazole (POS) are the most used antifungals, presenting general toxicity (Francisco, 2017, Viegas *et al.*, 2021a). ITR acts against a wide spectrum of fungi, including *Aspergillus* sp. (Francisco, 2017). VOR covers several species, being lethal for filamentous species as well as POS, capable of affecting a large group of fungal species (Odds *et al.*, 2003).

When it comes to antibacterial agents, the priority relies on targeting the antibiotic-resistant pathogens such as MRSA (Crowcroft *et al.*, 2013), as well as gram-negative bacteria, including carbapenem-resistant Pseudomonas, Acinetobacter and Enterobacterales (Theuretzbacher *et al.*, 2020). Some antibacterian agents are β- lactam- based, mostly focused on Enterobacterales and tetracycline derivates (Theuretzbacher *et al.*, 2020). Also quinolones and cephalosporins were associated to MRSA treatment (Crowcroft *et al.*, 2013).

3.3.2 Testing methods

Briefly, agar-based test methods, agar supplemented with resistant screening media, broth-based test methods (Sabino, 2017), and gradient concentration strips are some of the alternatives available for screen isolates (Ghelfenstein-Ferreira *et al.*, 2020). For the azole screening method, SDA supplemented with the most frequently used triazoles is prepared, in order to access fungal susceptibility, in case of growth, isolates are prepared and analyzed for susceptibility profiles (Viegas *et al.*, 2021a).

Through disk diffusion method it is possible to access susceptibility patter, in short, after inoculation, some paper disks soaked with a specific antibiotic solution are applied to the solid medium, since there is a higher antibiotic concentration near the disk drug resistance is determined by microbial growth around the disk (Sastry & Bhat, 2016).

Through dilution tests it is possible to access the minimum inhibitory concentration (MIC) of an antimicrobial agent, defined by the lowest concentration of the antibiotic in which there is no visible grow of the microorganism (Francisco, 2017). Serial drug dilutions are prepared in a broth and each tube is inoculated with the suspension of the microorganism being tested (Sastry & Bhat, 2016).In fact, EUCAST (European Comitte on Antimicrobial Susceptibility Testing) released a new screening method for susceptibility testing of moulds through broth dilution minimum inhibitory concentration (E.DEF 9.3.2) (EUCAST, 2020). Such methodology can be used to confirm disk diffusion test results, also despite traditional methods, some automated systems are available, such as Phoenix system or Micro Scan Walk Aways system, giving faster results, also through molecular methods it is possible to target specific drug resistant genes (Sastry & Bhat, 2016).

Chapter III: Workplace as a source of microbial exposure

1.Occupational exposure to microorganisms in Portugal

There are several studies conducted in Portugal assessing occupational exposure to microorganism (Pinto *et al.*, 2018, Ribeiro *et al.*, 2018, Saleiro *et al.*, 2019, Viegas *et al.*, 2019c, Gonçalves *et al.*, 2021). The presence of potential toxigenic species and environmental factors such as high exposure to bioaerosol are some of the driven forces for studies development (Viegas *et al.*, 2020d). Even at low doses, exposure may represent a risk to employees health, being frequently associated with respiratory symptoms (Saleiro *et al.*, 2019).

Aspergillus section *Fumigati* is one of the most common and resistant strains identified in clinical and environmental contexts in Portugal, such factor gains a major concern specially in professions where higher fungal loads are expected (Gonçalves *et al.*, 2021).

There is some literature indicating the intensive livestock production systems as a starting point for health hazards on laborers (Viegas *et al.*, 2013a) This might be related to the organic dust present in many activity sectors such as animal production, farming and waste management, consequently inhalation seems to be the main source of exposure (Viegas *et al.*, 2020d). For instance, fungal wide spread through dust aerosolization has been reported in swine farms and consequently workers can be expose to pathogenic microorganisms and their metabolites during certain operations (Viegas *et al.*, 2013b). Dust from poultry production is composed by residues, feathers and molds, being biologically active, also inflammatory agents may be present, as example endotoxins, β-D-glucans, promoting suppressive effects on the immune system (Viegas *et al.*, 2013a). Other important industrial activities in Portugal are bakeries, involving many human resources, being asthma a frequently reported work-related respiratory disease. Such reality might be justified by bakeries complex environment, having potential sensitizers in cereal flours, or even containing contaminants such as fungi and their metabolites, overall the highest levels of exposure seems to occur in mixing and baking or when opening the flour container (Viegas *et al.*, 2019c).

In a factory dedicated to the development and production of automobile equipment in the northern Portugal, about 85% of workers are exposed to dust and aerosolization of oil waste. Consequently, it was found that dyspnea was correlated to the duration of exposure to dust, evidencing the work environment as the responsible for the respiratory symptom reported (Saleiro *et al.*, 2019). Similar results were obtained in poultry farms, showing that the prevalence rate of obstructive pulmonary ventilator disturbance was directly correlated with a longer time of exposure to dust. Indeed, clinical data on respiratory symptoms highlighted the frequency of asthma and nasal symptoms in workers (Viegas *et al.*, 2013a).

According to Viegas and team members (2013b), elevated concentrations of *Aspergillus* section *Nidulantes* were detected after fungal contamination assessment in seven swine farms. Through a specific biomarker for AFB1 exposure it was found that about 75% of blood samples from workers exhibit high levels of the mycotoxin. Since the Portuguese system for pig production requires by majority, manual work, AFB1 concentration might be associated to the need of farmers intervention in several activities, promoting a longer exposure. Also, during bakeries assessment, fungal species with toxigenic potential were identified in all environments where fungal growth was detected, including *Fusarium* sp., *Penicillium* sp., and *Aspergillus* sections, emphasizing the need for further studies regarding occupational exposure (Viegas *et al.*, 2019c).

Aspergillus genera seem to dominate in Portuguese waste management facilities, presenting a large diversity in *Aspergillus* sections specially in waste sorting settings. Due to the favourable selective conditions such as high humidity, temperature level and azole pressure, resistant strains can evolve making waste sorting units the hotspot for the emergence of fungal resistance to triazole (Viegas *et al.*, 2017c). In fact, when it comes to resistance, one of the mechanisms is associated with a mutation in the

CYP51A gene (Snelders & Verweij, 2011). It was already reported pan-azole-resistant strains harboring the TR34/L98H mutations in Portuguese workplaces. Such findings were obtained from filtering respiratory protective devices used by workers of the waste sorting sector and one air sample from a dairy (Gonçalves *et al.*, 2021).

Medical facilities can also work as a source of contamination, according to Viegas and colaborators (2019a). During bioburden assessment in Portuguese health care settings, the most frequent fungi in air samples were *Chrysonilia sitophila, Cladosporium* sp. and *Penicillium* sp, also, some *Aspergillus* sections were reported in air samples, such as sections *Fumigati, Circumdati,* and *Nigri,* the last one was also found in HVAC filters samples. In addition, fungal growth was observed for *Chrysosporium* sp., *Cladosporium* sp., *Mucor* sp. and *Penicillium* sp. in SDA media supplemented with at least one azole. When it comes to mycotoxins, HVAC filters and air samples present some contamination, showing more than one mycotoxin in the same sample. Furthermore, FMs B_1, B_2, and B_3, roquefortine C, OTA and ochratoxin B were identified, being OTA the most prevalent and FMs B_2 presenting the highest measuring values (Viegas *et al.*, 2019a). Similar results for the most frequent fungal species were obtained on swabs from a clinical pathology service (Viegas *et al.*, 2020e).

Since Portuguese legislation concerning indoor air quality only include some fungal species during microbiota assessment, there might be some toxigenic species, already identify in clinical environments and equally harmful that go unnoticed, representing a health risk, as example *Aspergillus* sections *Nigri, Nidulantes* and *Aspergilli* (Viegas *et al.*, 2019a).

Due to the recurrent azole pressure in some occupational environments, the exposure assessment for cryptic species and azole resistance of *A.fumigatus* sensu stricto should be performed to ensure a continuous surveillance of *Aspergillus* sp., in order to control the emergence of resistance (Gonçalves *et al.*, 2021).

2. Firefighters occupational hazards

Firefighters are exposed to several chemicals and physical risks being of major interest to occupational health exposure assessors. It is fully recognized the adverse effects related to smoke exposure in firefighting profession, and some hazards may be categorized as physical, ergonomic, thermal, chemical or even psychological (Guidotti & Clough, 1992).

Since large part of firefighters shift is passed in the station, having overnight shifts, waiting for a call, consequently this settings need to be taken into consideration when assessing occupational hazards (Sparer *et al.*, 2017), pointing out another source of occupational hazards besides fire situations (Oliveira *et al.*, 2017).

Most studies related to occupational assessment are focus on the exposure of firefighters to combustion products and fire pollutants during work performance, neglecting the possible exposure to pathogenic microorganisms. Despite that reality, some papers have already reported the bacterial exposure (Sexton & Reynolds, 2010, Roberts & David, 2014, Farcas *et al.* 2019). Firefighters are subjected to MRSA exposure during medical emergencies, being already reported the bacteria presence in surfaces from ambulances (Viegas *et al.*, 2021b). Hospitals, communal environments and FFH, as well as on fireman turnout gear, even after sanitation (Farcas *et al.*, 2019).

In a study conducted on emergency medical responders facilities, through surface swabs, MRSA was identified, presenting the highest prevalence on couches (20%), class desks (10%) and offices (6.7%) (Sexton & Reynolds, 2010). Such results might be justified by the bacteria ability to survive on fomites, also the selected firefighters headquarter seems to be visibly soiled, demonstrating an deficient cleaning and disinfecting routine (Sexton & Reynolds, 2010). According to Roberts & David (2014), through surface sampling of 33 Washington State firefighters headquarters about 8% were contaminated with MRSA, where 61.5% of MRSA were detected in living areas. Still during the study, it was reported

that 12 stations had staff with MRSA infections that required health care provider. More recently, bacteria colonization was observed from biological samples among firefighters (21% MRSA) (Viegas *et al.*, 2021b).

Previous studies have already established the presence of pathogens in firefighters headquarters (McGuire-Wolfe, 2020), or even toxigenic fungi found in ambulance vehicles (Viegas *et al.*, 2021b). The existing knowledge gap related to the effectiveness of cleaning procedures was already emphasized (McGuire-Wolfe, 2020). Indeed, in emergency vehicles, when comparing fungal contamination on surfaces, there was some sites reporting an increase after cleaning procedures (Viegas *et al.*, 2021b).

Some hygiene measures were already referred such as the use of hand sanitizer systems in all firefighters headquarter entrances, daily disinfection of all commonly high-touch areas or even education increase in matters of infection control policies, with the main goal of minimize the potential cross contamination of MRSA (Sexton & Reynolds, 2010). Even though there are some preventive measures and protocols for cleaning, their effectiveness remains uncertain (Farcas *et al.*, 2019). In what concerns FFH, bioburden assessment it is mainly focused on bacteria (Nigam & Cutter, 2003, Sexton & Reynolds, 2010, Roberts & David, 2014, Farcas *et al.*, 2019). Therefore, further studies are needed covering a more complete microbial characterization including fungi and viruses.

Chapter IV: Research goal

1. Main objective

To characterize the microbiological contamination in Portuguese FFH including contamination by *SARS-CoV-2* and fungal resistant profile.

2. Specific objectives

- To assess microbial contamination through active and passive sampling methods.
- To characterize the resistance profile from fungal contamination.
- Molecular detection of *Aspergillus* sections *Fumigati* and *Nidulantes.*
- Assessment of *SARS-CoV-2* presence in air and surfaces.

Part II – Investigation framework
Chapter V: Materials and methods

1. Exposure assessment

1.1 Firefighters headquarters sampling locations

This study is incorporated on an enlarged exploratory study funded and supported by Instituto Politécnico de Lisboa, Portugal denominated " Occupational exposure of ambulance drivers to bioburden" (IPL/2020BIO-AmbuDrivers_ESTeSL) with the aim to assess occupational exposure of ambulance drivers to bioburden, being extended to microbial assessment in firefighters headquarters.

Initially a walkthrough survey was performed, registering cleaning procedures, the cleaning frequency, the number of workers present, the ventilation system adopted as well as building features (Table 5.1). In terms of cleaning routine all FFH (11) were cleaned and disinfected once a day, everyday.

Table 5.1- General caracterization of the 11 FFH sampled. Adapted from Viegas *et al.,* (2021c).

HEADQUARTER	BUILDING AGE	NUMBER OF OCCUPANTS	CRACKS ON THE WALLS/FLOOR	MOULD GROWTH	VISIBLE PROBLEMS
FFH1	100*	50	Frequently	Frequently	Damp spots
FFH2	100*	12	Frequently	Frequently	Damp spots
FFH3	37	30	Often	Often	Infiltrations
FFH4	20	11	-	Often	-
FFH5	100*	20	-	Frequently	Infiltrations
FFH6	100*	16	-	Often	Infiltrations
FFH7	2	7	-	-	-
FFH8	100*	16	-	-	-
FFH9	62	20	-	Often	Infiltrations
FFH10	30	20	-	-	Infiltrations
FFH11	4	7	-	-	-

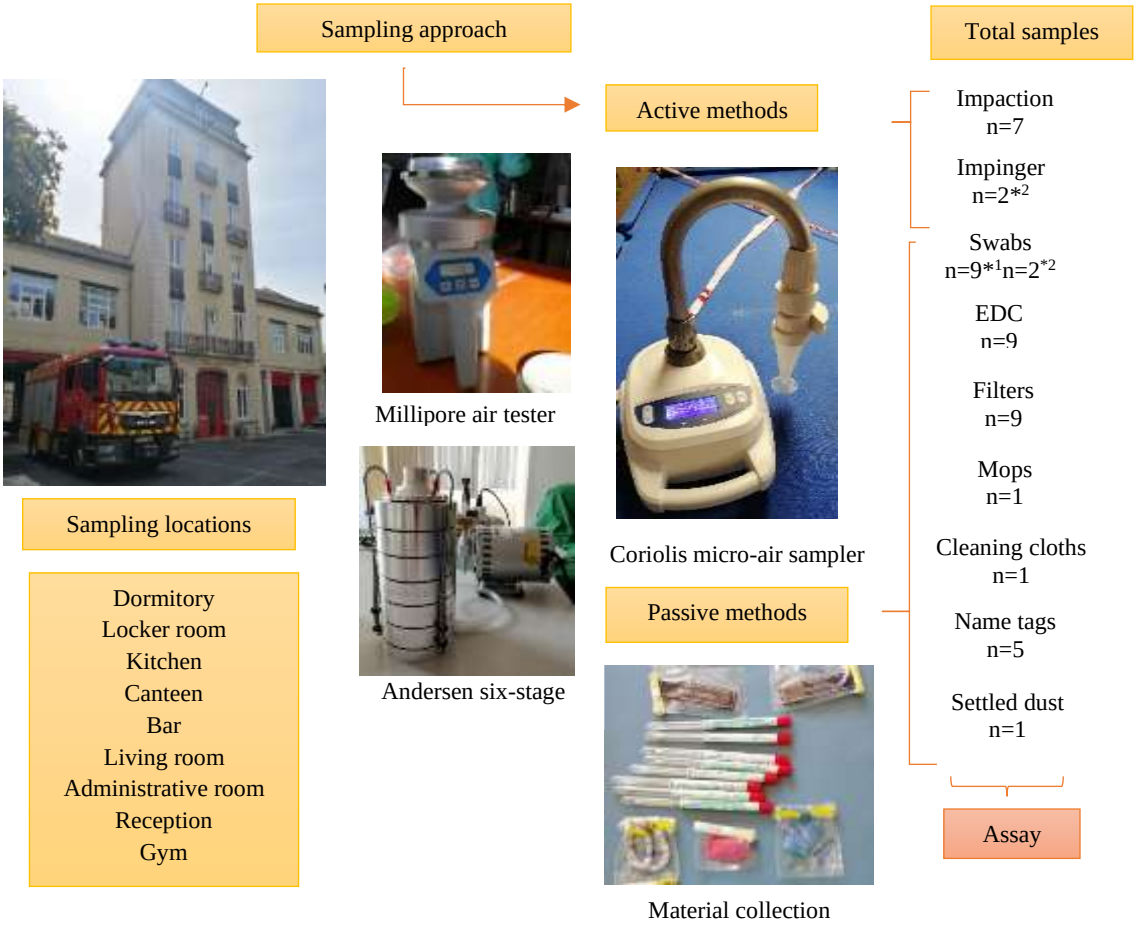
**Buildings over 100 years*

The work took place in 11 firefighters headquarters for 4 months, in Lisbon district, following a multi-approach protocol using active (impaction and impinger air sampling) and passive (EDC, floor surface swabs, filters, mops and cloths used on cleaning routine and uniform name tags) sampling procedures. The sampling campaign was performed during a normal working activity. Critical points associated to bioburden exposure were identified based on working activities. Several areas were accessed namely male dormitory, locker room, kitchen, canteen, bar, reception, gym and administrative and living rooms, being the designated locations for the sampling campaign in each FFH (Figure 5.1).

Concerning impaction methods, a total of 760 indoor air samples were obtained through two sampling devices (impaction method), onto each plate being 190 from each culture media used. Two media for fungi (MEA and DG18) and two for bacteria (TSA and VRBA). For *SARS-CoV-2* assessment, in similarity to Viegas *et al.*, (2021d) procedure, 23 indoor air samples were collected by the impinger,

in addition to the 21 swabs, rubbed in areas of greater use (door handles, buttons, surfaces). An outdoor air sample was collected trough Millipore air tester in the entrance of each FFH for reference (11 samples).

For passive methods about 82 EDC, 102 floor surface swabs, 90 filters, 67 uniform name tags, 14 mops and 25 cloths used for cleaning routine where collected. Finally, dust collected from each filter was grouped by FFH (11 samples).



*2*Air samples from impinger method and surface swabs were only for SARS-CoV-2 assessment*

Figure 5.1- Example of the sampling campaign for microbial assessment performed in FFH5 (n=total samples).

1.2 Sampling campaign

Indoor air was collected through the impactors Millipore and Andersen six-stage air sampler into four different culture media. Two for fungal assessment, namely MEA supplemented with chloramphenicol (0.05%) and DG18. In contrast to TSA supplemented with nystatin (0.2%) and VRBA for bacteria. About 250 L of air in a flow rate of 140 L/min was collected by Milipore Air Tester (Billerica, MA, USA) (Figure.5.2 -a), according to manufactures guidelines. The Andersen six-stage air sampler (Thermo-Andersen, USA) was also used with a flow rate of 28.3L/min for 9 minutes (Figure 5.2-b). The plates were then taken to the laboratory being incubated.

a) b)

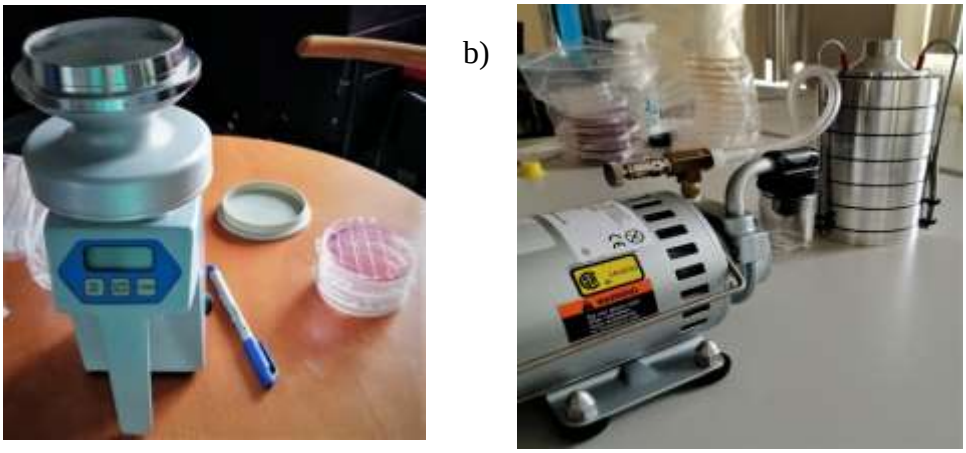


Figure 5.2- Impactors with the respective medium plates: a) Millipore air Tester b) Andersen 6- stage.

For *SARS-CoV-2* assessment the methodology followed the procedure applied in Viegas and team (2021d). Air sampling was performed by impinger method using the Coriolis micro air sampler (Bertin Technologies, Montigny-le-Bretonneux, France) Figure 5.3). About 600 L of air were collected by a flow rate of 300 L/min, onto 5 ml NVLinactivation buffer from NZY Viral RNA Isolation Kit (NZY Viral RNA Isolation kit (MB40701) component).

Figure 5.3- Liquid Impinger Coriolis with NVL inactivation buffer (Nzytech genes & enzymes) during a FFH *SARS-CoV-2* sampling campaign.

In addition to active sampling, according to Viegas (2021b) methodology, passive methods were also performed to assess microbial contamination. Floor surfaces were swabbed using a 10x10 cm square stencil, being disinfected with a 70% alcohol solution in each sampling. The dust was collected from each selected area through a vacuum cleaner (HOOVER Brave BV71_BV10 A2, USA) coupled with a collector filter. Also, EDC were used for settled dust assessment, each one having 90 mm surface exposed area, the plates were placed 1.5m heigh above the ground, for 30 days. Samples of cloths and mops used for common cleaning of FFH compartments were collected in addition to the uniform names tags used by the firemen. In addition to the collected air for *SARS-CoV-2* assessment, surface swabs moistened with saline solution were rubbed in areas frequently toutched and kept in eppendorfs with the same inactivation buffer (NVL).

All the collected samples were placed in sterilized bags, identified, and transported to the lab in refrigerated conditions (0 to 4ºC) proceeding to its treatment.

1.3 Samples treatment and inoculation

The analyze of the collected samples was performed by culture-based methods complemented with molecular procedures. Regarding *SARS-CoV-2*, the assessment was performed only through molecular tools in resemblance to Viegas *et al.*, (2021d) procedures (Figure 5.4).

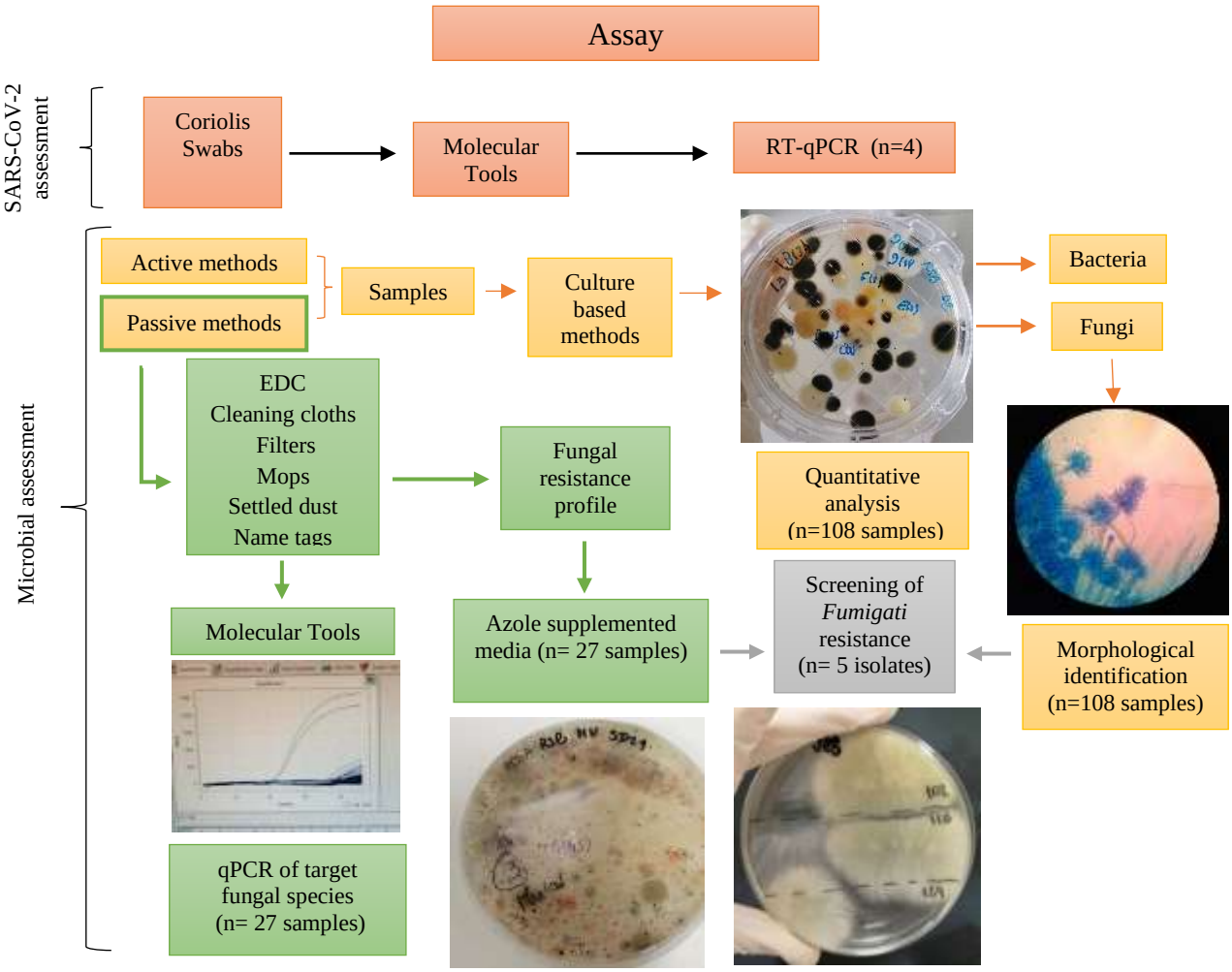


Figure 5.4- Example of the assay applied in samples collected from FFH5.

Floor surface samples, were extracted with 1 mL of 0.1% Tween 80 saline solution (NaCl 0.9%). Filters used for vacuuming, mops, cleaning cloths and uniform names tags, were extracted using 10 mL of 0.1% Tween 80 saline solution (NaCl 0.9%) based on the same methodology used in Viegas *et al.* (2019a).

EDC were weighed before extraction as Viegas *et al.* (2018b, 2021b) and in resemblance to the procedure applied in Viegas *et al.* (2018b), each sample was washed with 20 mL of 0.1% Tween 80 saline solution (NaCl 0.9%). After adding the solution, all samples were placed in the orbital laboratory shaker (Edmund Buhler SM-30, Hechingen, Germany) for 30 minutes at 250 rpm.

Dust from vacuum cleaner filters was separated in a falcon tube and washed in a ratio of 1g of the collected dust per 9.1 ml of NaCl (0.9%) with Tween 80(0.05%), during 30 minutes at 250 rpm in

similarity to the procedures applied in Viegas and team (2021b). As suggested in (EPA, 1995), a composite sample of the settled dust was performed in each FFH covering all the assessed facilities.

Followed by extraction, according to the procedures performed by Viegas *et al.*, (2018b, 2019a, 2021b), 150 µL of the obtained washed suspension of all extracted samples were plated into the same four culture media used in the impaction method (TSA,VRBA,MEA,DG18). After inoculation, plates of MEA and DG18 were then incubated for 5 to 7 days at 27ºC for fungi, and during 7 days for bacteria, being TSA at 30ºC for mesophilic bacteria and VRBA at 35ºC for coliforms Also, apart from swabs, 150 µL of the remaining samples were plated onto SDA supplemented with azoles, for screening of azole resistance (section 4.1).

The samples remaining suspension were freezed at -80ºC until DNA extraction (section 3.2). Samples processing for *SARS-CoV-2* assessment is described further (section 3.1).

2. Viable bioburden characterization

Fungal microscopic identification was performed through tease mount and lactophenol cotton blue procedures, also morphological identification was accomplished by macro and microscopic characteristics as noted by De Hoog (2016).

After incubation, bacterial and fungal quantitative results (Appendice 1: Equation 8.1-8.5) were obtained for samples from passive and active methods, in accordance to Viegas *et al.*, (2021b).

Despite the excessive microbial growth in some plates, it was decided to not dilute the liquid samples due to the possibility of sample loss as Franklin *et al.*(2001).

In cases of uncountable data due to fungal overgrowth on plates, the number 500 was used in the counts for air samples, as assumed in Viegas *et al.*, (2019c). For the remaining samples, filters, swabs, cleaning cloths, uniform name tags, EDC and mops the median of each matrix was used, in resemblance to the methods applied in Viegas *et al.*, (2015b, 2021d) .

3. Molecular tools

3.1 *SARS-CoV-2* virus assessment

For viral assessment surface swabs and air samples obtained by Coriolis air sampler were firstly decontaminated with paper towels soaked with 70% ethanol using complete personal protective equipment (PPE) composed by lab coat, disposable gown, gloves, surgical face mask, feet cover and glasses. Prior to RNA extraction all samples were heated at 56ºC to ensure *SARS-CoV-2* inactivation. Viral RNA extraction was carried out using NZY Viral RNA Isolation Kit (Nzytech genes & enzymes), conditions were based on manufacturers guidelines, standard operating procedure (SOP) based in Prudêncio *et al.* (2020).

Real time - quantitative polymerase chain reaction (RT-qPCR) was performed according to Molecular Medicine Institute SOP guidelines (Prudêncio et al., 2020), using the CFX96 (Bio-rad, Hercules, CA USA). Reactions included rRT-PCR primer/probe sets published by Centers for disease control and prevention (CDC) available on (https://www.cdc.gov/coronavirus/2019-ncov/lab/rt-pcr-panel-primer-probes.html). Each reaction was composed by two primers and probe sets for two different virus gene regions (N1 and N2) and also one spike in control and probe set. For assay control in all tested samples a positive template (EDX *SARS-CoV-2* Standard, ExactDiagnostics) and a negative template (no template addition) were used as positive and negative control respectively, also a spike in control was added in all the environmental samples to access the presence of potential inhibitors.

3.2 Detection of *Aspergillus* sections *Fumigati* and *Nidulantes*

All the material collected from passive sampling methods (EDC, filters, settled dust from filters, mops and cloths used for cleaning and name tags), apart from swabs, was used for molecular detection of *Aspergillus* sections namely: *Fumigati and Nidulantes*, known for their harmful health effects (Viegas *et al.*, 2021b). Fungal DNA extraction was performed using Quick-DNA™ Fungal/Bacterial Miniprep Kit (Zymo-research corp, Irvine, CA, USA), according to manufacturer's instructions. Also as performed in Viegas *et al.* (2019a), PCR grade water was used for negative control of each extraction batchUltra-pure DNA was kept at -20ºC for subsequent analysis .

After DNA extraction, qPCR was achieved using the CFX-Connect PCR System (Bio-rad, Hercules, CA USA). Reactions included 1x iQ Supermix (Bio-Rad, Hercules,CA, USA), 0,5µM of each primer and 0,375 µM of TaqMan, having a final volume of 20µL (Table 5.2). Also it was used a negative control, with no template, and a positive control, with DNA obtained from reference strains (sequenced for ITS, B-tubulin and Calmodulin) belonging to the culture collection of Parasitic and Fungal Infections unit – Infectious Diseases Department of the National Institute of Health Dr. Ricardo Jorge. PCR reaction conditions were based on the same methodology applied in Viegas, *et al.* (2020b), amplification was performed following a three-step PCR with 50 cycles, with denaturation at 95ºC for 30s, annealing at 52ºC for 30s and extension at 72ºC for 30s.

Table 5.2- Primers and TaqMan probes sequence used for real time PCR, adapted from Viegas *et al.* (2020b).

Aspergillus **Sections Targeted**		**Sequences**	**Reference**
Fumigati	Forward Primer	5′-CGCGTCCGGTCCTCG-3′	(Cruz-Perez *et al.*, 2001)
	Reverse Primer	5′-TTAGAAAAATAAAGTTGGGTGTCGG-3′	
	Probe	5′-TGTCACCTGCTCTGTAGGCCCG-3'	
Nidulantes	Forward Primer	5′-CGGCGGGGAGCCCT-3′	(EPA, 2017)
	Rever primer	5′-CCATTGTTGAAAGTTTTGACTGATcTTA-3′	
	Probe	5' – AGACTGCATCACTCTCAGGCATGAAGTTCAG-3'	

4. Fungal resistance profile

4.1 Azole resistance

The azole resistance was obtained through inoculation of 150µL of the extracted samples (filters, EDC, settle dust, uniform names, mops and cleaning cloths) in SDA media supplemented with azoles, namely, 4 mg/L itraconazole, 2mg/L voriconazole and 0.5 mg/L posaconazole (Viegas *et al.*, 2020b), with accordance with the proposed values of EUCAST norm for *A. fumigatus* by Guinea *et al.* (2019). Negative controls of all samples from passive methods, including SDA medium alone, were also prepared to ensure aseptic conditions. The plates were inoculated for 4 days at 27ºC, after the incubation period, fungal species were identified, as mentioned in section 2.

4.2. Susceptibility analysis of *Aspergillus* section *Fumigati* by the screening method

The isolates identified as belonging to section *Fumigati* (in section 2), were isolated in an eppendorf tube containing phosphate-buffered saline. After obtaining pure colonies through inoculation of the isolate in MEA medium, inoculum suspensions were prepared. The isolates were carefully scraped

with a cotton swab and transfer to a sterile tube containing saline (Guinea *et al.*, 2019). Tubes were placed on the spectrophotometer, and the concentration values were obtained at 600 nm, all the procedure was performed in accordance with the guidelines for inoculum preparation mention by EUCAST (Guinea *et al.*, 2019). After obtaining the fungal spore in saline solution at a final density of 0,5 McFarland (OD_{600} 0.060), based on Francisco (2017) methodology, a swab was soaked in the solution and through the streak technique, a inoculation was made onto the SDA media, supplemented with azoles.

5. Statistical Analysis

The data were analyzed following Jerrold (2010) guidelines, in similarity to the statistical tests applied in Viegas *et al*, (2021c). The analyses was performed through SPSS statistical software, V26.0 for Windows, the results were considered significant at the 5% significance level. To test data normality, the Shapiro-Wilk test (n's<50) or the Kolmogorov-Smirnov test (n's>50) was used. To compare the microbial contamination through sampling method, and *Aspergillus* section *Fumigati* molecular detection in all matrices, the Kruskal-Wallis test was used, since the assumption of normality was not verified. To study the relationship between sampling method and culture media in fungal and bacteria counts the Spearman's correlation coefficient was used, since the normality assumption was not verified.

Chapter VI: Results

1. Firefighters headquarters features

The 11 firefighter's headquarters were characterized. Indeed, some visible problems that might influence the results were detected as mould growth, dust and infiltrations (Figure 6.1).

Figure 6.1- Firefighters headquarters visible problems registered during the sampling campaign.

2. Viable Bacteria

Bacteria load (TSA) in indoor sites vary from $6.4x10^3$ to $2.8x10^4$ to CFU.m^{-3} in Andersen samples, while for Millipore the variation ranged from $1.2x10^3$ to $2.2\ x10^4$ CFU.m^{-3}. Coliforms load (VRBA) ranged from not detectable to $3.6x10^4$ and from 4 to $1.6x10^2$ CFU m^{-3} in Andersen and Millipore respectively.

Concerning passive methods, from all the matrices, floor surface samples presented the highest bacteria contamination ($1.2x10^6$ CFU.m^{-2}), ranging from $1.4x10^4$ to $1.2x10^6$ CFU.m^{-2}, while coliforms bacteria counts ranged from not detectable to $2.7x10^4$ CFU m^{-2}. Regarding filters, total bacteria counts vary from $1.5x10^3$ to $3.0\ x10^5$ CFU.m^{-2}, while coliforms had the highest value of $4.5x10^5$ CFU.m^{-2}. Cleaning cloths and mops presented similar values between total bacteria contamination (In cleaning cloths: $5.0\ x10^2$ – $5.5\ x10^5$ CFU.m^{-2}; In mops: $5.0\ x10^2$ – $1.9\ x10^5$ CFU.m^{-2}) and coliforms (In cleaning cloths: not detectable to $4.2\ x10^5$ CFU.m^{-2}; In mops: not detectable to $5.5\ x10^4$ CFU.m^{-2}). In the name tags from firefigthers uniforms, total bacteria ranged from $1.5\ x10^3$ to $3.4\ x10^4$ CFU.m^{-2}, while coliforms ranged from not detectable to $5.0\ x10^2$ CFU.m^{-2}. In EDC samples, bacteria counts vary from $2.1\ x10^1$ to $5\ x10^2$ CFU.m^{-2}.day^{-1} and coliforms from not detectable to $8.1x10^2$ CFU.m^{-2}.day^{-1}. Regarding total bacteria contamination in settled dust samples the values vary from 4 to $5.6\ x10^2$ CFU.g^{-1}. Same results were obtain for coliforms counts (Figure 6.2)

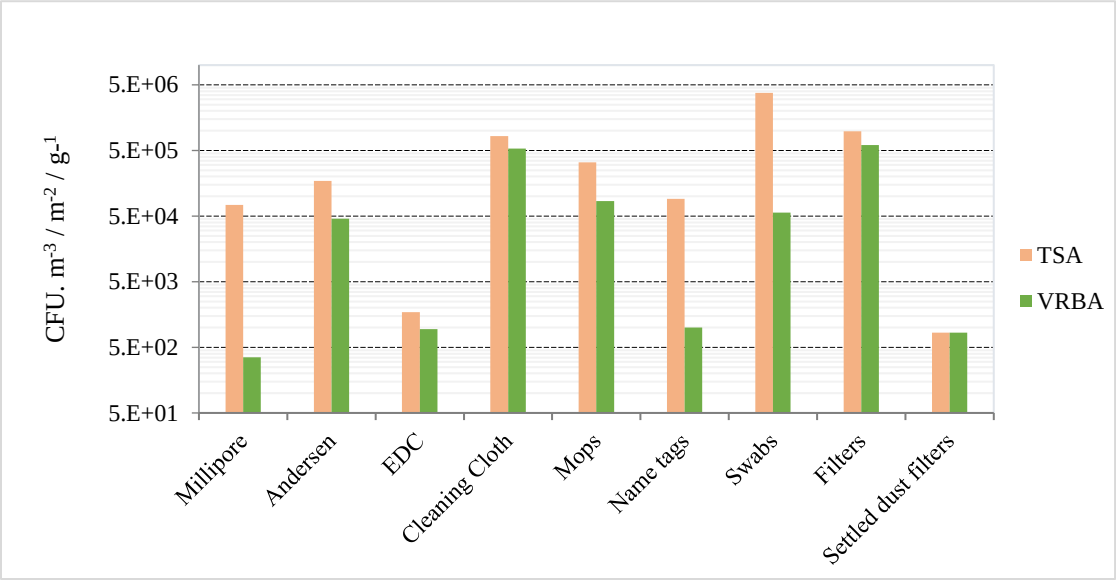


Figure 6.2- Total bacteria counts (TSA:tryptic soy agar) and coliforms bacteria (VRBA:violet red bile agar) in all matrices. Electrostatic dust collectors (EDC) results were presented as $CFU.m^{-2}.day^{-1}$.

3. Viable fungi

Regarding fungal loal in indoor sites, Millipore air samples ranged from $7.6x10^2$ to $6.5x10^4$ CFU m^{-3} on MEA and from $7.4x10^2$ to $2.0x10^4$ CFU m^{-3} in DG18. For Andersen six-stage sampler, the fungal counts vary from $7.4x10^3$ to $7.6x10^4$ CFU m^{-3} and from $1.5x10^3$ to $2.3x10^4$ CFU m^{-3} in the same culture media respectively.

Floor surface swabs had the highest count values among all the matrices. The values ranged from $7.5x10^3$ $CFU.m^{-2}$ to $1.2x10^6$ $CFU.m^{-2}$ on MEA and from $3x10^3$ $CFU.m^{-2}$ to $1.3x10^6$ $CFU.m^{-2}$ on DG18. The filters from the vacuum cleaner had the second highest fungal count among passive methods, with a ranged of total fungal contamination from $2.1x10^4$ to $1.6x10^6$ $CFU.m^{-2}$ on MEA and from $2.7x10^4$ to $8.7x10^5$ $CFU.m^{-2}$ on DG18. Fungal counts on name tags ranged from $2x10^3$ to $9.5x10^3$ $CFU.m^{-2}$ on MEA and from not detectable to $1.4\ x10^4$ on DG18.

Lowest values were obtained from mops (MEA: $5x10^2$ to $1.2x10^4$; DG18: 0 to $4x10^3$ $CFU.m^{-2}$;),cleaning cloths (MEA: 0 to $9.5x10^3$; DG18: 0 to $9.5x10^3$ $CFU.m^{-2}$;), EDC (MEA: $4.7x10^1$ to $2x10^3$;DG18: $1.4x10^1$ to $2.1x10^3$ $CFU.m^{-2}.day^{-1}$) and settled dust samples (MEA: 12 to $3.6x10^3$; DG18: 8 to $7.9x10^2$ $CFU.g^{-1}$;) (Figure 6.3).

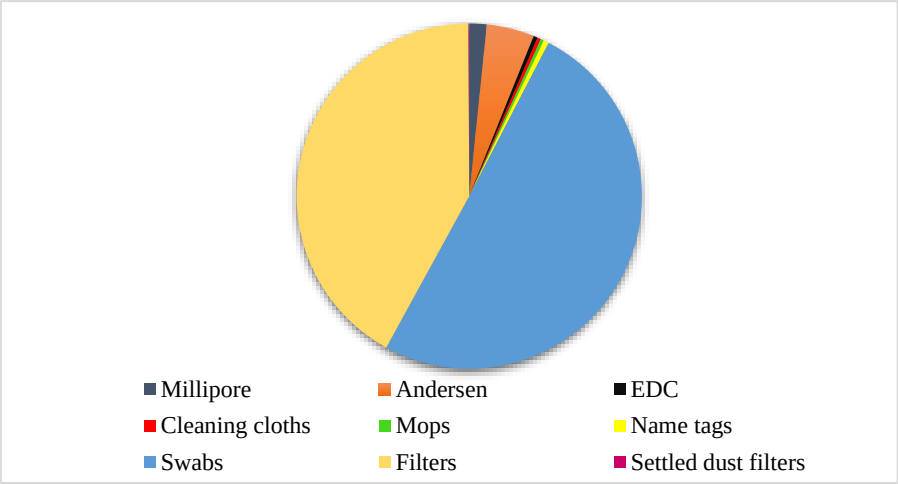


Figure 6.3- Distribution of total fungal counts (CFU. m^{-3} / m^{-2} / g^{-1} and CFU.m^{-2}.day^{-1} for electrostatic dust collectors (EDC)) per matrice in malt extract agar culture medium.

Regarding fungal diversity recovered through active sampling, 27 and 22 species were observed on MEA and DG18 respectively in air samples from Andersen six-stage, while 19 species were identified in Millipore air samples in both culture media (MEA: 19; DG18:19). Through passive sampling, the highest fungal diversity was obtained from EDC samples with 18 species on MEA and 15 on DG18, followed by filters (MEA: 13 species; DG18:10 species). In floor surface swabs 10 and 7 species were identified on MEA and DG18 respectively. In settled dust from filters 7 and 12 species were reported on MEA and DG18 respectively. Similar results were obtained between mops (MEA:5 species; DG18: 5 species), cleaning cloths (MEA:5 species; DG18: 4 species) and name tags (MEA:4 species; DG18: 4 species)

Concerning fungal identification, the most frequent species in air samples was *Chrysonilla sitophila* in Andersen (51.85% MEA; 52.00% DG18). Same results were obtained on DG18 (47.77%) in Millipore air samples. On MEA *Rhizopus* was predominant (40.07%). *Cladosporium* sp. Was the prevalent genera on EDC and filters (EDC: 72.68% MEA; 59.97% DG18; Filters: 62.89% MEA; 86.99% DG18). Similar fungal predominance was obtained from swabs, in both culture media (62.97% MEA; 67.74% DG18) and on DG18(59.99%) in settled dust. In contrast, *Penicillium* sp. was the most frequent genera on MEA (68.56%) in settled dust samples. Similar results were obtain in mops on both culture media (47.17% MEA; 46.88% DG18), in cleaning cloths (41.67% MEA; 59.73%) and on DG18 (38.46%) in name tags, while *C.sitophila* was the predomint species on MEA (54.95%) (Table 6.1).

Table 6.1 - Fungal quantitative (CFU. m^{-3} / m^{-2} / g^{-1} and CFU.m^{-2}.day^{-1} for electrostatic dust collectors (EDC)) and qualitative results from each sampling method. The data was obtained from samples inoculation in malt extract agar (MEA) and dichloran-glycerol agar (DG18) culture media.

	MEA			DG18		
Sample	Fungi	CFU. m^{-3} / m^{-2} / g^{-1} * CFU.m^{-2}.day^{-1}	%	Fungi	CFU. m^{-3} / m^{-2} / g^{-1} *CFU.m^{-2}.day^{-1}	%
Andersen	*C.sitophila*	2.1E+05	51.85	*C.sitophila*	3.6E+04	52.00
	Chrysosporium	6.6E+04	16.18	*Cladosporium* sp.	2.1E+04	21.04
	Penicillium sp.	8.5E+04	21.04	*Penicillium* sp.	1.0E+04	14.67
	Aspergillus sp.	4.9E+02	0.12	*Aspergillus* sp.	1.0E+03	1.51
	Other species	4.4E+04	10.82	Other species	5.3E+02	0.77
Millipore	*Rhizopus* sp.	5.9E+04	40.07	*C.sitophila*	2.4E+04	47.77
	C.sitophila	2.6E+04	17.55	*Cladosporium* sp.	1.5E+04	28.44
	Cladosporium sp.	2.1E+04	14.54	*Penicillium* sp.	9.3E+03	18.29
	Aspergillus sp.	1.6E+02	0.11	*Aspergillus* sp.	3.9E+02	0.77
	Other species	4.1E+04	27.73	Other species	2,4E+03	4.74
EDC*	*Cladosporium* sp.	5.2E+03	72.68	*Cladosporium* sp.	4.3E+03	59.97
	Penicillium sp.	9.2E+02	12.93	*Penicillium* sp.	1.8E+03	24.98
	Aspergillus sp.	2.5E+01	0.35	*Aspergillus* sp.	2.8E+03	2.16
	Other species	1.0E+03	14.04	Other species	4.6E+03	12.89
Cleaning cloths	*Penicillium* sp.	1.3E+04	41.67	*Penicillium* sp.	6.0E+01	59.43
	C.sitophila	1.2E+04	38.33	*Cladosporium* sp.	2.6E+01	26.41
	Cladosporium sp.	5.0E+03	16.667	*Aspergillus* sp.	7.1E+00	7.08
	Aspergillus sp.	5.0E+02	1.67	Other species	7.1E+00	7.08
	Other species	5.0E+02	1.67			
Mops	*Penicillium* sp.	1.3E+04	47.17	*Penicillium* sp.	7.5E+03	46.88
	C.sitophila	7.0E+03	26.42	*Cladosporium* sp.	5.0E+03	31.25
	Cladosporium sp.	4.0E+03	15.09	*Aspergillus* sp.	2.5E+03	15.63
	Other species	3.0E+03	11.32	Other species	1.0E+03	6.25
Name tags	*C.sitophila*	2.5E+04	54.95	*Penicillium* sp.	1.3E+04	38.46
	Cladosporium sp	1.6E+04	34.07	*C.sitophila*	1.1E+04	33.85
	Penicillium sp.	5.0E+03	10.99	*Cladosporium* sp.	8.5E+03	26.15
				Other species	5.0E+02	1.54
Filters	*Cladosporium* sp.	2.4E+06	62.89	*Cladosporium* sp.	2.4E+06	86.99
	Penicillium sp.	6.9E+05	18.16	*Penicillium* sp.	2.8E+05	10.05
	C.sitophila	5.7E+05	14.97	*Aspergillus* sp.	5.3E+04	1.91
	Aspergillus sp.	1.3E+05	3.37	*C.sitophila*	2.9E+04	1.05
	Other species	2.3E+04	0.60			
Settled dust	*Penicillium* sp.	4.5E+03	68.56	*Cladosporium* sp.	1.7E+03	56.99
	Cladosporium sp.	1.7E+03	25.81	*Penicillium* sp	1.2E+03	40.27
	Other species	3.7E+02	5.63	*Aspergillus* sp.	2.7E+01	0.90
				Other species	5.6E+01	1.84
Swabs	*Cladosporium* sp.	2.9E+06	62.97	*Cladosporium* sp.	2.9E+06	67.74
	Penicillium sp.	8.5E+05	18.69	*Penicillium* sp.	7.1E+05	16.85
	Phoma sp.	5.9E+05	12.90	*C.sitophila*	5.5E+05	13.04
	Aspergillus sp.	1.0E+04	0.22	*Aspergillus* sp.	1.0E+05	2.36
	Other species	2.4E+05	5.22	Other species	5.6E+01	0.001

In general, the predominant genera among all the matrices were *Cladosporium* (59.17% MEA; 73.43% DG18) and *Penicillium* (17.85% MEA; 14.83% DG18). Concerning *Aspergillus*, the genera had de highest values on DG18 (2.20%) when comparing with MEA counts (1.52%). In DG18, through active sampling, the genera was more frequently reported in air samples from Andersen six stage (1.51%) when comparing to Millipore (0.78%). For passive methods, mop samples presented the highest values (15.63%), followed by cleaning cloths (7.08%) and swabs (2.36%). The genera was also

identified in EDC (2.16%), filters (1.91%) and settled dust (0.90%). There was no evidence of the genera in name tag samples.

On MEA, the genera had a similar prevalence between air samplers (0.12% Andersen six stage ; 0.11% Millipore). *Aspergillus* sp. was more frequently detected in filter samples (3.37%), followed by cleaning cloths (1.67%), EDC (0.35%) and swabs (0.22%). *Aspergillus* sp. was not identified in samples from name tags, in addition to mops and settled dust samples (Figure 6.4).

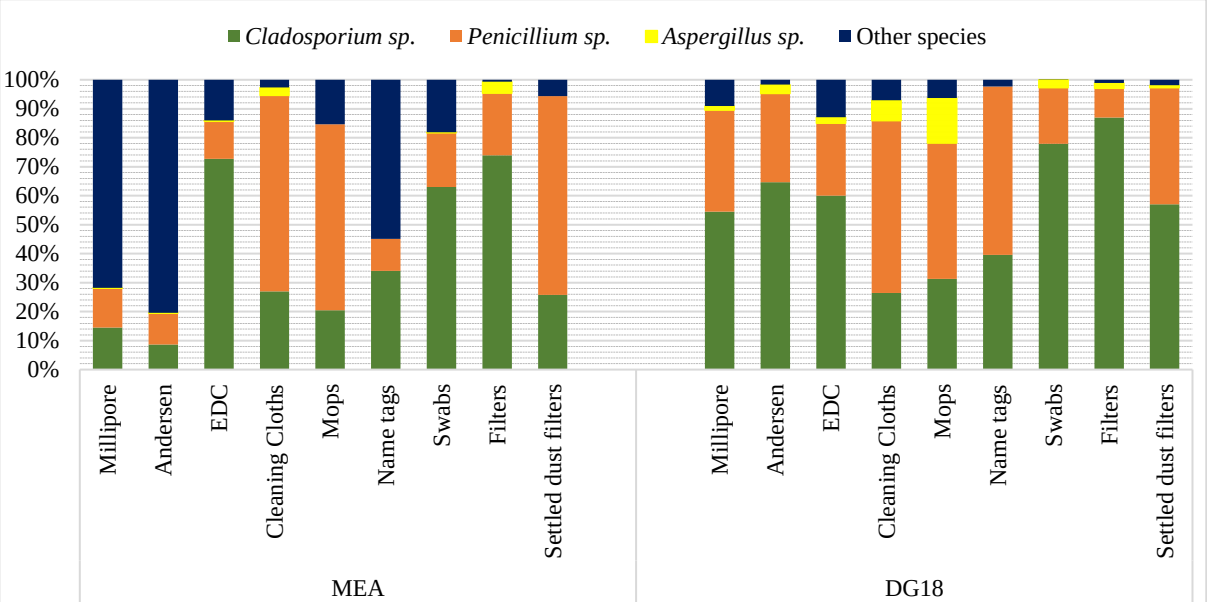


Figure 6.4 – Fungal prevalent genera in each matrice used (EDC: electrostatic dust collectors). The results were obtained from samples inoculation in malt extract agar (MEA) and dichloran-glycerol agar (DG18) culture media.

Among *Aspergillus* genera on DG18, the sections: *Fumigati* (33.01%), *Nidulantes* (29,38%), *Candidi* (20.22%), *Aspergilli* (7.68)%), *Circumdati* (6.11%), *Flavi* (3.23%), *Restricti* (0.27%) and *Nigri* (0.10%) were identified. On MEA, the prevalent sections were *Nidulantes* (57.31%), followed by *Circumdati* (32,49%) and *Candidi* (7.16%). Lower counts were obtained for *Nigri* (1.46%), *Flavi* (1.25%), *Fumigati* (0.33%) and *Aspergilli* (0.003%) *Aspergillus* section *Restricti* was not identified in MEA.

Concerning *Aspergillus* sp. in indoor air samples from Andersen six-stage the sections *Nidulantes* and *Candidi* were the most prevalent on MEA (45.04%) and DG18 (30.57%) respectively. Regarding *Nidulantes*, the section was reported in all the six stages, following the predominance: 1.79%; 5.38%: 7.18%; 44.85%, 21.53% and 10.76% in stage 1 (7 µm); 2 (4.7 µm); 3 (3.1 µm); 4 (2.1 µm); 5 (1.1 µm) and 6 (0,65 µm) respectively. *Aspergillus* section *Candidi* was also predominant between stages, following the prevalences: 2.47%; 6.17%; 7.41%; 64.20%; 17.28% and 2.47% in the 1st, 2nd, 3rd, 4th, 5th and 6th stage respectively.

Four *Aspergillus* sections were identified in Millipore air samples in MEA as follows: *Fumigati* (79.77%); *Nidulantes* (15.16%); *Aspergilli* (2.53%)*; Nigri* (2.53%). On DG18 six sections were reported, *Nigri* (36.64%), *Aspergilli* (24.43%), *Candidi* (14.25%), *Circumdati* (14.25%), *Nidulantes* (7.38%) and *Flavi* (3.05%).

Regarding samples from passive sampling methods, four sections were identified in EDC on MEA (28,57% *Fumigati; Nidulantes; Flavi;* and 14.29% *Circumdati*). In contrast to the six sections identified in DG18 (34.29% *Circumdati;* 29.75% *Aspergilli*; 13.73% *Flavi;* 10.78% *Candidi;* 9.16% *Restricti* and 2.28% *Nidulantes).* Sections *Candidi* (60%) and *Nidulantes* (40%) were observed on mop samples only on DG18. *Aspergillus* section *Nigri* had a prevalence of 100% on cleaning cloths on MEA,

while on DG18 *Flavi* was predominant (100%). Four sections were detected on filter samples on MEA (62.26% *Nidulantes*; 35.41% *Circumdati;* and 1.17% *Flavi*; *Nigri)*, while on DG18, six sections were reported (44.76% *Fumigati;* 20.95% *Aspergilli*; 15.24% *Circumdati;* 12.39% *Nidulantes;* 5.71% *Flavi;* 0.95% *Candidi*;). *Aspergillus* section *Aspergilli* was predominant on swabs (100%) on MEA, while on DG18 *Nidulantes* was more frequent (40%) followed by *Fumigati* (30%) and *Candidi* (30%). Two sections were identified on settled dust from filters on DG18 (96.30% *Nigri* and 3.70% *Flavi)* (Figure 6.5).

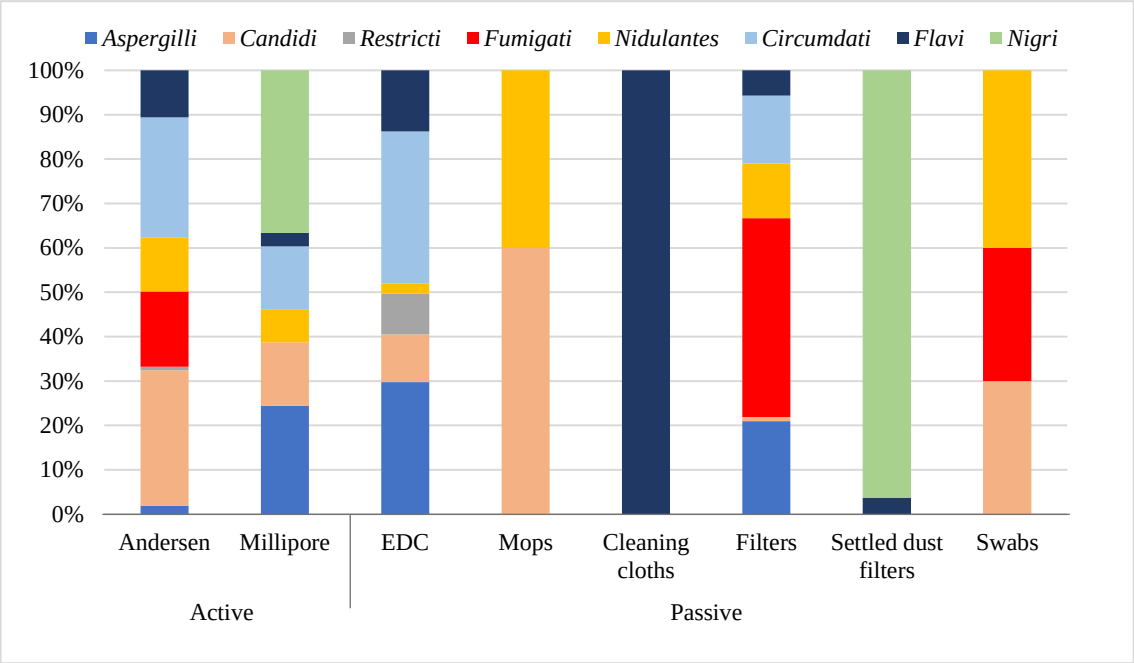


Figure 6.5- Distribution of *Aspergillus* sp. sections in the samples collected through active and passive sampling methods (EDC: electrostatic dust collectors). The results were obtained from samples inoculation onto dichloran-glycerol agar culture medium.

4. Compliance with legal and scientific criteria for microbiological contamination

Concerning the scientific criteria for occupational exposure assessment (I /O ≤1), among the 87 sampled sites, 38 (43.68%) were above 1 for fungi. Also, from the 11 FFH sampled, 5 (45.45%) surpassed the stipulated value. For bacteria, 44.68% of the sampling locations (39 out of 87) had a higher load indoor. The same trend was obtained from 63.63% of FFH (7 out of 11).

The limit proposed by WHO (maximum value of 150 CFU.m^{-3}) was used as guideline. From all the sampling sites (87), 28 (32.18%) were above the stipulated limite for bacteria. Also, 63.63% of FFH (7 out of 11) did not comply with the limit. Fungal load surpassed the imposed value in 37.93% of sampled locations (33 out of 87). Higher load indoor was obtain for 45.44% of FFH (5 out of 11) in case of fungi. Furthermore, species indicative of harmful fungal contamination as *Aspergillus fumigatus* complex were detected. Indeed, the section *Fumigati* contemplates the Portuguese legal list of biological agents requiring surveillance in occupational environments (*Directiva 2000/54/EC)*.

Regarding the Portuguese legal compliance (Portaria no353-A, 2013), the majority of the FFH analyzed (n=8, 72.72%) did not complied with bacteria first criteria ([indoor] + 350 CFU. m−3 < [outdoor]). When considering all sampling sites, 71 out of 87 were above the established limit (81.61%). For fungi, indoor first legal guideline, (I/O<1), evidence 56.32% of sampling sites within the limit (49 out of 87). From the 11 selected FFH, 6 comply with the legal guideline (54.55%).

When considering fungal identification, *Aspergillus* toxigenic species listed in the Portuguese legislation (Portaria no353-A, 2013), namely *Aspergillus versicolor* (*Aspergillus* section *Nidulantes*), *Aspergillus ochraceus* (*Aspergillus* section *Circumdati*), *Aspergillus flavus* (*Aspergillus* section *Flavi), Aspergillus fumigatus* (*Aspergillus* section *Fumigati*) were reported in air samples from all FFH. Indeed, the load of the sections *Nidulantes, Circumdati, Flavi* and *Fumigati* surpassed the limit of 12 CFU m^{-3} in 9 FFH.

5. Fungal azole resistance

All the samples from passive and active sampling were inoculated for azole resistance using SDA supplemented with three azoles (ITR; VOR and POS). Among fungal counts, *Cladosporium* sp., *C.sitophila* and *Penicillium* sp. were the most recurrent fungi identified. *Cladosporium* was the predominant genera in EDC (81.17% SDA; 58. 66% ITR; 65.05% VOR; 69.29% POS), in filters (41.72% SDA; 91.07% ITR; 72.06% VOR; 77.91% POS) and in settled dust (52.72% SDA; 79.73% ITR; 32.82% VOR; 88.03% POS). *C.sitophila*, was the most frequent species in cleaning cloths on SDA (78.95%) and in two azole-supplemented SDA media (42.86% ITR; 38.88% POS). The species was also predominant on mops on two azoles (71.43% ITR; 38.89% POS). *Penicillium* was also prevalent in cleaning cloths samples on VOR (83.57%) and on SDA media (67.69%) and VOR (97.38%) on mops (Table 6.2)

Table 6.2- Fungal quantitative (CFU. m^{-3} / m^{-2} / g^{-1} and CFU.m^{-2}.day^{-1} for electrostatic dust collectors (EDC)) and qualitative results from each sampling method. Samples extracts were inoculated in sabouraud dextrose agar (SDA) and azole supplemented media (Itraconazole (ITR), Voriconazole (VOR) and Posaconazole (POS).

		SDA		**ITR**		**VOR**		**POS**	
Sample	Fungi	CFU. m^{-3} / m^{-2} / g^{-1}	%	CFU. m^{-3} / m^{-2} / g^{-1}	%	CFU. m^{-3} / m^{-2} / g^{-1}	%	CFU. m^{-3} / m^{-2} / g^{-1}	%
EDC*	*Cladosporium* sp.	9.5E+03	81.00	6.2E+02	58.66	2.5E+03	65.05	8.8E+02	69.29
	Penicillium sp.	9.5E+02	8.11	1.2E+02	11.19	7.1E+02	18.88	1.1E+02	8.54
	C.sitophila	9.3E+02	7.93	2.2E+02	221.19	2.7E+02	7.20	1.3E+02	10.51
	*A.*section *Nidulantes*	3.7E+01	0.32	-	-	3.6E+00	0.10	-	-
	A.section Nigri	4.8E+01	0.41	-	-	-	-	-	-
	A. section Fumigati	3.6E+00	0.03	3.6E+01	3.39	1.2E+02	3.14	-	-
	Other species	2.4E+02	2.03	5.9E+01	5.57	2.1E+02	5.64	1.5E+02	11.66
	TOTAL	100.00	**TOTAL**	100.00	**TOTAL**	100.00	**TOTAL**	100.00	
Cleaning cloths	*C. sitophila*	1.1E+04	78.95	1.5E+03	42.86	-	-	3.5E+03	38.89
	Cladosporium sp.	2.5E+03	17.54	5.0E+02	14.29	2.1E+03	21.96	2.0E+03	22.22
	Penicillium sp.	5.0E+02	3.51	5.0E+02	14.29	9.0E+04	8.3.57	3.5E+03	38.99
	Other species	-	-	1.0E+03	28.57	1.6E+04	0.14	-	-
	TOTAL	100.00	**TOTAL**	100.00	**TOTAL**	100.00	**TOTAL**	100.00	
Mops	*Penicillium* sp.	2.2E+04	67.59	-	-	9.3E+04	97.38	3.5E+03	38.89
	C. sitophila	6.5E+03	20.00	2.5E+03	71.43	-	-	3.5E+03	38.89
	*A.*section *Fumigati*	2.5E+03	7.69	-	-	-	-	-	-
	Cladosporium sp.	-	-	1.0E+03	28.57	-	-	2.0E+03	22.22

Name tags	Other species	1.5E+03	4.62	-	-	2.5E+03	2.62	-	-
	TOTAL	100.00	**TOTAL**	100.00	**TOTAL**	100.00	**TOTAL**	100.00
	Penicillium sp.	3.5E+04	51.11	5.6E+03	32.75	-	-	3.0E+03	7.74
	C.sitophila	2.8E+04	40.74	7.5E+03	43.86	9.0E+03	6.6.67	3.5E+04	90.97
	Cladosporium sp.	5.0E+03	7.41	4.0E+03	23.89	1.5E+03	11.11	5.0E+02	1.29
	Other species	5.0E+02	0.74	-	-	3.0E+03	22.22	-	-
	TOTAL	100.00	**TOTAL**	100.00	**TOTAL**	100.00	**TOTAL**	100.00
Filters	*Cladosporium* sp.	1.6E+06	41.72	1.4E+06	91.07	2.6E+06	72.06	1.2E+06	77.91
	C.sitophila	1.2E+06	30.79	7.1E+04	4.71	4.0E+05	11.06	2.1E+05	13.44
	Penicillium sp.	9.8E+05	25.92	6.1E+04	4.05	5.9E+05	1.6.24	1.3E+05	8..55
	A section *Candidi*	5.0E+02	0.98	-	-	-	-	-	-
	A. section *Fumigati*	5.0E+02	0.03	-	-	-	-	-	-
	A. section *Nidulantes*	3.7E+04	0.01	-	-	-	-	-	-
	A. section *Flavi*	1.0E+03	0.01	5.0E+02	0.13	-	-	-	-
	A. section *Circumdati*	5.0E+02	0.01	-	-	-	-	-	-
	Other species	2.0E+04	0.52	2.0E+03	0.13	2.4E+04	0.65	1.5E+03	0.10
	TOTAL	100.00	**TOTAL**	100.00	**TOTAL**	100.00	**TOTAL**	100.00
Settled dust	*Cladosporium* sp.	8.7E+02	52.72	8.5E+02	79.74	1.9E+02	32.82	1.5E+03	88.03
	Penicillium sp.	5.5E+02	3.3E+01	1.3E+02	12.29	3.7E+02	62.60	6.7E+01	4.03
	C.sitophila	5.9E+01	33.27	3.6E+01	3.38	-	-	9.0E+01	5.46
	A. section *Candidi*	2.7E+01	1.64	-	-	-	-	-	-
	A. section *Nigri*	1.8E+01	1.10	-	-	-	-	-	-
	*A.*section *Circumdati*	4.0E+00	0.24	-	-	-	-	-	-
	Other species	1.2E+02	7.40	4.9E+01	4.60	2.7E+01	4.58	4.1E+01	2.49
	TOTAL	100.00	**TOTAL**	100.00	**TOTAL**	100.00	**TOTAL**	100.00

The genera *Aspergillus* was also detected in SDA (0.13%), being *Nigri* the predominant section on EDC (54.38%) followed by *Nidulantes* (41.24%) and *Fumigati* (4.38%). On filters, *A.* section *Candidi* was dominant (90.24%), followed by *Fumigati* (3.66%), *Circumdati* (3.66%) and *Flavi* (1.22%). *Fumigati* was the only section detected on mop samples (100%). *Candidi* (55.10%), *Nigri* (36.74%) and *Circumdati* (8.16%) were found in settled dust.

Regarding *Aspergillus* prevalence on azole-supplemented media, on ITR (0.03%), the section *Fumigati* was reported in EDC (100%) and filters (100%), while on VOR (0.03%), *Fumigati* was the prevalent sections in EDC (97.06%) followed by *Nidulantes* (2.94%). *Nidulantes* was only detected in filters (100%). The genera was not detected in POS (Figure 6.6).

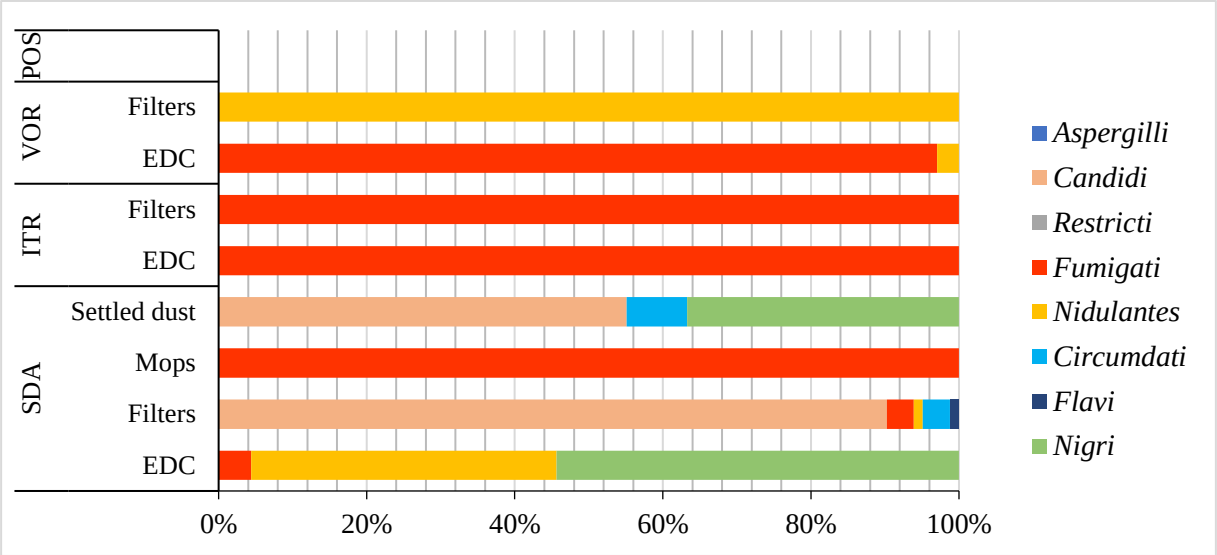


Figure 6.6- Prevalence of *Aspergillus* sp. sections in the samples inoculated onto sabouraud dextrose agar (SDA) and azole supplemented media (Itraconazole (ITR), Voriconazole (VOR) and Posaconazole (POS). Samples extracts were obtained from passive sampling methods.(EDC: electrostatic dust collectors).

6. Aspergillus susceptibility to azoles

Among the nineteen *A.*section *Fumigati* pure colonies isolated from Andersen (9), EDC (5), filters (3), and mops (1) samples, the azole screening results showed fungal development in azole supplemented media. With six isolates growing on ITR (samples from: Andersen (1), filters (1), mops (1), EDC(3)) and 3 isolates growing on VOR (samples from: filters(1), EDC (2)). No fungal growth was reported in POS (Appendix 2 – Table 10.1).

7. Molecular detection

7.1 *SARS-CoV-2* assessment

SARS-CoV-2 was not detected in the air samples obtained from Coriolis (23 samples) and surface swabs (21 samples).

7.2 *Aspergillus* sections

Through molecular detection, *Aspergillus* section *Fumigati* was detected in almost all samples from passive sampling methods. The highest prevalence was obtained in EDC (n=61; 67.8%), followed by filters (n=60; 66.6%), cleaning cloths (n=14, 56%), mops (n=7; 50%), and name tags (n=67, 5.97%). In contrast, the section *Nidulantes*, was only reported in settled dust from filters (n=1, 9.09%) (Table 6.3)

Table 6.3 – Detection of *Aspergillus* section *Fumigati* and *Nidulantes* by qPCR in each matrice (EDC: electrostatic dust collectors). The results ("+" :detected; "-": not detected) encompassed all firefighters headquarters (FFH), being compared with the detection obtain by culture dependent methods (MEA: malt extract agar; DG18:Dichloran-glycerol agar).

Aspergillus sp.	Matrice	Local	Sample	CFU.m^{-2} | CFU/day (MEA/DG18)	C_q	Result
Aspergillus section *Fumigati*	Names tags	FFH9	55	0/0	24.55	+
			56	0/0	25.19	+
			57	0/0	25.78	+
		FFH11	69	0/0	33.90	+
	Filters	FFH1	6	0/0	37.65	+
			7	0/0	34.86	+
			12A	0/0	37.64	+
		FFH2	13	0/0	34.66	+
			20	0/0	35.29	+
		FFH4	21	0/0	27.23	+
			22	0/0	25.71	+
			23	0/0	27.33	+
			24	0/0	26.31	+
			25	0/0	25.93	+
			26	0/0	26.84	+
			27	0/0	26.43	+
			28	0/0	26.00	+
			29	0/23500	28.56	+
		FFH5	40	0/0	35.78	+
			41	0/0	37.89	+
			42	0/0	14.77	+
			44	0/0	38.30	+
			45	0/0	38.38	+
		FFH6	46	0/0	35.46	+
			47	0/0	35.09	+
			48	0/0	37.09	+
			49	0/0	36.02	+
			50	0/0	35.92	+
			51	0/0	34.49	+
			52	0/0	35.57	+
			53	0/0	34.84	+
			54	0/0	33.67	+
		FFH7	55	0/0	35.96	+
			56	0/0	36.46	+
			57	0/0	38.39	+
		FFH8	61	0/0	34.78	+
			62	0/0	36.33	+
			63	500/0	37.61	+
			64	0/0	37.45	+
			65	0/0	38.03	+
			66	0/0	38.58	+
			67	0/0	37.89	+
			68	0/0	38.95	+
		FFH9	69	0/0	32.49	+
			70	0/0	33.88	+
			71	0/0	33.03	+
			72	0/0	33.29	+
			73	0/0	33.17	+
			74	0/0	33.14	+
			75	0/0	32.98	+
		FFH10	76	0/0	35.45	+
			77	0/0	37.47	+
			78	0/0	34.27	+
			79	0/0	35.98	+
			80	0/0	33.71	+
			81	0/0	35.24	+
			82	0/0	33.07	+
			83	0/0	36.47	+

	FFH11	84	0/0	34.59	+
		85	0/0	35.26	+
		86	0/0	35.70	+
		87	0/0	34.30	+
		88	0/0	32.77	+
		89	0/0	31.72	+
Mops	FFH1	2	0/0	38.09	+
		2A	0/0	35.49	+
	FFH3	5	0/0	34.88	+
		6	0/0	37.31	+
	FFH5	7	0/0	16.28	+
	FFH6	8	0/0	15.69	+
	FFH10	12	0/0	34.72	+
Cleaning Cloths	FFH2	8	0/0	35.42	+
	FFH4	10	0/0	36.13	+
	FFH3	12	0/0	32.93	+
		13	0/0	34.79	+
	FFH5	14	0/0	33.77	+
	FFH6	15	0/0	32.61	+
		16	0/0	31.99	+
	FFH7	17	0/0	36.45	+
	FFH8	19	0/0	32.28	+
	FFH9	20	0/0	30.21	+
		21	0/0	33.08	+
	FFH10	22	0/0	33.84	+
	FFH11	23	0/0	33.35	+
		24	0/0	33.83	+
EDC	FFH1	1.1B	0/0	36.55	+
		2.1A	0/0	36.09	+
		2.1B	0/0	34.94	+
		5	0/0	17.31	+
		6B	0/0	36.63	+
		10	0/0	36.71	+
	FFH2	2.1	0/0	35.72	+
		3.1	0/0	36.03	+
	FFH3	1.1	0/0	35.59	+
		2.1	0/0	33.96	+
		3.1	0/0	34.62	+
		4	0/0	34.42	+
		7	0/0	35.69	+
		10	0/0	33.85	+
	FFH4	4	0/0	34.28	+
		5	0/0	38.56	+
	FFH5	1.1	0/0	35.45	+
		2.1	0/0	34.98	+
		3.1	106.16/0	34.16	+
		4	0/0	35.81	+
		5	0/0	34.12	+
		6	0/0	35.99	+
		7	0/0	35.63	+
		10	0/0	35.37	+
	FFH6	1.1	0/0	36.91	+
		2.1	0/0	33.50	+
		3.2	0/0	35.17	+
		4	0/0	34.76	+
		6	0/0	35.92	+
		7	0/0	36.38	+
		10	0/0	37.54	+
	FFH7	1.1	0/0	32.63	+
		2.1	0/0	35.77	+
		3.1	0/0	35.34	+
		3.2	0/0	33.74	+
		7	0/0	35.33	+
		10	0/0	35.80	+
	FFH8	1.1	0/0	34.00	+
		3.1	0/0	33.92	+
		3.2	0/0	33.35	+

			4	0/0	32.50	+
			6	0/0	34.07	+
			7	0/0	33.95	+
			10	0/0	33.17	+
			1.1	0/0	33.80	+
			2.1	0/0	37.29	+
		FFH9	3.1	0/0	35.15	+
			3.2	0/0	26.74	+
			7	0/0	39.36	+
			1.1	0/0	35.43	+
			2.1	0/0	36.38	+
			3.1	0/0	35.07	+
		FFH10	3.2	0/0	34.85	+
			4	0/0	35.26	+
			5	0/0	33.52	+
			10	0/0	35.58	+
			1.1	0/0	36.58	+
			2.1	0/0	32.16	+
		FFH11	3.1	0/0	34.32	+
			3.2	0/0	36.77	+
			10	0/0	33.76	+
Aspergillus section *Nidulantes*	Settled Dust	FFH4	21	0/0	38.23	+

8. Statistical correlations

Concerning microbial contamination through each sampling method, statistically significant differences were detected between filters and cleaning cloths , mops and uniform name tags in i) MEA ($\chi^2_{K-W}(3) = 28.570$, p=0.000); ii) DG18 ($\chi^2_{K-W}(3) = 24.789$, p=0.000); iii) ITR ($\chi^2_{K-W}(3) = 28.121$, p=0.000); iv) VOR ($\chi^2_{K-W}(3) = 22.078$, p=0.000); v) POS ($\chi^2_{K-W}(3) = 26.295$, p=0.000). In all cases filters were the sampling method presenting the highest values (Table 6.4).

Table. 6.4 – Comparison of microbial contamination between sampling method (EDC: electrostatic dust collectors) by using the Kruskal-Wallis test (N- total number of Firefighters headquarters being considered in the statistic test). The results compared the fungal counts obtained from each culture media (malt extract agar (MEA), dichloran-glycerol agar (DG18), Itraconazole (ITR), Voriconazole (VOR) and Posaconazole (POS).

		Ranks		**Test Statistics[a,b]**		
Media	**Sampling method**	**N**	**Mean Rank**	**Kruskal-Wallis H**	**df**	**p**
MEA	Cleaning cloths	11	14.23	28.570	3	0.000*
	Mops	11	13.27			
	Name tags	11	23.50			
	Filters	11	39.00			
	Total	44				
DG18	Cleaning cloths	11	16.64	24.789	3	0.000*
	Mops	11	16.09			
	Name tags	11	18.27			
	Filters	11	39.00			
	Total	44				
ITR	Cleaning cloths	11	13.91	28.121	3	0.000*
	Mops	11	14.05			
	Name tags	11	23.23			
	Filters	11	38.82			
	Total	44				
	Cleaning cloths	11	16.68			
	Mops	11	15.50			

VOR	Name tags	11	20.09	22.078	3	0.000*
	Filters	11	37.73			
	Total	44				
POS	Cleaning cloths	11	17.00	26.295	3	0.000*
	Mops	11	12.59			
	Name tags	11	21.86			
	Filters	11	38.55			
	Total	44				

a. Kruskal Wallis Test. b. Grouping Variable: Sampler. **Statistical significant differences at 5% significance level

When it comes to molecular detection of *Aspergillus* statistically significant differences were identified for detection of section *Fumigati* in the matrices ($\chi^2_{K-W}(4) = 12.686$, p=0.041).EDC were the ones with the highest values. In contrast to the results obtained from name tags (Table 6.5).

Table. 6.5 – Comparison of fungal molecular detection in each matrice (EDC: electrostatic dust collectors by the Kruskal-Wallis test. (N: Number of samples were *Aspergillus* section *Fumigati* was detected through qPCR; df: Degrees of freedom, P: P-value).

		Ranks		Test Statistics[a,b]		
Molecular detection	**Matrice**	**N**	**Mean Rank**	**Kruskal-Wallis H**	**df**	**p**
Aspergillus section *Fumigati*	Name tags	4	17,50	12,686	4	0,013*
	Filters	60	75,83			
	Mops	7	72,57			
	Cleaning cloths	14	50,96			
	EDC	61	80,16			
	Total	146				

a. Kruskal Wallis Test. b. Grouping Variable: Matrice. *Statistical significant differences at 5% significance level

Significant correlations were obtained between sampling method (Millipore; EDC; cleaning cloths; mops; settled dust; swabs; name tags) and the culture media used for bacteria and fungal counts. No significant correlations were obtained for filters (Table 6.6).

Regarding air samples from millipore, statistically significant correlations were detected between bacteria values in TSA and fungal values in MEA (r_S=0,618 p=0.043), meaning that higher bacteria values are associated to higher fungal values in MEA.

In case of EDC, statistically significant correlations were detected between: i) bacteria counts in TSA and fungal counts in MEA (r_S =0.633, p=0.036) and DG18 (r_S =0.629, p=0.038); ii) Fungal counts in MEA and VOR (r_S =0.618, p=0.043); iii) Fungal counts in DG18 and VOR (r_S=0.636, p=0.035); iv) Fungal counts in ITR and POS (r_S =0.733 p=0.010). Revealing that higher values of bacteria in TSA media are related to higher values of fungal counts in MEA and DG18. Higher fungal counts on MEA and DG18 are related to higher values on VOR. Similar results were obtained between ITR and POS.

In case of cleaning cloths, statistically significant correlations were detected between: i) bacteria values in TSA and VRBA (r_S =0.689, p=0.036), same results were obtained for mops (r_S =0.701, p=0.016) ii) bacteria counts in VRBA and fungal counts in MEA (r_S =0.605, p=0.049). Suggesting that a higher bacteria contamination in TSA is related to a higher bacteria contamination in VRBA for

cleaning cloths and mops. Also, higher bacteria counts in VRBA is related to higher values of fungal counts in MEA for cleaning cloths.

In mops used for cleaning, statistically significant correlations were obtained between: bacteria counts in VRBA and fungal counts in VOR (r_S =0.631, p=0.037), being directly correlated. Similar associations were obtained for settled dust between: i) Bacteria values in TSA and VRBA and fungal values in MEA (r_S =0.647, p=0.031) (r_S =0.647, p=0.031) respectively. Results obtained from swabs reported statistically significant correlations between bacteria values in VRBA and fungal values in MEA (r_S =0.667, p=0.016).

Name tags results showed statistically significant correlations between: i) Bacterial contamination in VRBA and fungal counts in DG18 (r_S =-0.609, p=0.016) ITR (r_S =0.616, p=0.016) e VOR (r_S =-0.695, p=0.016) revealing that higher values of bacteria in VRBA are associated to lower fungal values in DG18 and VOR, while higher bacterial counts in VRBA are related to higher fungal counts in ITR.

Table. 6.6 – Relationship between sampling method (EDC:electrostatic dust collectors) and quantitative results obtained through culture dependent methods for bacteria (VRBA:violet red bile agar) and fungi (MEA:malt extract agar; DG18:dichloran-glycerol agar ; ITR:Itraconazole ; VOR:Voriconazole; POS: Posaconazole (POS) by Spearman's correlation coefficient.

Sampling method	Media	VRBA	MEA	DG18	ITR	VOR	POS
Millipore	TSA	-0.014	0.618*	-0.009	-	-	-
EDC	TSA	0.060	0.633*	0.629*	0.501	0.483	0.457
	MEA	-	-	0.900**	0.573	0.618*	0.314
	DG18	-	-	-	0.564	0.636*	0.442
	ITR	-	-	-	-	0.936**	0.733*
Cleaning cloths	TSA	0.689*	0.411	0.033	-0.028	0.167	-0.129
	VRBA	-	0.605*	-0.010	0.015	0.039	0.123
Mops	TSA	0.701*	0.233	0.399	-0.174	0.475	-0.434
	VRBA	-	-0.051	-0.039	0.070	0.631*	-0.268
Settled dust	TSA	1.000**	0.647*	0.543	-0.012	-0.168	-0.370
	VRBA	-	0.647*	0.543	-0.012	-0.168	-0.370
Swabs	VRBA	-	0.667*	0.516	-	-	-
Name tags	VRBA	-	-0.302	-0.609*	0.616*	-0.695*	0.424

*. Correlation is significant at the 0.05 level (2-tailed). **. Correlation is significant at the 0.01 level (2-tailed). a. Sampler = EDC

Chapter VII: Discussion

Nowadays, occupational infectious diseases are a rising research topic in the occupational medicine field, covering studies about biological hazards at work, their diagnosis, associated diseases and treatment (Rim & Lim, 2014). However, exposure assessment is difficult due to the lack of standard sampling protocols or guidelines (McDevitt *et al.*, 2007). Thus, most studies for evaluating the microbiological exposure in occupational environments are essentially exploratory (Viegas *et al.*, 2013a, Viegas *et al.*, 2017a, Viegas *et al.*, 2019c, Viegas *et al.*, 2021a, 2021b).

Given the nature of the workplace and tasks developed, firefighters, who must provide assistance in emergency situations, may be exposed to various hazards (Oliveira *et al.*, 2017, Sparer *et al.*, 2017), including biological ones (Sexton & Reynolds, 2010, Roberts & David, 2014 , Farcas *et al.*, 2019, Viegas *et al.*, 2021b). Nonetheless, occupational microbial exposure has been neglected in this occupational environment (Viegas *et al.*, 2021b).

Regarding fungal associated diseases, the genera *Aspergillus* is commonly related to serious illness in humans, with section *Fumigati* being frequently correlated to respiratory symptoms and therapeutic resistance (Viegas *et al.*, 2021a), widely reported in occupational environments (Viegas *et al.*, 2017a, Madsen *et al.*, 2020, Viegas *et al.*, 2020a, 2021a), and indicator of moisture damage buildings (Ramos *et al.*, 2016).

A multi-approach protocol was used in this study, applying active and passive sampling methods. Among the matrices, swabs presented the highest values of total bacteria and fungi, followed by filters. In fact, since passive sampling characterizes contamination from a wider period (Viegas *et al.*, 2021e), higher microbial counts are expected when comparing both methods (Viegas *et al.*,2021c). For microbial assessment impaction methods were selected due to the well known recovery effectiveness of microorganism, having better results when compared to other sampling methods (Viegas et al. 2010).

The most diversified fungal load was obtained from Andersen six-stage air samples, when comparing to Millipore. Being in accordance with the results obtained in some studies (Viegas *et al.*, 2020e, 2020f). The six-stages from Andersen simulates the human respiratory system (Viegas *et al.*, 2021b), and the presence of fungal species in all the six stages, such as *Aspergillus* section *Nidulantes* specially in the 4^{th}, 5^{th} and 6^{th} stage, might constitute a health hazard, since fungal presence on higher stages represents a higher penetrability in the lung (Viegas *et al.*, 2021b) highlighting fungal exposure through inhalation and constituting an additional risk factor to promote respiratory diseases (Viegas *et al.*, 2019a). Thus, due to their toxigenic potential, the detection of the sections *Candidi* and *Nidulantes* in indoor environment should be emphasized (Viegas *et al.*, 2021d). Regarding Andersen six-stage results, bioburden understimation was already reported (Chowdhary *et al.*, 2013, Mao *et al.*, 2019, Viegas *et al.*, 2021b). Such methods (active sampling), are limited when it comes to assessing long-term exposure due to the short time sampling of the instruments. In fact, some important toxigenic fungal species may not be detected due to low concentrations (Viegas *et al.*, 2019). Thus, other methods (passive) should be applied due to well-known variation of airborne bioburden (Jürgensen *et al.*, 2016, Viegas *et al.*, 2019), associated to the irregular release of microorganisms from surfaces (Reponen, 2017).

EDC presented the highest fungal diversity among samples from passive methods, in MEA and DG18, highlighting the potential of this matrix for fungal exposure assessment (Liebers *et al.*, 2012, Viegas *et al.*, 2018b). In fact, some studies have already pointed out the advantages associated to EDC, being a low-cost and low-maintenance method with a consistent fungal recovery (Adams *et al.*, 2021,Viegas *et al.*, 2021e,2021f).

The lack of standards for occupational exposure assessment in Portugal led to the use of scientific guidelines (Rao *et al.*, 1996,Cox *et al.*, 2020). Following the quantitative value (ratio I/O<1),

most of FFH were above the stipulated limit for bacteria load (63.63%), while some surpassed the limit for fungal load (45.45%), suggesting a critical environment (Cox *et al.*, 2020). When considering the limit proposed by the WHO (2009) (maximum concentration 150 CFU.m^{-3}), the majority of FFH failed to comply with bacteria (63.63%) and fungal (45.45%) levels. Also, *Aspergillus fumigatus* was identified among the air samples, being considered an indicative of harmful fungal contamination (Viegas *et al.*, 2015a,2017a), well known for their infectious potential (Bielawska-Drózd *et al.*, 2017,Viegas *et al.*, 2019b, Viegas *et al.*, 2020f). For additional information, the Portuguese legal compliance (Portaria no353-A, 2013) was applied. From all the FFH sampled, the greatest part (72.72%) did not comply with bacteria first criteria ([indoor] + 350 CFU. m−3 < [outdoor]).

The Portuguese legislation relies only on quantitative criteria for viable bacteriota. In fact, bacteria levels can be representative of non-pathogenic species, commonly found in the environments or bellonging to the skin flora (Nigam & Cutter, 2003). Due to survival abilities and considerable resistance, gram-positive bacteria represent the largest group present, already indicated as the most prevalent type indoor (Ramos *et al.*, 2016). However, the load obtained needs to be taken into consideration since the overuse of antibiotics increases the risk of exposure to drug resistant bacteria. (Sastry & Bhat, 2016). Also, bacteria components, as peptidoglycan, derived mainly from gram-positive, was already linked to imune inflammatory responses (Poole *et al.*, 2010). In what concerns gram-negative, bacteria levels might suggest the presence of potential pathogens (Nigam & Cutter, 2003), such as *Legionella* sp. (Barnes, 2017). Other possible hazard is associated to endotoxin production capacity (Ramos *et al.*, 2016), such as metabolites that can promote physiological responses interfering in metabolic processes (Poole *et al.*, 2010, Rogers, 2011). Additionally, biofilms are a major problem in several industries, enabling products contamination (Mai-Prochnow *et al.*, 2016) and promoting infections on humans (Barnes, 2017),

The fungal load is within the limits for most of FFH (54.55%). However, some toxigenic species from *Aspergillus* genera listed in *Portaria no353-A, 2013* were detected above the stipulated limits, as follows: *Aspergillus* section *Nidulantes, Circumdati, Flavi* and *Fumigati*. The sections were equally reported among the matrices from passive sampling methods, the majority identified as mycotoxins producers (*Circumdati, Nigri* and *Fumigati)* (Viegas *et al.*, 2021d). Indeed, the American Conference of Governmental Industrial Hygienists (ACGIH, 2009) emphasize the need of corrective measures when sections *Flavi, Fumigati* and *Nidulantes* are confirmed. Addittionally, a routine assessment for *Aspergillus* toxigenic sections has been indicated (Viegas *et al.*, 2019b) and a protocol for the assessment of *Aspergillus* sp. burden was suggested by Viegas and team members (2017a). The national guidelines for exposure assessment should not be restricted to the listed species, since some fungal species, commonly found and with toxigenic potential are not descriminated (Viegas *et al.*, 2019a). As example, the clinical revelant and with toxigenic potential *Aspergillus* sections *Aspergilli, Candidi, Restricti* identified in air and passive sampling methods (Viegas *et al.*, 2020a).

The predominant species in indoor air was *Chrysonilla sitophila* which accentuates a possible hazard since this species is often associated to asthma (Ramos *et al.*, 2016). Concerning *Aspergillus* sp., the genera was identified by both active and passive sampling, in accordance with the results obtained from other studies (Alberti *et al.*, 2001, Ramos *et al.*, 2016, Cox *et al.*, 2020, Viegas *et al.*, 2021a, Viegas *et al.*, 2021e).

Through passive methods the most frequent genera was *Cladosporium* followed by *Penicillium* in MEA and DG18, being already reported the dominance of *Cladosporium* sp. in work environments (Ramos *et al.*, 2016). Of note, the highest values of *Aspergillus* genera were obtained from mops and cloths samples. Considering the use of these materials for cleaning all FFH areas, they might be suggested as potential sources of cross-contamination (Viegas *et al.*, 2021b). The identification of common filamentous fungi (*Aspergillus* sp., *Penicillium* sp.), which are significant mycotoxin producers with pathogenic properties (Pereira *et al.*, 2012), might constitute a health risk regarding the potential

exposure of workers. Hence, other routes of exposure (inhalation), besides food consumption, should be taken into consideration when evaluating the risk associated to mycotoxins exposure (Viegas *et al.*, 2018a,2019a).

The variety of results emphasizes the benefits of using both active and passive methods for a proper replication of the real microbial exposure scenario (Cox *et al.*, 2020), contradicting the Portuguese regulatory framework of using only active sampling and culture-dependent methods for bioburden assessment (Viegas *et al.*, 2019a).

Building features, low ventilation, damage structures visible mold and dampness are some of the factors influencing fungal appearances, being largely associated with adverse health effects on the respiratory health (Strachan, 1988, Mendell *et al.*, 2011, Jürgensen *et al.*, 2016, Adams *et al.*, 2021). The causes associated to indoor moisture are varied, including leakages on roofs and structures, water accumulation and condensation due to poor ventilation and limit insulation. Indeed, wetting sites can promote microbial growth being an indoor source of biological exposure and their metabolites (Adams *et al.*, 2021). Therefore, the fungal contamination obtained in this occupational environment was expected when considering FFH conditions.

Fungal quantitative results vary depending on the selected culture media (MEA and DG18). Concerning *Aspergillus*, the prevalence of the genera was higher on DG18 when compared with MEA, already reported in some studies (Viegas *et al.*, 2020). Despite the recommendations to use MEA, as stand-alone culture media, for aerobiological studies (APA 2010), DG18 seems to be an efficient option, due its restrictive character, limiting the colonies size of fast growing fungal species (Viegas *et al.*, 2021a,2021c). Thus, both media (MEA and DG18) should be used when performing an exploratory exposure assessment (Viegas *et al.*, 2021f), in order to obtain a wider fungal characterization (Viegas *et al.*, 2017a, 2019b, 2020f).

The low prevalence of *Aspergillus* genera in air samples may be related to the conditions set on the impactor, such as air velocity, consequently microorganisms can be injured during impactation. Thus, these stressful conditions can compromise microorganisms culturability and, consequently, misleading the results (Reponen, 2017). In addition, *Aspergillus* sp. growth on plates may have been influenced by the overgrowth of some species due to chemical competition (Viegas, *et al.*, 2015a). Indeed, species overgrowth could have been avoided by the dilution method. However, the technique was not applied due to the significant differences in community structure that can entail, affecting the species richness and diversity of an original community, compromising the microbial content of a sample (Franklin *et al.*, 2001).

During the past decades resistant fungal strains have been increasing in the environment. *Cladosporium* sp., *Penicillium* sp. and *Aspergillus* sp. grow in more than one azole supplemented media suggests the presence of multidrug resistance (Viegas *et al.*, 2019a). Through the screening method, *Aspergillus* section *Fumigati* isolates (from Andersen, EDC, filters and mops samples), were able to grow on ITR and VOR. In accordance to the emergence of anti-fungal resistance associated to the section (Snelders *et al.*, 2008, Snelders & Verweij, 2011,Verweij *et al.*, 2016). Noteworthy, the significant positive correlations found between fungal ocurrence in ITR and POS in EDC, evidences the trend of azole resistance to more than one drug in this setting (Viegas *et al.*, 2021b). Also, positive correlation between fungal presence in regular media (MEA, DG18) and azole-supplemented media (ITR,VOR,POS) might suggest an increased fungal resistance in the sampled environment, as previously suggested (Viegas *et al.*, 2020b). Thus, further antifungal susceptibility tests should be preconized (Viegas *et al.*, 2021b). In fact, there is few data regarding azole resistance associated to the genera *Cladosporium* and *Penicillium* (Viegas *et al.*, 2020e).

Bacteria contamination on TSA and fungal contamination on MEA in air samples from millipore and samples from passive methods (EDC, mops, settled dust) seems to be associated. The positive significant correlations obtained might indicate that a higher bacteria contamination on TSA is related

to a higher fungal contamination on MEA, such results might be explained by the fact that fungal and bacteria possibly share the same source of contamination, has already reported (Viegas *et al.*, 2018b, 2020c). In contrast, bacteria counts on VRBA and fungal counts on DG18 seems to be negatively correlated in samples from name tags, suggesting that higher bacteria contamination on VRBA is associated to lower fungal contamination on DG18, in accordance with previous reports (Rajasekar & Balasubramanian, 2011). Indeed, bacteria presence can reduce the growth of pathogenic and saprotrophic fungi (Boer *et al.*, 2007). The negative interactions between both groups might be explained by the microbial competition, through bacteria antifungal activity (Boer *et al.*, 2007), or nutrients exploitation, depriving fungal resources (Rousk *et al.*, 2008).

To obtain more information concerning microbial biodiversity, molecular tools seem to be a suitable approach, overcoming culture-based methods limitations (Pitkäranta *et al.*, 2008). In fact, viable microbial contamination seems to represent up to 25% of the total microbial content (Heikkilä *et al.*, 2013, Jürgensen *et al.*, 2016). *Aspergillus* section *Fumigati* was detected through active (Andersen and Millipore) and passive methods (EDC, filters and swabs) by culture- dependent methods, while molecular tools allowed the detection in additional matrices (name tags, mops and cleaning cloths). Furthermore, section *Nidulantes* was detected by the same molecular method in one settle dust sample, being undetectable by culture. This method allows the detection of non-viable microorganisms (Franchitti *et al.*, 2020) and microbial components possibly having allergenic properties (Douwes *et al.*, 2003).

In culture-dependent methods, due to the selectivity of the media, only certain microorganisms will be able to grow, therefore the total microbial exposure can be underestimated (Viegas *et al.*, 2020a), perhaps overestimating the most tolerant species (Oppliger, 2014, Viegas *et al.*, 2019, 2021b). For instance, due to water activity and high sugar content of MEA media, fast growing fungal species are enhanced (Mensah-Attipoe & Taubel, 2017). Also, the absence of fungal viability (Viegas, *et al.*, 2015a, 2021c) might explain the detection only through qPCR . The fact that section *Fumigati* was not identified in floor surface samples through molecular methods might be related to an ineffective DNA extraction (Viegas *et al.*, 2020a,2020c), low specificity of primers for DNA amplification (Ribeiro & Faria, 2017) or the presence of contaminants in the sample (Sastry & Bhat, 2016, Viegas *et al.*, 2021a). Therefore, the use of both, culture-dependent and molecular methods can overcome the limitations of each assay (Ribeiro & Faria, 2017, Cox *et al.*, 2020).

This study was performed during the world pandemic COVID19 following the procedures already reported (Viegas *et al.*, 2021d), related to the determination of microbial contamination (*SARS-CoV-2*, fungal and bacterial) in higher education institutes. In times of pandemic crises, such as COVID19, a regular microbiological surveillance is important, especially in frontline medical care professions and essential workers as the firefighters, in order to prevent biological hazards and control the risk (Viegas *et al.*, 2021b).

The results regarding *SARS-CoV-2* assessment obtained through active (Coriolis) and passive sampling (swabs) suggest that the regular cleaning and desinfection procedures were effective for the virus, since it was not detected in the analysed samples. However, the sampling campaing covered only some of the assessed FFH, since firefighters from the remaining headquarters have already been covered by the vaccination campaign in place. Beside passive sampling methods, impinger method was preferentially selected for viral acessment since the the use of liquid medium potentiates the viral integrity, facilitating RNA extraction for molecular detection (Pena *et al.*, 2021).

Exposure assessment is difficult due to the lack of standard sampling protocols or guidelines (McDevitt *et al.*, 2007) . In what concerns the legal criteria the Portuguese legislation is not efficient to ensure a proper Indoor Air Quality (Viegas *et al.*, 2019a), since the majority of FFH meet fungal first quantitative criteria, but evidenced the presence of toxigenic fungal species above the stipulated limits. The lack of hygienic procedures in FFH might be suggested as a possible cause, justifying the

microbiological contamination (Viegas *et al.*, 2020a). In fact, clinically relevant *Aspergillus* sections (Viegas *et al.*, 2020a), were found on mops (*Candidi* and *Nidulantes*) and cleaning cloths (*Flavi)*. Thus, cleaning procedures need to be reviewed to reduce microbial levels (Nigam & Cutter, 2003). Also, ventilation rate appears to be an important role to improve Indoor Air Quality (Ki-Hyun *et al.*, 2017).

More research is needed to better characterize the microbial exposure in this specific occupational environment (Viegas *et al.*, 2020a). The sheer volume of microorganism in the environment makes it difficult to prevent the exposure. However, providing awareness (Barnes, 2017) by gathering more information about microbiological hazards and the identification of the activities which might enhanced the exposure is crucial to implement proper measures to reduce workers risk, minimize the exposure Viegas *et al*., 2020a) and control fungal (Verweij *et al*., 2016) and bacterial infections (Barnes, 2017). In fact, inadequated control measures seems to enhance the impact of infectious diseases on the public health (Viegas *et al*., 2021b).

There is a crescent need for the establishment of standardized sampling and assays protocols enabling the interpretation of data. An ideal standard would be based on scientific research evidencing fungal concentrations which cause adverse effects (Rao *et al.*, 1996). Thus, the risk evaluation should take into consideration the dose/response from microbiological exposure, as well as its metabolites and synergistic effects (Viegas *et al.*, 2018a).

Chapter VIII: Conclusion

Firefighter's headquarters are, indeed, an occupational environment to have in count, considering the microbiological contamination and load observed and the presence of potential azole resistant fungal species.

The diversity of results due the use of both active and passive sampling methods allowed a more reliable replication of the real exposure scenario. Also the use of culture-dependent methods, along with molecular tools, enable a wider characterization of *Aspergillus* sections with toxigenic potential (*Fumigati* and *Nidulantes*).

The quantitative value (ratio I/O) revealed that most of FFH had higher bacteria counts indoor when compared to the outdoor. The same trend was observed in several FFH regarding fungal load indoors. The obtained results need to be taken into consideration due to the well-known health hazards promoted by the exposure to microorganisms and their metabolites. Indeed, some toxigenic species belonging to *Aspergillus* genera were also reported . Also, the emergence of azole-resistance associated to *Aspergillus* section *Fumigati* is in fact a real scenario regarding this occupational environment. Other concerns are related to the anti-fungal resistance associated to other genera, such as *Penicillium*, a significant mycotoxin producer with pathogenic properties. Despite the effectiveness of cleaning procedures against *SARS-CoV-2*, the presence of clinically relevant *Aspergillus* sections (*Candidi* and *Nidulantes*) on mops and cleaning cloths should raise attention. Indeed, the identification of potential indoor contamination sources and activities that might enhanced the exposure, should comprise the first line of defense in order to reduce the microbial contamination and consequently workers exposure.

Overall, this study contributed to a wider knowledge regarding occupational exposure to microorganisms, being the first attempt to evaluate the microbiological exposure in FFH in Portugal. Additionally, also unveil the need of further investigation regarding this setting, contributing not only for the increase of knowledge on the topic but also to improve workers health.

1. Final remarks

The integration as team member in previous studies (Viegas *et al.*, 2021d), allowed me to raise skills for the development of this project. Also, technical scientific reports concerning *SARS-CoV-2* (Appendix 3), and fungal and bacterial (Appendix 4) contamination assessment were sent to each facilities assessed. The results obtained from the present work gave rise to scientific publications in international pre-reviewed journals (Viegas *et al.*, 2021c), as well as allowed to improve Firefighters working conditions through the recommendations raised in each scientific report.

2. Limitations and future considerations

During the use of culture-dependent methods, some limitations were associated to the plates contamination during the growth of *Aspergillus* section *Fumigati* isolates, when performing the screening method for azole resistance.

Species overgrowth in some plates could have been avoided by the dilution method, however the method was not performed in order to avoid sample loss. A better resolution would be the integration of a dilution along with a normal incubation, overcoming the limitations of both methodologies achieving a better microbial characterization of a sample. Also, the sampling campaign was carried out between late winter and early spring neglecting the influence of different seasons on the microbial content.

During the sampling campaign, the cleaning service was already being carried out in some of FFH. Thus, a study regarding the microbial assessment before and after the cleaning would be interesting to better characterize cleaning effectiveness. Additionally, the molecular detection of other potentially

toxigenic *Aspergillus* sections besides *Fumigati* and *Nidulantes* would be interesting for a broader fungal characterization.

This study corroborates the widespread of microbial contamination in FFH, as well as fungal multi-resistance potential to azoles. Thus, further studies are needed, taken into consideration microorganism's seasonal variations, species identification and characterization of the resistance profile. Bacteria identification and resistance profile should be also performed with deeper analysis.

The multi-approach sampling protocol allowed a better microbial characterization, since toxigenic fungal species were only detected in samples from passive methods. The use of culture dependent methods along with molecular tools allows to obtain a more accurate fungal characterization. In fact, molecular detection of harmful fungal species listed in the Portuguese legislation and other clinically relevant should be integrated in the exposure assessment.

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Chapter X: Appendices

Appendix 1 - Calculations for microbial contamination assessment

Assuming (x) the number of colonies counted in each media

- Samples from active sampling methods:

Equation 8.1 Millipore: CFU.m^{-3} =$\frac{x}{250}$ 1000

Equation 8.2 Andersen: CFU.m^{-3} = $\frac{x*10\hat{}3}{28,3*9}$

-Samples from passive sampling methods:

Equation 8.3 Surface swab : CFU.m^{-2}=$\frac{x}{0.0001}$ for fungi, CFU.m^{-2}=$x * 1000$ for bacteria

Equation 8.4 EDC: CFU. m^{-2}.day^{-1}=1 $\frac{xCFU}{3.14*0.003}$ / number of days

Equation 8.5 Filters, firefighter uniform names, mops and cleaning cloths : CFU.m^{-2}=0.1 $\frac{x}{2*10^{-4}}$

Equation 8.6 Settled dust samples: CFU.g^{-1}=x

Appendix 2 - Results supplementary data

Table 10.1- Results of *Aspergillus* section *Fumigati* by the screening method. The pure colonies isolated from the matrices (EDC:electrostatic dust collectors), were inoculated onto sabouraud dextrose agar (SDA) and azole supplemented media media (Itraconazole (ITR), Voriconazole (VOR) and Posaconazole (POS). (Fungal growth: detected (+); not detected (-)).

Matrice	ID	Media	SDA	ITR	VOR	POS
Andersen	78	MEA	+	+	-	-
Andersen	85	MEA	-	-	-	-
Andersen	100	MEA	+	-	-	-
Andersen	102	MEA	+	-	-	-
Andersen	104	DG18	+	-	-	-
Andersen	110	DG18	+	-	-	-
Andersen	113	DG18	-	-	-	-
Andersen	115	DG18	+	-	-	-
Andersen	124	MEA	+	-	-	-
Filter	126	SDA	+	-	-	-
Millipore	134	MEA	+	-	-	-
Mop	143	SDA	-	+	-	-
EDC	150	MEA	+	-	-	-
EDC	155B	ITR	+	+	+	-
EDC	156	ITR	-	-	+	-
EDC	160	VOR	+	+	-	-
EDC	161	VOR	-	+	-	-
Filter	185	DG18	+	+	+	-
Filter	241	MEA	+	-	-	-

Appendix 3 *SARS-CoV-2* Results Report

TECHNICAL AND SCIENTIFICAL REPORT

Executive commission: Carla Viegas, Edna Ribeiro and Miguel Brito

Task Force COVID19: Ana Almeida, Ana Margarida Costa, Anita Quintal Gomes, Bianca Gomes, Catarina Ginete, Ketlyn Oliveira, Mariana Delgadinho, Céu Leitão, Marta Dias, Raquel Pimenta, Renato Abreu, Sara Viegas

Date: 7 of January 2021

1 - Projects goals achievement

Overall, very promising results were obtained regarding place OP1 workers commitment with the project, since 766 volunteers were enrolled in the study (73% of the total expected). They have all received serologic and hematologic reports 1 to 2 days after performing the analysis. Regarding the environmental sampling campaign, all place OP1 (a total of 9 installations)) were assessed and environmental samples (air and surfaces samples) were isolated (an average of 10 samples in each installation) from workstations that had an increased risk due to occupation rates, activities performed or based on workers serologic surveillance results.

2 - Developed activities

More specifically, focusing on the tasks of the proposed project:

Task 1 *– Standard operation procedures*

All the activities comprised in the project followed the Standard Operation Procedures (SOP) in order to obtain the necessary efficacy and to perform all tasks with adequate safety measures. Several SOPs were established, namely: Samples collection, Samples decontamination, RNA extraction, *SARS-CoV-2* detection, Spill and Contact Emergencies, Surfaces cleaning and disinfection and Results communication to workers and Occupational Health Services.

Task 2 *– Serologic and hematologic surveillance from workers*

2.1 Serologic surveillance

All volunteers signed the informed consent and filed the demographic questionnaire. For rapid serologic screening, capillary blood was collected through capillary puncture with a lancet in which a 20ul of blood was collected for the Rapid detection kit for *SARS-CoV-2* IgM and IgG antibodies (NIMGENETICS). When a positive IgG was detected, venous blood was collected through venipuncture to a dry tube (serum) for IgG quantification by ELISA. As part of the laboratory procedures taking place at site C, the participants' serum was centrifugated and the analysis of the immune response (IgG) performed by ELISA technique on the Optic Ivyman microplate reader; System 2100C was performed.

2.2 – Hematologic surveillance

For hematologic screening, venous blood was collected through venipuncture to an EDTA tube (blood count) and the blood counts were processed in the Horiba 60 series automatic device and the hematological results analyzed. For each participant was determine complete blood count (RBC $x10^{12}$/L, Hgb g/dL, Hct %, VGM fL, HGM pg, CHGM g/dL, RDW %, Leukocytes (total), Neutrophils, Eosinophils, Basophils, Lymphocytes, and Monocytes and platelets).

Task 3 *– Environmental assessment of SARS-CoV-2*

3.1 – Sampling collection

Two sampling methods were applied combining active and passive methods. Regarding air samples 600 L were collected per sample into a conical vial containing 5 mL Buffer NVL (NZY Viral RNA Isolation kit (MB40701) component). Surface samples were collected by swabbing the areas of each local using flocked swabs moistened in sterilized water into a 15 mL falcon containing 1.5 mL Buffer NVL (NZY Viral RNA Isolation kit (MB40701) component). In some specific surfaces were performed composite samples (swabbing different surfaces with the same swab) since the type of use, surfaces material and cleaning procedures were common.

An average of 10 samples were performed in each faculty (Table 10.2) assessing the workplaces defined as having higher risk of exposure. The other criteria applied, and since we were assessing after the workers serologic surveillance, was having workers in the workplaces with the result of IgM+ or IgG +.

Table 10.2 – Sites assessed and number of environmental samples collected in OP1.

Site	Assessed areas	Air sampling (600 L)	Surfaces swabs
A	- Communication Office (Doorknob + Ventilation grids) - Drivers Reception (Counter) - Entrance (Coffee machine) - Lunchroom (Microwave + Coffee machine + Refrigerator + Ventilation grids) - Human Resources (Coffee machines + Access card + Staplers and stamps + Ventilation grids) - Financial Resources (Staplers and stamps + Ventilation grids in operation) - Human and Financial Resources (Windows + Doorknobs) - Reception (Printer + Table) - Shopping Department (1st floor) (Windows + Keys and card + - Ventilation grids in operation) - Male Bathroom (1st floor) (Doorknobs + Faucets) - Female Bathroom (1st floor) (Doorknobs + Faucets) - Printer (1st floor) - Academics, Quality and Planning Department (Mobile air conditioning equipment)	0*	25
B	- Lunchroom (Microwave + Refrigerator + Door) - Waiting Room / Classroom (Coffee Machine + Table) - Reception (Counter + Door + Water Machine) - Bathroom (Doors + Faucets) - Service Room (Table + Acrylic Protection) - Accounting (Printer + Doors)	6	10
C	- Logistics (Door + Window) - Accounting / Provisioning (Printer + Door + Stapler) - Academic Services (Printer (SA) + Printer (corridor)) - Reception (Door + Handrail + Balcony) - Bar (Chairs + Door) - Library (Printer + Computer) - Lunchroom (1st floor) (Fridge + microwave) - Lunchroom (2nd floor) (Fridge + microwave) - Printer (1st floor) - Bathroom (1st floor) (Doorknobs + Door + Flush toilet) - Office (1.16) (Mouse + Keyboard + Light switch + Door + Doorknob) - Printer (2nd floor) - Bathroom (2nd floor) (Doorknobs + Door + Flush toilet)	8	8
D e E	- Financial Services (Printers + Stapler) - Teachers Room 1 (Printers + Computers) - Teachers Room 2 (Printer + Computers + TV control) - Cafeteria / Bar (Counter + Tables) - Auditorium (Handrail + Light switch + Door) - Social Room (Door + Fridge + Microwave + Faucet+ Coffee Machine) - Library (Printer + Computers) - Human Resources (Printer + Door + Acrylics + Counter + Light switch)	8	8
F	- Library (Computer + Printer + Doorknob) - Lunchroom 1 (Faucet + Doorknob + Chair + Table + Microwave + Coffee machine + Faucet) - Lunchroom 2 (Microwave + Toaster + Kettle + Doorknob + Door + Table + Chair) - Bar 1 (Microwave + Refrigerator + Handles + Cash register + ATM) - Workshop (Machine + Doorknob + Door) - Bar 2 (Cash register + Control + Fridge + Microwave) - Organic Chemistry Lab (Scale + Faucets) - Organic Chemistry Lab - research (Equipment + Door + Doorknob) - Inorganic Chemistry Lab (Computer + Doorknobs + Equipment + Micropipettes + Refrigerator) - Canteen (Faucets + Counter + Acrylic + Cash Register) - Auditorium (Doorknobs + Door + Table + Chair + Eraser)	11	11

G	- Library (PC + Windows + Tables) - Canteen (Chairs + Table + PC + Doorknob) - Bar (Counter + Napkin Holder + PC + Coffee Machine + Table) - Grand Auditorium (Doorknob + PC + Chairs) - Small Auditorium - Scenic Interpretation Studio (Door + Table + Doorknob + Chairs) - Computer Room (Switch + Mouse + PC + Keyboard + Door + Doorknob) - Workshop (Doorknob + Tables + Lockers + Equipment)	7	7
H	- Bar / Cafeteria (Cash Register + Counters) - Science Laboratory (FQ) (Chairs + Scales + Faucets + Handle) - Social Room (Microwave + Faucet + Windows + Chairs) - Theatre and Choir Room (Switches + Windows + Chairs + Tables) - Changing room / dressing room (Doors + Doorknobs + Faucets) - Gym (Doors + Doorknobs + Table + Chair + Equipment) - Ceramic Workshop (Tables + Chairs + Faucets + Eraser + Doorknob + Door) - Dance Room (Doorknobs + Doors + Eraser) - Music Room (Switch + Handle + Window + Chairs + Piano)	9	9
I	- Grand Auditorium (Chairs + Tables + Piano + Tripods) - Small Auditorium (Switch + Window + Doorknob + Chairs) - Library (Computers + Tables) - Academic Services (Printer + Doorknob + Cabinets) - Printer (corridor) - Academic Services - Customer Service (Acrylic + Pens + Coffee Machine + Tables) - Lunchroom (Microwave + Tables + Chairs + Refrigerator + Doorknobs + Switch) - Choir Room (Chairs + Tables + Piano + Eraser + Switch + Door + Doorknobs) - Music Room (Doorknobs + Switch)	0*	9
J	- Bar (Chairs + Tables + Cash Register + Acrylic + Counter + Calculator) - Study room (Food and coffee machines + Chairs + Tables) - Printer (corridor) - “Home Food” Space (Microwave + Water machine + Tables + Chairs) - Auditorium (Door + Doorknob + Chairs) - Academic Services (Tables + Switch + Chairs + Acrylic + Printer + Stapler + Hole Puncher + Door + Doorknob) - Library (Tables + Chairs + Acrylic) - Multimedia Warehouse (Printer + Computers + Keyboards + Mouse + Barcode reader + Microwave + TV + Chairs)	0*	8
K	- Academic Services (Printer + Doorknob + Acrylic + Table) - Dance Reception (Doorknob + Computer + Telephone + Keys) - Teachers room (Doorknob + Printer + Switch + Flush toilet + Table + Chairs) - Atrium Studio C -1 (Bar + Sound System) - Studio D1 (Bar + Sound System) - Studio A1 (Bar + Sound system)	0*	6

* The sampling device was not operational

3.2 – RNA extraction and SARS-CoV-2 detection

RNA was extracted from the isolated sample (5 mL in air samples and 1.5 ml in surface samples) with the NZY Viral RNA Isolation kit, from Nzytech, according to manufacturer’s instructions. One step-RT qPCR was performed using NZYSpeedy One-step RT-qPCR probe Master Mix with primers and probes published by CDC (available on https://www.cdc.gov/coronavirus/2019-ncov/lab/rt-pcr-panel-primer-probes.html), which detect two viral gene regions (N1 and N2). qPCR was performed on BioRad CFX96 PCR machine. In each analysis a positive (a *SARS-CoV-2* positive sample) and a negative (water) sample was included. Moreover, to detect possible PCR inhibitors an internal control was added to each PCR.

Whenever a sample showed dubious results, this was repeated. All the results were analysed by two independent researchers.

Task 4 – Results analyses and interpretation

Serologic surveillance regarding IgG and IgM screening results were analyzed accordingly with the Kit manufacture guidelines. For positive IgM screening cases, nasopharynx swab was immediately performed for Real Time PCR diagnosis. For positive IgG screening cases, serum was collected for ELISA titers quantification. Enzyme-Linked Immunosorbent Assay SARSCoV-2, specifically designed against the S1 domain from SARS-CoV-2 SPIKE protein resulted in antibodies titers Index which is consider negative for values below 0.8 and positive for values above 1.1.

For hematologic surveillance, the collected blood was analyzed and complete blood count was performed. The hematological analyzed parameters indicated the presence of normal and abnormal hematological profiles. Among abnormal profiles we have assessed profiles with Anemia, including Microcytic and Hypochromic Anemia and Normocytic and Normochromic Anemia, Erythropenia, Erythrocytosis, Leukocytosis, Thrombocytopenia, Inverted differential leukocyte count.

The environmental samples were analyzed applying the protocol previously performed and the results were classified as positive or negative for the detection of *SARS-CoV-2*. Although the *SARS-CoV-2* viability was not assessed, the Task Force on COVID19 has agreed that the infection potential was not critical to recommend preventive/corrective measures. The *SARS-CoV-2* inactivation during sampling allowed to obtained safety levels for the professionals engaged in the field and lab work.

3 - Project deviations

The research team decided to assess *SARS-CoV-2* at workplaces also by active methods (air sampling) besides passive methods (surface methods), allowing to cover the aerosols droplets dissemination that can be contaminated with *SARS-CoV-2*. This was achieved by using a sampling device (impinger) that was able to collect 300L/min, and thus covering the criteria of high sample volume when targeting for virus. This was a deviation that made the results richer and allowed for more efficient sampling.

4- Results

4.1 *- Serologic and hematologic surveillance from workers*

4.1.1 *– Serologic surveillance*

Regarding workers serologic surveillance, we have observed an 1.96% of rapid test positivity for *SARS-CoV-2* IgM antibodies, in which, for every volunteer nasopharynx and oropharynx swabs were collected and stented for molecular biology diagnose trough PCR (Table 10.3; Figure 10.1). All samples were negative for *SARS-CoV-2* identification, which may indicate either a false positive due to cross reactions or due to an infection for which *SARS-CoV-2* is no longer detectable. On the other hand, we have reported a low prevalence of rapid test positivity for *SARS-CoV-2* IgG antibodies (0.91%) in which only 0.39% were confirmed by ELISA technique (Table 2; Figure 1). These results indicate the presence of false positive teste probably due to cross reactions. Nevertheless, these results indicate extremely low immunization levels of the local A workers, which suggests a low contact with *SARS-CoV-2* virus, and the effectiveness of the preventive measures endured by the individuals. The data presented also takes in consideration the local A organic units accessed and demonstrates that site C and J reported the higher IgM/IgG positive screening while organic units such as site I and site K had no identified cases. Moreover, ELISA confirmed IgG antibodies were detected in site L, site C and J (Figure 10.2).

Table 10.3. Serologic screening prevalence of place OP1 *SARS-CoV-2* IgM and IgG and ELISA IgG confirmation.

Parameter	*IgM (RDT)*	*RT PCR*	*IgG (RDT)*	*ELISA (IgG)*
Positive prevalence	*1.96 %*	*0.00 %*	*0.91 %*	*0.39%*

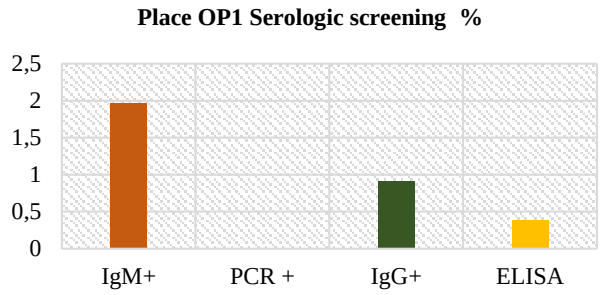

Figure 10.1. Graphic representation of place OP1 serologic screening prevalence data of *SARS-CoV-2* IgM and IgG and ELISA IgG confirmation.

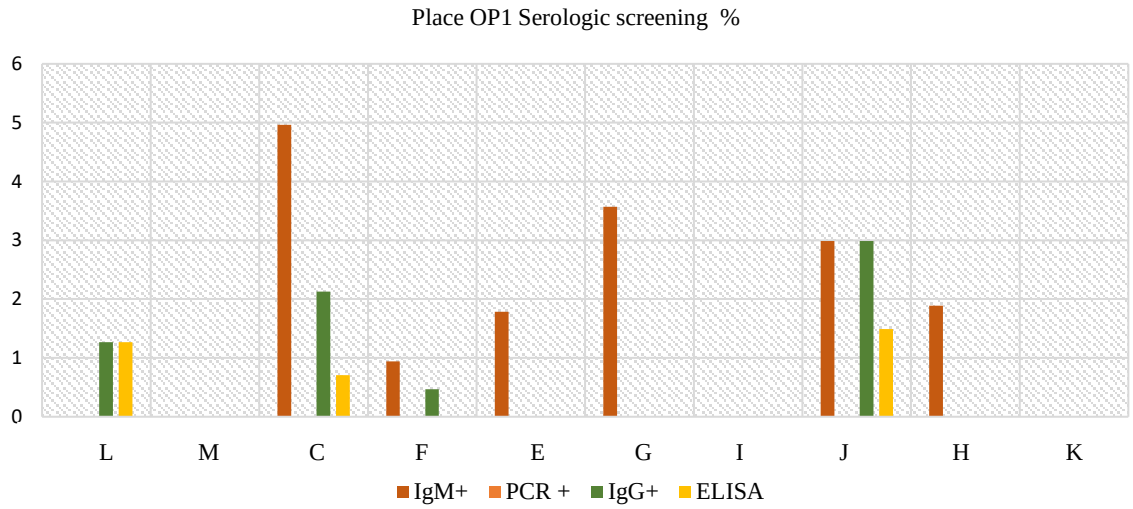

Figure 10.2. Graphic representation of place OP1 serologic screening prevalence dada of *SARS-CoV-2* IgM and IgG and ELISA IgG confirmation *per* organic units.

4.1.2 – Hematologic surveillance

Hematologic surveillance data revealed the presence of 10.44% of abnormal hematological profiles among the tested place OP1 community (Table 10.4; Figure 10.3). The most prominent and concerning hematological alteration is anemia (3.78%) which was classified in Microcytic and Hypochromic anemia (2.35%) and Normocytic and Normochromic Anemia (2.35%). Relevant levels of Erythropenia (2.48%) and Thrombocytopenia (2.09%) were also reported as well as other alterations such as increased red blood cells and white blood cells numbers (Erythrocytosis 0.26% and Leukocytosis 1.17%, respectively) (Table 10.4; Figure 10.3). Moreover, inverted differential leukocyte count (1.31%) (Table 10.4; Figure 10.3), which means that lymphocyte values were higher than neutrophils, was also reported. This variation may occur naturally in many people; however, it is advisable to understand if this

variation is physiological, or if there is any factor that may condition this inversion, thus we have recommended blood count repetition in a reference laboratory.
Furthermore, for hematologic surveillance data analysis, place OP1 organic units accessed potential divergences were also considered. Abnormal hematological profiles were reported in all accessed place OP1 organic units with divergent results between them. However, site J data demonstrated the higher hematological abnormal profiles, flowed by site C and F (Table 10.5; Figure 10.4). Relevantly, these results were not correlated with higher numbers of volunteers since siteJ had n= 67 and site C n= 141 and site F n=213.

Table 10.4. Hematologic screening data regarding local OP1 abnormal hematological profiles prevalence and hematological anomalies identification.

Abnormal hematological profile	*Prevalence (%)*
Abnormal blood count	*10.44*
Anemia	*3.78*
Microcytic and Hypochromic anemia	*2.35*
Normocytic and Normochromic Anemia	*1.44*
Erythropenia	*2.48*
Erythrocytosis	*0.26*
Leukocytosis	*1.17*
Thrombocytopenia	*2.09*
Inverted differential leukocyte count	*1.31*

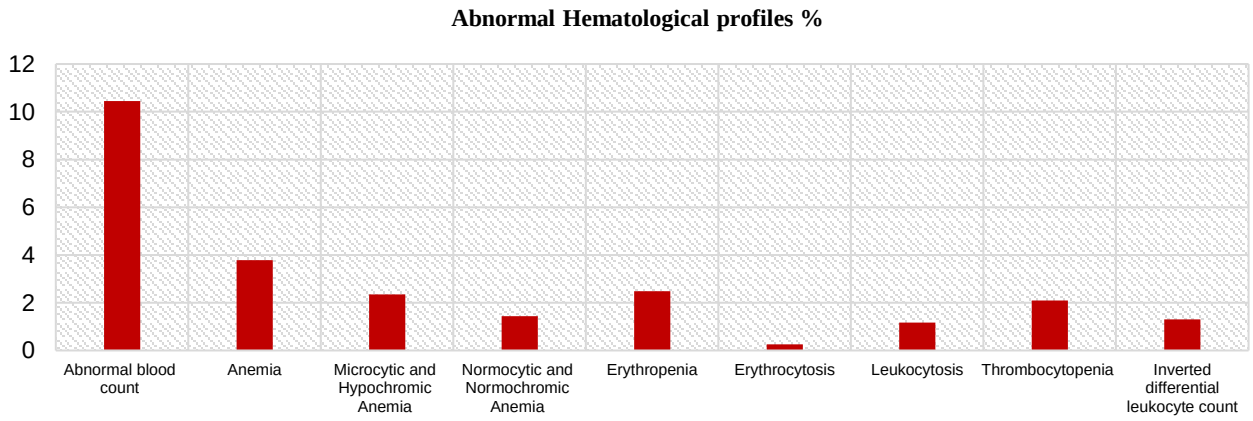

Figure 10.3. Graphic representation of place OP1 hematologic screening data regarding abnormal hematological profiles prevalence and hematological anomalies identification.

Table 10.5. Hematologic screening data regarding abnormal hematological profiles prevalence and hematological anomalies identification *per* organic units.

	Site									
Abnormal hematological profile (%)	L	M	C	F	E	G	I	J	H	K
Anemia	2.53	7.14	5.67	3.76	0.89	3.57	3.12	10.44	0.00	0.00
Microcytic and Hypochromic anemia	1.26	0.00	4.25	2.34	0.00	3.57	0.00	7.46	0.00	0.00

Normocytic and Normochromic Anemia	1.26	7.14	1.42	1.41	0.89	0.00	3.12	2.98	0.00	0.00
Erythropenia	0.00	0.00	3.55	2.35	1.78	3.57	6.25	2.98	3.77	0.00
Erythrocytosis	0.00	0.00	0.00	0.00	0.00	0.00	0.00	2.98	0.00	0.00
Leukocytosis	0.00	0.00	0.71	2.35	0.00	0.00	0.00	1.49	1.89	3.70
Thrombocytopenia	1.26	0.00	2.84	2.82	3.57	0.00	3.12	0.00	0.00	0.00
Inverted differential leukocyte count	0.00	0.00	2.13	0.47	1.78	0.00	3.12	1.49	3.77	0.00

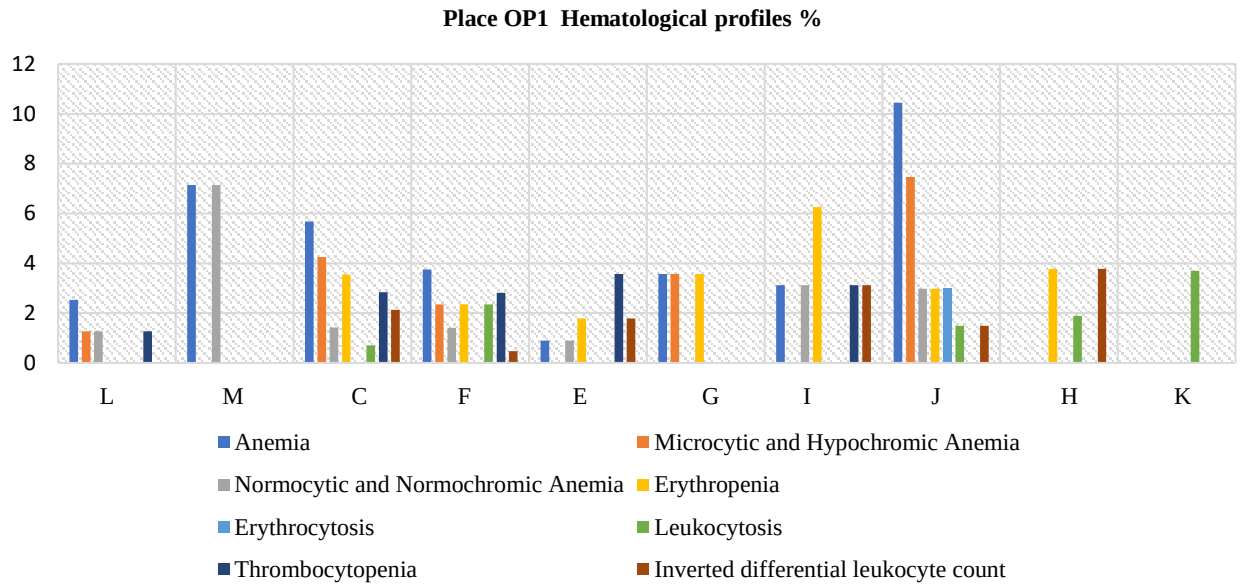

Figure 10.4. Graphic representation of place OP1 hematologic screening data regarding abnormal hematological profiles prevalence and hematological anomalies identification *per* organic units.

4.1.3 *– Serologic and hematologic surveillance correlation*

The performed correlation between serologic and hematologic surveillance results clearly sugests that *SARS-CoV-2* infection can affect hematological profile, as all individuals with *SARS-CoV-2* IgG and IgM positive antibodies, which is indicative of recent infection presented abnormal hematological profiles with decreased red blood cells (Erythropenia) and platelets (thrombocytopenia) (Figure 10.5).

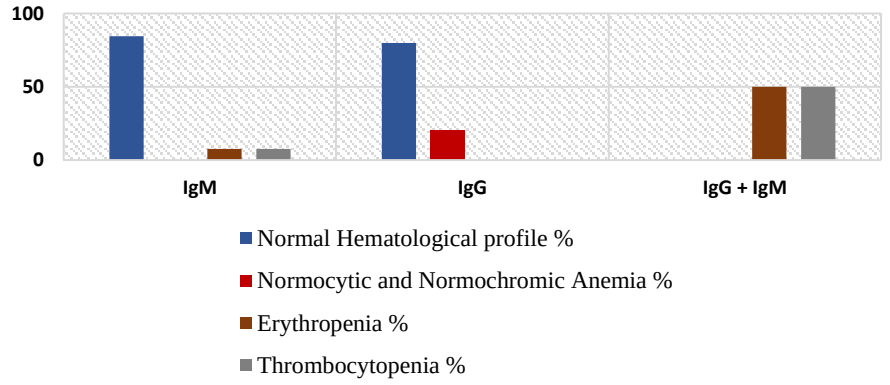

Figure 10.5. Graphic representation of place OP1 serologic and hematological anomalies identification correlation.

4.2 *- Environmental assessment from SARS-CoV-2*

In all the environmental samples analysed the results were negative regarding *SARS-CoV-2* detection corroborating the efficacy of the implemented preventive measures.

5. Concluding remarks

With this study we realized that the prevalence of *SARS-CoV-2* immunity is very low in place OP1 workers, (about 1%), and a lower value than the values referred by others in Portugal. Probably this is a specific population that followed the DGS recommendations and the incidence of infections was lower. Moreover, environmental samples collected from faculties facilities were all negative for *SARS-CoV-2* demonstrating once again that the cleaning activities seem to be in accordance with the recommendations.

Finally, in what concerns the hematologic study, two important concerns were raised. First, it is evident that COVID19 disease alters the hematologic parameters (we have a very small sample for positives that should be confirmed). Second the prevalence of abnormal hematological profiles is high, raising public health concerns, that should be evaluated and followed in the future.

In last, but not the least, this study highlights the importance of the academia response, not only for tackling the COVID19 pandemic crises, but also to ensure the workers 'health surveillance.

6 - Scientific production outputs

Viegas, C., Ribeiro, E., Quintal Gomes, A., Dias, M., Gomes, B., Pimenta, R., Oliveira, K., Delgadinho M., Ginete, C., Abreu, R., Almeida, A., Leitão, C., Viegas, S., Brito, M., Integrated approach on Occupational Health to tackle COVID19 pandemic. Virtual Conference on COVID19. MDPI

Appendix 4 - Environmental Assessment Report

TECHNICAL AND SCIENTIFICAL REPORT

1. Introduction

This report refers to the assessment of exposure/contamination carried out from February to July of the year 2021, in the occupational environment firefighter headquarter 1 (FFH1).

The collected samples were processed in the environmental microbiology laboratory between February 24, 2021 and July 20, 2021, in accordance with the protocols applied for the analysis of environmental samples.

This assessment stems from the research activities of the Environment and Health research area.

2. Context

This report describes the results from samples collected in the FFH1 occupational context by researchers from the environmental microbiology laboratory.

The samples collected reflect the diversity of microbial contamination obtained from FFH1, in the following spaces: dormitories, changing rooms, kitchen, canteen, bar, social and administrative room and gym.

The assessment of microbiological contamination in these locations was carried out as part of a project that aimed to assess occupational exposure to microorganisms in the locations described above. In addition to the detection of the pandemic virus, the results of which have already been sent in a specific report, an assessment of contamination by fungi and bacteria was also carried out in the same sampling sites.

3. Data

Table 10.6 shows the data relating to the entity where the assessment was carried out and to which the results presented in this Technical-Scientific Report relate.

Table 10.6 - General data

Sample identification	
Research project/External client:	Evaluation of microbiological contamination at FFH1
Start Date:	02/24/2021
Duration of analysis (months or days):	5 months
Report completion date:	09/22/2021
Report No.:	1/2021
Fieldwork carried out by:	Carla Viegas, Marta Dias, Bianca Gomes and Raquel Pimenta
Environmental harvests carried out by:	Marta Dias, Bianca Gomes and Raquel Pimenta
Laboratory work carried out by:	Marta Dias, Bianca Gomes and Raquel Pimenta
Report by:	Carla Viegas, Bianca Gomes and Sandra Ferreira

4. Acronyms and Definitions

Gram-negative bacteria: Bacteria consisting of a double layer of membranes, with a potent lipopolysaccharide (LPS) inducer expressed on the outside (Oliveira and Reygaert, 2020), are endotoxin producers (Maldonado et al., 2016 and Bertani & Ruiz, 2018). These bacteria do not retain the primary dye used in the Gram stain, retaining the second dye or counterstain usually Fuchsin or Safranin (Tripathi, and Sapra, 2020).

Bacteria: Unicellular prokaryotic organisms clinically classified on the basis of their forms (Sefton, 2019).

Bioaerosols: Aerosol particles of biological origin (eg bacteria, fungi, fungal spores, pollen, biofilm fragments (Liao et al., 2021).

Colony: Group of individuals of the same species, where the associated organisms are united through a common substrate (Tortora et al., 2009).

DG18: Dichloran glycerol chloramphenicol (Millipore 2021).

EDC: Electrostatic Dust Collectors (Viegas et al., 2018a).

Endotoxins: Lipopolysaccharide component of the Gram-negative bacterial cell that is released during active cell growth and after cell lysis (Stetzenbach, 2009)

Spores: Metabolically inactive structures that are tolerant to environmental stress (unlike vegetative cells). Important to organisms dispersal to other habitats (Setlow, 2006).

Fungi: Eukaryotic organisms that have a defined nucleus, which contains genetic material (DNA) surrounded by an envelope called the nuclear membrane (Tortora et al., 2009).

Environmental or geophilic fungi: These comprise fungi usually isolated in the soil, which are only occasionally pathogenic to man or other animals (Ferreira, 2000).

Potentially pathogenic fungi: These are fungi that cause disease only under certain circumstances, most often involving debilitation of the individual (Calo et al., 2013).

Relative humidity: Main environmental condition for the proliferation of mites and fungi indoors. It is recommended that the relative humidity indoors remain below 75% to prevent the proliferation of these organisms (WHO 2009)

Yeasts: Unicellular, non-filamentous fungi (Ferreira, 2000).

Reference location: Fresh air intake location (Outside the premises) (APA, 2010).

Particulate Matter: A mixture of solid particles and liquid droplets found in air. Some particles, such as dust, dirt, soot or smoke, are large or dark enough to be seen with the naked eye. Others are so small that they can only be detected with an electron microscope (U.S. EPA, 2020).

MEA: Malt Extract Agar (Millipore 2021).

Mycotoxins: Toxic secondary metabolites produced by filamentous fungi that contaminate various food and feed crops, presenting serious risks to human and animal health (Pankaj et al., 2018).

Particles (PM10): inhalable particles, with diameters generally 10 micrometers or smaller (U.S.EPA, 2020).

Particles (PM2.5): Inhalable fine particles, with diameters that are generally 2.5 micrometers and smaller (U.S. EPA, 2020).

PMC: Particulate matter – mass value (Soppa et al., 2014).

PNC: Particle number count (Soppa et al., 2014).

Proliferation: Rapid reproduction of microorganisms (Eduard, 2009).

qPCR: Quantitative Polymerase Chain Reaction (Forero et al., 2019).

Indoor air quality (IAQ): It is the set of physical, chemical and biological characteristics of the building's internal spaces, eg offices, rooms, offices, bedrooms. Does not include the industrial interior space (APA, 2009).

Temperature: Environmental factor that affects all terrestrial organisms, due to the relationship of rates of biochemical reactions and biological processes with temperature. Increased rates of biochemical reactions are enhanced with increasing temperature (Klepsatel et al., 2019)
TSA: Tryptone Soya Agar (Millipore 2021).
CFU: Colony-forming units, commonly abbreviated as CFU, refer to the individual colonies of microorganisms. For example, a colony of bacteria or yeast refers to a mass of individual cells from the same organism that grow together. A colony is a group of cells that grow together. Colony forming units are used as a measure of the number of microorganisms present in a sample (Tortora et al., 2009).
VRBA: Violet Red Bile Agar (Millipore 2021).

5. Methodological approach

The sampling sites at each facility were previously selected by the Occupational Health Services based on defined criteria, in order to prioritize the most critical work areas. The criteria applied were: 50-80% occupancy of facilities, activities carried out, and spaces common to all FFH.

Sampling was carried out in the period between winter and spring, during the daytime and during the course of normal activities.

6. Equipment and Materials

Table 10.7 presents the information regarding the sampling strategy and the microbiological variables analyzed.

Table 10.7 – Procedures and material used and microbiological parameters analyzed. (TSA tryptic soy agar; VRBA: violet red bile agar; MEA: Malt extract agar; DG18: dichloran-glycerol agar).

Harvest Method	Variable	Edquipment	Brand	Associated Material
Sample collection: air (Andersen)	Fungi (UFC/m³) Bacteria (UFC/m³)	Andersen six-stage air sampler	Thermo-Andersen, USA	Culture media MEA; DG18; TSA; VRRB; Gloves; Alcohol.
Sample collection: air (Millipore)	Fungi (UFC/m³) Bacteria (UFC/m³)³)	Milipore Air Tester	Billerica, MA, USA	Culture media MEA; DG18; TSA; VRRB; Gloves; Alcohol.
Surface sample (swab)	Fungi (CFU/m2) Bacteria (UFC/m2)	X	X	Swabs; Metal form for delimitation of the area to be sampled; Saline solution; Gloves; Alcohol; Cotton.
Electrostatic dust collectors	Fungi (CFU m-2 / day) Bacteria (CFU. m-2 / day)	x	x	Petri dish Filter; Gloves; Alcohol; Cotton.
Settled dust (Vaccum cleaner)	Fungi (CFU/m2) Bacteria (UFC/m2)	Vaccum cleaner	HOOVER Brave BV71_BV10 A2, USA	Filters; Gloves; Alcohol; Plastic bags; Glue tape; Scissors; Cotton.
Material collection: Uniform name ags Cleaning cloths mops	Fungi (CFU/m2) Bacteria (UFC/m2)	x	x	Gloves; Alcohol; Plastic bags; Scissors; Cotton.

7. Results

Table 10.8 shows the results by sampling method used.

Regarding the matrices with higher microbial contamination, the cloths used in cleaning had high amount of total and coliforms bacteria, followed by mops for total bacteria. The air samples collected by the Andersen impactor equipment obtained the second highest value of gram-negative bacteria.

In what concerns the fungal contamination, in MEA medium, surface samples, followed by filters were the matrices with the greatest contamination. In DG18, the highest values obtained was in filters followed by surface samples.

Table. 10.8 – Sites with greater microbial contamination by sampling method used (CFU. m^{-3} / m^{-2} .day^{-1} /m^{-2} / g^{-1}). (TSA:tryptic soy agar; VRBA:violet red bile agar; MEA: Malt extract agar; DG18: dichloran-glycerol agar).

Matrice	Total bacterial (TSA)	Coliforms (VRBA)	Fungi (MEA)	Fungi (DG18)
	*CFU. m^{-3} / m^{-2} .day^{-1} /m^{-2} / g^{-1}			
Andersen	1.60E+04	3.60E+04	5.32E+04	4.51E+03
Millipore	1.47E+04	1.04E+02	1.90E+04	1.84E+03
Electrostatic dust collectors	5.12E+02	7.32E+01	4.08E+02	4.13E+02
Cloths	5.50E+05	4.22E+05	8.50E+03	3.00E+03
Mops	1.94E+05	4.00E+03	2.50E+03	4.00E+03
Mops	3.35E+04	-	9.50E+03	5.50E+03
Surface samples (swabs)	1.19E+05	1.00E+03	3.10E+05	5.90E+04
Filters	1.93E+05	2.25E+04	1.48E+05	1.88E+05
Settled dust (vacum cleaner)	6.00E+00	6.00E+00	1.62E+02	2.28E+02

Compliance with scientific references:

Considering the quantitative comparison (indoor/outdoor) of the microbiological load (CFU/m^3), a higher bacterial load indoor was observed compared to the outdoors in the following sampled locations: Canteen, Bar, Reception, Gym, Dormitory, social and administrative room of FFH1 (Division A) and also the Dormitory, Balneary, social and administrative room of FFH1 (Division B) (Figure.1).

The World Health Organization suggests a maximum limit of 150 CFU/m^3 for indoor concentration). Of the 14 locations sampled, 7 did not comply with the established limit, namely: Bar, Reception, Dormitory, Administrative and social room (Division A) and administrative room (Division B).

Regarding fungi, considering the indoor load, of the 14 sampled locations, 4 exceed the outdoor load, namely: Dormitory, Balneary, social and administrative room of FFH1 (Division B) (Figure 10.6). According to the limit imposed by WHO, 3 places had a fungal load above the stipulated value, namely: Balneary, social and administrative room of FFH1 (Division B).

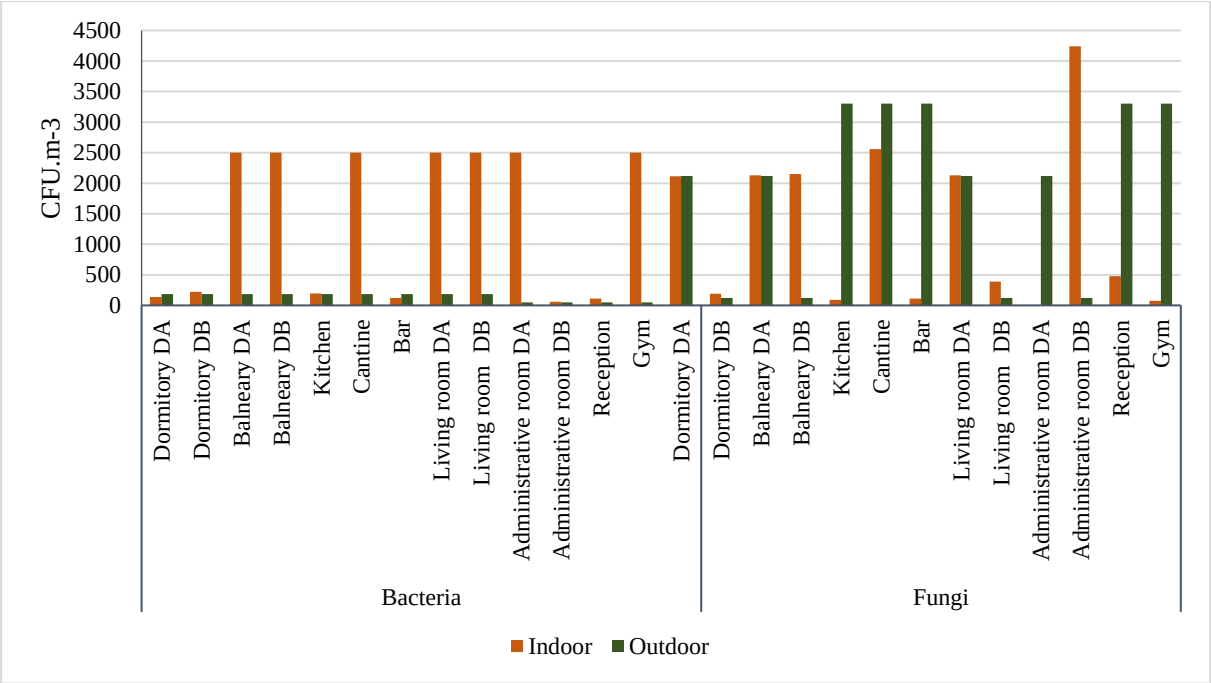

Figure 10.6 - Summary of quantitative results of the bacteria and fungal load present in the air samples collected by Millipore (CFU.m^{-3}) indoor and outdoor the building for common areas, division A(DA) and division B(DB) of FFH1.

For a better characterization of fungal contamination, the fungal species observed in the sampling methods used were identified (Table 10.9).

In total, 25 different fungal species were identified in MEA and 16 species in DG18. The most prevalent fungI in both culture media was *Chrysonilia sitophila* (81.02% MEA; 58.84% DG18). In addition to *Cladosporium sp* in both media,(14.56 % MEA; 32.51% DG18).

Table 10.9 - Results obtained regarding fungal distribution (CFU. m-3 / m-2.day-1 /m-2 / g-1) in samples inoculated in malt extract agar (MEA) and dichloran glycerol chloramphenicol (DG18). Common areas of firefighter headquarter 1, Division A and Division B.

	MEA			DG18		
Matrice	ID	CFU. m^{-3} / m^{-2} .day^{-1} /m^{-2} / g^{-1}	%	ID	CFU. m^{-3} / m^{-2} .day^{-1} /m^{-2} / g^{-1}	%
Andersen Common areas	*Chrysosporium* sp.	7856.30	41.89	*Cladosporium* sp.	372.99	80.51
	C.sitophila	5901.06	31.46	*Penicillium* sp.	82.45	17.80
	Penicillium sp.	4267.77	22.75	*Chrysosporium* sp.	3.93	0.85
	Outras espécies	679.23	3.62	Outras espécies	3.93	0.85
	*A.*section *Nidulantes*	51.04	0.27			
	Total	18755.40	100.00	Total	463.29	100.00
Andersen Division A	*Chrysosporium* sp.	7875.93	25.92	*C.sitophila*	1970.95	62.67
	Cladosporium	6431.10	21.17	*Cladosporium* sp.	592.85	18.85
	Penicillium sp.	6223.01	20.48	*Penicillium*	530.04	16.85
	Outras espécies	9850.80	32.42	Outras espécies	35.34	1.12
	*A.*section *Nidulantes*	3.93	0.01	*A.*section *Nidulantes*	3.93	0.12
				A. section *Flavi*	11.78	0.37
	Total	30384.77	100.00	Total	3144.88	100.00
Andersen Division B	*Scopulariopsis candida*	3926.19	96.43	*Cladosporium* sp.	757.75	84.28
	Cladosporium sp.	70.67	1.74	*Penicillium* sp.	113.86	12.66
	Penicillium sp.	39.26	0.96	Outras espécies	15.70	1.75
	Outras espécies	19.63	0.48	*Chrysosporium* sp.	11.78	1.31
	*A.*section *Nidulantes*	15.70	0.39			

Millipore Division A	Total	4071.46	100.00	Total	899.10	100.00
	Ulocladium sp.	2004.00	24.29	*Penicillium* sp.	152.00	65.52
	Fusarium verticilloides	2000.00	24.24	*Cladosporium* sp.	72.00	31.03
	Aureobasidium sp.	2004.00	24.29	*Chrysosporium* sp.	8.00	3.45
	Outras espécies	2232.00	27.05			
	*A.*section *Nidulantes*	8.00	0.10			
	*A.*section *Aspergilli*	4.00	0.05			
Millipore Division B	Total	8252.00	100.00	Total	232.00	100.00
	Geotrichum sp.	2204.00	46.42	*Penicillium* sp.	108.00	47.37
	Rhizopus sp.	2000.00	42.12	*Cladosporium* sp.	68.00	29.82
	Cladosporium sp.	364.00	7.67	*A.*section *Nidulantes*	24.00	10.53
	Outras espécies	180.00	3.79	*A.* section *Circumdati*	12.00	5.26
				Outras espécies	12.00	5,26
				A section *Candidi*	4.00	1.75
Millipore Common areas	Total	4748.00	10.00	Total	228.00	100.00
	Cladosporium sp.	1832.00	95.22	*Cladosporium* sp.	1124.00	81.45
	Penicillium sp.	92.00	4.78	*Penicillium* sp.	92.00	6.67
				Paecilomyces sp.	44.00	3.19
				Outras espécies	16.00	1.16
				A section *Candidi*	8.00	0.58
				*A.*section *Aspergilli*	92.00	6.67
				*A.*section *Niger*	4.00	0.29
Electrostatic dust collectors	Total	1924.00	100,00	Total	1380.00	100.00
	Cladosporium sp.	309.32	75.78	*Cladosporium* sp.	355.08	85.92
	Penicillium sp.	40.27	9.87	*Penicillium* sp.	29.28	7.09
	Trichoderna sp.	29.28	7.17	*C.sitophila*	18.30	4.43
	Outras espécies	29.28	7.17	Outras espécies	7.32	1.77
				A. *section Candidi*	3.28	0.79
Cleaning cloths	Total	408.16	100.00	Total	413.27	100.00
	Cladosporium sp.	2000.00	23.53	*Penicillium* sp.	2000.00	66.67
	Penicillium sp.	4500.00	52.94	*Cladosporium* sp.	1000.00	33.33
	C.sitophila	1500.00	17.65			
	Outras espécies	500.00	5.88			
Name tags	Total	8500.00	100.00	Total	3000.00	100.00
	C.sitophila	7500.00	78.95	*Cladosporium* sp.	1500.00	27.27
	Cladosporium sp.	1000.00	10.53	*Penicillium* sp.	2500.00	45.45
	Penicillium sp.	1000.00	10.53	*C.sitophila*	1500.00	27.27
Mops	Total	9500.00	100.00	Total	5500.00	100.00
	C.sitophila	500.00	20.00	*A. section Candidi*	1500.00	60.00
	Aureobasium sp.	500.00	20.00	*Cladosporium* sp.	1000.00	40.00
	Cladosporium sp.	1500.00	60.00			
Filters (vacum cleaner)	Total	2500.00	100.00	Total	2500.00	100.00
	Cladosporium sp.	92000.00	73.02	*Cladosporium* sp.	183000.00	96.32
	Penicillium sp.	19000.00	15.08	*Penicillium* sp.	6000.00	3.16
	C.sitophila	11000.00	8.73	*C.sitophila*	1000.00	0.53
	Outras espécies	4000.00	3.17			
Settled dust (vacum cleaner)	Total	15000.00	100.00	Total	190000.00	100.00
	C.sitophila	159.00	98.15	Penicillium sp.	98.00	42.98
	Penicillium sp.	2.00	1.23	Cladosporium sp.	125.00	54.82
	Cladosporium sp.	1.00	0.62	*A.* section *Nidulantes*	5.00	2.19
Surface samples	Total	162.00	100.00	Total	228.00	100.00
	C.sitophila	530000.00	94.64	*C.sitophila*	340000.00	91.89
	Cladosporium sp.	10000.00	1.79	*Cladosporium* sp.	10000.00	2.70
	Penicillium sp.	10000.00	1.79	*Penicillium* sp.	10000.00	2.70
	Outras espécies	10000.00	1.79	*A.* section *Candidi*	10000.00	2.70
	Total	560000.00	100.00	Total	370000.00	100.00

Distribution of *Aspergillus* genera

Concerning *Aspergillus* genera (0.01% MEA; 1.76% DG18), 6 distinct sections were obtained, with the section Nidulantes showing the highest prevalence in both media (0.01% MEA; 3.95% DG18).

The air collected by the Andersen six-stage impactor allowed the assessment of the prevalence of *Aspergillus* sp. and the size of the fungi particles obtained. *Aspergillus* section *Nidulantes* was found in 3 dimensions (4.7 µm; 2.1 µm and 0.65 µm) in DG18 with a prevalence of 11.11%; 11.11% and 16.67%, respectively. *Aspergillus* section *Aspergilli* was detected in the 2.1 µm dimension with a 50% predominance in the same medium.

In MEA, section *Nidulantes* was prevalent (100%), being detected in the dimensions: 4.7 µm (11.11%); 3.3 µm (0.60%); 2.1 µm (0.39%); 0.65 µm (1.51%) (Figure 10.7).

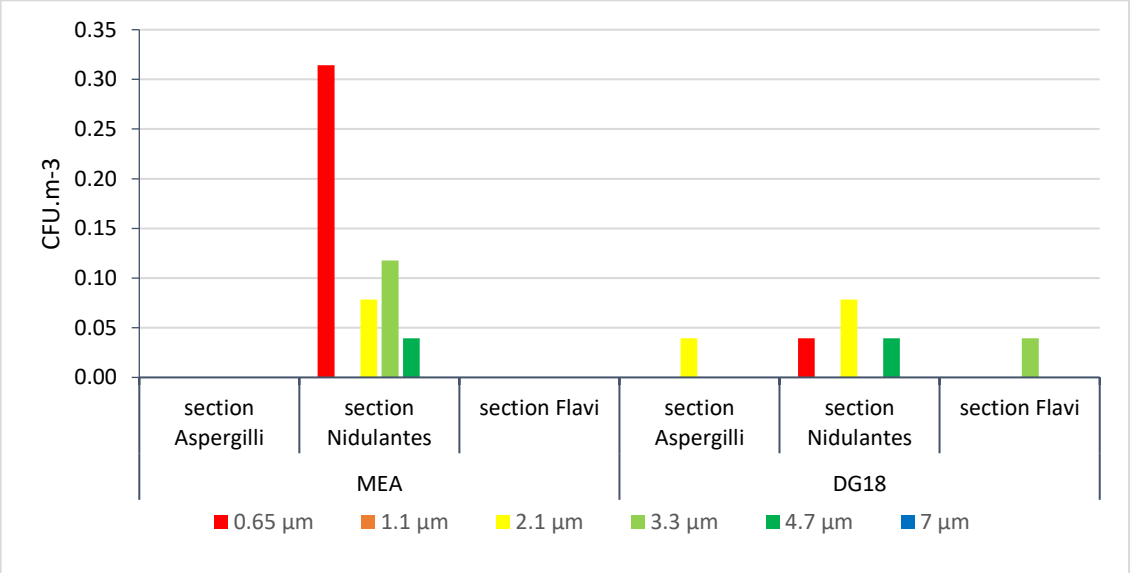

Figure 10.7 – Distribution of Aspergillus sections in the air samples collected by Andersen six-stage (CFU.m-3) and inoculated in malt extract agar (MEA) and Dichloran glycerol chloramphenicol (DG18).

Molecular identification of *Aspergillus* section *Fumigati* and *Nidulantes* through real-time PCR was performed on the collected samples, due to their clinical relevance. *A.* section *Fumigati* was detected on 3 filters (23.08% 3 of 13 samples), 2 mops (66.67% 2 of 3 samples) and 6 EDC (46.15% 6 of 13 samples). The sections *Nidulantes* was not detected in any of the samples.

8. Discussion

Despite the lack of national guidelines concerning microorganisms presence in samples from passive methods, it is important to emphasize that the aerosolization of species found on surfaces will depend on the influence of environmental variables and fungal characteristics (Roussel et al., 2008). The results of these methods are essential to achieve the characterization and evaluation of contamination by fungi and bacteria, and can be used to identify the sources of contamination (Stetzenbach et al., 2004; Klánová and Hollerová 2003).

It was possible to verify bacteria and fungi coexistence in the sampled places. As expected, a lower concentration of coliforms bacteria was evident compared to total bacteria, since total bacterial represent the sum of Gram-positive and coliforms. Furthermore, coliforms are also more sensitive to environmental variables, not being as persistent in the environment. It was also possible to verify that the total bacteria are widely distributed in the collected matrices. A higher bacterial concentration was detected in the cloths and mops used for cleaning, suggesting a potential source of cross contamination for several areas of the firefighters headquarters (Viegas et al., 2021a).

Inadequate personal hygiene can increase the diversity of microorganisms on hands and, by extension, on contact surfaces (Ross and Neufeld 2015). Most bacteria found inside buildings belong to

the normal microflora of the skin, mouth and nose, and are continuously emitted by people, accumulating indoors (Nigam & Cutter, 2003; APA, 2010; Moldoveanu et al.et al. . 2015). These bacteria generally do not pose a health hazard (APA, 2010). The existence of Gram-negative bacteria must be considered, as these bacteria can produce endotoxins, which are harmful toxins to health, causing respiratory problems (Moldoveanu et al. et al. 2015; WHO 2009). Consequently, the higher concentration of coliforms detected in air samples collected by Andersen six-stage could pose a health risk (Viegas et al. et al., 2019). In air samples, the higher bacterial and fungal load inside indoor may indicate a possible source of internal contamination, given the higher microbiological load obtained compared to the outside (Viegas et al. et al. 2017). Thus, the sampling sites where this situation was verified should be the target of intervention, in order to verify the source of internal contamination. The characteristics of each location, such as infiltration and humidity, may be associated with the fungal load found (WHO 2009).

The Andersen six-stage impactor equipment intends to simulate the respiratory system through the 6 dimensions it presents, making it possible to measure the pulmonary penetration of a given microorganism, taking into account its distribution over the different dimensions. The prevalence of *Aspergillus* section *Nidulantes* in 3 dimensions, especially in dimension 6 (0.65 µm), which can penetrate the alveoli, may indicate a potential factor for the development of respiratory diseases (Bowyer et al., 2019). The presence of species indicator of fungal contamination species such as *Aspergillus* sp., namely the *Nidulantes* section, suggests the implementation of corrective measures (Viegas et al. et al. 2017).

The results obtained in both culture media (DG18 and MEA), may be due to ineffective cleaning and ventilation in the places accessed (Viegas et al. et al. 2015; Ordinance 353-A of 2013 of December 4th) . Temperature and humidity can also promote fungal growth and mycotoxin production (Boudra et al. et al., 2005).

Fungi were found to grow on both media in all the collected matrices. However, the quantitative variation in results may be due to the ability of some fungi to grow differently in DG18 and MEA. For a better characterization of fungal contamination, the most prevalent fungi and those with clinical and toxicological relevance were listed, as shown in table 10.10.

Table 10.10 - Fungi with higher prevalence and clinical and toxicological relevance.

Fungi with > prevalence in the samples (passive methods) (Viegas et al., 2020)	*Cladosporium* sp., *Aspergillus* sp.
Fungi with clinical relevance (according to Decree-Law No. 102-A/2020)	*Aspergillus* sp.
Fungi with toxicological relevance (Serra, 2005; Varga et al. 2015)	*Aspergillus* sp., *Fusarium* sp., *Penicillium* sp.

Cladosporium sp., *Penicillium* sp., *and Aspergillus* sp. are fungi commonly found indoors (Stryjakowska-Sekulska, 2007).

Cladosporium sp. in indoor environments it mainly originates from external sources (Fukutomi & Taniguchi 2015). Due to the small size of their spores, they are easily spread over long distances (Bensch *et al*., 2012). It is usually associated with allergic rhinitis or with localized superficial or deep lesions, but rarely cause disseminated infections (Sandoval-Denis *et al*., 2015). Some species can cause allergic pulmonary mycosis (Bensch *et al*., 2012; Ziaee *et al.*, 2018).

Penicillium sp. is more common in indoor when compared with outdoor levels (Reboux *et al.*, 2019). They are found where organic matter is available, and can grow in conditions with very little water (Ziaee *et al.*, 2018). Increased relative humidity contributes to high concentrations of *Penicillium* sp. (Reboux *et al.*, 2019). Despite being distributed across different environments, they are generally not associated with infections for humans and animals (Egbuta *et al*., 2017). However, some species can cause infections and other diseases, such as pneumonia, urinary tract infections and asthma (Egbuta *et al.*, 2017). Furthermore, they can produce mycotoxins such as ochratoxin A, considered to be potentially carcinogenic (Egbuta *et al.*, 2017).

Aspergillus sp. can grow at high temperatures and in moist environments (Sabino *et al.*, 2019). They have a high nutritional versatility and are able to use various organic compounds and substances as a carbon source (Sabino *et al.*, 2019). The reduced size of spores facilitates their spread through the air (Sabino *et al.*, 2019). Many species have been reported as causative agents of opportunistic infections in man (Egbuta *et al.*, 2017). The most common diseases associated with occupational exposure to *Aspergillus* sp. are: allergic bronchopulmonary aspergillosis, rhinosinusitis, rhinitis and severe asthma due to fungal sensitization (Sabino *et al.*, 2019). In addition, some species are capable of producing mycotoxins, which are harmful to health (Egbuta *et al.*, 2017).

The *Fusarium* sp. is widely distributed in soil, water, and this ability to diffuse into different environments is attributed to its ability to grow on different substrates. This species can cause superficial infections as well as allergic diseases (Nucci & Anaissie, 2007). Species of the *Fusarium* genus are considered to be indicators of moisture problems or health risks (Goyer *et al*., 2001) and also producers of mycotoxins (Varga *et al*., 2015).

Although species from section *Fumigati* were identified in surface samples using culture-based methods, molecular identification allowed detecting their presence in 11 different samples, as happened previously in other studies (Viegas *et al.*, 2018b; Viegas *et al*., 2021b). These results support the need to use the classical and molecular microbiology tools in parallel, in order to allow a better characterization of the exposure.

9. Recommendations

In all facilities, the reported procedures adopted were normal cleaning and disinfection. The cleaning products used were bactericidal and virucidal, bleach and multipurpose detergent. Although regular cleaning of all spaces is evident, it was clear that most of the units and surfaces sampled had microbiological contamination. In general, and considering the results obtained, evaluations should be carried out before and after cleaning, in order to verify if the procedures and products used are the most adequate.

Another important aspect is to ensure the adequate training for workers on the most effective cleaning procedures to ensure proper hygiene.

It is crucial to identify the indoor sources of contamination, in order to guarantee the resolution of situations that are increasing the microbiological contamination of the analyzed spaces. In addition, regular monitoring of facilities to assess microbiological contamination, and especially after the changes implemented, would be crucial to ensure the health and performance of all workers.

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Conclusion of the environmental and laboratory assessments: The results found show that the procedures and/or products being used may not be the most suitable and that there are sources of internal contamination. Future evaluations of microbiological contamination should focus on evaluating the effectiveness of the procedures and products used in the cleaning operations of interior spaces and after intervention in possible sources of internal contamination

Conclusão do relatório em:

Prepared by:	**Approved by:**
Bianca Gomes	
Sandra Ferreira	Project cordinatoor