INSTITUTO POLITÉCNICO DE LISBOA

ESCOLA SUPERIOR DE TECNOLOGIA DA SAÚDE DE LISBOA

EXPOSIÇÃO OCUPACIONAL A FUNGOS NUMA TRIAGEM DE RESÍDUOS NO CANADÁ

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Orientadora: Prof.ª Doutora Carla Viegas

Mestrado em Tecnologias Clínico Laboratoriais

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OCCUPATIONAL EXPOSURE TO FUNGI IN WASTE SORTING FROM CANADA

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Abstract

Globally, a significant number of people deal with waste management, and microorganism contamination is seen as an occupational health issue for those who treat solid waste. Waste-related microbiological exposures may occur indoors where waste is processed, or outdoors during its processing and may be caused by the sorting, transfer or cleaning process. The aim of the study is to assess forklift drivers' exposure by inhalation to fungi, during waste management. A total of 41 samples was used, 16 Cabin Ventilation Filters (ventilation filters from vehicle cabinets of waste management trucks) and 25 filters from air microbiological sampler Surface Air System Super (SASS). The SASS Filters and the Cabin Ventilation Filters extracts were analyzed by culture-based methods (including azole-resistance screening) and qPCR, targeting four *Aspergillus* sections (*Fumigati*, *Flavi*, *Nidulantes* and *Circumdati*). To improve the selectivity for fungal growth, two different culture media were used: malt extract agar (MEA) supplemented with chloramphenicol (0.05%) and dichloran-glycerol agar (DG18). For antifungal resistance screening, samples were spread (0.15 ml) onto Sabouraud dextrose agar media supplemented with 4 mg L^{-1} itraconazole (ITR), 1 mg L^{-1} voriconazole (VOR), or 0.5 mg L^{-1} posaconazole (POS) (protocol adapted from the EUCAST 2017 guidelines).

Aspergillus sp. presented the highest prevalence in Cabin Ventilation Filters on MEA (35.8%), while in DG18, the highest prevalence belongs to *Penicillium* genera (66.5%). In SASS Filters, *Aspergillus* sp. presented the highest prevalence on MEA (67.6%), whereas on DG18, the highest prevalence belongs to *Penicillium* sp. (31.1%). Thirteen of the 16 cabin ventilation filter samples (81.2%) presented fungal growth on 4 mg/L ITR, 10 out of 16 (62.5%) samples presented two additional fungal species on 1 mg/L VOR; and in 9 out of 16 (56.2%) samples was observed one additional fungal species on 0.5 mg/L POS. Among *Aspergillus* genus, *Aspergillus* sections *Fumigati, Flavi* and *Nidulantes* presented the highest prevalence in Cabin ventilation Filters (2 out of 16; 12.5%), whereas in SASS Filters, the highest prevalence belongs to *Aspergillus* section *Fumigati* (3 out of 25; 12%). Regarding molecular tools, detected by PCR in the Cabin Ventilation Filters and in SASS Filter samples, all four sections targeted were detected.

Overall, it was possible to assess fungal contamination in the matrices assessed and conclude that the use of different sampling methods such as the Cabin Ventilation Filters and the SASS Filters to assess the occupational exposure to fungi in this type of industry allowed to perform a more complete characterization of the fungal

contamination. This study also reinforces the need to assess the exposure to fungal contamination in this specific occupational environment.

Keywords: Occupational exposure; Waste Industry; Risk Assessment; Bioaerosols; Fungi

Resumo

Globalmente, um número significativo de pessoas lida com gestão de resíduos, e a contaminação por microrganismos é vista como um problema de saúde ocupacional para quem trata de resíduos sólidos. As exposições microbiológicas relacionadas aos resíduos podem ocorrer em ambientes internos, onde os resíduos são processados, ou ao ar livre, durante o processamento e podem ser causados pelo processo de separação, transferência ou limpeza. O objetivo deste estudo é avaliar a exposição inalação a fungos dos condutores de empilhadores, durante a gestão de resíduos. Foram utilizadas 41 amostras, 16 Filtros de Ventilação de Cabine (filtros de ventilação de cabines de empilhadores) e 25 filtros de amostrador microbiológico de ar Super Sistema de Ar de Superfície (SSAS). Os extratos dos filtros SSAS e dos Filtros de Ventilação da Cabine foram analisados por métodos baseados em cultura (incluindo resistência a azóis) e qPCR, visando a deteção de quatro espécies de *Aspergillus* (*Fumigati, Flavi, Nidulantes* e *Circumdati*). Para melhorar a seletividade para o crescimento fúngico, foram utilizados dois meios de cultura diferentes: ágar extrato de malte (MEA) suplementado com cloranfenicol (0,05%) e ágar diclorano-glicerol (DG18). Para a triagem de resistência antifúngica, as amostras foram espalhadas (0,15 ml) em meio de ágar Sabouraud dextrose suplementado com 4 mg L^{-1} itraconazol, 1 mg L^{-1} voriconazol ou 0,5 mg L^{-1} posaconazol (protocolo adaptado das diretrizes EUCAST 2017).

Nos Filtros de Ventilação de Cabine a espécie com maior prevalência em MEA (35,8%) foi *Aspergillus sp.*. Em DG18, a maior prevalência pertence ao *Penicillium* sp. (66,5%). Nos Filtros SSAS, *Aspergillus sp*. apresentou a maior prevalência em MEA (67,6%), e em DG18, a maior prevalência pertence a *Penicillium sp.* (31,1%). Treze das 16 amostras de Filtro de Ventilação de Cabine (81,2%) apresentaram crescimento fúngico em 4 mg/L ITR, 10 de 16 amostras (62,5%) apresentaram mais duas espécies fúngicas em 1 mg / L VORI; e 9 de 16 amostras (56,2%) apresentaram mais uma espécie fúngica em 0,5 mg / L POSA. Relativamente às ferramentas moleculares, *Aspergillus* sections *Fumigati, Flavi* e *Nidulantes* foram as que apresentaram a maior prevalência nos Filtros de Ventilação de Cabine (2 de 16; 12,5%), enquanto nos Filtros SASS, a maior prevalência pertence à section *Fumigati* (3 de 25; 12%).

De forma geral, foi possível concluir que o uso de diferentes métodos de amostragem como os Filtros de Ventilação de Cabine e os Filtros SSAS, para avaliar a exposição ocupacional a fungos neste tipo de indústria é um bom método, uma vez que nos permite realizar uma caracterização mais completa da contaminação fúngica.

Este estudo também reforça a necessidade de avaliar a exposição à contaminação fúngica neste ambiente ocupacional especificamente.

Palavras-Chave: Exposição Ocupacional; Indústria de Resíduos; Avaliação de Riscos; Bioaerossóis; Fungos

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Abbreviations

CFU - Colony Forming Unit

DG18 – Dichloran (18%) Glycerol Agar Base

ITR – Itraconazol

MEA – Malt Extract Agar

POS – Posaconazol

SAB – Sabouraud Dextrose Agar

TSA – Tryptic Soy Agar

VOR – Voriconazol

VRBA – Violet Red Bile Agar

SASS - Surface Air System Super

PCR - Polymerase Chain Reaction

FRPD - Filtering Respiratory Protecting Devices

EDC - Electrostatic Dust Cloths

MPG - Mechanical Protection Gloves

1. Chapter I: Bibliographic Review

Globally, a significant number of people deal with waste management, and microorganism contamination is seen as an occupational health issue for those who handle solid waste (1). Organic dust is commonly characterized as an airborne mixture of viable and non-viable microorganisms (bacteria, fungi, viruses, protozoa), their metabolites (endotoxins, (1-3)-β-D51 glucans, mycotoxins, peptidoglycans, enzymes, etc.) and solid vegetable and animal particles (allergens, including pollen, crops, etc.) (2). The handling of residues and the consequent microbial exposure, such as fungi and bacteria, is associated with several health effects, such as asthma, diarrhea and organic powder syndrome (3).

Regarding mycobiota, one of the most prevalent genera in waste industries is *Aspergillus sp.* (4) *w*ith allergic and toxigenic potential (5,6). In addition to the workers involved in handling and sorting waste, there are still other workers with different roles in this sector who are also exposed to organic substances. Among them are the drivers of garbage collection trucks, recyclable materials and organic waste. Although these professionals are not in direct contact with the waste, an exposure can occur during the waste handling, through contamination of the components of the cabin's ventilation and air conditioning system or even contamination of the vehicle cabin (4,7–9). Therefore, an exposure assessment to fungal contamination of these workers is crucial to ensure their safety.

1.1. Exposure to Microbiological Agents in Waste Industry

Waste-related microbiological exposures may occur indoors where waste is processed, or outdoors during its processing and may be caused by the sorting, transfer or cleaning process (10–12).

Organic dust, including microorganisms, may become aerosolized as biodegradable waste is treated, and waste handlers may be exposed to several biological agents (13,14). Exposure to this multitude of bioaerosols can present waste handlers with a high risk of occupational health problems (14).

Bioaerosols consist of living and dead micro-organisms, either as individual micro-organisms or as components of aggregates, fragments, and micro-organisms, such as endotoxins and mycotoxins. Owing to the wide variety of adverse health effects associated with exposure in occupational and residential settings such as

infections, immuno-allergic, non-allergic inflammatory and toxic effects, the interest of scientists and health authorities in bioaerosols has grown over the past two decades (15–21).

1.1.1. Occupational Exposure to Fungi in Waste Industry

There are some genera/species of fungi that are considerably prevalent in this type of industry such as *Aureobasidium pullulans, Epicoccum nigrum, Fusarium sambucinum, Beauveria bassiana, Rhizopus stolonifer, Phoma, Absidia, Mucor, Rhizopus, Penicillium*, and yeasts (22,23). However, in this highly contaminated environment, one of the most prevalent genera is *Aspergillus* sp. (6,23).

Aspergillus sp. are commonly found in different environmental compartments. Some species among the *Aspergillus* genera can be harmful to humans and only a few species are considered as significant opportunistic pathogens in humans. This type of fungus has been subdivided into 22 distinct sections, being the following the clinically relevant species: *Aspergilli, Fumigati, Circumdati, Terrei, Nidulantes, Ornati, Warcupi, Candidi, Restricti, Usti, and Flavi* (6,24). Among the *Aspergillus* genera, *Fumigati* section is the one that causes most diseases followed by the *Flavi* section. Furthermore, as previously reported, *Aspergillus* is one of the most prevalent fungi in several highly contaminated occupational environments, including waste industry (6).

It is important to highlight that most of these settings tend to form dust and resuspension, and that these aspects along with the humidity and temperature changes may increase occupational exposure to fungi and their metabolites. Since small sized particles can carry them, the coexistence of dust and fungi in these settings contributes for the increase of fungal burden and presents a higher risk factor for the exposure of workers to fungi by inhalation (6). The exposure to *Aspergillus* conidia may cause adverse human health effects such as harmful immune responses (e.g., allergy or hypersensitivity pneumonitis), direct infection by the organism, and toxic-irritant effects from *Aspergillus* by-products, such as mycotoxins. Allergic bronchopulmonary aspergillosis affects individuals with asthma or cystic fibrosis, therefore allergic fungal sinusitis are manifestations of significant hypersensitivity to fungi, particularly *Aspergillus* species (6).

Furthermore, it is important to mention that workers may be exposed to the same potential risks as the general population, although exposure and hazards may vary due to worker waste handle and proximity to sources of contamination and a more contaminated occupational environment (23,25). Waste management has been shown

to release airborne microorganisms and bioaerosols into the environment surrounding waste facilities (23,26) and that the concentration and viability of airborne microorganisms are influenced by temperature, relative humidity and wind speed (23,27). Therefore, in terms of occupational exposure to fungal infection, this type of industry and facilities are deemed critical (23,28,29) as they have ideal conditions for fungal growth (23,30).

1.1.2. Fungal Azole Resistance

There is a lack of understanding about the epidemiology of fungal infections globally because there are no reporting commitments (31). The last human-approved antifungals were defined in 2002 but that is not the only problem. Medically authorized antifungal medicines have been used for agricultural purposes for centuries and most pathogens have environmental niches, which implies that the agricultural use of medically approved drugs is a real danger since they encourage drug resistance (32). We know that systemic antifungals and fungicides are used as frontline medicines for fungal illnesses in humans and crops. However, fungal pathogen control may be ephemeral due to the fast growth of chemical resistance. Fungi have extremely plastic genomes and reproduce quickly. The combination of these characteristics produces variations chosen for resistance rapidly (33). Thus, we can consider that the emergence of antifungal resistance can jeopardize therapy alternatives that are already restricted, with calamitous impacts on therapy results (34).

Resistant fungal isolates have been found in many countries worldwide since the introduction of the first antifungal compounds in the late 1960s (35–40). As a result, over recent decades, the incidence of invasive infections caused by resistant fungi has increased (40–42) and clinicians are increasingly worried about antifungal resistance.

Fungal infections are usually efficiently eradicated by the immune system in healthy humans. Fungal infections are also more common in immunodeficiency patients or in patients with underlying chronic disorders such as asthma, leukemia or cystic fibrosis (40,43,44).

While it is important to treat invasive fungal infections with antifungals to improve survival, there are still limited options for treatment. This is because, due to their shared cellular eukaryotic structures, most compounds that are effective against fungi are toxic to mammalian cells. More treatment options became available in the 1990s with the advent of triazoles and echinocandins, but these advances were quickly

accompanied by the appearance of triazole and echinocandin resistant fungal pathogens (40,45–49). Current antifungal therapy includes five primary groups of antifungal agents, including polyenes, imidazoles and trizoles, allylamines, echinocandins, and the 5-flucytosine compound (40,50,51). Although some fungal species present inherent resistance to azole, acquired resistance to azole may also be found in occupational fungi, such as sawmills, where some selection pressure can be imposed on fungal colonies by azole fungicides (14-alpha demethylase inhibitors, DMI) used for wood preservation. DMI is used against phytopathogenic fungi and is believed to be an environmental cause of secondary antifungal resistance worldwide (52–55).

The relation between the selection pressure due to the use of azole fungicides in agriculture and floriculture and the growth of fungal resistance in *Aspergillus Fumigatus* has been described in recent years (52,56,57). It is crucial to assess fungal resistance profile in the environment of specific working environments, not only in high-load settings such as waste industry, but also in working environments that use azole fungicides for protection of spruce and pine fields, and other activities such as wood processing, preservation and maintenance (52,58). Sawmills processing resinous woods for example, frequently use azole fungicides to protect wood from phytopathogenic fungi that cause wood deterioration making wood unworkable (52,59). As another example, growing antifungal resistance in agricultural fields can cause significant problems not only in agriculture but also in human health, particularly in the case of opportunistic human fungal pathogens with a primary ecological niche in soil, including agricultural soil. It is therefore very important to examine the occurrence and molecular mechanisms of azole resistance in order to better understand the origin and evolution of azole resistance and to better respond to the continued development of azole-resistant aspergillosis (60).

1.1.3. Mycotoxins

Mycotoxins are metabolites produced by specific fungal species mainly by *Aspergillus*, *Penicillium, Alternaria, Fusarium, and Claviceps* by specific fungal genera (3,61). Under the influence of different kinds of environmental stress, unique fungal species can produce many different mycotoxins. These low molecular mass natural toxins are very durable and can withstand unfavourable environmental conditions such as high or low temperatures and may be present in the environment due to their persistence long after fungal death and disintegration, even in the absence of any

observable fungi (3,62). Some mycotoxins, when ingested, cause severe effects on human health, but their impact on human health after inhalation or dermal contact is inadequately recorded (63). In highly contaminated occupational environments, such as the waste sorting industry, where fungi, namely *Aspergillus* sp., are prevalent, potential hazards may arise from inhalation exposure to fungi and mycotoxins (3,5,63–70). For example, in the assessment of mechanical protective gloves from waste sorting workers by Viegas et al. (2020) (70), it was possible to detect mycotoxins in 89,6% of the samples, which reinforces the idea that mycotoxins are highly present in this type of industry.

Some biomonitoring campaigns in waste workers showed that an occupational exposure to aflatoxin B1 (AFB1) was occurring (68,71). Moreover, the biomonitoring data available, allowed the confirmation that regardless the activity developed by these workers, all of them were exposed, even the ones that move the waste mechanically in a closed environment, like forklifts drivers that work inside the forklift cabinets (70).

1.2. Assessment of Occupational Exposure to Fungi

1.2.1. Active and Passive Sampling Methods

Active and passive sampling approaches are used to characterize occupational exposure to culturable bioburden (72). Even though personal or stationary sampling of airborne bioburden can be used to quantify inhalation exposure, it may be significantly affected by some environmental variables, such as seasonal variation and ventilation (73–76) number and hygiene of people who occupy the building (77,78), the physical layout of the building, and procedures for cleaning and products applied (18,78). Additionally, inadequate maintenance of heating, ventilation, and air conditioning system can boost the hazardous effect of many biological contaminants and nonbiological pollutants (78,79).

Active sampling methods may use impaction air samplers, impinger air samplers and button air samplers. This type of sampling method is always dependent of the use of an air sampling pump to actively pull air through a collection device. Microbiological air samplers physically draw a known volume of air through or over a particle collection device which can be a liquid (impinger) or a solid culture media (impaction) or a nitrocellulose membrane (button sampler) and the quantity of microorganisms present is measured in CFU (colony forming units)/m^3 of air. This method is applicable when the concentration of microorganisms is not very high, for

example in operating theatres and other hospital-controlled environments. (80–84). Nevertheless, there are some studies that applied this type of sampling method in waste industry (eg. 25,81,82), the counterpart is that in this highly contaminated environment, the collection of a big amount of air may cause the rapid growth of some species that consequently may inhibit the growth of other species and that may lead to inaccurate results (23,86).

Passive sampling methods do not require the use of air pumps which enables the use of a broader range of assays due to the ease with which samples can be handled, without the logistical constraints of more sophisticated sampling devices (87), such as HVAC Filters (Viegas et al. 2019b), Filtering Respiratory Protecting Devices (FRPD) (Viegas et al. 2020d), Settled Dust (Viegas et al., 2019c, 2019e, 2020e) Electrostatic Dust Cloths (EDC) (Viegas et al., 2018b; 2019e,f; 2020e), Mechanical Protection Gloves (MPG) (Viegas et al., 2020g) and Feed Viegas et al., (2018b) depending always on the assessed environment.

While active sampling methods only reflect the load of a shorter period (mostly minutes) corresponding to the sampling duration, passive sampling methods allow the collection of contamination over a longer period (days, weeks or several months) (23,52,88–90). Therefore, passive and active sampling methods should always complement each other to ensure a more precise assessment of occupational exposure to bioburden (74,78,91). Considering that by increasing the number of different sampling methods, the data available will consequently increase and it will allow the industrial hygienists to perform a more accurate risk characterization (74,78,91).

1.2.2. Culture-Based Methods and Molecular Tools

Culture-based methods, like any other method, has its own advantages and disadvantages. It is important to use culture-based methods to assess the viable component of the bioburden (89,92) since its viability affects biological mechanisms, such as the inflammatory and cytotoxic responses (93,94). This means that the inflammatory potential of a single environmental sample (air, settled dust, etc.) can vary depending on its microbial composition and the biological activity of the microorganisms present in that sample (70,93,95,96). However, there are some drawbacks, such as how the growth rate and requirements of different fungal species influence other species in mixed cultures. For example, the rapid growth of a particular

species may cause overgrowth and, therefore, chemical competition, which may inhibit the growth of other species and lead to a lack of accuracy in the results of the analysis (23,86). The selective incubation temperature (85,97) and culture media used (85,98,99), are also disadvantages of using only culture-based methods (85,100,101).

On the other hand, molecular tools have certain characteristics that allow them to be more effective than culture-based methods, such as its precision, speed, extreme analytical sensitivity of detection and the fact that it allows the detection and identification of dead or dormant microorganisms, as well as toxigenic strains of specific fungal species (23,86,102,103). However, molecular tools have also its own disadvantages such as not being able to give details on the viable component of the bioburden, which is crucial to estimate health risks by affecting biological mechanisms such as cytotoxic and inflammatory responses (94). Another disadvantage is the fact that false negatives in qPCR assays for microbial detection may occur as a result of insufficient removal of PCR inhibitors from the tested sample, inefficient release of microbial DNA content from cells, or poor DNA recovery after extraction and purification steps (5,104). It has also been confirmed that the presence of inhibitor sources may be caused by particles in the air (5,105).

Therefore, these evidence show that culture-based methods and molecular tools should be combined whenever it’s possible (23,86).

2. Chapter II: Materials and Methods

This study was conducted between January and October 2019 in one waste-sorting industry located in Canada, as part of an extended study financed by the Institut de Recherche Robert-Sauvé en Santé et Sécurité du Travail (IRSST) to assess occupational exposure to microbiologic agents in waste management units.

The aim of the study is to assess forklift drivers' exposure by inhalation to fungi, during waste management. To accomplish that main purpose, there are some specific goals that were achieved, such as the characterization of fungal contamination present in the filters of individual sampling filters, the characterization of fungal contamination present in the filters of the ventilation and air conditioning system of the truck cabins and the identification of possible correlations between fungal contamination from both environmental matrices.

It is considered a cross-sectional study since it does not require manipulation of variables, it is a study aimed at assessing whether the exposure to a particular risk factor (occupational exposure to fungi) can be associated with specific outcomes and it is a type of observational analysis that analyses data from a population or a representative subset at a specific period of time.

2.1. Sampling

2.1.1. Samples Collection and Extraction

In order to assess forklift drivers' occupational exposure to fungal load and contamination, a total of 41 samples, 16 Cabin Ventilation Filters (ventilation filters from vehicle cabinets of waste management trucks) (Figure 2.1) and 25 filters from air microbiological sampler Surface Air System Super (SASS) (Figure 2.2) corresponded to airborne microorganisms collected, were collected during summer season in Canada.

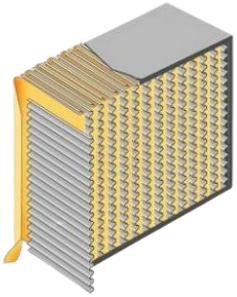

Figure 2.1 - Cabin Ventilation Filter Martix

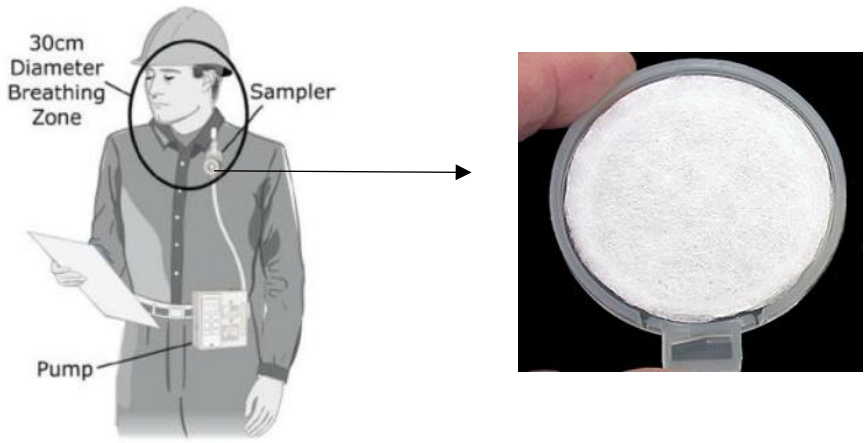

Figure 2.2 - SASS equipment and Filter martix

After sampling and extraction, according to a pre-established protocol (Figure 2.1), the samples were sent to ESTeSL in Lisbon, Portugal to be processed. The extraction was performed in Canada to allow the samples extracts shipment. The laboratory protocol used to perform the samples extraction, was the one already published and used to assess waste industry workers' exposure, through their Filtering Respiratory Protective Devices by Viegas et al. 2020 (3) (Figure 2.3). It is important to highlight that, even though the Mycotoxins assay and the Cytotoxic assay are contemplated in the Samples Processing Scheme, they are not included in this work.

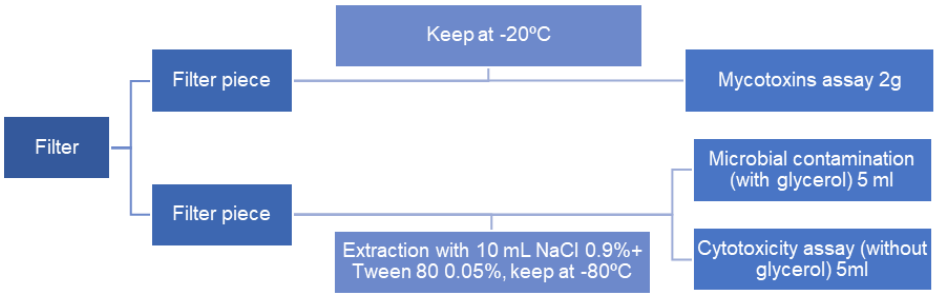

Figure 2.3 - Samples processing protocol

2.2. Characterization of Viable Bioburden

2.2.1. Inoculation

Two different culture media were used to enhance the selectivity for fungal growth: malt extract agar (MEA) supplemented with chloramphenicol (0.05%) this media allows the growth of most yeasts and molds whilst inhibiting most bacteria. It is

only used for cultivations of fungi and it is not recommended for use in the diagnose of human's disease. On the other hand, dichloran-glycerol agar (DG18), allows the growth of yeasts and molds in a laboratory setting. It is used for a selective isolation of fungi and it is not recommended for use in the diagnose of human disease or conditions. Samples were also inoculated (0.15 ml) onto Sabouraud dextrose agar media supplemented with 4 $\mathrm{mg.L^{-1}}$ itraconazole (ITR), which is an antifungal (1st generation) used to tread a wide variety of fungal infections, 1 $\mathrm{mg.L^{-1}}$ voriconazole (VOR), antifungal (2nd generation) used to tread a wide variety of fungal infections, or 0.5 $\mathrm{mg.L^{-1}}$ posaconazole (POS), antifungal (3rd generation) used to tread a wide variety of fungal infections (protocol adapted from the EUCAST 2017 guidelines) (106) for the screening of antifungal resistance (3,70,92).

2.2.2. Fungal Quantification and Identification

After incubation of MEA, DG18 and Sabouraud at 27 Cº for 5 to 7 days for fungi and fungal densities (colony-forming units, CFU.$\mathrm{m^{-2}}$) were calculated. Fungal species were identified microscopically by an experienced environmental and occupational mycologist using tease mount or Scotch tape mount and lactophenol cotton blue mount procedures. Morphological identification was achieved through macro and microscopic characteristics as noted by De Hoog (107). The identification was followed by the MsC student.

2.3. Molecular Detection of *Aspergillus* Sections

All samples (16 Cabin Ventilation Filters; 25 SASS Filters) were used for the molecular detection of fungal species/strains, with reported toxigenic potential, namely Aspergillus sections (*Flavi, Fumigati, Circumdati and Nidulantes*) as previously described (89).

Fungal DNA was extracted using the ZR Fungal/Bacterial DNA MiniPrep Kit (Zymo Research, Irvine, USA) according to the manufacturer's instructions (Table 2.1), and molecular identification of target species was achieved by Real Time PCR (qPCR) using the CFX-Connect PCR System (Bio-Rad). Reactions were performed as previously reported (89); for each gene amplified, a non-template control and a positive control consisting of DNA obtained from reference strains (kindly provided by the Mycology laboratory of the National Institute of Health Dr. Ricardo Jorge) were included.

Table 2.1 - Sequence of primers and TaqMan probes used for Real Time PCR

Fungal species/sections targeted	Sequences	Reference
***Flavi* (Toxigenic Strains)**		
Forward Primer	5‘-GTCCAAGCAACAGGCCAAGT-3‘	
Reverse Primer	5‘-TCGTGCATGTTGGTGATGGT-3‘	(108)
Probe	5‘-TGTCTTGATCGGCGCCCG-3‘	
Fumigati		
Forward Primer	5‘-CGCGTCCGGTCCTCG-3‘	
Reverse Primer	5‘-TTAGAAAAATAAAGTTGGGTGTCGG -3‘	(109)
Probe	5‘-TGTCACCTGCTCTGTAGGCCCG -3‘	
Circumdati		
Forward Primer	5‘-CGGGTCTAATGCAGCTCCAA-3‘	
Reverse Primer	5‘-CGGGCACCAATCCTTTCA-3‘	(6)
Probe	5‘-CGTCAATAAGCGCTTTT-3‘	
Nidulantes		
Forward Primer	5’ – CGGCGGGGAGCCCT-3’	(110)
Reverse Primer	5’ – CCATTGTTGAAAGTTTTGACTGATcTTA-3’	
Probe	5’ –AGACTGCATCACTCTCAGGCATGAAGTTCAG-3’	

2.4. Statistical Analysis

The data were analysed using SPSS statistical software, version 26.0. The results were considered significant at the 5% significance level. To test the normality of the data, the Shapiro-Wilk test was used. For the study of the relationship between fungal contamination, azoles resistance and molecular tools, Spearman's correlation coefficient was used since the assumption of normality was not verified. To compare fungal contamination, azoles resistance and molecular tools between the two types of sample, the Mann-Whitney test was used, since the assumption of normality was not verified.

3. Chapter III: Results

This chapter is dedicated to the results of the project, the aim is to show the findings throughout the research for further discussion.

3.1. Fungal Contamination Characterization by Culture-Based Methods

In Cabin Ventilation Filters, the highest percentage of fungal burden was found on MEA media (61.9%; $2.2x10^2$ ($3.4x10^2$)), whereas in SASS Filters the highest percentage belongs to xerophilic fungi (DG8 61.9%; $1.3x10^2$ ($1.4x10^2$)) (Table 3.1).

Table 3.1 - Fungal contamination on Cabin Ventilation Filters and SASS Filters

Cabin Ventilation Filters	
	Mean (SD) CFU
MEA	2.2×10^2 (3.4×10^2)
DG18	1.1×10^2 (1.2×10^2)
SASS Filters	
	Mean (SD) CFU.m^{-2}
MEA	0.8×10^2 (1.9×10^2)
DG18	1.3×10^2 (1.4×10^2)

Aspergillus sp. presented the highest prevalence in Cabin Ventilation Filters on MEA media (35.8%) followed by *Chrysonilia sitophila* (28.7%). Concerning the xerophilic fungi (DG18), the highest prevalence belongs to *Penicillium* genera (66.5%) followed by *Mucor* sp. (18%). In SASS Filters, *Aspergillus* sp. presented the highest prevalence on MEA media (67.6%) followed by *Penicillium* sp. (23.7%), while on DG18, the highest prevalence belongs to *Penicillium* sp. (31.1%) followed by *Aspergillus* sp. (27.5%) (Figure 3.1).

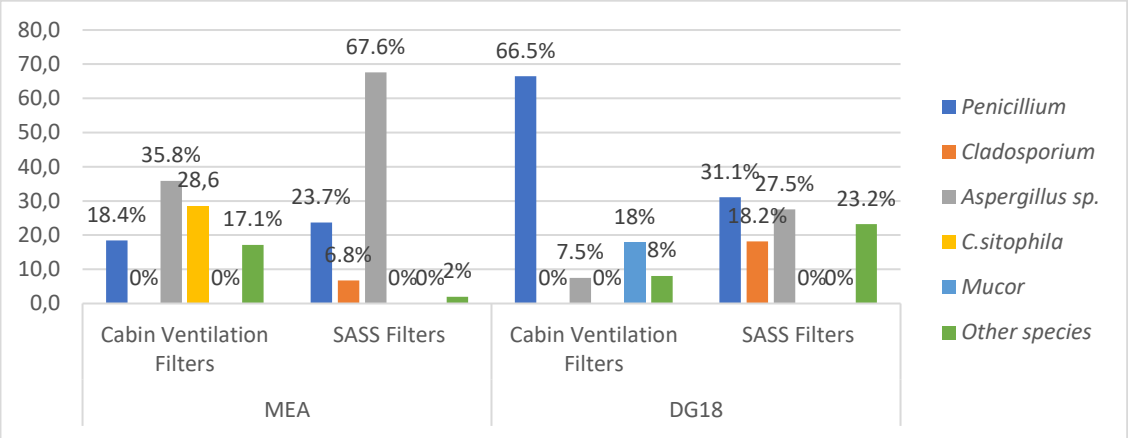

Figure 3.1 Fungal distribution in both matrices

Among *Aspergillus* genera, section *Fumigati* was the one with the highest prevalence in Cabin Ventilation Filters on MEA media (41.41%), whereas on DG18 it was section *Flavi* (92.13%). However, other *Aspergillus* sections were also detected on MEA media such as *Nigri* (32.81%) and *Flavi* (25.78%), and in DG18 such as *Aspergilli* (0.79%), *Fumigati* (3.15%) and *Nigri* (3.94%). Concerning the SASS Filters, the highest prevalence was *Nigri* section on MEA media (61.54%) and *Circumdati* (94.69%) on DG18. Some species were also detected in both media such as *Fumigati* (18% MEA; 0.1% DG18), *Flavi* (15.4% MEA; 4.6% DG18) and *Nigri* (61.5% MEA; 0.2% DG18) (Table 3.2).

Table 3.2 - Aspergillus sections distribution on both matrices

Cabin Ventilation Filters					
MEA			**DG18**		
Sections	**CFU/m²**	**%**	**Sections**	**CFU/m²**	**%**
Nigri	42	32.8	*Aspergilli*	1	0.8
Fumigati	53	41.4	*Flavi*	117	92.1
Flavi	33	25.8	*Fumigati*	4	3.2
			Nigri	5	3.9
Total	**128**	**100**	**Total**	**127**	**100**
SASS Filters					
MEA			**DG18**		
Sections	**CFU/m²**	**%**	**Sections**	**CFU/m²**	**%**
Nigri	24	61.5	*Flavi*	41	4.6
Flavi	6	15.4	*Circumdati*	838	94.7
Restricti	2	5.1	*Nidulantes*	1	0.1
Fumigati	7	18.0	*Aspergilli*	2	0.2
			Nigri	2	0.2
			Fumigati	1	0.1
Total	**39**	**100**	**Total**	**885**	**100**

3.2. Fungal Azole Resistance

Sixteen Cabin Ventilation Filters and 25 SASS filter samples were screened for the assessment of azole resistance. Thirteen of the 16 cabin ventilation filter samples (81.2%) presented fungal growth on 4 mg/L ITR including three fungal species (*Penicillium* sp., *Cladosporium* sp., and *Chrysosporium* sp.); 10 out of 16 (62.5%) samples presented two additional fungal species (*C. sitophila*, and *Rhizopus* sp.) on 1 mg/L VOR; and 9 out of 16 (56.2%) samples one additional fungal species (*Fusarium solani*) on 0.5 mg/L POS. Six out of the 16 samples (37.5%) presented the same fungal species in different azole-supplemented media (Table 3.3).

Table 3.3 - Fungal species/sections distribution in azole-supplemented media

	SAB		**ITR**		**VOR**		**POS**	
Fungal species/sections	**CFU/cm²**	**%**	**CFU/cm²**	**%**	**CFU/cm²**	**%**	**CFU/cm²**	**%**
A. section *Circumdati*	4	0.55		0.00		0.00		0.00
A. section *Flavi*	0.3	0.03		0.00		0.00		0.00
A. section *Fumigati*	1	0.09		0.00		0.00		0.00
A. section *Nigri*	135	17.00		0.00		0.00		0.00
A. section *Candidi*	63	7.98		0.00		0.00		0.00
C. sitophila	438	55.19		0.00	63	30.85	125	49.43
Chrysosporium sp.	63	7.90	0.3	2.90	0.4	0.19	1	0.20
Cladosporium sp.	3	0.35	3	37.68	2	0.99	0.5	0.05
Fusarium solani	0.1	0.02		0.00		0.00	0.5	0.05
Mucor sp.	63	7.88		0.00		0.00		0.00
Penicillium sp.	24	3.01	5	59.42	10	5.00	2	0.84
Rhizopus sp.		0.00		0.00	128	62.99	125	49.43
Total	**793**	**100**	**9**	**100**	**203**	**100**	**254**	**100**

3.3. Molecular Detection

Among the four *Aspergillus* sections investigated by PCR in the Cabin Ventilation Filters and in SASS Filter samples, all four were detected. *Aspergillus* section *Fumigati* was detected in 2 Cabin Ventilation Filters samples (2 out of 16; 12.5%), and 3 SASS Filter samples (3 out of 25; 12%) (Table 3.4); in this case, *Fumigati* was detected in more samples by culture-based methods (6 out of 16 Cabin Ventilation Filters samples; 37.5%, and 4 out of 25 swab samples; 16%).

Aspergillus section *Flavi* was detected in 2 Cabin Ventilation Filters samples (2 out of 16; 12.5%) and 1 SASS Filter sample (1 out of 25; 4%) (Table 3.4); molecularly this section was also detected in more samples by culture-based methods (5 out of 16 Cabin Ventilation Filters samples; 31.25%, and 8 out of 25 swab samples; 32%).

*S*ection *Nidulantes* was detected in 2 Cabin Ventilation Filters samples (2 out of 16; 12.5%) and 2 SASS Filter samples (2 out of 16; 12.5%); interestingly, *Nidulantes* was detected in also 2 Cabin Ventilation Filters samples (2 out of 16; 12.5%) and it wasn't detected by culture-based methods in none of the SASS Filter samples.

Aspergillus section *Circumdati* was only detected in one Cabin Ventilation Filter sample (1 out of 16; 6.25%) (Table 3.4); section *Circumdati was* detected by culture-based methods in both matrixes (3 out of 16 Cabin Ventilation Filters samples; 18.75%, and 9 out of 25 swab samples; 36%).

Table 3.4 - Molecular detection results from the samples analysed

***Aspergillus* sections**	**Matrices**	**CFU.m^{-2} (MEA/DG18)**	**C_q**
Aspergillus section *Fumigati*	Cabin Ventilation Filter	0/0	24.99
		0/0	33.69
	SASS Filter	0/0	35.04
		0/0	33.57
		0/0	32.37
Aspergillus section *Flavi*	Cabin Ventilation Filter	0/0	29.21
		1/58	40.35
	SASS Filter	0/0	38.25
Aspergillus section *Nidulantes*	Cabin Ventilation Filter	0/0	37.69
		0/0	38.9
	SASS Filter	0/0	39.55
		0/0	38.08
Aspergillus section *Circumdati*	Cabin Ventilation Filter	0/0	35.6

3.4. Statistical Analysis

Regarding fungal contamination in MEA, correlations were detected with fungal contamination in DG18 (rS=0.844, p=0.000), with Azoles resistance in ITR (rS=0.660, p=0.000), VOR (rS=0.776, p=0.000) and in POS rS=0.758, p=0.000), revealing that higher fungal counts in MEA is related with higher fungal counts in DG18, higher Azoles resistance in ITR, VOR and POS.

With respect to fungal contamination in DG18, correlations were detected with Azoles resistance in ITR (rS=0.615, p=0.000), in VOR rS=0.749, p=0.000) and in POS rS=0.745, p=0.000), indicating that higher fungal contamination in DG18 is related with higher counts in ITR, VOR and POS.

Regarding Azoles resistance in ITR, correlations were detected with Azoles resistance in VOR (rS=0.550, p=0.000) and in POS (rS=0.550, p=0.000), indicating that higher counts in ITR is related with higher counts in VOR and POS. As for Azoles resistance in VOR, a correlation was detected with Azoles resistance in POS (rS=0.680, p=0.000), revealing that higher Azoles resistance in VOR is related with higher counts in POS.

Finally, regarding molecular tools (*Flavi, Fumigati, Nidulantes and Circumdati*), no significant correlations were detected (Table 3.5).

Table 3.5 - Study of the relationship between fungal contamination (MEA and DG18), Azoles resistance (ITR, VOR and POS) and Molecular tools (Flavi, Fumigati, Nidulantes and Circumdati). results from Spearman's correlation coefficient

		Fungal contamination		**Azoles resistance**			**Molecular tools**			
		MEA	**DG18**	**ITR**	**VOR**	**POS**	***Flavi***	***Fumigati***	***Nidulantes***	***Circumdati***
Fungal contamination	MEA		0.844**	0.660**	0.776**	0.758**	-0.500	-0.200	0.400	-
	DG18			0.615**	0.749**	0.745**	-0.500	0.000	0.400	-
Azoles resistance	ITR				0.550**	0.550**	-0.500	-0.100	0.400	-
	VOR					0.680**	-0.500	0.000	0.400	-
	POS						-0.500	0.300	0.000	-
Molecular tools	*Flavi*							1.000	-	-
	Fumigati								-	-
	Nidulantes									-

Comparing fungal contamination, Azoles resistance and molecular tools between the sample type sample, the following statistically significant differences were found: i) fungal contamination in MEA (U = 2.0, p = 0.000) and in DG18 (U = 15.5, p = 0.000); ii) Azoles resistance in ITR (U = 116.0, p = 0.000), in VOR (U = 32.0, p = 0.000) and in POS (U25.0, p = 0.000). In either case, the sample type SASS Filters was the one that showed the highest fungal contamination in both MEA and DG18 and the highest Azoles resistance in ITR, VOR and POS.

Regarding molecular tools (*Flavi, Fumigati, Circumdati and Nidulantes*), no statistically significant differences were detected between the two sample types, what was expected given the residual number of data (Table 3.6).

Table 3.6 - Comparison of fungal contamination (MEA and DG18), Azoles resistance (ITR, VOR and POS) and molecular tools (Flavi, Fumigati, Circumdati and Nidulantes. Mann-Whitney test results

		Sample Type	N	Ranks		Test Statistics	
				Mean Rank	Sum of Ranks	Mann-Whitney U	p
Fungal contamination	MEA	Cabin Ventilation Filters	16	8,63	138,00	2,000	0.000*
		SASS Filters	25	28,92	723,00		
		Total	41				
	DG18	Cabin Ventilation Filters	16	9,47	151,50	15,500	0.000*
		SASS Filters	25	28,38	709,50		
		Total	41				
Azoles resistance	ITR	Cabin Ventilation Filters	16	15,75	252,00	116,000	0.023*
		SASS Filters	25	24,36	609,00		
		Total	41				
	VOR	Cabin Ventilation Filters	16	10,50	168,00	32,000	0.000*
		SASS Filters	25	27,72	693,00		
		Total	41				
	POS	Cabin Ventilation Filters	16	10,06	161,00	25,000	0.000*
		SASS Filters	25	28,00	700,00		
		Total	41				
Molecular tools	*Flavi*	Cabin Ventilation Filters	2	2,00	4,00	1,000	1,000
		SASS Filters	1	2,00	2,00		
		Total	3				
	Fumigati	Cabin Ventilation Filters	2	2,50	5,00	2,000	0,564
		SASS Filters	3	3,33	10,00		
		Total	5				
	Nidulantes	Cabin Ventilation Filters	2	2,00	4,00	1,000	0,439
		SASS Filters	2	3,00	6,00		
		Total	4				

*Statistically significant differences at the 5% significance level.

4. Chapter IV: Discussion

Fungal exposure is considered an occupational health threat, and it is highly associated with waste sorting (3,111). Waste handling has an extreme impact as a release source for a wide variety of microbiological agents (23,26,112), such as fungi. Fungi can influence human health in various ways that result in health outcomes such as infections, allergic reactions, irritation, and toxic reactions (23,113–115).

Waste industry is known to have a high load of fungal burden, being *Aspergillus sp.* between the most prevalent species identified along with other toxigenic species (3,6,67,86,116,117). In fact, *Aspergillus* sections *Fumigati* and *Flavi* were the most prevalent in Cabin Ventilation Filters, whereas in SASS Filters, the highest prevalence was seen in *Aspergillus* sections *Nigri* and *Circumdati*. This is particularly relevant since three of them (*Fumigati, Flavi and Circumdati*) are considered clinically relevant species (6,24) because of their toxigenical potential and since they work as opportunistic pathogens (61).

Additionally, it is important to highlight that *Aspergillus* section *Fumigati* is considered an opportunistic pathogen of immunocompromised hosts, and the *Aspergillus* species that cause an added wide range of respiratory disorders (5,118), being responsible for 90% of the cases of the most severe invasive aspergillosis (119). *Aspergillus* sections *Flavi* and *Circumdati* are known for their toxigenic potential (61). Ochratoxin A exposure should be considered in *Aspergillus* section *Circumdati*, and Aflatoxin B1 is the main toxigenic threat to be considered in *Aspergillus* section *Flavi*. In fact, both mycotoxins have already been detected in biomonitoring campaigns designed by the same waste sorting industry to assess occupational exposure to mycotoxins (68,71,120). The biomonitoring analyses also revealed that workers were exposed to multiple mycotoxins at the same time (71,120).

Contrary to what was seen in previous studies developed in this type of industry (eg. (3,70)), no *Aspergillus* sections presented azole resistance and the reason might be the overgrowth of *C. sitophila* and Mucorales order (*Rhizopus* sp.). Both species presented the highest prevalence in two of the three azoles (VOR: 62.99% *Rhizopus sp.;* 30.85% *C.sitophila* and POS: 49.43% *Rhizopus sp.;* 49.43% *C.sitophila*) and their overgrowth may inhibit the growth of *Aspergillus sp.* species. This is considered a limitation since the azole screening can only be made through culture-based methods. It is, therefore, possible that this limitation underestimates the presence of resistant

Aspergillus species which may lead to misleading results (86). However, to overcome this limitation, the isolates with higher clinical relevance (*Aspergillus* sections *Fumigati* and *Nigri*) are also recovered from the other media applied (MEA and DG18) and inoculated in the supplemented media used for the azole screening.

Although *Aspergillus* sections *Fumigati* and *Flavi* contamination were detected in a higher number through culture-based methods in both matrixes, molecular biology allowed the detection of this section in different samples. It was also interesting to acknowledge that *Aspergillus* section *Nidulantes* was detected in both matrixes by molecular tools and it was only detected in Cabin ventilations Filter samples by culture-based methods, being undetectable by culture-based methods in all SASS Filter Samples. That may happen because those species could be unviable for culture, but its presence was still detectable through its DNA by RT-PCR. Furthermore, *Aspergillus* section *Circumdati* was detected in both matrixes through culture-based methods, but it was only detected through molecular tools in one Ventilation Filter. Developments in molecular biology and high-performance sequencing offer new methods for biodiversity studies (69,121). Refined molecular instruments, such as high-performance sequencing, allow for rapid detection and are increasingly being used to collect information on a wide variety of aerosolized microbial biodiversity in several environments, including high contaminated areas such as waste sorting industries (3,69,70,117). Overall, high-throughput sequencing will reveal a more detailed measure of microbial exposure, including biodiversity knowledge (69,122). However, as all methods, this one also presents disadvantages such as the fact that it is only possible to make a microorganism identification but only at the level of the genus, this is a problem because to characterize the exposure risk, it is crucial to identify the microorganism to the species level (69). Furthermore, sequencing does not provide details on the viable component of the fungal contamination, which is crucial to estimate health risks by affecting biological mechanisms such as cytotoxic and inflammatory responses (94), besides presenting bias in calculation due variable DNA extraction efficiency, polymerase chain reaction (PCR) biases, copy number variance, and short size of amplified DNA fragments (69,123).

This study combines the use of culture-based methods and molecular tools, which is something important to highlight since complementing those methods is the only way to reduce the “misleading” results that each method can give when used individually. As previously reported in literature, culture-based methods are commonly used to perform

fungal identification, however, this method has its own drawbacks, such as how the growth rate and requirements of various fungal species impact other species in mixed cultures (23,86).

On the other hand, molecular tools are known to have certain characteristics that allow them to be more effective than other approaches, such as its precision, speed, intense analytical sensitivity of detection and the fact that it enables the detection and identification of dead or dormant microorganisms, as well as toxigenic strains of some fungal species (23,86,102,103). This corroborate the idea that culture-based methods and molecular tools should always be used together.

It is also important to highlight the use of more than one culture media in laboratory routine when culture-based methods are applied for fungal assessment (3). Given the diversity of fungal genera found in the air, malt extract agar (MEA) is unquestionably the culture medium most commonly recommended (24,124–127) and used in aerobiological research. It's a nutrient-rich medium for growing environmental fungi (24,126,128). MEA is ideal for yeasts and molds due to its high concentration of maltose and saccharides as energy sources. The acidic pH promotes fungal growth while inhibiting bacterial growth (24,126,128). However, there are many other effective culture media, such as DG18 (dichloran-glycerol Agar), which was originally used to count xerophilic molds and osmophilic yeasts. DG18 contains glycerol at a concentration of 16 percent (w/w), which reduces the water activity (aW) from 0.999 to 0.95 (24,125). Glycerol was selected over sodium chloride and sugars, which have historically been used to formulate reduced aW media, because of its benefits in the culture of a broader range of xerophilic fungi (24,125). Several studies have shown that this medium is a better choice for colony counting and obtaining a greater diversity of genera since it includes dichloran, which prevents the spread of fungi belonging to the Mucorales order, such as the *Rhizopus* and *Mucor* genera, and limits the colony size of other genera (24,52,70,89,90,129,130). Because of this restricting property, the medium is particularly suited for enumeration because it allows for the unobstructed growth of organisms that normally form small colonies (24,125,131). In this study Cabin Ventilation Filters presented a higher diversity of species on MEA media, whereas SASS Filters presented a higher number of species on DG18, which reinforces the idea that different (selective and non-selective) culture media should always be used as a complement (3,23,115).

Fungal contamination in MEA, and fungal contamination in DG18 seem to be related, since results showed significant positive correlations revealing that higher fungal counts in MEA is related with higher fungal counts in DG18 and higher Azoles resistance in ITR, VOR and POS. These findings demonstrate that both Cabin Ventilation Filters and SASS Filters, can give valuable information regarding not only the fungal contamination that these workers are exposed, but also the resistant profile of the species that are present in this type of environment. In either case, SASS Filters showed the highest fungal contamination in both MEA and DG18 and the highest Azoles resistance in ITR, VOR and POS. This is extremely important since this sampling device is placed in a 30 cm diameter of the workers' breathing zone, which, therefore, may allow a better representation of the worker's exposure through their airways. The same tendency was already found in a previous study (90).

The use of SASS Filters and Cabin Ventilation Filters, which are active and passive sampling methods respectively, is one of the this study features since the use of both methods combined, increases the sensitivity of the assessment (115) which allows a more accurate replication of the real scenario exposure to fungal contamination. It was possible to find different species in each matrix, for example, *Cladosporium sp.* was only detected in SASS Filters, whereas species like *C.sitophila* and *Mucor sp.* were only found in Cabin Ventilation Filters. *Aspergillus sp.* was detected in both matrixes but with a higher prevalence in SASS Filters. The same trend was observed in other studies where different sampling methods were applied (90,92) and it was already reported that the sampling methods and culture based-methods define the *Aspergillus sp.* distribution(8).

The assessment of the occupational exposure of workers in waste industries is crucial, since it is considered a highly contaminated environment. Employers are required to evaluate the risks associated with biological agent exposure under the 2000/54/EC Directive, which summarizes the concepts of risk assessment, prevention, and biological agent control (5,132). As a result, they can gather information about the harmful biological agents present in the workplace, identify them, and list the activities performed by workers that may increase exposure as well as the time of exposure to each biological agent. Then, appropriate measures to minimize worker risks must be enacted, as well as exposure prevention and control (5).

Taking into account the contamination of *Aspergillus* sections found in this study, and in waste industries in general (3,6,67,86,116,117), it is crucial to use a

stablished protocol like suggested by Viegas et al. (2017) (6), to perform a proper *Aspergillus* sp. burden exposure assessment in this type of highly contaminated industry. The suggested protocol is suitable for highly contaminated settings, as waste industry, and it takes into account the drawbacks of using only culture-based methods (Figure 4.1).

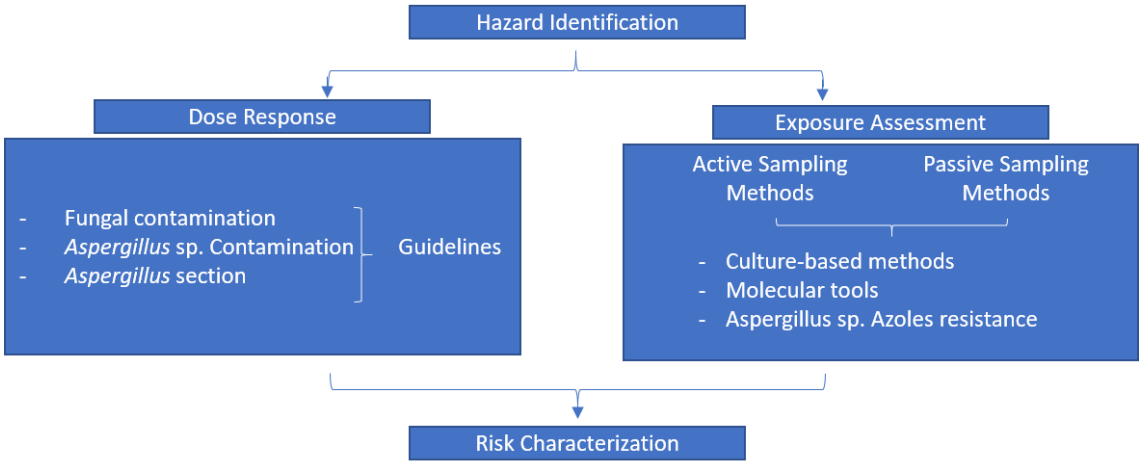

Figure 4.1 - Protocol to ensure a proper Aspergillus sp. assessment adopted from Viegas et al. 2017 (6)

5. Chapter V: Final Considerations

This chapter is dedicated to the conclusions, the expected limitations of the study and some suggestions for futures studies.

5.1. Conclusions

Overall, it was possible to conclude that the use of different sampling methods such as the Cabin Ventilation Filters and the SASS Filters to assess the occupational exposure to fungi in this type of industry is a good method since it allows us to perform a more complete characterization of the fungal contamination. Furthermore, it is also clear that the complement between culture-based methods and molecular tools allows us to perform a more accurate characterization of the fungal contamination.

Waste industry is, indeed, an environment with a high fungal burden, as it was possible to confirm with this study, which consequently increases the risk of health problems in these workers. Therefore, this study reinforces the need to assess the exposure to fungal contamination in this specific occupational environmental.

5.2. Study Limitations

Some of the limitations detected in this study are the fact that the assessment of azoles resistance is dependent on the growth of the isolates, due to the impossibility to detect mutations directly in the sample; the fact that sampling was only carried out in the summer, thus neglecting the influence of different seasons conditions; and, the fact that the team that performed the field work and the laboratory work was not the same, may bring some limitations in the interpretation of results.

5.3. Suggestions for Future Studies

This project will bring innovative aspects since after the assessment of occupational exposure, and depending on the results obtained, a sampling and analysis protocol will be performed for the assessment of occupational exposure to fungi, with the aim of assessing exposure in drivers in order to decrease workers exposure.

As a suggestion for future studies, it would be interesting to re-evaluate exposure to

microbiological agents after presenting the results to workers It would also be interesting to add another sampling method such as Filtering Respiratory Protective Devices (FRPD) and mechanic protection gloves (MPG) as previously performed (3,70). Both protection devices are used as personal protective devices, and therefore can mimic the actual inhalation exposure situation (FRPD) or the fungal contamination present in the workplace (MPG) (3). Thus, it would be interesting to compare the results obtained with the results from the Sampling methods applied in this study to verify if the results follow the same trend.

5.4. Ethical and Legal Considerations

Ethical and legal considerations do not apply to this study.

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