Universidade de Aveiro Departamento de Biologia
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Raquel do Nascimento Amaro

Characterization of fungal community associated with house dust

Caracterização da comunidade fúngica associada ao pó doméstico

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Toxicologia e Ecotoxicologia, realizada sob a orientação científica da Doutora Ana Catarina Almeida Sousa, Investigadora em pós-doutoramento do CICECO (Centro de Investigação em Materiais Cerâmicos e Compósitos), no Departamento de Química da Universidade de Aveiro, e co-orientação do Doutor Carlos Miguez Barroso, Professor Auxiliar no Departamento de Biologia da Universidade de Aveiro.

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“Deus Quer, o Homem sonha e a Obra nasce.”

Fernando Pessoa

o júri

presidente

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palavras-chave Comunidade fúngica, pó doméstico, asma, qualidade do ar interior.

resumo

Com a crescente urbanização, a população mundial passa cada vez mais tempo no interior de edifícios, onde a exposição a contaminantes pode ser elevada. Este cenário leva a uma degradação da Qualidade do Ar Interior (QAI) que, em casos extremos, pode conduzir ao Síndrome do Edifício Doente (SED).

É possível encontrar fungos em todo o tipo de ambientes e a comunidade fúngica encontrada no interior dos edifícios desempenha um papel essencial no estado de saúde dos indivíduos que frequentam esses locais. O pó em específico, atua como um reservatório de todos contaminantes presentes no interior dos edifícios, incluindo fungos, e pode ser utilizado para caracterizar o ambiente interior dos respetivos locais.

A convivência dos indivíduos com os fungos no interior de um edifício nem sempre é benéfica para a saúde. Existe uma forte associação entre os pacientes com doenças respiratórias alérgicas e a sensibilização a fungos, onde os últimos desempenham um papel importante no desenvolvimento, persistência e gravidade das primeiras. Para este tipo de indivíduos imunologicamente sensíveis, a exposição à contaminação fúngica pode desencadear os sintomas respiratórios como a asma.

Deste modo, este trabalho pretendeu numa 1ª fase caracterizar a comunidade fúngica do pó em ambiente doméstico de habitações construídas em diferentes décadas utilizando diferentes técnicas de amostragem de pó e numa 2ª fase caracterizar a comunidade fúngica presente em ambiente doméstico de doentes asmáticos e respetivos controlos por forma a avaliar possíveis associações. Este trabalho foi dividido em dois pontos fulcrais: i) identificar a comunidade fúngica no pó doméstico e a sua abundância ii) associar os géneros fúngicos encontrados com a severidade de asma.

Os géneros fúngicos maioritariamente encontrados foram *Aspergillus*, *Penicillium*, *Cladosporium*, Alternaria e leveduras. Quanto à associação à exacerbação da asma, não foi encontrada nenhuma relação. No entanto, dada a natureza preliminar do ponto ii), será necessário um número maior de amostras, por forma a tirar conclusões mais robustas.

keywords Fungal community, house dust, asthma, indoor air quality.

abstract

With the increasing urbanization, the world population spends more and more time indoors, where exposure to contaminants inside buildings can be high. This scenario leads to a degradation of Indoor Air Quality (IAQ), which, in extreme cases, can lead to Sick Building Syndrome (SBS).

Fungi can be found in all types of environments and the fungal community found inside buildings plays an essential role in the health of individuals that use these locations. Dust in particular, acts as a reservoir of all contaminants inside buildings, including fungi and can be used to characterize the indoor environment.

The coexistence of individuals with the fungi in the interior of a building is not always beneficial to health. There are strong associations between the patients with respiratory allergies and sensitization to molds where the latter play an important role in the development, persistence and severity of the former. For this type of immunologically susceptible individuals, exposure to fungal contamination can trigger respiratory symptoms such as asthma.

Thus, this work aims in a first stage to characterize the fungal community in house dust samples from houses built along different decades using different dust sampling procedures and in a second stage to characterize the fungal community in dust from the houses of asthmatic patients and respective controls in order to unravel possible associations. This work was divided into two key points: i) to identify the fungal community in house dust and its abundance ii) to associate the fungal genera found with the severity of asthma.

The most abundant fungal genera found were *Aspergillus*, *Penicillium*, *Cladosporium*, *Alternaria* and yeast. As for the association to asthma exacerbations, no association was found. However, given the preliminary nature of point ii), a larger number of samples will be necessary in order to draw any robust conclusions.

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General Introduction

1. Indoor Environment

With modernization of the society, individuals spend more time inside buildings. Currently people spend a large amount of their daily time indoors, from 85 to 90%, wherein 2/3 of this time is spent at home (European Environment Agency, 2013).

Indoor environment is defined by the environment inside homes, workplaces, schools, vehicles and all the other closed environments (Viegi et al., 2004). Its constitution is strongly influenced by the outdoors contaminants (Lee et al., 2000), but usually, indoors have higher concentrations of contaminants than outdoors (Hulin et al., 2012; Sundell, 2004; Viegi et al., 2004). In fact, studies by the United States Environmental Protection Agency (US-EPA) demonstrated that indoor pollutants' levels could be 2 to 5 times higher than outdoors, therefore indoor contamination now ranks in top five amongst public health risks (US EPA, 2013). Hereupon, a good Indoor Air Quality (IAQ) is an essential determinant to an healthy life (WHO, 2010).

Particulate matter (PM), carbon monoxide, the different ways of exposure to tobacco smoke, pesticides, solvents, Volatile Organic Compounds (VOCs), radon, asbestos, metals, occupation-related contaminants and biological allergens, such mites, allergens and moulds, are some of the indoor contaminants that are possible to address through environmental analysis (WHO, 2008).

Table 1 Main indoor pollutants, related sources and some threats towards human health (International Agency for Research on Cancer, 1987; Kim et al., 2013; Le Cann et al., 2011; Mercier et al., 2011; Tischer & Heinrich, 2013; US EPA, 2016; Viegi et al., 2004; WHO, 2000).

Indoor Pollutants	Typical source	Threats
Carbon Monoxide (CO)	Fuel, tobacco, wood and coal combustion; gas ranges and pilot lights; unvented kerosene and gas heaters	Deleterious health effects related to the lack oxygen in tissues/organs; dead
Nitrogen Dioxide (NO_2)	Gas ranges and pilot lights; unvented kerosene and gas heaters	Respiratory effects Asthma exacerbation
Inhalable Particles	Fireplaces; tobacco, wood and coal combustion	Increase respiratory symptoms; decrease lung function; asthma exacerbation; hearth attacks and irregular heartbeat; premature death on people with lung/ hearth diseases
Environmental Tobacco Smoke	Tobacco cigarettes; cigars and pipes	Mutagenic, Carcinogenic and cardiovascular effects; Chronic diseases as lung cancer and heart disease
VOCs (Aldehydes)	Furniture; solvents; paints; adhesives; cleaning products; insulation materials; tobacco smoke	Respiratory effects; Asthma
Aliphatic Halogenated Hydrocarbons	Furniture, solvents, paints, adhesives, cleaning products, tobacco smoke, insulation materials	Headaches; eyes and nose irritation; Carcinogenic
Aromatic Hydrocarbons (PAHs)	Outdoor air; cooking, smoking, unvented combustion appliances	Skin irritation and inflammation; eye irritation; nausea and vomiting; carcinogenic; DNA, cataracts, kidney and liver damage; gene mutation; cell damage; cardiopulmonary mortality
Metals (Lead)	Lead-based paints; diet; toys	Affect nervous and immune systems, the kidney's function, and the oxygen transportation; neurological effects on children; cardiovascular effects on adults
House Dust Mites	Dust; bedding; carpeting	Respiratory symptoms
Small animals	Dandruff; Feathers	Respiratory tract infections
Cockroaches	Floors	Respiratory tract infections
Fungi	Dampness; Dust	Respiratory and allergic health disorders; asthma exacerbation
Pollens	Plants	Respiratory tract infections
Bacteria	Dust	Respiratory tract infections
Endotoxins	Air; dust	Increase severity of asthma

All those contaminants can lead to a poor Indoor Air Quality (IAQ), causing complains about indoor air, which sometimes leads to nonspecific building-related symptoms, commonly called Sick Building Syndrome (SBS).

1.1. Indoor Air Quality and Sick building Syndrome

Indoor Air Quality is influenced by chemical, physical factors and bioaerosols and an upward of one of those factors can lead to Sick Building Syndrome (Di Giulio et al., 2010). The definition of SBS appeared on early 1970s (Chang et al., 2015) and it includes a range of health symptoms of unclear aetiology associated with the environment of a specific building, among them mucous membrane symptoms (eye, nose, and throat irritation), skin irritation, fatigue and headache (Burge, 2004; Fisk et al., 2009; Tsai et al., 2005). Usually there is a temporal relationship between the individuals' symptoms and the building, with an improvement in the symptoms after the individuals leave a particular building (Burge, 2004; Crook & Burton., 2010). Traditionally, SBS was an occupational related problem but nowadays this term is commonly applied to domestic dwellings, especially those with water damage problems (Burge, 2004).

There are several parameters evaluated during the monitoring of IAQ: temperature, humidity, formaldehyde, carbon dioxide, particulate matter, volatile organic compounds, odours, bacteria, fungi, amongst others (Chang et al., 2015; WHO, 2010; Wolkoff et al., 2006). The quantity of microorganisms is determinant for air quality. In a normal environment, there are few airborne microorganisms, but an increase in the number of those microorganisms may represent a disease risk factor (Di Giulio et al., 2010). One important group of those microorganisms are fungi. The exposure to airborne fungi can lead to several health effects on humans (Li & Yang, 2004) and there are some studies relating levels of indoor airborne fungi with the prevalence of some SBS symptoms (see eg. Chang et al., 2015; Crook & Burton, 2010).

1.2. Fungal community

Fungi communities are ubiquitous and easily found in all types of environments. About 100,000 species of fungi have been described including mushrooms, moulds and yeasts (Madigan et al., 2009; Prillinger et al., 2002).

Fungi are non-motile eukaryotic organisms so they have membrane-enclosed nucleus which contains genetic material and several membrane-bound cytoplasmic organelles (Deacon, 2005; Madigan et al., 2009). Therefore, most of them are multicellular. Fungi are classified as heterotrophs – they nourish through different organic means, by absorbing them from substrate. Some are saprophytes – the ones that digest dead organic matter and

wastes – and others are parasites, and obtain nutrients from tissues of other organisms (Black, 2002; Fisher & Cook, 1998).

A typical fungus has a body called thallus, consisting of a network termed mycelium formed by a mass of threadlike structures – the hyphae, with an apical growth in branches or by elongation at the tips. These type of organisms are called filamentous fungi (figure 1). But some fungi replaced their typical growth as hyphae for yeasts – usually unicellular cells (Black, 2002; Deacon, 2005; Fisher & Cook, 1998; Madigan et al., 2009).

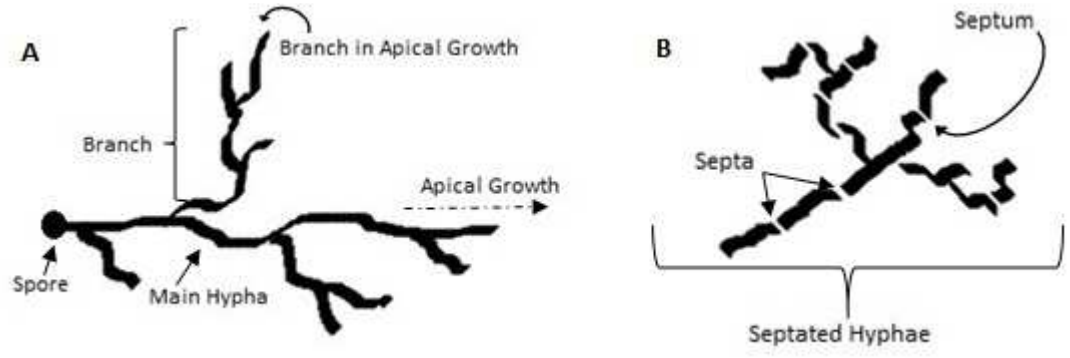

Figure 1 Representation of a mycelium (A) with septated hyphae, adapted from (Fisher & Cook, 1998); (B) with aseptated hyphae and his apical growth, adapted from Vergara-Fernández et al. (2011).

Hyphae are tubular cell walls that surround the cytoplasmic membrane (Madigan et al., 2009). They can be septated or aseptated (Figures 1a and 1b), when septated, there are cross walls called septa which divide the hyphae into small fragments (Fisher & Cook, 1998).

The septa have pores to help the transition of cytoplasm and nuclei between cells. Some fungi only have one septal pore, with an organelle called Woronin body, this organelle acts like a pore-blocker when a hyphal cell is damaged or old, so the tainted material cannot enter in a healthy cell (Black, 2002).

The mycelium has the function of nourishing the fungus, absorbing small nutrients, molecules through the enzymes released by mycelial cells (Black, 2002). There are three types of mycelia, classified according to where they grow: vegetative, aerial and reproductive mycelium (figure 2). The vegetative ones grow in or on the substrate and their function is to absorb nutrients. Aerial mycelia have aerial hyphae which grow above the surface of the substrate, almost all these constitute the visible colony. Reproductive mycelia develop where the reproductive structures grow (Fisher & Cook, 1998).

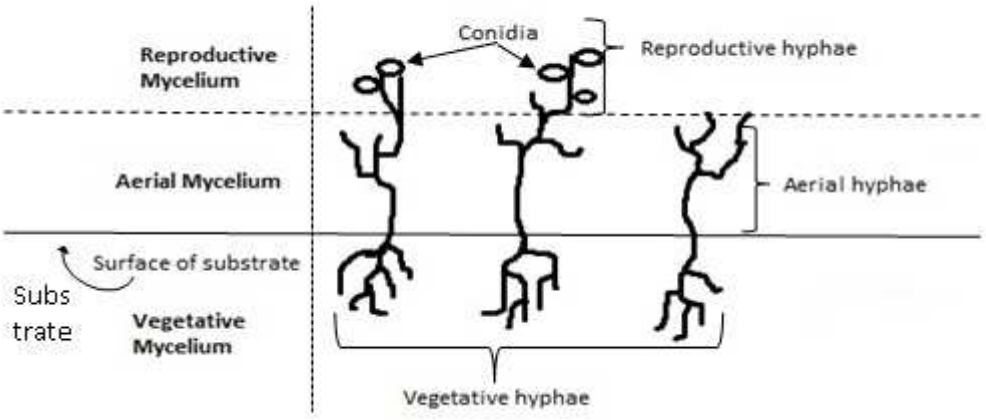

Figure 2 Representation of the tree different types of mycelia, adapted from Fisher et al. (1998).

The majority of fungi's cell walls contain chitin, and only a few fungi have cellulose (Fisher & Cook, 1998). All fungi have lysosomal enzymes to digest damaged cells and also to invade hosts in the case of parasitic fungi. The yeasts, and other types of fungi, may also have plasmids, which have the ability to clone foreign genes into yeasts cells (Black, 2002). Almost all fungi can reproduce both sexually and asexually, however there are a few that only reproduce by asexual way (Black, 2002). These are coined as imperfect fungi, and they only have an anamorph form – just the asexual form of reproduction. Perfect fungi or teleomorphs reproduce by both ways, having more than one sexual form. They can also have at least one anamorph form. If there are more than one anamorph form associated to a teleomorph form, the first one are called synanamorphs and all the forms together are called holomorphs (Fisher & Cook, 1998).

Therefore, the taxonomy of fungi is based on the sexual reproduction and it is possible to separate these organisms into 4 main categories: Zygomycetes, Ascomycetes, Basidiomycetes and Deuteromycetes (table 2) (Fisher & Cook, 1998; Prescott et al., 2005).

Table 2 Classification of Fungi, adapted from Fisher & Cook (1998) and Prescott et al., (2005)

Class	Sexual Reproduction (Teleomorph)	Asexual Reproduction (Anamorph)	Common Name	Hyphae	Approximate Nr. Species
		Perfect Fungi			
Ascomycota	Ascospores	Conidia	Sac Fungi	Septated	35,000
Basidiomycota	Basidiospores	Conidia	Club Fungi	Septated	30,000
Zygomycota	Zygospores	Sporangiospores	Zygomycetes	Aseptated	600
		Imperfect Fungi			
Deuteromycota		Conidia	Fungi Imperfecti	Septated	30,000

Zygomycetes are multinucleate fungi or called coenocytic. They differ from the others by forming zygospores. These structures, which also produce spores, enclose zygotes which are the result of the fusion of the nuclei from multinucleate cells (Black, 2002; Madigan et al., 2009)

Ascomycetes range from single-celled species, from yeasts, to species which grow as filaments. They produce a sac-like asci cells with diploid nucleus, resulting from the fusion of two haploid cells from different mating during sexual reproduction. These diploid nucleuses go through meiosis and forms haploid ascospores, which are then released into environment. Asci can also be formed in a fruiting body called ascocarp. This type of fungi also reproduces asexually through the production of spores called conidia (Black, 2002; Madigan et al., 2009).

Basidiomycetes are usually the mushrooms, rusts, smuts and toadstools, yet in this group there are also yeasts and plant's and human's pathogens. They have a basidium, a single-cell structure in which basidiospores are formed. Basidiospores are sexual spores which germinate forming a septate mycelium. Mycelium cells unite into a dikaryotic form which grows and produces basidium that in turn produces basidiospores (Black, 2002; Madigan et al., 2009).

Deuteromycetes, also called the imperfect fungi, do not have any sexual stage in their life cycle, but due to their vegetative characteristics and their ability to produce asexual spores, most of these fungi are often associated to the Ascomycota group (Black, 2002).

Although fungal communities vary due to several indoor factors, such as occupation or heating ventilation and air-conditioning systems (HAVAC), at a global scale, indoor fungal diversity is also strongly influenced by the outdoor environment (Amend et al., 2010). Thus, the main indoor taxa are species also found in the outside of the dwellings – *Cladosporium*

sp., *Aspergillus* sp. *Alternaria alternata* and *Penicillium* sp. (Augustyniuk-Kram, 2013; Fairs, 2010).

1.3. Evaluation of the fungal community in indoor environments

There are several methods to collect samples to assess the diversity of the fungal community in indoor environments including: i) air sampling; ii) settled dust sampling and; iii) house dust vacuuming.

Air sampling collection implies a special device – a portable air sampler – to filter the air during a specific time period (Hicks et al., 2005).

Settled dust sampling is considered a passive method consisting in the placement of a sterile empty petri-dish at a specific location during a certain period of time in which dust falls into it. After the sampling period, the petri-dish is sealed and sent to the laboratory to analyse the fungal communities by seeding the dust in appropriate culture media (Adams et al., 2013; Barberán et al., 2015). Other passive methods include the use of Electrostatic Dust Fall Collectors, collectors that consist of electrostatic cloths used as collectors for dust sedimentation (Madsen et al., 2012).

In house dust vacuuming, a vacuum cleaner is used to collect the house dust during a specific time period and a specific area. In some cases the vacuum cleaner may have a special dust collector and filters attached, but most commonly vacuum cleaner used is the one that the house inhabitants' use to regularly vacuum their houses. (Norbäck et al., 2014; Pitkäranta et al., 2008). After collection, the dust is removed from the vacuum cleaner bag and samples are sieved before being analysed.

1.4. Indoor dust

Indoor dust works as a long-term reservoir of all kind of indoor contaminants, including fungi, being a useful tool for the characterization of indoor environments (Sousa et al., 2014a).

In fact, house dust is a matrix relatively easy to collect. Hence, it's application on human exposure to environmental contaminants studies has been increasing and recently its usage in large epidemiological studies has been recommended (Rintala et al., 2008; Sousa et al., 2014a; Sousa et al., 2014b; Whitehead et al., 2011) and should also be considered in Indoor Air Quality monitoring (Pan et al., 2000).

Dust sampling is carried out since 1940s (Conant et al., 1936; Rintala et al., 2012) and since then there is no consensus about the best collection methodology. Therefore there is no standard protocol for human exposure validated for the measurement of allergens and other contaminants in house dust (Mansour et al., 2001; Paustenbach et al., 1997). Despite the

knowledge on house dust potentialities, there are only few studies using dust as an indicator of microbial communities.

Exposure to house dust can result in several health effects on humans. In fact, there are several studies that demonstrated an association between certain dust properties and the Sick Building Syndrome symptoms (Gyntelberg et al., 1994; Mendell et al., 2002; Niven et al., 2000; Pan et al., 2000; Skov et al., 1987). Furthermore, there are several reports in the scientific literature relating the human exposure to house dust and the development and/or exacerbation of respiratory symptoms and diseases such as asthma (Calderón et al., 2015; Douwes et al., 2000; Van Dyken et al., 2011).

1.4.1. Characterization of indoor dust

Indoor dust is by definition a complex mixture of particles and biological material, that is found settled in all indoor surfaces, floors and carpets, most of the times brought from outdoors by indoor occupants or carried by indoor aerosols (US EPA, 1997, 2011). In fact, about 45 to 50% of the house dust comes from soil and street dust, 2 to 3% comes from tyre wear, cement and car emissions, 1% is salt and 43% of the house dust is material presumably organic (Fergusson et al., 1986). Recently, indoor sources of dust are also being considered as well as their contribution to the levels of contaminants present in dust. The major indoor sources include microorganisms, furniture, household objects, pollen and other allergens, smoke, the occupants (including pet's) and their activities (Butte et al., 2002).

House dust works as an adsorbent of organic and inorganic contaminants, so it can be considered one of the most important sources of human exposure to a large number of pollutants. Furthermore, dust can be considered an "archive" of indoor contamination, due to its ability to accumulate all kind of matter, and to its low capacity for degradation of contaminants, which leads to a continuous enrichment of chemicals (Butte et al., 2002; Cizdziel & Hodge, 2000).

There are several dust characteristics that influences the exposure potential to humans: i) the size of the particles; ii) the concentration of the contaminants; iii) the fine particle enrichment; and iv) the bioavailability of the fine particles and the larger particles (Paustenbach et al., 1997). Fine particles adhere more effectively to skin than the biggest ones (Kissel et al., 1996). In fact 50% of house dust particles have less than 50 µm of diameter (Roberts et al., 1991). And the fine particle enrichment has an impact on the amount of contaminants on house dust. The concentration of the contaminants is higher on the smaller size fraction of the particles (Beamer et al., 2012; Paustenbach et al., 1997).

2. Health impacts of fungal exposure

Individuals are continuously exposed to airborne fungi and generally this exposure doesn't translate into adverse effects in humans. In fact, there are some studies that support the called hygiene hypothesis (Gereda et al., 2000; Slameňová et al., 2003), which holds that a premature exposure to a rich microbial environment can decrease the risk of allergic diseases later in life, due to a stimulation of the immune-system to fight the allergy, so later the individual may have a non-allergic immune response (Strachan, 1989).

However, some fungi are in fact responsible for adverse health effects, especially related with respiratory diseases (Pei-Chih et al., 2000). The most frequent fungi found in indoor environments, especially in house dust, are: *Alternaria, Cladosporium, Aspergillus* and *Penicillium* and even yeasts, causing several allergic reactions on humans (Augustyniuk-Kram, 2013). These genera of fungi are consistent with those found outdoors, which suggests that indoors' fungal communities are influenced by fungal taxa from outdoors (Chew et al., 2003).

2.1. Human Respiratory System

The respiratory system is a complex system responsible for gas exchanges between living beings and the environment (Foster & Costa, 2005; Tu et al., 2013). Oxygen, which is essential for cellular function, enters the organism through nose or mouth during inhalation. Then, the inhaled oxygen passes through pharynx, larynx and reaches trachea that splits into two branches – the bronchi – which penetrate into the lungs. Once in the lungs, each bronchus bifurcates and forms bronchial tubes. These bronchial tubes form a network inside the lungs – the bronchioles – culminating in tinny sacs – the alveoli (see figure 3) (Netter, 2010; Tu et al., 2013).

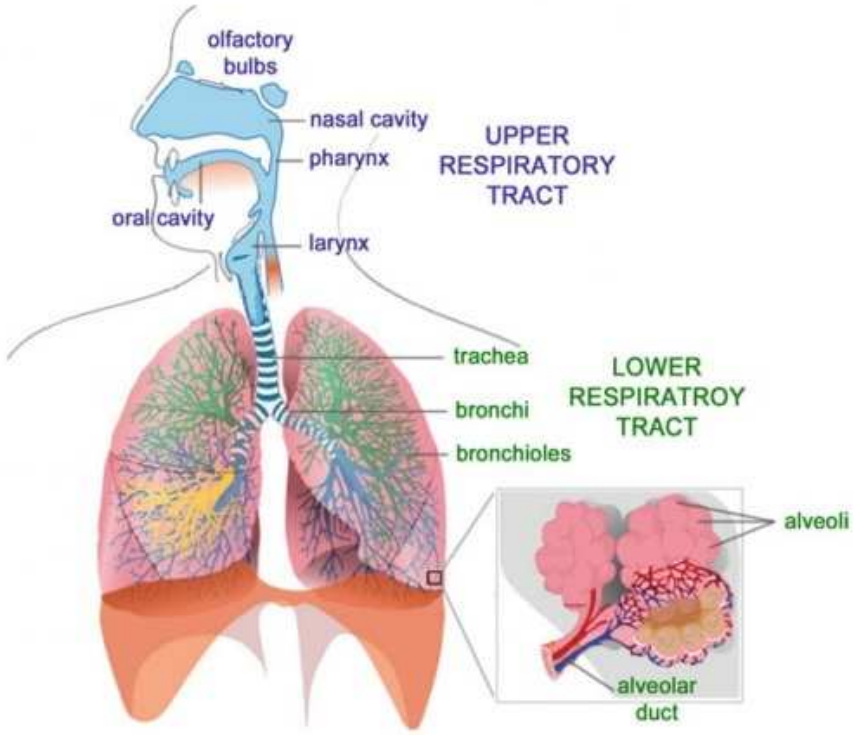

Figure 3 Human Respiratory System displayed by upper and lower respiratory tract, retrieved from Tu et al. (2013).

Once in the alveoli, the oxygen passes to the bloodstream to reach all the tissues in organism through the blood capillary network aggregated on the alveolar wall. At the same time, carbon dioxide (a waste product derived from cellular function) is excreted into the bloodstream to perform the inverse route and being exhaled from the organism to the environment. This gas exchange on alveoli is called hematosis (see Figure 4) (Tu et al., 2013).

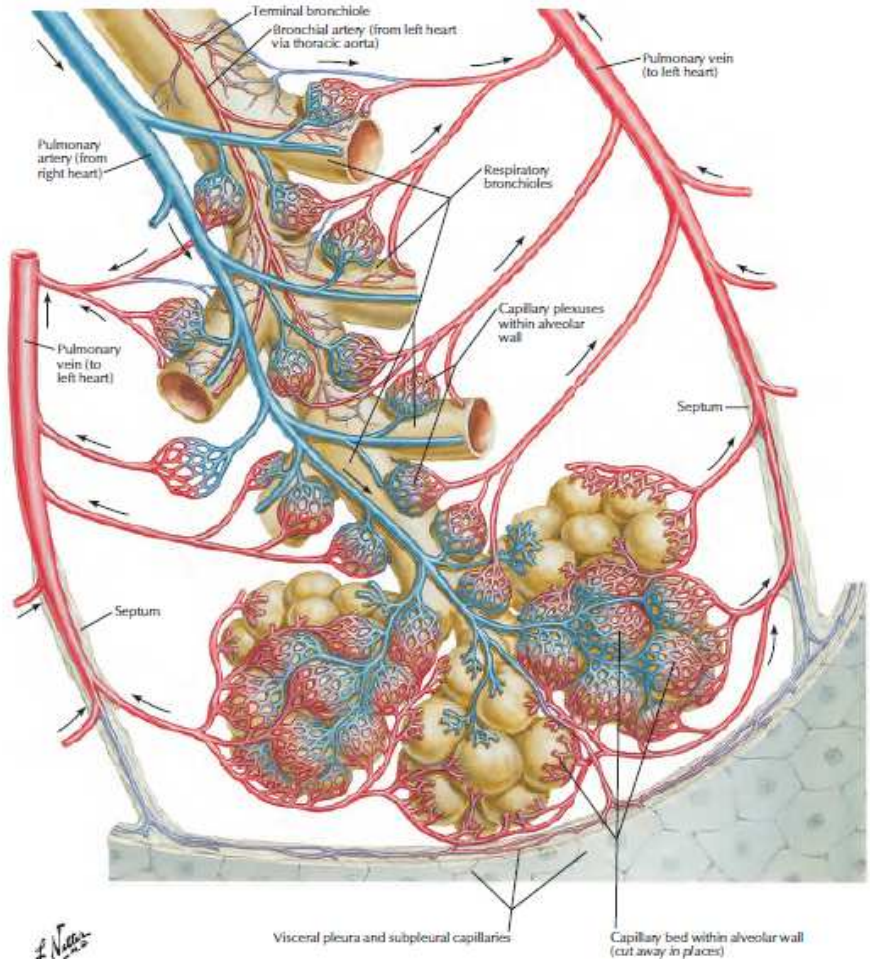

Figure 4 Pulmonary hematosis on alveoli, retrieved from Netter (2014).

Due to its function, the respiratory system can be divided into two sections: upper airways and lower airways. The upper airways include all organs that are outside de thorax – mouth, nose, pharynx and larynx -, and the lower ones are the organs located inside the last one – trachea, lungs, bronchi, bronchiole and alveoli (Tu et al., 2013).

Although the main function is gas exchange, this system is still responsible for the filtration, heating and humidification of inhaled air; for the smell, through the passage of air on olfactory bulbs; for the production of the vocal sound on larynx; for homeostasis of the pH of the organism; and for the protection of the organism, against microorganisms and particles (Tu et al., 2013).

2.2. Inhalation of organic particulate matter and fungal allergens

The surface of the respiratory system is one of the largest interfaces between humans and their environment, being constantly in contact with gaseous contaminants and particulate matter. The inhaled particles usually deposits on respiratory tract, leading to a great number of allergic reactions and cardio respiratory diseases (Foster & Costa, 2005).

Particulate matter (PM) availability is an important factor to determine whether it might be dangerous for health or not. The greater the amount of particles in the environment, the greater it is the possibility of particle inhalation and thus, the greater the probability of cardio and respiratory damage, since those are the primary targets of those pollutants (Kodavanti & Watkinson, 2005).

Particle size is a key aspect on particles' toxicity, as those particles are small enough to be respirable and to penetrate into the lungs are of major concern towards human health. Particles can be divided in three groups, regarding their sizes: coarse particles (from 10.0 to 2.5 µm of diameter); fine particles (from 2.5 to 0.1 µm of diameter); and the ultrafine particles (with <100 nm of diameter). In terms of health impacts, the particles with diameter between 10 µm and 10 nm are the ones of more concern (Heyder, 2004; Kodavanti & Watkinson, 2005; Sierra-Vargas & Teran, 2012).

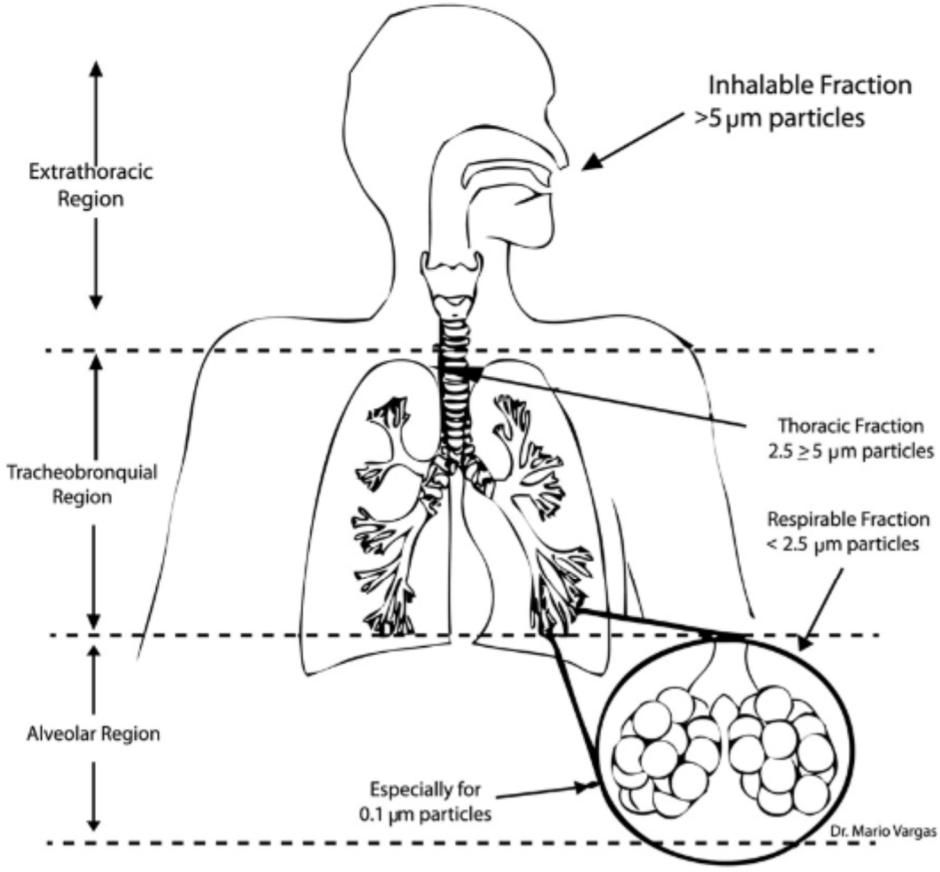

Figure 5 Regional deposition of particles on human respiratory tract, retrieved from Sierra-Vargas & Teran (2012).

Besides particle size there are other factors which determine the amount of inhaled particles, including their deposition location on respiratory system which is dependent on the particles themselves (chemical and other physical characteristics), and the individual

anatomical and physiological characteristics (airways anatomy, inhalation routes and biological factors) (Agnew, 1984).

Once on the respiratory system, there are three types of mechanisms for particle deposition: i) diffusion; ii) impaction; and iii) sedimentation. Diffusion occurs when, after inhalation, the particles target the surface of the airway through Brownian movement - aleatory movement in a fluid. Impaction is related with the inertia, ie, the particles are not able to follow the air flow and reach the airways' surface. Sedimentation occurs on airways wall (Foster & Costa, 2005). The particles with diameters ranging between 0.5 and 3 µm usually get deposited on the nose by impaction and sedimentation (Heyder et al., 1985; Heyder, 2004). Those that aren't retained on the nose, pass through larynx, provoking cough and bronchospasms and may reach the tracheobronchial tree (Bennett & Brown, 2005). Once there, the particles can deposit by impaction and by diffusion on bronchi, and/or by sedimentation and diffusion on bronchiole. The ones which diameter ranges between 2 µm and 0.1 µm can reach the alveoli and deposit by sedimentation (Heyder, 2004; Lippmann et al., 1980).

Due to its reduced size, fungal spores can penetrate the airways and cause illicit adverse reactions. The fungal spores size can range between 2 to 500 µm, but the average size range between 2 to 10 µm (Simon-Nobbe et al., 2008) and once on the respiratory tract, the spores germinate and only then release allergens (Burge, 2005). Moreover inhalation of fungal fragments with size in the order of submicrometer can also occur (Green et al., 2006). The presence of allergens on human organism initiates a specific immune response through the linkage between those allergens to antibody IgE (Crameri, 2015). The number of fungal proteins found to be able to bind IgE is around 200 and according to some authors this number is far to be a realistic one (Simon-Nobbe et al., 2008). Most of the fungal allergens are strongly associated to allergic diseases such as asthma (Burge, 2005; Crameri, 2015; Simon-Nobbe et al., 2008).

3. Asthma

In 2014, and according to the Global Asthma Network (Global Asthma Network, 2014), the number of individuals reported with asthma was over 334 million.

In Portugal, the disease has a great impact on population's health and the tendency is to increase. In the latest report from the National Observatory of Respiratory Diseases (Observatório Nacional das Doenças Respiratórias, 2016) it was estimated that there are over a million of asthmatic Portuguese with different gravity levels. In the same report, although Portugal was considered the EU country with the lowest rate of hospitalization for asthma, an increase in the number of hospitalizations from 2005 to 2014 was registered

and an increase in the number of deaths with this condition also increased during the same time period. In 2014, the prevalence of the disease varied between regions, with a sharpest increase rate of hospitalizations in the Center, followed by Algarve, North, Lisbon and Vale do Tejo and Alentejo. The rate of hospitalizations by asthma was higher on the group under 18. The group with 40 to 64 years suffered a decrease in the hospitalization rates, as well as the group of 65 to 79. There was a small increase in the hospitalizations for the group above 79, but this could be a consequence of the increase in the average life expectancy (Observatório Nacional das Doenças Respiratórias, 2016).

Asthma is an immunologically mediated hypersensitivity disease of the airways (WHO, 2003). It's a chronic inflammation of the bronchial tubes in the lungs, provoking a reversible obstruction of the flow of air in and out of airways, when exposed to a certain stimuli (see figure 6). The characteristic symptom of asthma is wheezing – a high-pitched whistling sound heard during breathing. However, not always this symptomatology occurs. Other asthma symptoms include breathless, chest tightness and coughing (Global Asthma Network, 2014).

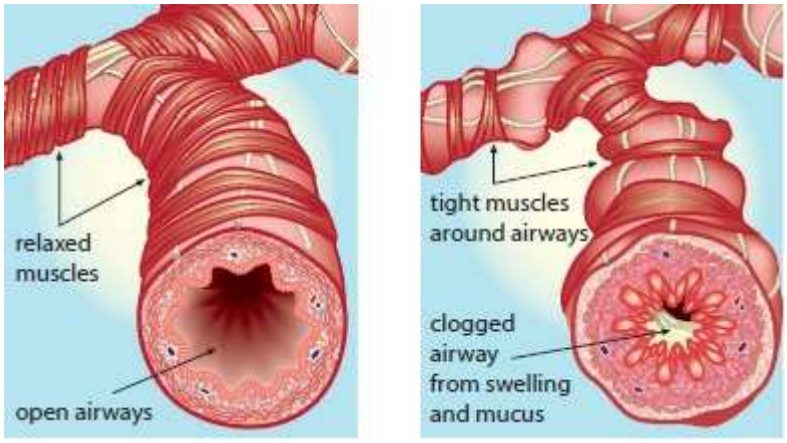

Figure 6 Healthy bronchial tube (A); and Asthmatic Bronchial tube (B), retrieved from American Thoracic Society (2013).

Asthma can affect all ages, but is more common in early childhood (before age 7) and usually the symptoms disappear around the age of 16. The deaths for asthma are uncommon and usually occur in elderly individuals. Even so, it just represents less than 1% of the total deaths worldwide and most of the times can be preventable (Global Asthma Network, 2014).

There are several factors affecting asthma condition, and they can be divided in two major groups: genetic and environmental factors. Individuals with a family history of allergic disease are more prone to develop the same condition (Global Asthma Network, 2014; WHO, 2003) and therefore genetic predisposition is considered a risk factor for asthma.

There are strong literature that evidences a small number of genetic variants that influence asthma risk – especially in children.

Concerning the environment, there are according to the Global Asthma Network, several factors to be considered:

• Common cold and exercise: the infection of the upper airways that occurs during a cold can trigger an asthma attack. The same background of infection can occur by effort, during exercise and thus initiate an asthma attack.

• Irritants: exposure to cigarette smoke, different vapours from cooking, heating or from vehicles can lead to an asthma attack. The same can occur by exposure to certain cosmetics and aerosol sprays.

• Second-hand smoke: this air pollutant can induce the appearance of asthma symptoms either in children and adults. It is particularly dangerous during prenatal exposure.

• Pharmaceuticals: Asthma attacks are more common in children who were treated with antibiotics in early stages of life. However this factor is still unclear mostly because wheezing symptoms are usually treated well before the recognition of asthma manifestations with drugs.

• Occupational exposure: bakers, woodworkers, farmers and professionals exposed to laboratory animals and to a certain chemicals are associated with the development of occupational asthma.

• Allergens: allergen exposure causes IgE sensitization, and the continued exposure can result in asthma.

• Animals: there are no consistent evidences that animals are either a risk factor or a protective one.

• Dampness and moulds: according to the Global Asthma Network its contribution is still unclear, because only few individuals are demonstrably allergic to fungal moulds and because dampness can occur in the houses of both allergic and non-allergic forms of asthma patients. Nevertheless, dampness and moulds appears more in asthmatic patients' homes.

Several studies performed over the last years examined the association between asthma and fungal exposure and provided further evidences on the role of these biological contaminants in the development and progression of this disease.

The first evidences of fungal sensitization of asthmatic patients dates from 1698, when a famous asthmatic physician, John Floyer noticed a deterioration of asthma, when exposed

to damp on houses (Sakula, 1984). Thenceforth, mould sensitivity has been associated all over the years to triggering of asthma attacks, and its consequences.

In a work performed by Zureik et al. (2002), the authors concluded that sensitization to moulds was significantly associated with severity of asthma, for the genera *Alternaria* and *Cladosporium*. In a meta-analysis carried out by Mendell et al. (2011), several fungal species were able to induce inflammatory reactions. Sensitivity to fungi of the genus *Alternaria* and *Cladosporium* was also associated with persistence and severity of allergic asthma by Knutsen et al. (2012). Markowicz et al. (2014) identified *Aspergillus versicolor* as a crucial fungus in triggering symptoms of asthma. There was a positive association between the *Aspergillus versicolor* DNA concentrations and the lack of air during the day in the participants. It was also possible to validate that the concentration of ergosterol (often used as fungal marker) was related to the diagnosis of asthma, proving to be a risk factor for this disease.

The presence of fungi inside the home and the diagnosis of asthma in the house inhabitants was also studied. Blanc et al. (2013) used the Environmental Relative Moldiness Index (ERMI), this index is a quantification method of the exposure to mould inside the buildings in order to assess the effect of fungi in patients with asthma and rhinitis. The authors concluded - that the average value ERMI in homes of adults diagnosed with asthma was significantly higher than the average value of ERMI in households selected at random (control group) in the same geographic area of study in Northern California.

Fungal exposure can require multiple hospital admissions by asthmatic individuals (O'Driscoll et al., 2005). In a study conducted by Black et al. (2000), about fungal allergens sensitization on patients with asthma attacks admitted on Intensive Care Units (ICU), it was concluded that there was an association between life-threatening asthma and sensitization to one or more fungal allergens.

Some fungi, such as *Aspergillus*, can even act as opportunistic pathogens and attack the respiratory system of patients with previous lung diseases (Ader, 2010; Smarakoon & Soubani, 2008).

In the worst case scenario, fungal allergens derived from environmental moulds can play a role on asthma-related mortality (Targonski et al., 1995).

4. Aim and organization of the thesis

The aim of this thesis is to characterize the fungal communities in house dust samples from households located in Covilhã municipality. Hence two different studies were performed: the first one aimed to characterize the fungal community in dust samples from houses

constructed in different decades (from 1960s to 2010s) from the 6X60X6 project and to compare two dust sampling strategies. The second study aimed to characterize the fungal community in dust samples from the houses of asthmatic patients and controls in order to understand possible associations between the fungal composition and asthma exacerbations.

The present dissertation is organized in 4 different Chapters. Chapter 1 provides a general introduction about fungal communities on house dust and their relationship with asthma exacerbation. Chapter 2 and 3 are structured as research papers and correspond to the 6X60X6 Project and to the asthma study, respectively. In chapter 4, general remarks are presented and the obtained results are discussed and compared with previous works.

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Chapter II:
House dust fungal communities' characterization: a double take on the six by sixty by six project (6x60x6)

Based on the submitted manuscript:

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HOUSE DUST FUNGAL COMMUNITIES' CHARACTERIZATION: A DOUBLE TAKE ON THE SIX BY SIXTY BY SIX PROJECT (6X60X6)

1. Abstract

Fungi are a group microbes that are found with particular incidence in the indoor environment. Their direct toxicity or capability of generating toxic compounds has been associated with a large number of adverse health effects, such as infectious diseases, allergies and other toxic effects. Given that in modern society people spend a large part of their time indoors; fungal communities' characterization of this environmental compartment assumes paramount importance in the comprehension of health effects. House dust is an easy to obtain, time-integrative matrix, being its use in epidemiological studies on human exposure to environmental contaminants highly recommended. Furthermore, dust can carry a great variety of fungal content that undergoes a large number of processes that modulate and further complexify human exposure. Our study aims to quantify and identify the fungal community on house dust samples collected using two different methodologies (an approach not often seen in the literature): active (vacuum cleaner bags) and passive sampling (dust settled in petri dishes). Sampling was performed as part of the ongoing 6X60X6 Project in which six houses from Covilhã (Portugal), with building dates representative of six decades, were studied for a period of sixty days.

Keywords: Indoor environmental quality; Fungi; House dust

2. Introduction

In modern society, most people spend a large part of their time indoors, being exposed to a broad number of contaminants, which may come from the outdoors or be locally generated as the result of household activities and building materials as well as from the decay of consumer products (WHO, 2010). The built environment air pollution is considered a major cause of morbidity and mortality all over the world (WHO, 2009) and as such the study of indoor environmental quality is of great importance.

Fungi are a group of well-known microbes that are easily found in all types of environments (Madigan et al., 2009) with particular incidence in the indoor environment. Their direct toxicity or capability of generating toxic compounds (e.g., mycotoxins and harmful antigens)

has been associated with a large number of adverse health effects in humans, such as infectious diseases, allergies and other toxic effects (Méheust et al., 2014). Fungi produce tiny spores with those smaller than 10 µm being particularly hazardous to human health, as they can enter the respiratory tract and reach the alveoli (the gaseous exchange areas of the lung), which may lead to respiratory infections and allergic (Araujo & Cabral, 2010; Stetzenbach et al., 2004).

Spores can be suspended in the air, deposited on various surfaces and included within different matrices such as house dust (Nevalainen et al., 2015). This matrix results essentially from materials tracked indoors and the settling of airborne particles, a process that can take weeks or even months (especially the latter), being therefore regarded as a time-integrated sample (Dallongeville et al., 2015; Rintala et al., 2012). Furthermore, house dust is an easy sample to obtain and its use in epidemiological studies on human exposure to environmental contaminants, has been highly recommended (Sousa et al., 2014a; Sousa et al., 2014b). Its relevance as an important exposure pathway is exacerbated by the fact that in general, adults may ingest 50 mg of dust per day and inhale 0.8 mg, and children (a risk group) may ingest 100 mg per day and inhale 2 mg (see for example (Coelho et al., 2014)).

Dust can carry a great variety of fungal content - intact fungal conidia, spores, hyphae and other. This microbial content undergoes processes of deposition, removal, proliferation, death and degradation, contributing towards the content and diversity of fungi in this type of sample (Rintala et al., 2012).

To date several papers have been published on the fungal community in house dust samples (see e.g. the review by Rintala et al. (2012)) but there is still limited information on this topic, particularly for Portuguese households. Furthermore, comparisons between sampling strategies are scarce in the literature. Hence, our study aims to quantify and identify the fungal community on house dust samples collected using two different methodologies: active and passive sampling. For this purpose we analyzed dust collected from vacuum cleaner bags and dust settled in petri dishes. The surveyed houses are part of the ongoing 6X60X6 Project in which six houses from Covilhã (Portugal), with building dates representative of six decades, were studied for a period of sixty days.

3. Materials and Methods

3.1. Sampling

Under the framework of the 6x60x6 project, six houses built from 1960 to 2010 in the urban area of Covilhã were studied for a period of sixty days. Covilhã is located in the interior center of Portugal in the Cova da Beira Region at an average altitude of 7000m. For decades the Municipality had a very strong textile industry, and to this day Covilhã is synonym of fabrics. However, the crisis experienced by the sector in the 1980's, led to a profound reconversion of the local economy, being led nowadays by the tertiary sector (PORDATA, 2015).

The houses were selected by convenience and each participant signed an informed consent and completed a questionnaire about the household characteristics. At each house the master bedroom temperature and humidity values were recorded continuously using a temperature and relative humidity data logger (EasyLog - EL-GFX-2).

During the period of the study the wind regime varied. May was characterized by a dominant wind direction from NW with an average speed of 6.3 Km/h. In June the predominant wind direction was WNW with average speed of 3.9 Km/h, shifting in July to a W dominance and an average speed of 3.3 Km/h (GOA-UVa, 2015).

House dust samples were collected by means of active and passive sampling. Active sampling included the use of the household vacuum cleaner. At the beginning of the study a new vacuum cleaner bag (Wonderbag Compact WB 305120) was fitted and the participants were asked to vacuum only inside the house (excluding e.g. garage and cars). At the end of the 60 days the bag was removed, sealed and transported to the CICS-UBI laboratory (Figure 7).

Figure 7 House dust sampling. (A) Household vacuum cleaner used for active sampling; (B) Petri dishes placed on the top of a shelf; (C) Vacuum cleaner bag retrieved after 60 days of sampling.

Passive sampling was performed in the master bedroom using sterile glass petri dishes that remained unlidded at the selected sampling sites during 60 days. The petri dishes were

placed at sites that minimized possible disturbances by the normal routine of the inhabitants (e.g. on top of shelves, figure 7B). At the end of the sampling period the dishes were retrieved by the researchers, sealed and transported to the reference laboratory for fungal analysis at the National Institute of Health (INSA), in thermal bags and processed immediately upon arrival. Table 3 describes the characteristics of the houses and the sampling details.

3.2. Treatment of samples

In the laboratory the vacuum cleaner bags were opened and the samples sieved twice through stainless steel sieves of decreasing mesh (5 mm and 500 µm) to remove fibrous material and large pieces of debris in order to obtain a suitable degree of homogeneity. Samples were then stored in polyethylene tubes and transported to the INSA laboratory where they were analyzed.

3.3. Culture Methods: Fungal Culture and Identification

For vacuum cleaner samples, we followed the procedure proposed by Verhoeff et al.(1994). Three different culture methods were used, in order to achieve an optimal growth for analysis purposes: i) Direct plating: 30 mg representative aliquot of sieved dust was plated directly onto Malt Extract Agar with 1% cloramphenicol (MEA) plates using a sterile plastic spreader; ii) Suspension: 100 mg representative aliquot of sieved dust was mixed with 5 ml of liquid Sabouraud medium. The solution was shaken for 10 minutes. Subsequently, 100 µl of the prepared suspension was plated onto MEA plates with a sterile plastic spreader; iii) Dilution: 1 ml of the previous suspension was diluted in 9 ml of liquid Sabouraud and shaken for 10 minutes. Afterwards, 100 µl of the diluted suspension was plated onto MEA plates through a sterile plastic spreader.

As a measure of quality assurance, duplicates were made for each method/sample. All samples were incubated at 25±3ºC for 72 ± 3 hours.

For passive samples, each petri dish was washed with 1 mL of liquid culture medium – sabouraud with 1% cloramphenicol, and the obtained suspension was used for seeding over malt extract agar (MEA) and dichloran glycerol agar (DG-18) plates. Five plates of MEA and 5 plates of DG-18 were seeded with 100 uL of the suspension each, and incubated for 72h ± 3h at 25ºC±3ºC.

Quantification was performed by naked eye count. Fungal identification was performed either on the original sampling media (MEA) plates or after subculturing procedures, whenever colony isolation and growth observation were needed. Subculture was made on

MEA plates and incubated, at 25 ± 3 ºC, for periods ranging from 3 days to 3 weeks (Figure 8).

Fungal samples were mounted on lactophenol blue and visualized under optical microscope and identification of fungal colonies was based upon phenotypic characteristics and followed standard mycological procedures according to their micro and macro-morphological characteristics (Fisher & Cook, 1998).

Figure 8 *Cladosporium* sp., 20 days growth on MEA plate.

4. Results and Discussion

The total number of cultivable fungi found in the analyzed dust samples along with some of the house characteristics are depicted in Table 3. The average temperature was 25ºC in the majority of the houses whereas the relative humidity varied from 37.5% in house A to 47.3% in house D. Despite such differences in relative humidity and in the number of CFUs (Table 3) there was no significant correlation between the average humidity found in bedrooms and the number of CFUs at the same location (Spearman correlation, p=0.242).

Table 3 Characteristics of the surveyed houses with the indication of: sampling season, number of occupants, area (m^2), construction year (Const. year), temperature (°C) and relative humidity (%) registered in the master bedroom (min-max, average ± stdev) and the number of total Colony Forming Units (CFU) using active and passive sampling methods. For the active sampling method the results are shown for the three different culture techniques used (direct plating, suspension and dilution).

Sample ID	Sampling season	No. of occupants	House area (m^2)	Const. year	Bedroom		Active sampling			Passive sampling
					Temperature °C (min-max, average±stdev)	Relative humidity % (min-max, average±stdev)	Direct Plating (CFU/g)	Suspension (1:50) (CFU/g)	Dilution (1:10) (CFU/g)	Suspension (CFU/g)
House A	Spring/ summer	3	58.8	1961	20.0-33.5 27.4±3.3	21.8-57.5 37.5±5.8	Overgrowth	123 750	450 000	2 494
House B	Spring/ summer	2	112.3	1973	20.8-29.8 25.6±2.4	18.1-54.0 39.0±5.7	Overgrowth	49 750	235 000	4 333
House C	Spring/ summer	2	141.5	1983	19.7-30.3 25.4±2.7	21.3-60.2 46.9±6.6	Overgrowth	63 000	260 000	2 313
House D	Spring/ summer	3	139.1	1994	21.9-28.1 25.0±1.9	30.9-75.8 47.3±4.2	3550	49 000	155 000	1 090
House E	Spring/ summer	4	255.4	2000	20.8-29.6 25.7±2.4	27.2-60.8 42.4±5.2	2850	24 000	2 975 000	4 598
House F	Spring/ summer	1	109.4	2011	22.0-28.4 25.2±1.9	31.9-55.0 45.1±3.6	Overgrowth	35 750	117 500	3 115

When comparing the two sampling methods, there are clear differences between them, with a higher amount of CFU per gram of dust when dust is collected by means of active sampling. Such differences are easily explained when one considers the differences between the two methods: passive sampling reflects only the airborne fungi from the main bedroom settled in the petri dish during 60 days, whereas the vacuum cleaner samples concern the entire house and even though the sampling period was the same (60 days), the collected dust might corresponded to a longer period as for example carpets and rugs tend to trap dust for several months.

Overall our results are consistent with other studies on fungal communities' in house dust (Table 4).

Table 4 Comparison of the total amount of fungi detected in different surveys worldwide. Total CFU/g: Total number of Colony Forming Units (CFU) per gram of dust. *average values. na: information not available

Location	Sampling and culture method	No. samples	Total CFU/g	Reference
Boston, USA	Portable canister vacuum cleaner with a cellulose thimble; suspension	na	355, 756*	(Chao, Milton, Schwartz, & Burge, 2002)
Boston, USA	Portable canister vacuum cleaner with a cellulose extraction thimble; suspension	397	200 473*	(Chew et al., 2003)
Baden-Württemberg, Germa	Vacuum cleaner with special filter holder and gelatin filter; suspension	397	1 500 – 1 235 000	(Jovanovic et al., 2004)
Brittany, France	Dustream Collector sampler-fitted vacuum cleaner; Suspension	133	1 000 – 3 800 000	(Dallongeville et al., 2015)
Covilhã, Portugal	Vacuum cleaner bags; suspension	6	24 000 – 123 750	This study
Covilhã, Portugal	Passive sampling; suspension	6	1 090 - 4 598	This study

Generally, the most frequent fungi genera found in all samples were *Alternaria* sp., *Aspergillus* sp., *Cladosporium* sp., *Penicillium* sp., and yeasts (Figure 9). *Aspergillus* sp. and *Penicillum* sp. are found both in outdoor and indoor environments, where they are considered common fungi species (Fairs, 2010). Nevertheless, these genera also comprise species that are important allergic agents with implications in human respiratory health (Araujo & Cabral, 2010; Rintala et al., 2012).

In a previous study conducted by our team in two Portuguese cities (Aveiro and Coimbra, n= 28), *Aspergillus* and *Penicillium* were also the most abundant genera found (Sousa et al., 2014a). However, *Alternaria* sp., present in all the houses in the present study, was not detected in our previous study. Furthermore, when comparing samples obtained by active sampling in both studies, a higher diversity in the present study is evident. Such outcome is probably a consequence of an optimization of the protocol used in the current study, especially the aspect concerning dust samples being processed immediately after collection (instead of being preserved at -20º C).

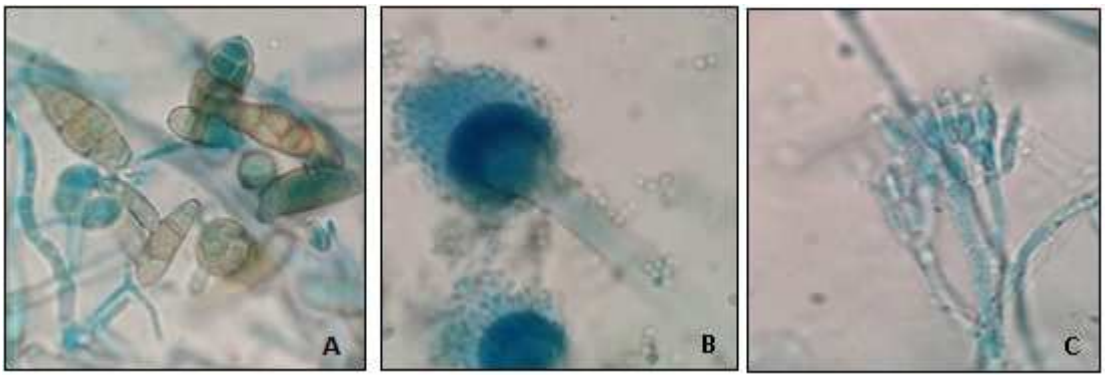

Figure 9 Most frequent genus detected. A) *Alternaria* sp.; B) *Aspergillus* sp.; C) *Penicillium* sp.

Regarding the taxon characterization, the passive sampling method proved to be more effective for the identification of fungi found in each sample (Table 5). Such results are foremost a consequence of the lower counts of fungi obtained with this method, thus enabling a greater rate of success in obtaining isolated and identifiable colonies. Also, suspension procedures may lead to breakage of suspended fungal spores, preventing their growth. Furthermore the low diversity of fungi found using the active method might be a consequence of the complex matrix that we are dealing with. Besides fungi, this dust also includes a large variety of other biological and chemical contaminants that may work as inhibitors and affect the viability of some fungal species.

The passive sampling technique using petri dishes provides a useful, simple and cost effective alternative for the fungal characterization of a particular set of the indoor environment and it should be considered in future monitoring studies.

Table 5 Identification of fungi found at each house using dust samples from the vacuum cleaner bag (active sampling) and from the deposited dust on petri dishes (passive sampling).

Sample ID	Active sampling	Passive sampling
House A	*Alternaria* sp. *Aspergillus fumigatus* *Penicillium* sp. *Rhodotorula* sp.	*Cladosporium* sp. Yeasts *Penicillium* sp. *Rhodotorula* sp. *Geotrichum* sp. *Acremonium* sp. *Fusarium* sp. *Alternaria* sp.
House B	*Penicillium* sp. *Aspergillus niger* *Mucor* sp. *Alternaria* sp. *Chrysosporium* sp.	*Cladosporum* sp. *Penicillium* sp. *Acremonium* sp. Yeasts *Fusarium* sp. *Alternaria* sp. *Aspergillus niger* *Geotrichum* sp. *Chrysonilia sitophila*
House C	*Aspergillus fumigatus* *Aspergillus niger* *Cladosporium* sp. *Penicillium* sp. *Rhodotorula* sp. *Chrysonilia sitophila*	*Cladosporium* sp. Yeasts *Geotrichum* sp. *Penicillium* sp. *Acremonium* sp. *Alternaria* sp. *Aspergillus* sp. *Aspergillus fumigatus* *Chaetomium* sp. *Fusarium* sp.
House D	*Alternaria* sp. *Chrysosporium* sp. *Aspergillus* sp. *Penicillium* sp. *Aerobasidium pullulans* *Trichophyto*n sp.	*Cladosporium* sp. *Penicillium* sp. Yeasts *Alternaria* sp. *Aspergllus flavus* *Aspergillus niger* *Fusarium* sp. *Acremonium* sp. *Aspergillus fumigatus* *Rhodotorula* sp. *Geotrichum* sp.
House E	*Penicillium* sp. *Alternaria* sp. *Trichoderma* sp. *Fusarium solani* *Aerobasidium pullulans* *Chrysosporum* sp.	*Penicillium* sp. *Cladosporium* sp. *Rhodotorula* sp. Yeasts *Alternaria* sp. *Acremonium* sp. *Aspergillus niger* *Geotrichum* sp. *Aspergillus fumigatus* *Fusarium* sp. *Mucor* sp. *Rhizopus* sp.
House F	*Alternaria* sp. *Chrysosporium* sp. *Penicillium* sp. *Paecilomyces* sp. *Trichophyton verrucosum*	*Cladosporium* sp. Yeasts *Penicillium* sp. *Fusarium* sp. *Geotrichum* sp. *Acremonium* sp. *Aspergillus niger*

5. Conclusions and future perspectives

House dust is a repository and concentrator of many contaminants including biological ones such as fungi. The obtained results showed that house dust samples obtained through active sampling are very complex and should not be assessed by direct plating. Based on the results from the suspension and dilution methods we recommend the use of the dilution method. When aiming to analyze specific locations inside a house, passive sampling using Petri dishes is a cost-effective and useful technique and should be used as a complement to vacuum cleaner bags (that are able to integrate the dust borne fungi of the entire household).

A future sampling campaign will be performed in the studied houses during winter in order to evaluate the seasonal trends in dustborn fungi. Furthermore, the obtained results (in terms of species distribution and richness) will be correlated with the respiratory health of the participants and a set of recommendations in order to reduce exposure will be prepared.

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Chapter III: Fungal communities in house dust samples from asthmatic patients

CARACTERIZATION OF FUNGAL COMMUNITIES' IN HOUSE DUST SAMPLES FROM ASTHMATIC PATIENTS

1. Abstract

Fungi are associated with a wide range of adverse health effects, including the risk of asthma onset or exacerbation. Several studies disclosed associations between the exposure to indoor damp and mould and the risk of asthma. Hence, the study of the indoor fungal biome is of particular importance, particularly for susceptible individuals such as asthmatic patients. With this study the fungal communities from houses of asthmatic patients and controls (non-asthmatics) were characterized in dust samples collected from the participants' vacuum cleaner bags. The number of Colony Forming Units of fungi per gram (CFU/g) ranged from 833 to 1583 CFU/g for asthmatics and from 933 to 2767 CFU/g for the controls. No significant differences (Mann Whitney U test, $p>0.05$) were found between the CFUs in the house dust from the two groups. *Aspergillus niger, Penicillium* sp., *Alternaria alternata* and yeasts were the most common fungi, being detected in both groups of samples.

Keywords: Fungi, house dust, asthma.

2. Introduction

Environmental exposure to fungi is associated with the onset of allergic asthma or to an increase in asthma severity (Dannemiller et al., 2016). This occurs trough spore and hyphae inhalation, fungal colonization of the lungs or through fungal infection outside respiratory tract and consequent sensitization (Bateman et al., 2008; Maurya et al., 2005; O'Driscoll et al., 2005). Furthermore, for individuals with allergic respiratory tract diseases, sensitization to fungi can develop, exacerbate or increase the persistence of lower airways diseases (Knutsen et al., 2012).

Asthma is a chronic disease characterized by recurrent attacks of breathlessness and wheezing (WHO, 2003). This disease affects around 334 million people worldwide, with great incidence on children and on elderly ages (Global Asthma Network, 2014).

The presence of fungal spores on indoor environments are not particularly dangerous for individual's health, when the fungal source comes from the interaction of the outside

environment and when the amount and the type of spores indoors are the same or less than the ones outdoors (Rabinovitch, 2012). The adverse effects on health depends on the total concentration of fungi and the specific fungal genera composition of the environments (Norbäck & Cai, 2015). Exposure to fungal allergens is related with asthma severity, leading to an increased number of hospital admissions, even admissions on Intensive Care Units and in some cases can result in premature death (Black et al., 2000; O'Driscoll et al., 2005; Targonski et al., 1995).

Additionally, dampness and mouldy environments are considered a risk factor for respiratory health, especially for asthmatic individuals (WHO, 2009). Some works described an association between dampness in houses and indoor moulds and the onset or the exacerbation of asthma (Quansah et al., 2012). A longitudinal European study found that asthmatic patients living in damped houses had increased lung function decline (FEV 1), when compared with asthmatic patients who lived on houses free of mould/dampness (Williamson et al., 1997). However, the specific causal agents for asthma exacerbation on this type of indoor environments has not been identified yet (Kanchongkittiphon et al., 2015).

Overall, studies on the characterization of the indoor dust microbiome from urban environments and its influence on the occurrence and development of allergic diseases is limited (Tischer et al., 2016). A previous study by this team demonstrated that house dust is a suitable matrix to evaluate indoor fungal communities (Sousa et al., 2014).

Thereby, our study aims to quantify and identify the fungal community in dust samples collected in the houses of asthmatics patients and controls.

3. Materials and methods

3.1. Sampling collection

House dust samples were collected from the vacuum cleaner bags donated by the participants. Asthmatic patients were recruited at *Centro Hospitalar Cova da Beira*, by a medical doctor (Allergist) during their routine consultation and those willing to participate signed an informed consent. Controls were recruited from a pool of volunteers at *Centro Hospitalar Cova da Beira* and at the Faculty of Health Sciences, University of Beira Interior. The study protocol was approved by the ethic committee of the Faculty of Health Sciences, University of Beira Interior (CE-FCS-2012-034).

The results hereby presented correspond to the first group of samples analysed: 6 samples from asthmatic patients' houses and 7 samples from controls.

3.2. Samples' treatment

In laboratory the vacuum cleaner bags were opened and the samples sieved twice through stainless steel sieves of decreasing mesh (5 mm and 500 µm) to remove fibrous material and large pieces of debris in order to obtain a suitable degree of homogeneity. Samples were then stored in polyethylene sterile tubes and transported to the INSA laboratory where they were analysed.

3.3. Fungal characterization

For the fungal identification, the samples were treated by three different culture methods (direct plating, suspension and dilution) following the protocol proposed by Verhoeff and collaborators (Verhoeff et al., 1994). Fungal quantification was performed by naked eye and the identification was performed based on the phenotypic characteristics of each fungus, identified through an optical microscope. For direct plating an aliquot representative of sieved dust about (ca 30 mg) was plated directly onto Malt Extract Agar with 1% cloramphenicol (MEA) plates using a sterile plastic spreader; for the suspension method, 100 mg of sieved dust was mixed with 5 ml of liquid Sabouraud medium. The solution was shaken for 10 minutes. Subsequently, 100 µl of the prepared suspension was plated onto MEA plates with a sterile plastic spreader; for the dilution method 1 ml of the previous suspension was diluted in 9 ml of liquid Sabouraud and shaken for 10 minutes. Afterwards, 100 µl of the diluted suspension was plated onto MEA plates through a sterile plastic spreader.

As a measure of quality assurance, duplicates were performed for each method/sample. All samples were incubated at 25±3°C for 72±3 hours.

Quantification was performed by naked eye count. Fungal identification was performed either on the original sampling media (MEA) plates or after sub culturing procedures, whenever colony isolation and growth observation were required. Subculture was performed on MEA plates and incubated, at 25±3°C, for periods ranging from 3 days to 3 weeks.

Fungal samples were mounted on lactophenol blue and visualized under optical microscope and identification of fungal colonies was based upon phenotypic characteristics and followed standard mycological procedures according to their micro and macro-morphological characteristics (Fisher & Cook, 1998).

3.4. Statistical analysis

Mann Whitney U test was used to compare the the number of Colony Forming Units (CFU) from asthmatic patients' house dust samples and controls. The significance level was set at 0.05

4. Results and discussion

Fungi were detected in all samples analysed and no significant differences between the number of Colony Forming units (CFUs) per gram of dust were found between the samples from house dust of asthmatic individuals and controls (Mann Whitney U test, $p>0.05$). Such results are in accordance with a previous study on indoor fungi levels on house dust from homes of children with and without allergy history (Jovanovic et al., 2004), in which no significant differences were found between the concentrations of fungi on both groups of houses.

In the asthmatic patients' house dust, the CFUs/g ranged from 833 to 1583. In controls, the number of CFUs per gram varied between 933 and 2767 (see Table 6). The highest numbers of CFUs were found on dust from houses of controls (See Table 6).

Overall, our range of CFUs values are lower than the ones previously reported by other works that characterized fungal communities in house dust samples. Beguin & Nolard (1996), for example, found concentrations of fungi ranging between $5x10^3$ to $66x10^6$ CFU/g in dust samples from carpeted floor environments covering living-rooms, bedrooms, offices and school classrooms. In a study conducted by (Chao et al., 2002) on dust borne fungi in office buildings, the mean of CFU/g was 355 756. High counts of CFUs ($1x10^3$ to $38x10^5$ CFUs/g) were also obtained in a recent study that evaluated the concentration of moulds and allergens on dust from children's bedrooms (Dallongeville et al., 2015).

Table 6 Colony Forming Units of fungi per gram of dust in each sample with the indication of the corresponding dominant fungal genera.

Asthmatic Patients (n=6)	**CFU/g**	**Dominant Fungal Genera**
A1	1517	*Aspergillus niger*; *Paecilomyces*
A2	833	*Aspergillus niger*
A3	1458	*Aspergillus niger*; *Penicillium* sp.
A4	1583	*Aspergillus niger*; *Penicillium* sp.
A5	1300	*Aspergillus niger*
A6	Overgrowth	*Penicillium* sp.
Controls (n=7)	**CFU/g**	**Dominant Fungal Genera**
C1	1583	*Aspergillus niger*; *Penicillium* sp.
C2	1950	*Aspergillus niger*; *Penicillium* sp.
C3	933	*Aspergillus fumigatus*
C4	1417	*Aspergillus niger*; *Penicillium* sp.
C5	1967	*Chrysonilia sitophyla*
C6	Overgrowth	*Aspergillus niger*; *Mucor* sp.
C7	2767	*Aspergillus niger*; *Mucor* sp.

Regarding fungal identification, the most common fungi found was *Aspergillus niger* (detected in 77% of the houses), followed by *Penicillium* sp. (detected in 46% of the houses) and *Alternaria alternata* (detected in 46% of the houses). Furthermore, yeasts were detected in all samples analysed (see Table 7). *Aspergillus* sp. and *Penicillium* sp. are considered to be the main fungi in indoor environment and their sources are mainly associated with outdoors (Adams et al., 2013; Shelton et al., 2002).

The fungal taxa with higher counts were *Aspergillus niger*, *Peacilomyces*, *Penicillium* sp., *Aspergillus fumigatus*, *Chrysonilia sitophyla* and *Mucor* sp. (see table 6 and table 7).

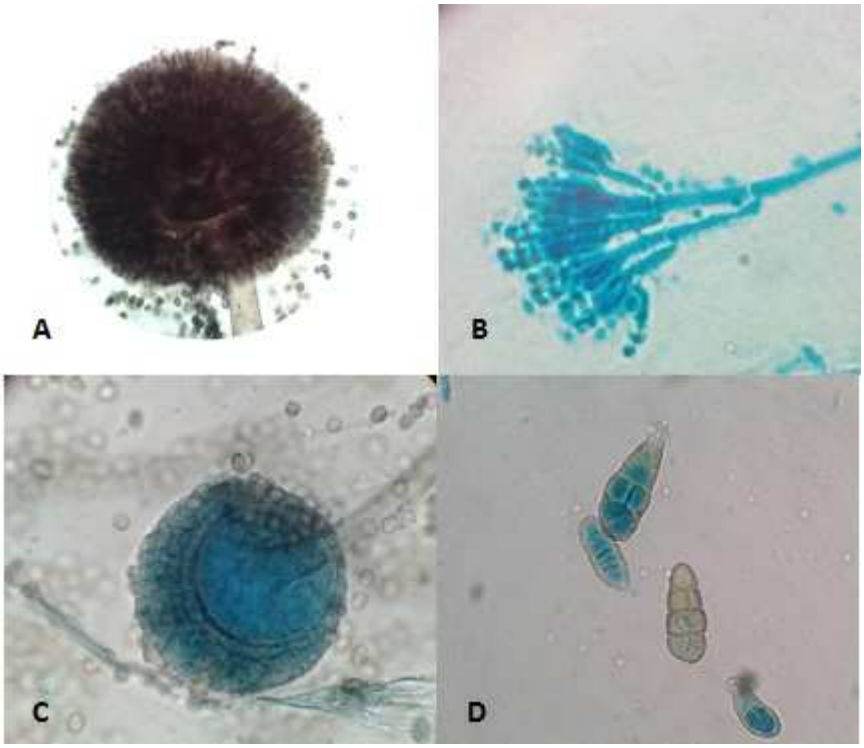

Figure 10 Common genera found on house dust samples in both groups of houses: A) *Aspergillus niger*; B) *Penicillium* sp.; C) *Mucor* sp.; and D) *Alternaria alternata.*

Our results are in accordance with previously published ones where yeasts and the following genera of fungi were also detected in house dust: *Penicillium, Cladosporium, Aspergillus, Uclocladium, Fusarium, Epicoccum, Trichoderma, Curvularia, Drechslera, Aerobasidium, Eurotium, Mucor, Paecilomyces, Chrysosporium, Acremonium* (Basilico et al., 2007; Chao et al., 2002; Chew et al., 2003; Dallongeville et al., 2015; Hicks et al., 2005; Sousa et al., 2014).

Some studies identified the sensitivity to fungal allergens as a cause of allergic asthma (Beguin & Nolard, 1996; Dannemiller et al., 2014), especially on children. In fact, in those surveys it was possible to establish an association with the risk of asthma on children and the following fungal genera in house dust generas *Alternaria, Cladosporium, Epicoccum, Penicillium, Ulocladium, Malassezia, Rhodotorula, Aspergillus, Curvularia, Fusarium, Pleospora* and *Stachybotrys*. The risk was greater with higher concentrations of fungi in dust (Dannemiller et al., 2016). Such results however were in disagreement with other studies, namely Sharpe et al. (2015) that suggested that the allergic risk doesn't increase with the increasing exposure to multiple biologic agents and that exposure to multiple allergens in house dust may also be associated to a reduced risk of asthma on adults.

Behbod et al. (2013) revealed that fungal dust exposure to *Alternaria* sp., *Aspergillus* sp., *Cladosporium* sp. and *Penicillium* sp. improved the risk of wheeze in 2-3 months infants. However, yeasts in indoor dust seemed to have a protective effect. In an update to the 2000

review by the Institute of Medicine, on exacerbation of asthma, Kanchongkittiphon et al. (2015) disclosed that fungal exposure to the genus *Penicillium* contributes to asthma recrudescence on children. Pongracic et al. (2010) also associated *Penicillium* sp. to asthma morbidity in inner-city children. Furthermore, Reponen et al. (2012) reported that the combination of some *Penicillium* and *Aspergillus* species could be a predictive for risk of asthma in children by the age 7. This study concluded that children that live in houses with increased levels of these two fungi genera on house dust can have twice or more probability of asthma onset than children who live in dwellings with lower mould levels detected on dust. Further evidences on the associations between asthma symptoms and fungi exposure were provided by Snelgrove et al. (2014) that proved that *Alternaria alternata* was responsible for severe asthma exacerbations, through interaction of *Alternaria*-specific serine protease on mice's organism. A recent meta-analysis and systematic review performed by Sharpe et al. (2014) also showed that the exposure to *Aspergillus* sp., *Penicillium* sp. and *Cladosporium* sp. represents an health risk for susceptible populations and these fungi can increase the exacerbation of asthma symptoms on children and on adults, as well as *Alternaria* species. Despite such evidences further work is necessary in order to fully understand the role of fungi exposure on asthma.

Table 7 Dust samples analysis: Colony Forming Units per gram of dust (CFU/g) in each dust sample based on direct plating results and respective fungal colony identification. Bold letters denote those species or genera that were more abundant.

Sample code	CFU/g (Direct plating)	Colonies identification
A1	1517	***Aspergillus niger,*** *Aspergillus fumigatus,* ***Paecilomyces variotii,*** *Penicillium sp., Chrysonilia, Sitophila, Mucor sp., Yeasts*
A2	833	*Scytalidium sp., Bipolaris sp., Alternaria alternata,* ***Aspergillus niger,*** *Trichophyton rubrum, Fusarium oxysporum, Sepedonium sp., Mucor sp., Yeasts*
A3	1458	***Aspergillus niger, Penicillium sp.,*** *Fusarium oxysporum, Alternaria alternate, Yeasts*
A4	1583	***Aspergillus niger,*** *Penicillium sp., Fusarium solani, Yeasts*
A5	1300	*Mucor sp.,* ***Aspergillus niger,*** *Alternaria alternata, Penicillium sp., Yeasts*
A6	overgrow	***Penicillium sp.,*** *Mucor sp., Aspergillus niger, Yeasts*
C1	1583	***Aspergillus niger, Penicillium sp.,*** *Trichophyton violaceum, Crysosporium sp., Yeasts*
C2	1950	***Aspergillus niger,*** *Penicillium sp., Yeasts*
C3	933	***Aspergillus fumigatus,*** *Fusarium solani, Aspergillus niger, Alternaria alternata, Trichophyton sp., Penicillium sp., Mucor sp., Yeasts*
C4	1417	***Aspergillus niger, Penicillium sp.,*** *Exophiala sp.,Mucor sp., Alternaria alternate, Yeasts*
C5	1967	***Chrysonilia sitophyla***
C6	overgrow	***Aspergillus niger, Mucor sp.****, Alternaria alternata, Penicillium sp., Yeasts*
C7	2767	***Aspergillus niger, Mucor sp.,*** *Penicillium sp., Exophiala sp., Epidermophyton sp., Rizophus sp., Yeasts*

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Chapter IV:
Final Remarks

1. General Conclusion

The surveys presented in this dissertation, contributes for increasing the scientific knowledge on the characterization of fungal communities in house dust samples, especially on Portuguese dwellings and asthmatic patients.

Regardless its small sample universe, chapter 2 presented important and novel results. There are few works reported on the characterization of house dust fungal communities based on quantification of CFU/g through the suspension method. Those studies include: i) a survey about dust borne fungi in large office buildings in Boston, USA (Chao et al., 2002); ii) a study about the relation of airborne and dust borne fungi and their variation according home characteristics in Boston, USA (Chew et al., 2003); iii) a study about the levels of indoor fungi found on homes of children with and without allergy history in Germany (Jovanovic et al., 2004) and iv) a study about the concentrations and determinants of moulds and allergens in indoor air and in house dust of French dwellings (Dallongeville et al., 2015). With this study it was even possible to select the best culture method (dilution) for the identification of fungal colonies in house dust samples collected by active sampling. Furthermore, this study was the first of its kind to comparing two sampling methods (active *vs* passive sampling). With this strategy it was possible to conclude that despite active sampling was a good methodology to study house dust fungal communities of indoor environments as a whole, for more accurate results, this methodology should be complemented with a passive sampling analysis. The passive sampling allows to better understand which specific fungal communities are present in each particular site of the indoor environment.

In chapter 3 the house dust fungal community in houses of asthmatic and controls was studied. The influence of fungal communities on house dust and their influence on asthma onset, development and persistence, is still a research field with many unanswered questions. In fact, as recently stated by Tischer et al. (2016) “evidence regarding the microbiome composition in dust from urban environments and its influence on the occurrence and development of allergic diseases is still scarce and comprehensive understanding is lacking”.

The results presented in this chapter refer only to 13 samples and are a part of an ongoing work. Due to the limited sample size it is difficult to draw robust conclusions. Based on the results obtained no significant differences could be found in the number of CFUs in the samples from houses of asthmatic patient’s and the control group. As previously mentioned, no consensus on the associations between fungi in dust and asthma exists. Nevertheless,

most of the recent reports disclose a fungal taxa and the exacerbation, occurrence and onset of asthma symptoms. In the works reviewed by Denning et al. (2006), associations between *Mucor* sp.*, Alternaria* sp.*, Aspergillus* sp.*, Cladosporium* sp.*, Penicillium* sp. and other fungi and the development, persistence and severity of the disease were established. For example, *Alternaria* species were identified as common allergens on development of respiratory symptoms and asthma onset and severity (Bush & Prochnau, 2004; Kilic et al., 2010). *Aspergillus* hypersensitivity was often associated to asthmatic patients and could lead to Allergic Bronchopulmonary Aspergillosis (Agarwal et al., 2009; Knutsen & Slavin, 2011). *Penicillium* sp. exposure was associated with the variance of the expiratory peak flow in asthmatic children (Bundy et al., 2009) and allergic asthmatics with sensitization to *Cladosporium* had more predisposition to have severe asthma attacks (Hayes et al., 2013). Overall, the available evidences seems to suggest that fungi are important factors in asthma, however further research is necessary.

2. Future work

This work represents the starting point of the research on an emerging research field. Taking account the results obtained with this thesis, further investigations will be performed. On study performed on chapter 2, a sampling campaign on winter will be carried out, in order to evaluate the seasonal trends in dust borne fungi. On chapter 3, the sample universe needs to be extended, in order to obtain robust results. Additionally, it would be important to perform Skin Prick Tests to–diagnose allergies, particularly to fungi, in order to set recommendations to reduce the exposure to these allergens

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