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House dust fungal communities' characterization: a double take on the six by sixty by six (6 × 60 × 6) project

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Abstract: Fungi are a group of 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 and allergies. 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 identify and quantify 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 6 × 60 × 6 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 active sampling; passive sampling

1 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 [1]. The indoor air pollution is considered a major cause of morbidity and mortality all over the world [2] 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 [3] 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 [4]. Fungi produce tiny spores with those smaller than 10 µm being particularly hazardous to human health, as they

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can enter the respiratory tract and reach the alveoli (the gaseous exchange areas of the lung), which may lead to respiratory infections and allergic reactions [5, 6].

Spores can be suspended in the air, deposited on various surfaces and included within different matrices such as house dust [7]. 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 [8, 9]. 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 [10, 11]. Its relevance as an important exposure source 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 [12].

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 [8].

To date several papers have been published on the fungal community in house dust samples (see e.g. Rintala et al. [8] , Sousa et al. [11], Chew et al. [16]). However, 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 identify and quantify the fungal community on house dust samples collected using two different methodologies: active and passive sampling. For this purpose, we analysed 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 Covilhă (Portugal), with building dates representative of six decades, were studied for a period of sixty days.

2 Materials and Methods

2.1 Sampling

Under the framework of the 6 × 60 × 6 project, six houses built from 1960 to 2010 in the urban area of Covilhă were studied for a period of sixty days. Covilhă is located in the interior center of Portugal in the Cova da Beira Region at an average altitude of 7000 m. During the period of the study the wind regime varied. The month of 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 (http://webx.ubi.pt/~goa).

The Covilhă Municipality had for decades a very strong textile industry, and to this day Covilhă 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 (http://www.pordata.pt).

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 (°C) and relative humidity (RH) data logger (EasyLog - EL-GFX-2, Lascar Electronics, accuracy: 0.35 °C; 0.1%RH).

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.

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). At the end of the sampling period the petri dishes were retrieved by the researchers, sealed and transported to the reference laboratory for fungal analysis at the National Institute of Health – Porto, Portugal (INSA) in thermal bags, and processed immediately upon arrival. Table 1 describes the characteristics of the houses and the sampling details.

2.2 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 analysed.

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Table 1. Characteristics of the surveyed houses with the indication of: 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	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	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	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	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	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	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	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

3 Culture Methods: Fungal Culture and Identification

For vacuum cleaner samples, we followed the procedure proposed by Verhoeff and collaborators [13]. Three different culture methods were used, in order to achieve an optimal growth for analysis purposes:

1. Direct plating: 30 mg representative aliquot of sieved dust was plated directly onto Malt Extract Agar (MEA) with 1% cloramphenicol (MEA) plates using a sterile plastic spreader;
2. 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;
3. 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 ± 3 °C 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 72 ± 3 hours at 25 ± 3 °C.

The quantification of the number of Colonies Forming Units (CFUs) was performed by naked eye count following the ISO 4833:2003 guideline. 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 °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 [4].

4 Results and Discussion

The total number of cultivable fungi found in the analysed dust samples along with some of the house characteristics is depicted in Table 1. The average temperature was 25 °C 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 1) 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).

When comparing the two sampling methods clear differences were noticed 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 2).

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Table 2. 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	N0. samples	Total CFU/g	Reference
Boston, USA	Portable canister vacuum cleaner with a cellulose thimble; suspension	na	355 756*	Chao et al., 2002 [15]
Boston, USA	Portable canister vacuum cleaner with a cellulose extraction thimble; suspension	397	200 473*	Chew et al., 2003 [16]
Baden-Württemberg, Germany	Vacuum cleaner with special filter holder and gelatin filter; suspension	397	1 500 – 1 235 000	Jovanovic et al., 2004 [17]
Brittany, France	Dustream Collector sampler-fitted vacuum cleaner; Suspension	133	1 000 – 3 800 000	Dallongeville et al., 2015 [9]
Covilhă, Portugal	Vacuum cleaner bags; suspension	6	24 000 – 123 750	This study
Covilhă, 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 1). *Aspergillus* sp. and *Penicillum* sp. are found both in outdoor and indoor environments, where they are considered common fungi species [18]. Nevertheless, these genera also comprise species that are important allergic agents with implications in human respiratory health [5, 8].

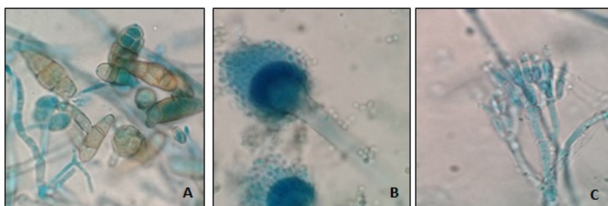

Fig. 1. Most frequent genus detected. A) *Alternaria* sp.; B) *Aspergillus* sp.; C) *Penicillium* sp.

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 [11]. 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 −20 °C). Regarding the taxon characterization, the passive sampling method proved to be more effective for the identification of fungi found in each sample (Table 3). 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, such metals, organometals, semi volatile organic compounds, including some antimicrobials, 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.

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 analyse specific locations inside a house, passive sampling using Petri dishes is a cost-effective and useful technique and should be used

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Table 3. 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.	*Cladosporium* sp.
	Aspergillus fumigatus	Leveduras
	Penicillium sp.	*Penicillium* sp.
	Rhodotorula sp.	*Rhodotorula* sp.
		Geotrichum sp.
		Acremonium sp.
		Fusarium sp.
		Alternaria sp.
House B	*Penicillium* sp.	*Cladosporum* sp.
	Aspergillus niger	*Penicillium* sp.
	Mucor sp.	*Acremonium* sp.
	Alternaria sp.	Leveduras
	Chrysosporium sp.	*Fusarium* sp.
		Alternaria sp.
		Aspergillus niger
		Geotrichum sp.
		Chrysonilia sitophila
House C	*Aspergillus fumigatus*	*Cladosporium* sp.
	Aspergillus niger	Leveduras
	Cladosporium sp.	*Geotrichum* sp.
	Penicillium sp.	*Penicillium* sp.
	Rhodotorula sp.	*Acremonium* sp.
	Chrysonilia sitophila	*Alternaria* sp.
		Aspergillus sp.
		Aspergillus fumigatus
		Chaetomium sp.
		Fusarium sp.
House D	*Alternaria* sp.	*Cladosporium* sp.
	Chrysosporium sp.	*Penicillium* sp.
	Aspergillus sp.	Leveduras
	Penicillium sp.	*Alternaria* sp.
	Aerobasidium pullulans	*Aspergllus flavus*
	Trichophyton sp.	*Aspergillus niger*
		Fusarium sp.
		Acremonium sp.
		Aspergillus fumigatus
		Rhodotorula sp.
		Geotrichum sp.
House E	*Penicillium* sp.	*Penicillium* sp.
	Alternaria sp.	*Cladosporium* sp.
	Trichoderma sp.	*Rhodotorula* sp.
	Fusarium solani	Leveduras
	Aerobasidium pullulans	*Alternaria* sp.
	Chrysosporum sp.	*Acremonium* sp.
		Aspergillus niger
		Geotrichum sp.
		Aspergillus fumigatus
		Fusarium sp.
		Mucor sp.
		Rhizopus sp.
House F	*Alternaria* sp.	*Cladosporium* sp.
	Chrysosporium sp.	Leveduras
	Penicillium sp.	*Penicillium* sp.
	Paecilomyces sp.	*Fusarium* sp.
	Trichophyton verrucosum	*Geotrichum* sp.
		Acremonium sp.
		Aspergillus niger

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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