

Article

Loading Rates of Dust and Bioburden in Dwellings in an Inland City of Southern Europe

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Abstract: Sampling campaigns indoors have shown that occupants exposed to contaminated air generally exhibit diverse health outcomes. This study intends to assess the deposition rates of total settleable dust and bioburden in the indoor air of dwellings onto quartz fiber filters and electrostatic dust collectors (EDCs), respectively. EDC extracts were inoculated onto malt extract agar (MEA) and dichloran glycerol (DG18) agar-based media used for fungal contamination characterization, while tryptic soy agar (TSA) was applied for total bacteria assessment, and violet red bile agar (VRBA) for Gram-negative bacteria. Azole-resistance screening and molecular detection by qPCR was also performed. Dust loading rates ranged from 0.111 to 3.52, averaging 0.675 $\mu\text{g cm}^{-2} \text{ day}^{-1}$. Bacterial counts ranged from undetectable to 16.3 colony-forming units (CFU) $\text{m}^{-2} \text{ day}^{-1}$ and to 2.95 CFU $\text{m}^{-2} \text{ day}^{-1}$ in TSA and VRBA, respectively. Fungal contamination ranged from 1.97 to 35.4 CFU $\text{m}^{-2} \text{ day}^{-1}$ in MEA, and from undetectable to 48.8 CFU $\text{m}^{-2} \text{ day}^{-1}$ in DG18. *Penicillium* sp. presented the highest prevalence in MEA media (36.2%) and *Cladosporium* sp. in DG18 (39.2%). It was possible to observe: (a) settleable dust loadings and fungal contamination higher in dwellings with pets; (b) fungal species considered indicators of harmful fungal contamination; (c) *Aspergillus* section *Candidi* identified in supplemented media with voriconazole and posaconazole; (d) specific housing typologies and (e) specific housing characteristics influencing the microbial contamination.

Keywords: dwellings; settleable dust; bioburden; electrostatic dust collector; toxigenic fungi; antifungal resistance

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1. Introduction

Bioburden (comprising bacterial and fungal genera) is very common in dwellings. The most prevalent fungal genera found in this indoor environment are *Cladosporium*, *Penicillium*, *Alternaria* and *Aspergillus* [1,2]. In fact, in Portuguese dwellings, located in the districts of Lisbon and Aveiro, *Penicillium* and *Aspergillus* have been also reported as the dominant fungi [3,4]. Lately, exposure to fungal contamination has gained increasing importance, since correlations with respiratory diseases and atopic dermatitis have been often observed [5–7]. Recent studies have also documented the emerging worldwide

problem of azole drug resistance of *A. fumigatus* isolates [8–12]. The prevalence of azole resistant fungi in dwellings, especially in homes of immunocompromised individuals or patients with respiratory diseases, represents a significant health risk that supports the need for assessments of the prevalence of azole resistance in indoor environments of different regions [13,14].

Several studies have reported that dust is the most suitable matrix to characterize bioburden, so it has been used by several researchers in this type of evaluation [15,16]. Furthermore, settled dust has been considered a reservoir of bacterial contamination [17]. Thus, although some researchers argue that active sampling methods (air pumping) represent a better surrogate of exposure, numerous advantages can be pointed out to passive sampling methods. Indeed, active air sampling is limited by short sampling periods (minutes to hours), representing only a snapshot of bioburden exposure [18]. Electrostatic dust collectors (EDCs) constitute a simple and cost-effective passive sampling method able to characterize bioburden present in settleable dust [3,4,19–23]. Given their simplicity, low cost, independence from noisy pumps and power supply, possibility of allowing simultaneous sampling in multiple locations, and ability to provide indications of exposure over longer time frames, passive samplers are regarded as a good alternative in indoor air quality and exposure assessment studies [24,25].

Bioburden assessment with EDCs has been previously carried out in occupational environments, such as schools [3], health care centers [26] and swine farms [27], as well as in residential settings of Portuguese coastal cities [3,4]. However, outdoor fungi and bacteria are mainly affected by local climate conditions [28], while in indoor environments different factors, such as the thermal comfort, dwelling characteristics and occupant activities, may play a key role in bioburden [3,4,29,30]. Thus, results of a given region may not be generalizable to other target populations.

In this study, the deposition rates of total settleable dust and bioburden were assessed in the indoor air of dwellings in a small urban area within a touching distance from the highest mountain range in mainland Portugal. The investigation also included the molecular detection of toxigenic fungal species and the analysis of antifungal resistance profiles.

2. Materials and Methods

2.1. Location of the Studied Dwellings

This study was conducted in the living rooms of 30 dwellings located in the city of Guarda (Figure 1), from mid-June to early October 2018. The characteristics of each dwelling are listed in the supplementary material. Figure 2 shows the frequencies of the main features. With a population of about 50,000 inhabitants, Guarda is the city located at the highest altitude in continental Portugal (around 1000 m). It has a continental cool Mediterranean climate. The average temperature of the coldest month (January) is of 4 °C, that of the warmest month (August) is of 19.2 °C. Precipitation ranges from 10 mm in the driest month (August) to 140 mm in the wettest (December).



Figure 1. Location of the studied dwellings in Guarda, Portugal.

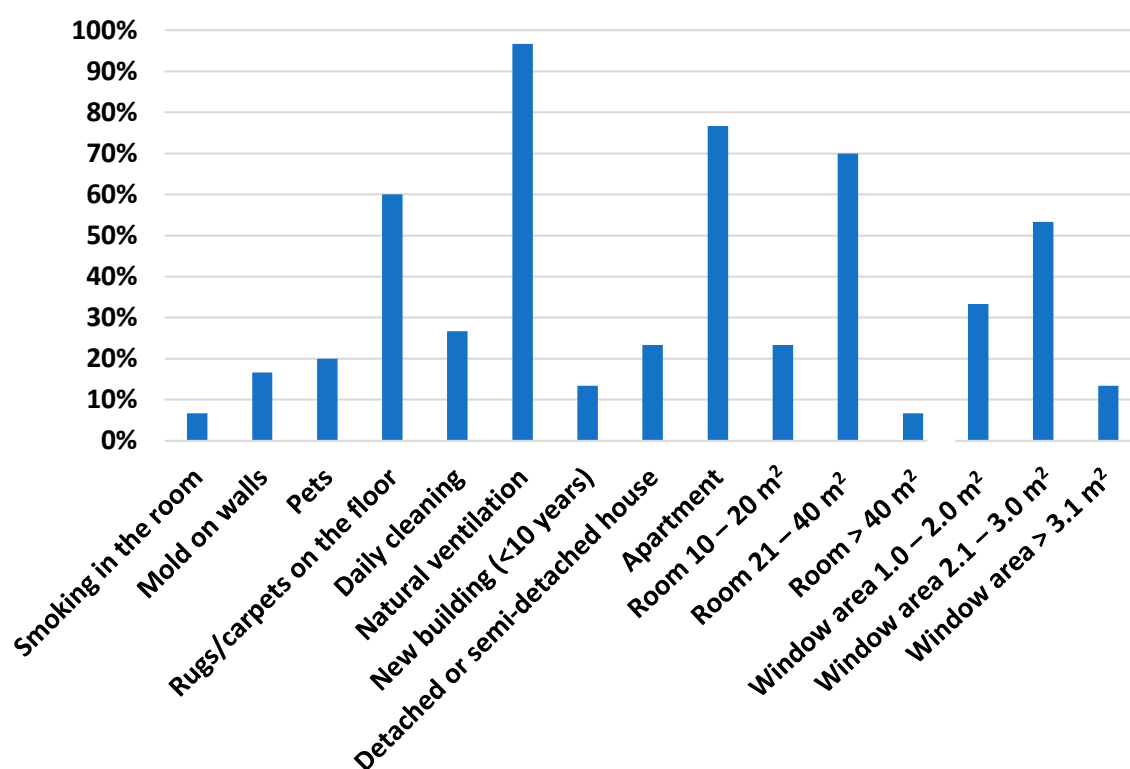


Figure 2. Frequencies of the main features of the houses and rooms where samples were obtained.

2.2. Settleable Dust Sampling and Quantification

To collect particulate matter, 47 mm diameter quartz fiber filters (Pallflex® Putnam, CT, USA) were exposed in uncovered petri dishes (Analyslide® Pall, München, Germany) over periods from 105 to 160 days, between June and early October 2018, under typical summer conditions. Filters were weighted, before and after exposure, in a microbalance (RADWAG 5/2Y, Radom, Poland) after conditioning for 24 h in a room with controlled humidity (50%) and temperature (20 °C). Each filter was weighted at least 6 times. The final value resulted from the average of these 6 readings.

Electrostatic dust collectors (EDC), which comprise electrostatic polypropylene clothes, were exposed to particulate fallout over the same time as quartz filters. One EDC was placed in each sampling location. EDCs were placed in open petri dishes (surface area of 154 cm²) side by side with the quartz filters, on top of the furniture, at 1.3–1.5 m above the ground, which corresponds approximately to the breathing zone height. A member of the research team, together with the family nurse, explained to the members of each dwelling about the importance of not touching the samplers and that information was also passed on to the cleaning lady. After transport in refrigerated conditions (<4 °C), EDCs were then used to assess bioburden.

2.3. Electrostatic Dust Cloth Extraction and Bioburden Analyses

Settled dust collected by EDCs was analyzed by culture-based methods and using Real Time PCR (qPCR), targeting 4 indicators of harmful fungal contamination belonging to *Aspergillus* genera, namely sections *Flavi*, *Fumigati*, *Circumdati* and *Nidulantes* [4].

EDC extracts (0.15 mL) were obtained following the procedures already published [23] and inoculated onto 2% malt extract agar (MEA) with 0.05 g L⁻¹ chloramphenicol media and dichloran glycerol (DG18) agar-based media were used for fungal contamination characterization, while tryptic soy agar (TSA) with 0.2% nystatin was applied for total bacteria assessment, and violet red bile agar (VRBA) for Gram-negative bacteria.

Samples were also seeded (0.15 mL) onto Sabouraud dextrose agar (SAB) media supplemented with 4 mg L⁻¹ itraconazole (ITR), 1 mg L⁻¹ voriconazole (VOR), or 0.5 mg L⁻¹ posaconazole (POS) (protocol adapted from the EUCAST 2017 guidelines) [31] for the screening of antifungal resistance [32].

Incubation of MEA, DG18 and azole screening plates at 27 °C for 5 to 7 days and TSA and VRBA plates at 30 and 35 °C for 7 days, respectively, was ensured. Bioburden densities (colony-forming units, CFU m⁻² day⁻¹) were calculated following the same procedures already published [3,4].

Molecular detection of the different *Aspergillus* sections was accomplished by qPCR using the CFX-Connect PCR System (Bio-Rad). Reactions included 1× iQ Supermix (Bio-Rad), 0.5 µm of each primer (Table 1), and 0.375 µm of TaqMan probe in a total volume of 20 µL. Amplification followed a three-step PCR: 50 cycles with denaturation at 95 °C for 30 s, annealing at 52 °C for 30 s, and extension at 72 °C for 30 s. A non-template control was used in every PCR reaction. For each gene that was amplified, a non-template control and a positive control were used, consisting of DNA obtained from a reference that belonged to the culture collection of the Reference Unit for Parasitic and Fungal Infections, Department of Infectious Diseases of Dr. Ricardo Jorge National Institute of Health. These strains have been sequenced for ITS B-tubulin, and calmodulin.

Table 1. Sequence of primers and TaqMan probes used for real time PCR.

| <i>Aspergillus</i> Sections Targeted | Sequences | Reference |
|--------------------------------------|---|-----------|
| <i>Flavi</i> (Toxigenic Strains) | 5'-GTCCAAGCAACAGGCCAAGT-3' | |
| Forward Primer | | |
| Reverse Primer | 5'-TCGTGCATGTTGGTGATGGT-3' | [33] |
| Probe | 5'-TGTCTTGATCGGCGCCCG-3' | |
| <i>Fumigati</i> | | |
| Forward Primer | 5'-CGCGTCCGGTCCTCG-3' | |
| Reverse Primer | 5'-TTAGAAAAATAAAGTTGGGTGTCG G-3' | [34] |
| Probe | 5'-TGTCACCTGCTCTGTAGGCCCG-3' | |
| <i>Circumdati</i> | | |
| Forward Primer | 5'-CGGGTCTAATGCAGCTCCAA-3' | |
| Reverse Primer | 5'-CGGGCACCAATCCTTTCA-3' | [35] |
| Probe | 5'-CGTCAATAAGCGCTTTT-3' | |
| | 5' – CGGCGGGGAGCCCT-3' | |
| <i>Nidulantes</i> | | |
| Forward Primer | 5'– CCATTGTTGAAAGTTTTGACTGATcTT | |
| Reverse Primer | A-3' | [36] |
| Probe | 5'– AGACTGCATCACTCTCAGGCATGAA GTTCAG-3' | |

2.4. Statistical Analysis

The data were analyzed using the statistical software SPSS, V26.0. The results were considered significant at the 5% significance level. To test the normality of the data, the Shapiro–Wilk test was used. For the characterization of the sample, frequency analysis (n, %) was used for qualitative data and the minimum, maximum, mean or median and standard deviation for quantitative data. Graphical representations were also used according to the nature of the data. The Spearman's correlation coefficient was used to study the relationship between housing characteristics, bacterial and fungal counts and azoles screening, since the assumption of normality was not verified. For the comparison of bacterial counts, fungal counts and azoles screening between the heating systems (which was dichotomized, in central and others, due to the reduced number of responses), between wall materials (also dichotomized, brick/stone and brick + stone), between window frames, between rugs (yes/no), between smoke in the room (yes/no), between pets (yes/no), between cleaning frequency (daily/weekly) and between fungi (yes/no), the Mann–Whitney test was employed, since the assumption of normality was not verified. To compare the bacterial counts, fungal counts and screening azoles between the types of frames, the Kruskal–Wallis test was used, since the assumption of normality was not verified. Neural networks were used to assess which housing characteristics are most important for bacterial and fungal counts and azoles screening.

3. Results and Discussion

3.1. Dust Loading Rates

Dust loading rates ranged from 0.111 to 3.52, averaging 0.675 $\mu\text{g cm}^{-2} \text{ day}^{-1}$. These mass loadings of household dust are in line with the mean values (0.429 $\mu\text{g cm}^{-2} \text{ day}^{-1}$ in winter, 0.570 $\mu\text{g cm}^{-2} \text{ day}^{-1}$ in summer) reported for different dwellings located in the coastal district of Aveiro, also in Portugal [3,4]. Slightly lower deposition rates were documented for four New Jersey homes, where mean values of $0.37 \pm 0.13 \mu\text{g cm}^{-2} \text{ day}^{-1}$ during the summer and $0.22 \pm 0.13 \mu\text{g cm}^{-2} \text{ day}^{-1}$ during the winter were obtained [37].

Shraim et al. [38] collected dust samples from residential houses in the city of Almadinah Almunawarah, Saudi Arabia, reporting loading rates from $0.489 \mu\text{g cm}^{-2} \text{day}^{-1}$ to $1.53 \mu\text{g cm}^{-2} \text{day}^{-1}$. Much higher mean dust loading rates of 22.6 and $117 \mu\text{g cm}^{-2} \text{day}^{-1}$ were measured by Khoder et al. [39] inside and outside of domestic houses, respectively, in an urban area of Giza, Egypt. In the present study, on average, settleable dust loadings in dwellings with dogs ($1.25 \mu\text{g cm}^{-2} \text{day}^{-1}$) were approximately double those in homes that did not have that pet ($0.576 \mu\text{g cm}^{-2} \text{day}^{-1}$). No conclusions could be drawn regarding the influence of other characteristics.

3.2. Bacterial Contamination Assessment

Bacterial counts ranged from undetectable to $16.3 \text{ CFU m}^{-2} \text{day}^{-1}$ and to $2.95 \text{ CFU m}^{-2} \text{day}^{-1}$ in TSA and VRBA, respectively. Median values were $5.82 \text{ CFU m}^{-2} \text{day}^{-1}$ in TSA and $0.00 \text{ CFU m}^{-2} \text{day}^{-1}$ in VRBA. The lower counts of Gram-negative bacteria followed the same trend as in previous studies conducted in dwellings from two different Portuguese cities [4], which can in part be explained by less tolerance to the environmental factors of these species [40].

3.3. Fungal Contamination Assessment

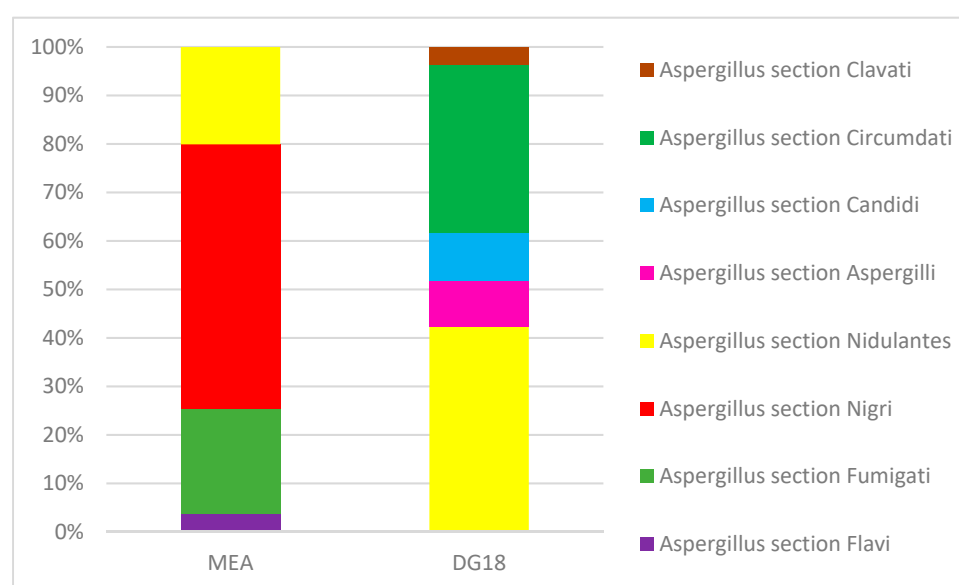
Fungal contamination ranged from 1.97 to $35.4 \text{ CFU m}^{-2} \text{day}^{-1}$ in MEA, and from undetectable to $48.8 \text{ CFU m}^{-2} \text{day}^{-1}$ in DG18. These counts are lower than those obtained in previous studies conducted in Lisbon city [23], in French dwellings [22] and in Danish homes [41]. The differences can be due to dissimilar contamination sources, or even environmental variables that can impact the fungal profile indoors [42]. Additionally, the differences observed between the results of this study and those reported in the literature may also arise from aspects related to seasonality that may have an influence on microbial contamination [23,28,42].

Penicillium sp. presented the highest prevalence in MEA media ($143 \text{ CFU m}^{-2} \text{day}^{-1}$; 36.2%), followed by *Aspergillus* sp. ($104 \text{ CFU m}^{-2} \text{day}^{-1}$; 26.4%) and *Cladosporium* sp. ($75.7 \text{ CFU m}^{-2} \text{day}^{-1}$; 19.1%). In DG18 media, the highest prevalence belonged to *Cladosporium* sp. ($202 \text{ CFU m}^{-2} \text{day}^{-1}$; 39.2%), followed by *Aspergillus* sp. ($149 \text{ CFU m}^{-2} \text{day}^{-1}$; 28.8%) and *Penicillium* sp. ($109 \text{ CFU m}^{-2} \text{day}^{-1}$; 21.2%) (Table 2). The most prevalent fungi followed the same trend of other studies conducted in different Portuguese cities, where *Aspergillus*, *Penicillium* and *Cladosporium* genera also dominated [3,4]. As expected, depending of the culture media, the fungal characterization presented different results in counts and species. Indeed, the exclusive identification by MEA of *Mucor* sp., *Paecilomyces* sp. and *Trichothecium roseum* with clinical relevance and/or with toxigenic potential [43–45], should be highlighted.

Table 2. Distribution of fungal species in both culture media applied.

| Genus/Species | MEA | | | DG18 | | |
|-----------------------------|-----|---------------------------------------|-------|------|---------------------------------------|-------|
| | N | CFU m ⁻² day ⁻¹ | % | N | CFU m ⁻² day ⁻¹ | % |
| <i>Alternaria</i> sp. | 17 | 4.84 × 10 ¹ | 11.3 | 2 | 4.02 × 10 ⁰ | 0.777 |
| <i>Aspergillus</i> sp. | 29 | 1.04 × 10 ² | 26.4 | 24 | 1.49 × 10 ² | 28.8 |
| <i>Aureobasidium</i> sp. | 2 | 8.28 × 10 ⁰ | 2.09 | 3 | 7.46 × 10 ⁰ | 1.44 |
| <i>C. sitophila</i> | 2 | 3.78 × 10 ⁰ | 0.956 | 3 | 5.778 × 10 ⁰ | 1.12 |
| <i>Chrysosporium</i> sp. | 2 | 1.90 × 10 ⁰ | 0.480 | 5 | 2.44 × 10 ¹ | 4.73 |
| <i>Cladosporium</i> sp. | 19 | 7.57 × 10 ¹ | 19.1 | 30 | 2.02 × 10 ² | 39.2 |
| <i>F. oxysporum</i> | 1 | 9.83 × 10 ¹ | 0.248 | 2 | 4.98 × 10 ⁰ | 0.964 |
| <i>Mucor</i> sp. | 5 | 7.48 × 10 ⁰ | 1.89 | 0 | 9.48 × 10 ⁰ | 1.83 |
| <i>Paecilomyces</i> sp. | 1 | 9.07 × 10 ¹ | 0.229 | 0 | Not detected | - |
| <i>Penicillium</i> sp. | 33 | 1.43 × 10 ² | 36.2 | 27 | 1.09 × 10 ² | 21.2 |
| <i>Trichothecium roseum</i> | 1 | 9.83 × 10 ¹ | 0.248 | 0 | Not detected | - |
| <i>Ulocladium</i> sp. | 2 | 2.98 × 10 ⁰ | 0.753 | 2 | Not detected | - |

Regarding *Aspergillus* sp., a total of eight sections were identified in these samples. In MEA media, *Aspergillus* section *Nigri* presented the highest prevalence (54.5%), followed by *Aspergillus* section *Fumigati* (21.7%) and *Aspergillus* section *Nidulantes* (20.1%). Concerning DG18, *Aspergillus* section *Nidulantes* showed the highest prevalence (42.4%), followed by *Aspergillus* section *Circumdati* (34.5%) and *Aspergillus* section *Candidi* (9.89%) (Figure 3). The identification of *Aspergillus* sections (*Fumigati*, *Nidulantes* and *Circumdati*) should be stressed since they are considered indicators of harmful fungal contamination [46]. Additionally, the exclusive identification by MEA of *Aspergillus* sections *Fumigati* and *Flavi* and *Circumdati* section in DG18 increases the importance of applying different culture media to acknowledge the *Aspergillus* contribution to fungal contamination [32]. In fact, the presence indoors of *Aspergillus* section *Fumigati* represents a major risk for the onset and development of respiratory diseases in immunocompromised individuals [47,48], while sections *Flavi* and *Circumdati*, major producers of aflatoxin B1 and ochratoxin, respectively, represent the potential exposure to these mycotoxins [3,44,49].

**Figure 3.** *Aspergillus* sections identified in EDC samples.

3.4. Screening of Azole-Resistance

Fungal contamination in EDC samples ranged from 0.99 to 21.4 CFU m⁻² day⁻¹ in Sabouraud dextrose agar media (SAB), followed by maxima of 3.93, 5.54 and 1.98 CFU m⁻² day⁻¹ in ITR, VOR and POS, respectively. *Penicillium* spp. was also the most frequent in SAB media (82.2 CFU m⁻² day⁻¹; 40.7%), followed by *Aspergillus* spp. (44.6 CFU m⁻² day⁻¹; 22.1%) and *Cladosporium* spp. (27.8 CFU m⁻² day⁻¹; 13.8%), with a fungal distribution similar to that observed in MEA. In azole-supplemented media, a higher fungal diversity was observed in VOR (seven genus/species ranging from 0.98 to 27.8 CFU m⁻² day⁻¹), followed by POS (four genus/species ranging from 0.98 to 2.97 CFU m⁻² day⁻¹) and ITR (three genus/species ranging from 0.93 to 6.79 CFU m⁻² day⁻¹) (Table 3).

Table 3. Distribution of fungal species through azole screening of electrostatic dust collectors (EDC) samples.

| Genus/Species | SAB | | | ITR | | | VOR | | | POS | | |
|---------------------------|-----|---------------------------------------|-------|-----|---------------------------------------|------|-----|---------------------------------------|------|-----|---------------------------------------|------|
| | N | CFU m ⁻² day ⁻¹ | % | N | CFU m ⁻² day ⁻¹ | % | N | CFU m ⁻² day ⁻¹ | % | N | CFU m ⁻² day ⁻¹ | % |
| <i>Alternaria</i> sp. | 5 | 4.74 | 2.35 | 3 | 2.95 | 27.6 | 2 | 3.91 | 8.68 | | | |
| <i>Aspergillus</i> sp. | 24 | 44.6 | 22.1 | 0 | | | 1 | 0.983 | 2.19 | 1 | 1.91 | 24.7 |
| <i>Aureobasidium</i> sp. | 1 | 1.80 | 0.892 | 0 | | | 0 | | | | | |
| <i>C. sitophila</i> | 18 | 27.3 | 13.5 | 1 | | | 0 | | | 2 | 2.97 | 38.2 |
| <i>Cladosporium</i> sp. | 10 | 27.8 | 13.8 | 0 | | | 6 | 7.67 | 17.0 | | | |
| <i>Fusarium oxysporum</i> | 0 | | | 1 | 0.931 | 8.72 | 1 | 0.983 | 2.19 | 1 | 1.90 | 24.4 |
| <i>Fusarium poae</i> | 1 | 1.97 | 0.974 | 0 | | | 0 | | | | | |
| <i>Mucor</i> sp. | 3 | 5.70 | 2.82 | 0 | | | 1 | 1.78 | 3.97 | | | |
| <i>Penicillium</i> sp. | 32 | 82.2 | 40.7 | 6 | 6.79 | 63.7 | 18 | 25.9 | 57.5 | 1 | 0.983 | 12.7 |
| <i>Rhizopus</i> sp. | 3 | 4.76 | 2.36 | 0 | | | 1 | 1.82 | 4.03 | | | |
| <i>Ulocladium</i> sp. | 1 | 0.983 | 0.487 | 0 | | | 0 | | | | | |

SAB, Sabouraud dextrose agar media; ITR, 4 mg L⁻¹ itraconazole SAB media; VOR, 1 mg L⁻¹ voriconazole SAB media; POS—0.5 mg L⁻¹ posaconazole SAB media.

Five *Aspergillus* sections were identified in SAB in 17 (32.7%) of the EDC samples, with a sixth section (*Candidi*) identified in VOR and POS. *Aspergillus* section *Nigri* was also (as in MEA) the most frequent in SAB (28.9%), followed by *Aspergillus* sections *Clavati* (8.38%) and *Fumigati* (4.85%). No *Aspergillus* sections were identified in ITR (Figure 4).

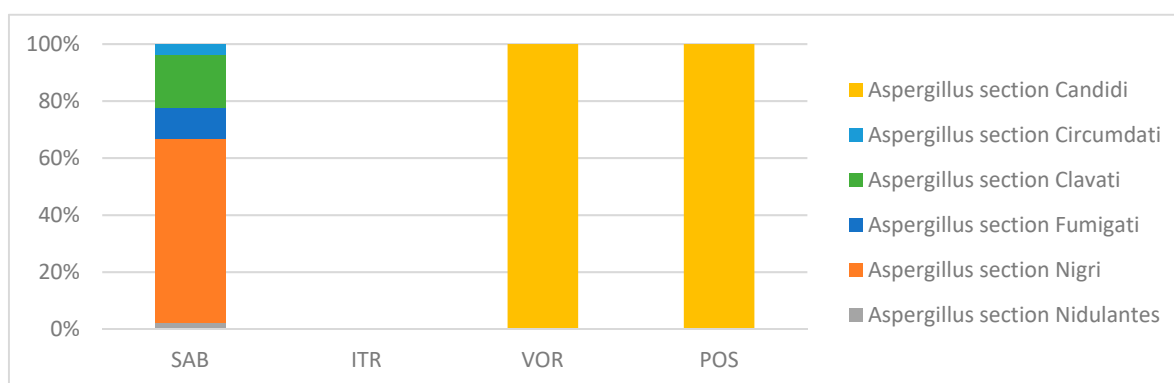


Figure 4. *Aspergillus* sections identified through azole screening of EDC samples.

3.5. Molecular Assessment

None of the fungal species/strains (*Aspergillus* sections *Fumigati*, *Flavi*, *Nidulantes* and *Circumdati*) have been amplified by RT-PCR, while culture-based methods were able to provide positive results within *Aspergillus* genera. False negatives in the qPCR assays for fungal detection can occur. This can be due to inadequate removal of PCR inhibitors from the sample (such as particles present in the EDC), ineffective release of microbial DNA content from the cells, or poor DNA recovery after extraction and purification steps [50,51]. Although these observations, molecular tools are generally an appropriate solu-

tion to overcome the limitations of culture-based methods as they might also provide a more complete diversity profile (e.g., high throughput sequencing), unlike culture methods that might reveal less abundant taxa in an environment or with slower growth rates [52]. Nevertheless, molecular methods often only identify most of the organisms until taxonomic levels [52,53] and this identification level is not exhaustive enough to perform exposure assessment [32].

3.6. Correlation Analysis

Between housing characteristics and bacterial counts (TSA and VRBA), fungal counts (MEA and DG18) and azoles screening (SAB, ITR, VOR and POS), only the following correlations were detected: (i) area of the living rooms and fungal counts in MEA ($r_s = -0.500$, $p = 0.005$) and in DG18 ($r_s = -0.512$, $p = 0.004$); (ii) ceiling height and the fungal counts in MEA ($r_s = -0.464$, $p = 0.010$); (iii) number of floors and fungal counts in SAB ($r_s = 0.368$, $p = 0.045$) and in POS ($r_s = 0.467$, $p = 0.009$) (Table 4). These results reveal that larger dimensions of the rooms and higher ceiling heights are related to less fungal counts, whilst a higher number of floors of the building is associated with higher fungal counts in SAB and in POS.

Fungal proliferation indoors could be a result of water leakage or inadequate ventilation [54]. In fact, larger spaces can favor better ventilation and dilution of the microbial contamination [54]. On the other hand, house typologies with a higher number of floors, thus, more compartmentalized, might have poorer ventilation and promote the accumulation of dust, as all residential surfaces may act as passive collectors of airborne fungi of outdoor origin [55]. This phenomenon has already been described in previous studies [56]. Noteworthy, the observed correlation between higher fungal counts in POS and higher number of floors suggests that these housing typologies may favor the development indoors of fungal resistance. Dust and moisture are strongly associated with fungal concentration [57]. However, there are other important determinants, such as type of building material [58], air temperature and moisture levels [59] and also some type of carpet fiber that can affect fungal growth [57]. Additionally, occupants' behavior can also influence the indoor microbiome through the different frequency of window opening, the use of exhaust fans [60] or of humidifiers [60,61]. Significant correlations were only found between bacterial counts in TSA and in VRBA ($r_s = 0.440$, $p = 0.015$) and fungal counts in SAB ($r_s = 0.416$, $p = 0.022$), revealing that higher bacterial counts in TSA are related to higher bacterial counts in VRBA and higher counts in SAB (Table 4). Whereas the origin of bacteria indoors is linked to a mixture of bacteria from outdoor air, house inhabitants, and bacterial growth on surfaces, fungal contamination originates mostly from outdoor air, with some fungal growth on surfaces with high levels of moisture [55].

The EDC sampler, as used in this study, collects airborne bacteria and fungi that are allowed to deposit on its surface during the exposure period (about 3 months, in the present case). The fact that a relation between bacteria and fungi was observed only in SAB (and not in MEA or DG18) might be related to the substrate nature of these fungal media. Sabouraud's agar is a type of agar growth medium containing peptones that is selective to certain fungi due to its low pH (5.6) and high glucose concentration (3–4%). DG18 media has dichloran added to limit the spread of some fast-growing fungal colonies, limiting their diameter. As for MEA media, they have been extensively used for decades due to its low cost and high availability of malt extract. However, for the wide range of fungi growing in microenvironments such as damp cardboard or carpets in water damaged buildings, the use of maltose as substrate is not likely given its typical absence in those ecological niches [62].

Table 4. Study of the relationship between housing characteristics, bacterial counts (tryptic soy agar (TSA) and violet red bile agar (VRBA)) and fungal counts (malt extract agar (MEA) and DG18) and azoles screening (in SAB, ITR, VOR and POS): Spearman correlation results.

| | | | Bacterial Counts | | Fungal Counts | | Azoles Screening (CFU m ⁻² | | | |
|--|---------------------|--------|------------------|--|---------------|--|---------------------------------------|---------------------|---------|----------|
| | | | Deposit Rate | (CFU m ⁻² day ⁻¹) | | (CFU m ⁻² day ⁻¹) | | day ⁻¹) | | |
| | | | | TSA | VRBA | MEA | DG18 | SAB | ITR | VOR |
| Characteristics of dwellings | | | −0.133 | 0.115 | −0.044 | −0.043 | 0.179 | 0.181 | 0.299 | −0.310 |
| | Household | 0.033 | −0.258 | −0.015 | −0.234 | −0.298 | −0.193 | −0.096 | −0.091 | −0.103 |
| | Housing age in 2019 | 0.230 | 0.056 | −0.188 | 0.265 | 0.192 | −0.006 | 0.052 | 0.096 | −0.134 |
| | Number of floors | −0.051 | 0.284 | 0.205 | −0.152 | 0.049 | 0.368 * | 0.021 | 0.093 | 0.467 ** |
| | Floor | −0.043 | 0.074 | 0.040 | 0.248 | 0.256 | −0.007 | 0.061 | 0.168 | −0.209 |
| | Division area | 0.160 | −0.319 | −0.195 | −0.500 ** | −0.512 ** | −0.245 | 0.069 | 0.078 | −0.168 |
| | Ceiling height | −0.080 | −0.099 | 0.010 | −0.464 ** | −0.301 | −0.048 | −0.196 | −0.179 | 0.152 |
| | Window dimension | 0.008 | −0.123 | 0.362 | −0.351 | −0.165 | −0.145 | −0.235 | 0.167 | 0.112 |
| Bacterial counts | TSA | | | 0.440* | 0.294 | 0.352 | 0.416 * | −0.103 | 0.063 | 0.291 |
| (CFU m ⁻² day ⁻¹) | VRBA | | | | 0.206 | 0.339 | 0.121 | 0.116 | 0.065 | −0.065 |
| Fungal counts | MEA | | | | | 0.779 ** | 0.272 | 0.285 | 0.320 | 0.016 |
| (CFU m ⁻² day ⁻¹) | DG18 | | | | | | 0.199 | 0.184 | 0.233 | −0.031 |
| Azoles | SAB | | | | | | | 0.277 | 0.350 | 0.220 |
| screening | ITR | | | | | | | | 0.402 * | −0.243 |
| (CFU m ⁻² day ⁻¹) | VOR | | | | | | | | | 0.038 |

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

3.7. Comparison between Sampling Locations

From the comparison of bacterial counts, fungal counts and azoles screening, among the type of housing, only statistically significant differences were detected in relation to the bacterial counts in VRBA ($U = 45.5$, $p = 0.041$). It was found that the bacterial counts in buildings were higher, probably because detached houses have more solar radiation than buildings [63], decreasing the counts of Gram-negative bacteria due to their less tolerance to environmental factors [40].

Among the types of heating system, wall materials, window frames, types of frames, presence or absence of rugs, smoke in the room and the presence/absence of fungi, no statistically significant differences were detected in terms of bacterial and fungal counts and azoles screening.

Statistically significant differences were detected between fungal counts in DG18 and the presence or absence of pets ($U = 25,000$, $p = 0.015$). Higher fungal contamination in DG18 was found when pets are present. Indeed, pets have been described as a contamination source of fungi indoors [64].

With regard to the frequency of cleaning, statistically significant differences were detected for fungal counts in VOR ($U = 45.5$, $p = 0.042$), showing higher values when cleaning was weekly, in opposition to daily. While this correlation may suggest that a reduced frequency in cleaning favors the development or persistence of fungal resistance indoors, it is noteworthy that many fungal species present intrinsic resistance to voriconazole, namely, of the *Mucor* genera [43], as found in this study (Table 4). It is also important to have information on the microbiome outdoors in order to determine the origin of azole resistance. Other fungal species observed in VOR media, such as *Aspergillus* sp., raise concern on the origin of fungal resistance and its implications for human health [8–10,12–14,58,65,66].

3.8. Importance of Housing Characteristics for Bacterial Counts, Fungal Counts and Azoles Screening

Figures 5–7 show the importance (normalized) of the characteristics of the dwellings for the bacterial counts, fungal counts, and screening azoles. From their analysis, it can be seen that regarding the counts in TSA, the heating system is the one that contributes the most, while in VRBA it is the frequency of cleaning, followed by the heating system and pets. In the case of fungal counts in MEA, the factor that contributed most was the type of ventilation. In DG18, the heating system was the most influential, followed by the type of house, rugs and cleaning frequency. These results corroborate the role of all these factors as determinants of microbial contamination indoors [54,59–61,65–67].

Finally, for fungal counts in SAB, what contributed most was housing type, ventilation type and cleaning frequency. In ITR, the most influential factor was the material of the walls. This is in accordance with the fungal species detected in ITR (*Alternaria* sp., *Fusarium oxysporum*, and *Penicillium* sp., (Table 4), which are commonly found in ceramic tiles, concrete efflorescences, brick and stone [59,68,69]. In VOR, what contributed most was the cleaning frequency (as abovementioned), followed by the rugs, ventilation type, housing type and pets. The effects of carpet fiber materials and dust loading on fungal growth has been recently described [57]. Nastasi and colleagues concluded that elevated moisture, followed by the presence of house dust are the main contributors to fungal growth in carpets. In POS, the major determinant was the type of ventilation (natural, in 29 out of 30 cases), followed by pets and housing type [57]. Fungal species observed in POS (*C. sitophila*, *Aspergillus* sp., *Fusarium oxysporum* and *Penicillium* sp.) are commonly found outdoors. Natural ventilation, prevalent in the dwellings of this study, can be a source of microorganisms, especially in the countryside or near nature, and promote their way into buildings during ventilation periods, thus, contaminating the indoor environments [59]. The fact that they were able to grow in a fixed concentration of posaconazole is of concern for *Aspergillus* sp. and must be further explored in order to determine fungal susceptibility and consequent risk for the health of house inhabitants, especially if immunocompromised or with respiratory diseases [13,14].

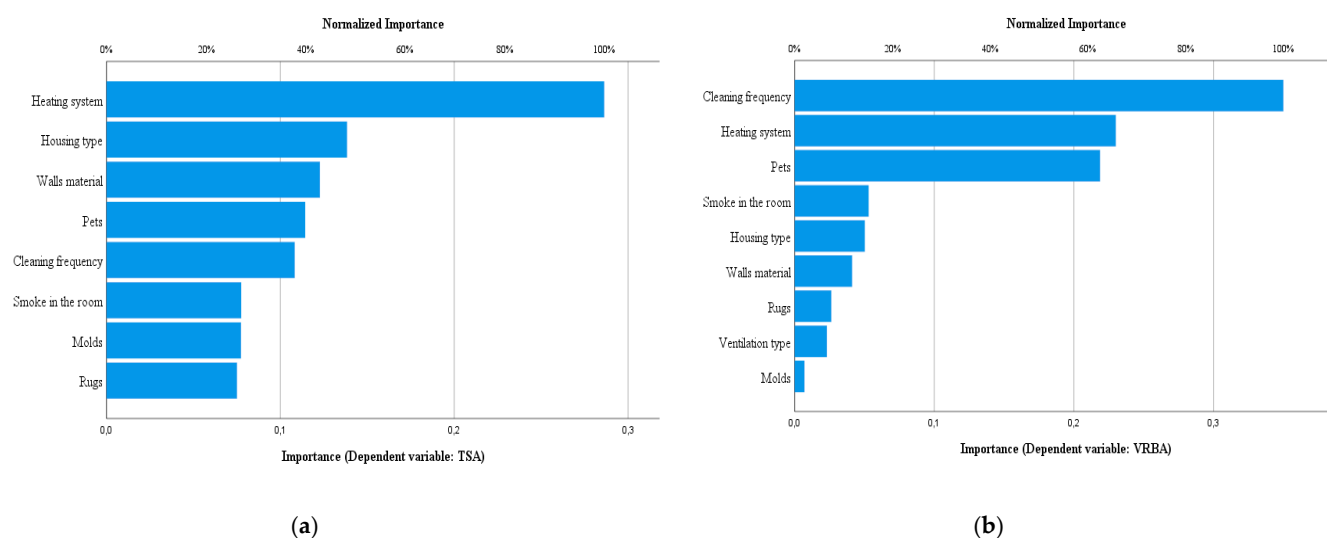
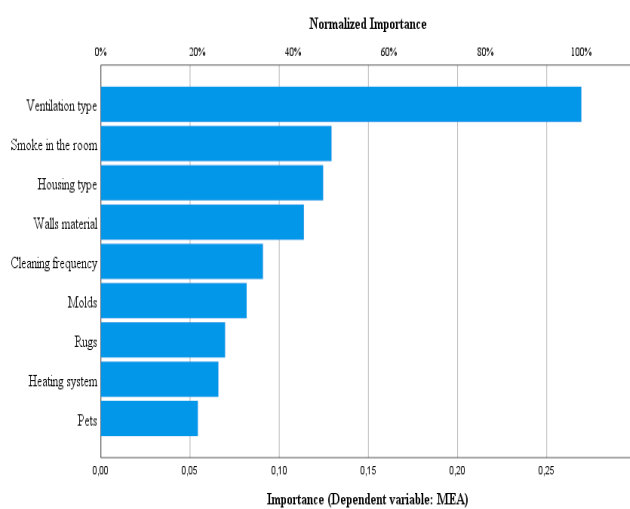
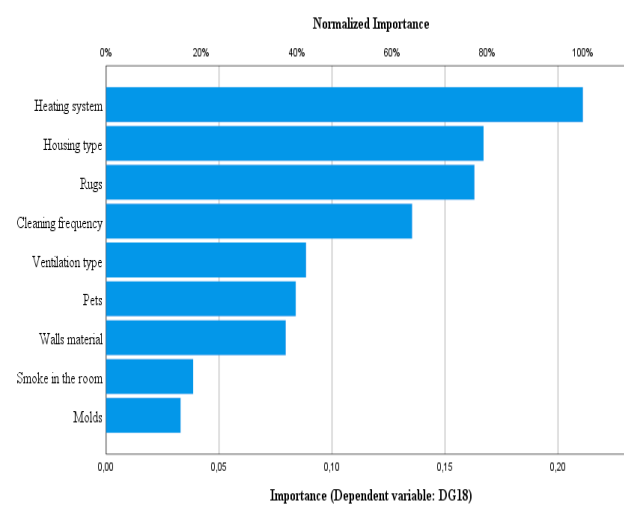


Figure 5. Normalized importance of housing characteristics for bacterial counts. (a)TSA, (b)VRBA.

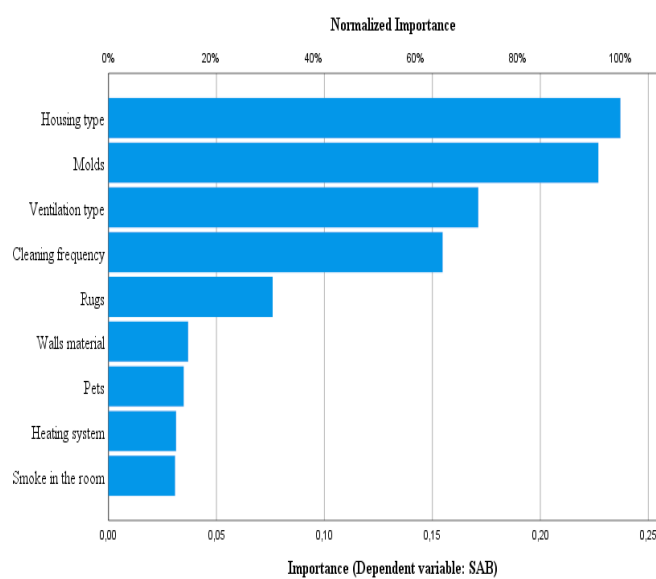


(a)

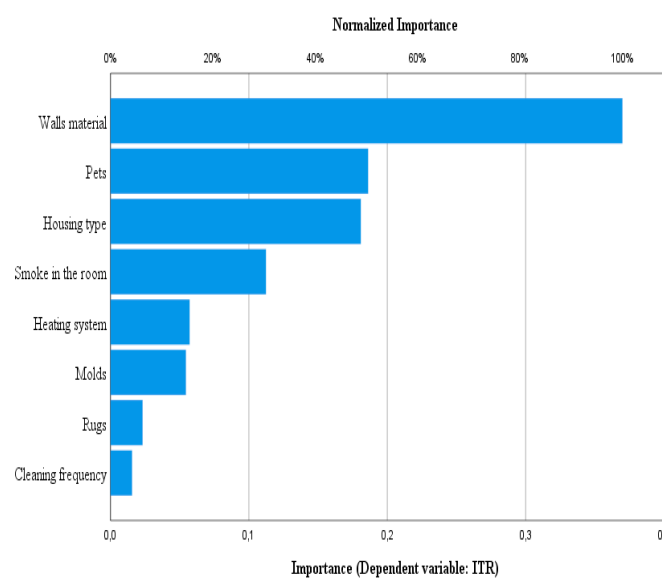


(b)

Figure 6. Normalized importance of housing characteristics for fungal counts. (a) MEA, (b) DG18.



(a)



(b)

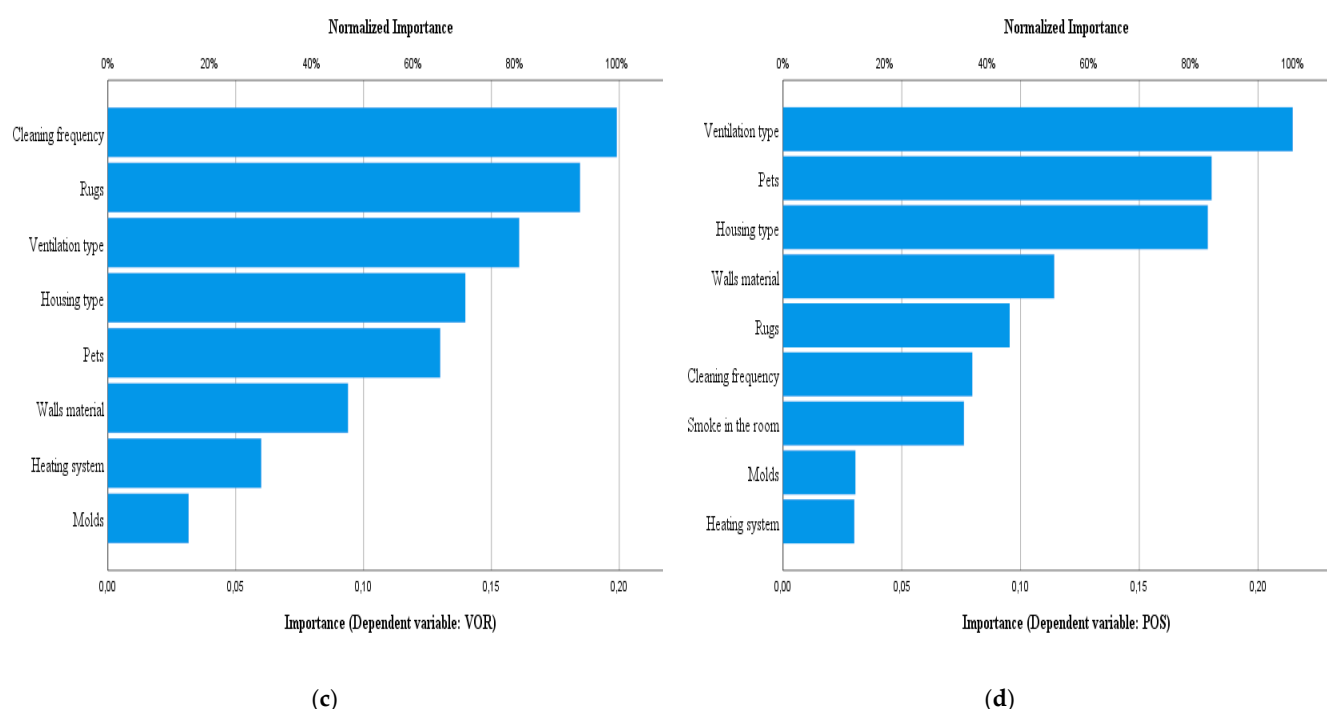


Figure 7. Normalized importance of housing characteristics for azoles screening. (a) SAB, (b) ITR, (c) VOR, (d) POS.

It was possible to obtain information on the housing typologies and characteristics that most influence microbial contamination, including azole resistance. The influence of all these variables was previously studied [54–70]. In the present study, results correlated with several physical variables. However, the results of microbial counts can be underestimated due to the extended sampling duration that can decrease microbial viability and because of the aforementioned limitations of culture-based methods [52,70].

In future studies, the record on opening windows should be included, since the promotion of natural ventilation contributes to the decrease of microbial loads in the indoor air [71].

4. Conclusions

The use of electrostatic dust collectors allowed the assessment of settleable dust and bioburden, and to identify the variables that most influence them, in the assessed dwellings. Overall, it was possible to observe:

1. Higher settleable dust loadings and fungal contamination levels in dwellings with pets;
2. Fungal species considered indicators of harmful fungal contamination, namely *Aspergillus* sections (*Fumigati*, *Nidulantes* and *Circumdati*);
3. The presence in dwellings of *Aspergillus* sp. (namely, *Aspergillus* section *Candidi*) with reduced susceptibility to azoles, thus, with potential risk for immunocompromised inhabitants or individuals with respiratory diseases, corroborating the need to assess the prevalence of azole resistance also in indoor environments;
4. Specific housing typologies, such as larger dimensions of the rooms and higher ceiling heights, leading to less fungal counts;
5. Specific housing characteristics favoring the development of microbial communities in the indoor air, namely heating system, cleaning frequency and pets, in the case of bacterial contamination, and ventilation type, heating system, house type, rugs and cleaning frequency with regard to fungal contamination.

Supplementary Materials: The following are available online at www.mdpi.com/2073-4433/12/3/378/s1, Table S1: Characteristics of dwellings where the samples were taken.

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