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ABSTRACT

The collection and analysis of settled dust samples from indoor environments has become one of several environmental sampling methods used to assess bioburden indoors. The aim of the study was to characterize the bioburden in vacuumed settled dust from 10 Primary Health Care Centers by culture based and molecular methods. Results for bacterial load ranged from 1 to 12 $CFU.g^{-1}$ of dust and Gram-negative bacteria ranged between 1 to 344 $CFU.g^{-1}$ of dust. Fungal load ranged from 0 $CFU.g^{-1}$ of dust to uncountable. *Aspergillus* section *Fumigati* was detected in 4 sampling sites where culture base-methods could not identify this section. Mucorales (*Rhizopus* sp.) was observed on 1 mg/L voriconazole. Three out of 10 settled dust samples were contaminated by mycotoxins. Settled dust sampling coupled with air sampling in a routine way might provide useful information about bioburden exposure.

Introduction

Occupational exposure to bioburden, defined as microbial contaminants such as fungi and bacteria (Cabo Verde et al. 2015; Viegas et al. 2018a), in the workplaces is a major concern (Kettleson et al. 2015; Nevalainen et al. 2015) with increased relevance in clinical environments (Cabo Verde et al. 2015). Clinical settings must provide a clean and safe environment to protect patients and staff from nosocomial infections and occupational diseases (Leung and Chan 2006). Occupational Health and Indoor Air Quality (IAQ) studies have an important role in the exposure and risk assessment and, consequently, in management strategies (Cabo Verde et al. 2015).

Active and passive sampling methods have been used to characterize occupational exposure to culturable bioburden (Reponen 2017). However, although personal or stationary sampling of airborne bioburden can be performed to quantify exposure by inhalation, may be

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extensively influenced by environmental variables, such as seasonal variation and ventilation (Flannigan 1997).

Whereas passive-collection methods allow the collection of contamination over a longer period (days, weeks or several months), air samples can only reflect the load of a shorter period (mostly minutes) corresponding to the sampling duration (Badyda et al. 2016; Viegas et al. 2018b). Passive and active methods should, therefore, be used in parallel to ensure a more precise assessment of occupational exposure to bioburden (Reponen 2017; Viegas 2018). Increasing the number of different sampling methods will increase and enrich the data, enabling industrial hygienists to perform a more accurate risk characterization (Reponen 2017; Viegas 2018).

The collection and analysis of settled dust samples from indoor environments has become one of several environmental sampling methods used during bioburden evaluations by several researchers (Leppänen et al. 2018; Park et al. 2018). Settled dust has also been used for the assessment of mycotoxins contamination (Halstensen et al. 2006; Tangni and Pussemier 2007; Viegas et al. 2018e). In addition, settled dust was reported as an environmental support for bacterial development and is therefore considered a reservoir of bacterial contamination (Bouillard et al. 2005).

Passively settled dust can be sampled by directly vacuuming carpets, furniture or floors into filters, tubes or nylon sampling socks (Leppänen et al. 2014). The origins of bioburden in floor dust include the deposition of microbiota from outdoor air, humans, pets and pests, and also from growth in building material or furniture (Dunn et al. 2013). Settled dust can be used as an inhalation exposure surrogate marker as it presents the crucial feature of being less influenced by the short-term variability in indoor activities and ventilation (Meyer et al. 2004). The presence of bioburden in settled dust holds has an additional significance due to the possibility of dust-borne microorganisms and their metabolites to become resuspended and inhaled (Aleksic et al. 2017).

Culturable fungal loads exceeding 10^5 colony forming units per gram (CFU.g^{-1}) of dust collected from carpet or furniture surfaces have been suggested as evidence that an indoor environment has been contaminated with fungi (Hodgson and Scott 1999). However, the data from their control group is limited and may not be representative (Hicks et al. 2005). Besides this quantitative criteria, qualitative criteria should also be considered. Hodgson and Scott have proposed the identification of the present genera, particularly *Penicillium* sp. and *Aspergillus* sp., as indicators of indoor fungal contamination (Hodgson and Scott 1999). Non-problematic indoor environments do not present a dominant genera when three or more genera are present, unless *Cladosporium* or *Alternaria* genera are more prevalent (Hodgson and Scott 1999).

Airborne bioburden levels have already been described for some health care environments, such as hospital lobbies (Park et al. 2013), Portuguese operating theatres (Cabo Verde et al. 2015), bronchoscopy operating room (Marchand et al. 2016), patient rooms (Klánová and Hollerová 2003), haematological wards and surrounding areas (Tang and Wan 2013), and Portuguese hospital canteens (Viegas et al. 2011). However, until now, bioburden assessment has not been reported for Primary Health Care Centers in Portugal, even though patients attending such facilities routinely present a vulnerable health status.

The qualitative criteria, besides the quantitative, in the assessment of IAQ is of utmost importance in clinical settings, since not only different species may be related to different health outcomes, and the prevalence of antimicrobial resistance in human pathogens is also a global health challenge, with increased risk for subsequent occurrence of clinical diseases (up to 10-fold) (WHO 2016). The increasingly emergence of antifungal resistant *Aspergillus* sp., in the clinical context and in the environment should be considered (Perlin 2017). The increasing occurrence of cryptic species (i.e. morphologically indistinguishable species that can only be recognized by molecular phylogenetic methods and their DNA sequences) is also of concern as they may represent significant differences in the severity of diseases they cause (Lamoth 2016). The importance of cryptic species, often toxigenic (i.e. producers of mycotoxins) and/or drug resistant, reinforces the need to use complementary strategies, such as the measurement of mycotoxins or molecular detection of toxigenic species, for a broader characterization of the existent microbiota

(Viegas et al. 2012). Therefore, using settled dust, which represents the cumulative indoor contamination of both bacteria and fungi (Meyer et al. 2004; Bouillard et al. 2005), for molecular detection and antimicrobial resistance should be considered in IAQ assessments (Viegas et al. 2017).

Thus, the aim of the study was to characterize the bioburden in settled dust from 10 Primary Health Care Centers by culture based and molecular methods. Additionally, screening of azole-resistance and mycotoxins detection was also performed to allow a detailed assessment of the bioburden. The data obtained will provide useful information for future recommendations and guidelines on occupational exposure and IAQ and support preventive actions to protect the health of healthcare workers and patients.

Materials and methods

Primary health care centers assessed

This exploratory study aimed to establish protocols to assess occupational exposure to bioburden in clinical settings. The study was conducted between June and September 2018 in 10 Portuguese Primary Health Care Centers (PHCC) located in the Lisbon district. The 10 assessed were the ones that accepted to participate in the study.

The sites selected for sampling (Table 1) were the ones classified as more critical regarding occupational exposure to bioburden (information retrieved by a walkthrough survey). Most PHCC were organized in two different zones: one used by the clinical staff and one used by the patients. In this study, the bioburden assessment was performed in the zone used by the workers, comprising different areas (Table 1). In each PHCC, a composite sample of settled dust was obtained covering different sampling areas and surfaces (Table 1). Additionally, dust from the vacuum bag used in all PHCC was collected at the end, for an additional characterization of the assessed PHCC.

A prior walkthrough survey was performed, as mentioned, to collect information regarding cleaning routines and procedures and to observe any type of water infiltration and fungal growth (Table 2). Sampling sites (floors and surfaces) are cleaned daily after patient service and working hours, and are performed by an external company. The treatment room due to the type of activity performed is cleaned several times a day by the workers of each PHCC or by an external company after working hours. The waste is collected, the floor is always cleaned and the surfaces when are visibly contaminated with organic fluids. The floor is cleaned with mop and surfaces with cloths. Some of the cleaning products contained disinfectants; however no information on the frequency and conditions of use (e.g. dilution factor) was obtained.

Table 1. Different areas covered by the composite sample in each PHCC.

Areas	Number of covered areas	Surfaces
Medical office	12[#]	Chairs, floor, desk, cabinets, digital scales, skirting board, window sills, door jamb, portable massage tables, keyboard, telephone, computer and printer
Technical office	1[*]	Chairs, floor, desk, cabinets, digital scales, skirting board, window sills, door jamb, keyboard, telephone, computer and printer
Vaccination room	10	Chairs, floor, desk, cabinets, digital scales, skirting board, window sills, door jamb, portable massage tables, keyboard, telephone, computer and printer
Treatment room	10	Chairs, floor, desk, cabinets, digital scales, skirting board, window sills, door jamb, portable massage tables, keyboard, telephone, computer and printer
Corridor	10	Floor and skirting board
Warehouse/Cleaning room	8	Floor, skirting board, cabinets and workbench

PHCC 1 and 9 had 2 medical office assessed
* PHCC1

Table 2. Vacuumed settled dust weights and cleaning routines and observations obtained during the walkthrough survey.

PHCC	SETTLE DUST SAMPLES	SETTLED DUST WEIGHT (G)	CLEANING ROUTINES	OBSERVATIONS
1	D-HU1	0.34	All areas cleaned once a day	Cracks on the walls of the vaccination room
2	D-HU2	0.3	All areas cleaned once a day except treatments room (cleaned twice)	Visible mold growth in some corridors
3	D-HU3	0.75	All areas cleaned once a day except vaccination treatments rooms and medical office (cleaned twice)	Cracks on the walls of the vaccination room and treatments room; visible mold growth in the vaccination room and medical office
4	D-HU4	0.66	All areas cleaned once a day except treatments room (cleaned four times a day)	
5	D-HU5	0.24	All areas cleaned once a day except treatments room (cleaned twice)	Cracks on the walls and visible mold growth in some corridors
6	D-HU6	0.6	All areas cleaned once a day except treatments room (cleaned twice)	
7	D-HU7	0.51	All areas cleaned once a day except treatments room (cleaned four times a day)	Cracks on the walls in some corridors
8	D-HU8	0.48	All areas cleaned once a day except treatments room (cleaned twice)	Cracks on some walls and floors
9	D-HU9	0.36	All areas cleaned once a day except treatments room (cleaned four times a day)	Visible mold growth and cracks on some walls
10	D-HU10	1.52	All areas cleaned once a day except treatments room (cleaned twice)	Visible mold growth and discoloration on the walls of the medical office

Settled dust sampling

Sampling of the settled dust was performed by a vacuum cleaner (HOOVER Brave BV71_BV10 A2, USA) equipped with a 40 micron nylon mesh inserted in a DUSTREAM™ collector (Indoor Biotechnologies, USA). Vacuuming was conducted for 10 minutes in different areas (Table 1) and a DUSTREAM™ filter tube was collected in each PHCC (n = 10). Settled dust was collected from several surfaces for a better representation of airborne contamination. The weight of the 10 PHCC dust samples was measured (Table 2) and analysed for microbial load and mycotoxins.

Bioburden isolation

The vacuumed dust (retained in the filter of nylon mesh) collected in each PHCC, as well as one piece (2 cm^2) of the vacuuming bag used in all assessments, were used for bioburden character-ization. All the samples were weighted and washed in a ratio of 1 g (dust) or 2 cm^2 (vacuum bag) per 9.1 ml of NaCl 0.9% with 0.05% Tween 80 (10 μl) for 60 min at 250 rpm and 0.15 mL of this suspension were spread onto four media: 2% malt extract agar (MEA) with 0.05 g.L^{-1} chloram-phenicol media (Frilabo, Portugal); dichloran glycerol (DG18) agar-based media (Frilabo, Portugal); tryptic soy agar (TSA) with 0.2% nystatin (Frilabo, Portugal); and Violet Red bile agar (VRBA) (Frilabo, Portugal). Samples were also spread (0.15 ml) onto Sabouraud agar media supplemented with 4 mg.L^{-1} itraconazole, 1 mg.L^{-1} voriconazole, or 0.5 mg.L^{-1} posaconazole (protocol adapted from the EUCAST 2017 guidelines) (Frilabo, Portugal) (Arendrup et al. 2013) for the screening of antifungal resistance.

After incubation of MEA, DG18 and Sabouraud at 27 ∘C for 5 to 7 days for fungi and TSA and VRBA at 30 ∘C and 35 ∘C for 7 days for mesophilic bacteria and coliforms (Gram-negative

bacteria), respectively, bioburden densities (colony-forming units, CFU·g^{-1} of dust or CFU.m^{-2}) were calculated. Fungal species were identified microscopically using tease mount or Scotch tape mount and lactophenol cotton blue mount procedures. Morphological identification was achieved through macro and microscopic characteristics as noted by De Hoog et al. (2000).

When colonies overgrowth was observed due to fungi with fast growing rates (*Chrysonilia sitophila*), making it impossible to count colonies, a quantitative cut off was applied of 30 CFU (highest counts of colonies on the 10 settled dust samples excluding *Chrysonilia sitophila*) to allow obtaining fungal densities.

Fungal biomass, Aspergillus/Penicillium/Paecilomyces and Aspergillus section Fumigati molecular detection

In average 10 mg of dust was extracted in 200 μl of PCR grade water using the Quick-DNA™ Fungal/Bacterial Miniprep Kit (Zymo-research corp, Irvine, CA, USA). For each extraction batch, a negative control of extraction was performed using only PCR grade water. The DNA extracts were kept at −20°C until analysis. The primers and probes of the amplification systems used are shown in Table 3. The digital droplet PCR assay (ddPCR) was performed with a PCR reaction volume of 25 μL using the ddPCR™ Supermix for Probes (no dUTP) master mix (Bio-Rad, Hercules, CA) including 2 μL of the template DNA. Positive controls were done in the same conditions with *Penicillium digitatum* or *Aspergillus fumigatus* DNA depending on the target assay. For the negative controls (NTC), the DNA was replaced by PCR grade water.

The fungal biomass, *Aspergillus/Penicillium/Paecilomyces* and the *Aspergillus* section *Fumigati* assays performed by dd-PCR were amplified in a Bio-Rad T-100 thermal cycler using the following cycling conditions: an initial denaturation step at 95°C for 10 minutes, followed by 40 cycles consisting of denaturation at 94°C for 30 seconds, annealing step at 49°C for Afumi and PenAsp or at 52°C for fungal biomass for 1 minute and extension at 72°C for 30 seconds, followed by a final extension step at 98°C for 10 minutes and a 12°C indefinite hold.

Amplicons (samples, NTC and PC) were immediately analyzed on the QX200™ Droplet Reader. Amplicons were classified as positive or negative according to a threshold manually set at 300 across all wells based upon results of the NTC and PC results. The QuantaSoft™ v.1.7.4.0917 (Bio-Rad) software was used to calculate the concentration of each amplicon, along with their Poisson-based 95% confidence intervals (Hindson et al. 2011).

In order to ensure reliable results, the manufacturer recommends to only analyze wells having more than 10,000 counted droplets, so only those were included in the analysis.

Molecular detection of other Aspergillus sections and Stachybotrys chartarum species complex

Molecular identification from specific species/strains recognized by their toxigenic potential was achieved by Real Time PCR (qPCR) using the CFX-Connect PCR System (Bio-Rad) on settled dust and vacuuming bag as previously indicated (Viegas et al. 2018c). 20ul Reactions included

Table 3. Primers and TaqMan® probe used in the dd-PCR for *Aspergillus* section *Fumigati*, *Aspergillus/Penicillium/Paecilomyces* and the global fungi assays.

Target	Primers/probes names	Sequences	Reference
Aspergillus section *Fumigati* (*Aspergillus fumigatus* and *Neosartorya fischeri*)	AfumiF1	5'-GCC CGC CGT TTC GAC-3'	Haugland et al. 2002
	AfumiR1	5'-CCG TTG TTG AAA GTT TTA ACT GAT TAC-3'	
	AfumiP1	5'-CCC GCC GAA GAC CCC AAC ATG-3'	
Aspergillus/ Penicillium/Paecilomyces	PenAspF1	5'-CGG AAG GAT CAT TAC TGA GTG-3'	Haugland et al. 2004
	PenAspR1	5'-GCC CGC CGA AGC AAC-3'	
	PenAspP1 mgb	5'-CCA ACC TCC CAC CCG TG-3'	
Fungal biomass	FungiQuant-F	5'-GGR AAA CTC ACC AGG TCC AG-3'	Liu et al. 2014
	FungiQuant-R	5'-GSW CTA TCC CCA KCA CGA-3'	
	FungiQuant-Probe	5'-TGG TGC ATG GCC GTT-3'	

R = A or G (puRine); W = A or T (Weak – 2 H bonds); K = G or T (Keto)

1× iQ Supermix (Bio-Rad), 0.5 µM of each primer (Table 4), and 0.375 µM of TaqMan probe and 4 ul of DNA template. 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. As positive controls of amplification, DNA samples were obtained from reference strains from the Mycology Laboratory from the National Institute of Health Doctor Ricardo Jorge (INSA).

Mycotoxins analysis

Ten settled dust samples and one filter from the dust bag were screened for mycotoxins presence. Settled dust samples (0.20 g) were extracted with 2.0 mL of ACN: H_2O: AcOH (79:20:1) for 90 minutes using Multi Reax shaker (Heidolph, Schwabach, Germany). Raw extracts were diluted with the same amount of water (total sample-to-solvent ratio was 1:20 (w/v), mixed, centrifuged and injected into the LC-MS/MS system. Detection of mycotoxins was carried out using high performance liquid chromatograph (HPLC) Nexera (Shimadzu, Tokyo, Japan) with a mass detector API 4000 (Sciex, Foster City, CA, USA).

Mycotoxins were separated on a chromatographic column Gemini NX-C18 (150 × 4.6 mm, 3 µm) (Phenomenex, Torrance, CA, USA); mobile phase (A: water + 5 mM ammonium acetate + 1% acetic acid, B: methanol + 5 mM ammonium acetate + 1% acetic acid) mobile phase flow rate: 0.75 mL.min^{-1}, injection volume: 7 µL. Several mycotoxins were assessed, namely: patulin, nivalenol, deoxynivalenol-3-glucoside, deoxynivalenol, fusarenon-X, α-zearalanol, β-zearalanol, β-zearalenol, α-zearalenol, zearalanone, zearalenone, T2 tetraol, deepoxydeoxynivalenol, neosolaniol, 15-acetyldeoxynivalenol, 3-acetyldeoxynivalenol, monoacetoxyscirpenol, diacetoxyscirpenol, aflatoxin M1, aflatoxin B1, aflatoxin B2, aflatoxin G1, aflatoxin G2, fumonisin B1, fumonisin B2, fumonisin B3, T2 triol, roquefortine C, griseofulvin, T2 toxin, HT2 toxin, ochratoxin A, ochratoxin B, mycophenolic acid, mevinolin and sterigmatocystin. The limits of detection (LOD) and quantification (LOQ) obtained for each mycotoxin with the analytical method used are presented in Table 5.

The LOD (signal-to-noise ratio of 3) and LOQ (signal-to-noise ratio of 10), respectively, were calculated (using the 'S-To-N' script of Analyst® 1.6.2 software) by spiking blank dust extract at low concentrations.

Statistical analysis

The data were analyzed in the statistical software SPSS, v 23.0 for Windows. The results were considered significant at the significance level of 5%. The Shapiro-Wilk test was used to test the normality of the data. Frequency analysis (n; %), and the calculation of the minimum and maximum, were performed for the qualitative and quantitative data, respectively. Since the assumption of normality was not verified, the Spearman correlation coefficient was used to study the relationship between fungal load, fungal biomass and bacterial load and with the dust screened for fungal biomass assay and copies.g^{-1}.

Results

Bioburden characterization

Results for bacterial load (in TSA media) in settled dust samples ranged from 1 CFU.g^{-1} to 12 CFU.g^{-1}, excluding one PHCC (PHCC 10) with uncountable result (Figure 1). Gram-negative bacteria load (in VRBA media) in settled dust samples ranged between 1 to 344 CFU.g^{-1}, with PHCC 1 presenting the highest value (Figure 1). In the vacuuming bag sample, the bacterial load was 55×10^2 CFU.m^{-2} in TSA, and 25×10^2 CFU.m^{-2} in VRBA.

Table 4. Sequence of primers and TaqMan probes used for real time PCR.

Target	Sequences	Reference
***Aspergillus* section *Flavi* (Toxigenic Strain – nor 1 gene)**		
Forward Primer	5′-GTCCAAGCAACAGGCCAAGT-3′	
Reverse Primer	5′-TCGTGCATGTTGGTGATGGT-3′	(Mayer et al. 2003)
Probe	5′-TGTCTTGATCGGCGCCCG-3′	
***Aspergillus* section *Circumdati* – 18SrDNA**		
Forward Primer	5′-CGGGTCTAATGCAGCTCCAA-3′	
Reverse Primer	5′-CGGGCACCAATCCTTTCA-3′	(Viegas et al. 2017)
Probe	5′-CGTCAATAAGGCGTTTT-3′	
***Aspergillus* section *Versicolores* -ITS region**		
	5 – CGGCGGGGAGCCCT-3	
	5 – CCATTGTTGAAAGTTTTGACTGATcTTA-3	(EPA 2017)
	5 – AGACTGCATCACTCTCAGGCATGAAGTTCAG-3′	
***Stachybotrys chartarum* complex – 18SrDNA**		
Forward Primer	5′- GTTGCTTCGGCGGGAAC – 3′	
Primer Forward	5′- TTTGCGTTTGCCACTCAGAG – 3′	(Cruz-Perez et al. 2001)
Primer Reverse	5′- CTGCGCCCGGATCCAGGC – 3′	
Probe		

Table 5. Limits of detection (LOD) and quantification (LOQ) for dust samples.

Mycotoxin	LOD (μg.kg-1)	LOQ (μg.kg^{-1})
Patulin	2.5	8.4
Nivalenol	10.8	36.0
Deoxynivalenol-3-glucoside	12.6	42.0
Deoxynivalenol	5.6	18.8
Fusarenon-X	9.5	31.8
α-Zearalanol	4.2	14.0
β-Zearalanol	2.2	7.2
β-Zearalenol	3.4	11.2
α-Zearalenol	2.2	7.2
Zearalanone	1.1	3.6
Zearalenone	0.5	1.5
T2 Tetraol	12.3	41.0
Deepoxydeoxynivalenol	1.0	3.3
Neosolaniol	0.4	1.2
15-Acetyldeoxynivalenol	2.3	7.6
3-Acetyldeoxynivalenol	2.1	6.9
Monoacetoxyscirpenol	0.5	1.6
Diacetoxyscirpenol	0.7	2.4
Aflatoxin M1	0.2	0.6
Aflatoxin B1	0.2	0.5
Aflatoxin B2	0.2	0.6
Aflatoxin G1	0.1	0.4
Aflatoxin G2	0.3	1.1
Fumonisin B1	1.6	5.3
Fumonisin B2	1.1	3.8
Fumonisin B3	1.4	4.6
T2 Triol	0.9	3.0
Roquefortine C	0.7	2.2
Griseofulvin	0.4	1.2
T2 toxin	0.4	1.2
HT2 toxin	0.8	2.6
Ochratoxin A	0.2	0.7
Ochratoxin B	0.3	1.1
Mycophenolic acid	0.7	2.2
Mevinolin	0.4	1.2
Sterigmatocystin	0.5	1.6

Fungal load in settled dust samples ranged from 0 CFU.g^{-1} (PHCC 3) to uncountable (PHCC 2, 4, 8 and 9) in MEA, and in DG18 (PHCC 7 presented the lowest load and PHCC 1 and 6 the highest load) (Figure 1). The vacuuming bag sample presented total fungal loads of 37×10^3 CFU. m^{-2} in MEA, and 264×10^3 CFU.m^{-2} in DG18.

Nine different fungal species were found on MEA and 7 in DG18. In dust samples, *Chrysonilia sitophila* was the most prominent species found in MEA (57.42%), and in DG18 the most common species was *Penicillium* sp. (36.17%) (Table 6). In the vacuuming bag sample, the most common species in MEA were *Cladosporium* sp. and *Penicillium* sp. (48.65%), and the most common species in DG18 was *Cladosporium* sp. (94.70%).

In MEA, *Aspergillus* section *Nigri* was found in settled dust samples from PHCC 1 and 9 (1 CFU. g^{-1}), *Aspergillus* section *Fumigati* in PHCC 6 (2 CFU.g^{-1}) and *Aspergillus* section *Versicolores* in PHCC 7 and 8 (1 CFU.g^{-1}); *Aspergillus* sections *Nigri* and *Versicolores* (5×10^2 CFU.m^{-2}) were also found in the vacuuming bag sample. In DG18, *Aspergillus* section *Versicolores* was found in the settled dust samples from PHCC 7 (1 CFU.g^{-1}), and *Aspergillus* section *Candidi* (4×10^3 CFU.m^{-2}) was found in the vacuuming bag sample (Table 6).

Table 7 shows the fungal distribution in settled dust and vacuuming bag samples through growth in azole-supplemented Sabouraud media as a screening tool for resistance. Eight different fungal species were found in Sabouraud (*Alternaria* sp., *C. sitophila*, *Chrysosporium* sp., *Cladosporium* sp., *Aspergillus* section *Fumigati*, *Penicillium* sp., *Stemphilium* sp. and *Rhizopus* sp.). Fungal growth in at least one azole-supplemented media was observed for 6
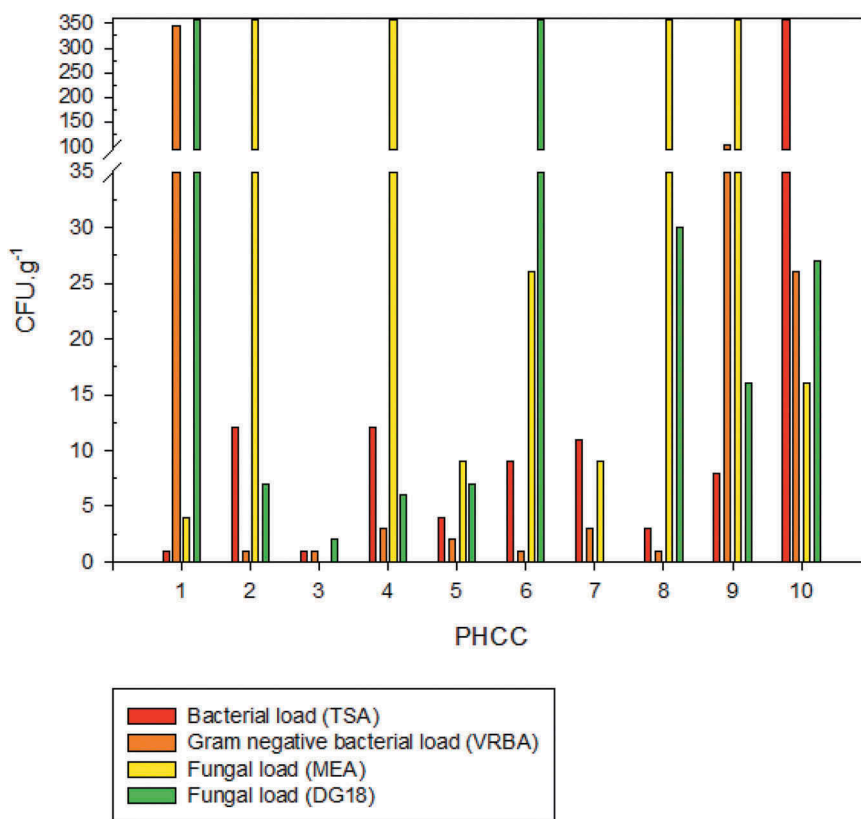
Figure 1. Bioburden obtained from settled dust samples from the 10 PHCC studied.

fungal species (*Alternaria* sp., *C. sitophila*, *Chrysosporium* sp., *Cladosporium* sp., *Penicillium* sp., and *Rhizopus* sp.) in settled dust samples from 7 out of 10 assessed PHCC (except for PHCC 3, 6 and 9).

The burden of *Aspergillus (*section *Fumigati)* was 3 CFU.g^{-1} (PHCC 9) on Sabouraud, with no growth observed in azole-supplemented media; the burden of Mucorales (*Rhizopus* sp.) was 3×10^3 CFU.m^{-2} (vacuuming bag) on 1 mg/l VORI only. Of note, fungal growth of *Penicillium* sp. was observed in ITRA and VORI in settled dust from PHCC 2 and 4 (ranging from 1 to 2 CFU.g^{-1}), and in vacuuming bag sample (ranging from 15×10^2 to 65×10^2 CFU.m^{-2}).

Fungal biomass, Aspergillus/Penicillium/Paecilomyces and Aspergillus section Fumigati dd-PCR molecular detection assays

Fungal biomass levels were above the detection limit (1 copy/2 µL) and varied from 3.76×10^4 to 6.00×10^6 copy.g^{-1} (Figure 2). *Aspergillus/Penicilliium/Paecilomyces* levels were above the detection limits except in PHCC 7 and in the vacuuming bag. The levels in all the others PHCC varied from 9.88×10^3 to 1.83×10^6 copy.g-1 (Figure 2). Regarding the *Aspergillus section Fumigati* assay detection was positive in 5 of the 10 PHCC. The detected levels varied from 2.64×10^3 to 1.30×10^5 copy.g^{-1} (Figure 2).

Molecular detection of specific Aspergillus sections and Stachybotrys chartarum species complex

qPCR analysis of DNA directly extracted from the 10 settled dust samples revealed no detectable levels of the targeted *Aspergillus* species/complexes (*Flavi, Versicolores* or *Circumdati*) and from *Stachybotrys chartarum* complex. *Aspergillus* section *Versicolores* was detected on the vacuuming bag sample.

Table 6. Fungal distribution on MEA and DG18 from all the settled dust samples.

|	PHCC	MEA		DG18	
		Species	n (CFU.g^{-1}/CFU. m^{-2}); %	Species	n (CFU.g^{-1}/CFU. m^{-2}); %
Settled dust samples	1	*Penicillium* sp.	2; 50	*C. sitophila*	30; 93.75
		Chrysonilia *sp.*	1; 25	*Cladosporium* sp.	2; 6.25
		Aspergillus section *Versicolores*	1; 25		
	2	*C. sitophila*	30; 73.17	*Ulocladium* sp.	4; 57.14
		Penicillium sp.	9; 21.95	*Cladosporium* sp.	3; 42.86
		Chrysosporium sp.	1; 2.44		
		Stemphilium sp.	1; 2.44		
	3			*Penicillium* sp.	1; 50
				Cladosporium sp.	1; 50
	4	*C. sitophila*	30; 96.77	*Ulocladium* sp.	4; 66.67
		Aspergillus section *Nigri*	1; 3.23	*Penicillium* sp.	2; 33.33
	5	*Penicillium* sp.	5; 55.56	*Penicillium* sp.	4; 57.14
		Chrysosporium sp.	2; 22.22	*Cladosporium* sp.	2; 28.57
		Ulocladium sp.	1; 11.11	*Alternaria* sp.	1; 14.29
		Cladosporium sp.	1; 11.11		
	6	*Penicillium* sp.	18; 69.23	*C. sitophila*	30; 49.18
		Cladosporium sp.	8; 30.77	*Penicillium* sp.	19; 31.15
				Cladosporium sp.	12; 19.67
	7	*Penicillium* sp.	5; 55.56		
		Aspergillus section *Fumigati*	2; 22.22		
		Chrysosporium sp.	1; 11.11		
		Cladosporium sp.	1; 11.11		
	8	*C.sitophila*	30; 69.77	*Penicillium* sp.	20; 66.67
		Penicillium sp.	11; 25.58	*Cladosporium* sp.	10; 33.33
		Aspergillus section *Nigri*	1; 2.33		
		Alternaria sp.	1; 2.33		
	9	*C. sitophila*	30; 100	*Penicillium* sp.	13; 81.25
				Cladosporium sp.	3; 18.75
	10	*Penicillium* sp.	12; 75.00	*Cladosporium* sp.	17; 62.96
		Cladosporium sp.	2; 12.50	*Penicillium* sp.	9; 33.33
		Alternaria sp.	1; 6.25	*Aspergillus* section *Versicolores*	1; 3.70
		Aspergillus section *Versicolores*	1; 6.25		
Vacuuming bag		*Cladosporium* sp.	18x10^3; 48.65	*Cladosporium* sp.	25x10^4; 94.70
		Penicillium sp.	18x10^3; 48.65	*Penicillium* sp.	1x10^4; 3.79
		Aspergillus section *Nigri*	5x10^2; 1.35	*Aspergillus* section *Candidi*	4x10^3; 1.52
		Aspergillus section *Versicolores*	5x10^2; 1.35		

Correlation analyses

No significant correlations were observed between fungal biomass levels and bacteria counts, Gram – counts, fungal counts on MEA and DG18 (Table 8). However, although not significant, there were a correlation between higher levels of fungal biomass and lower counts of bacteria (r_S = −0.316, p = 0.344), lower concentrations of fungal counts on MEA (r_S = −0.326, p = 0.327) and higher concentrations of fungi on DG18 (r_S = 0.539, p = 0.087) (Table 8).

Mycotoxins contamination

Three out of ten settled dust samples were contaminated by mycotoxins: one, the PHCC 9, with three mycotoxins (roquefortine C: <2.2 μg.kg^{-1}; griseofulvin: <1.2 μg.kg^{-1}; mycophenolic acid: 2.5 μg.kg^{-1}), and two with one mycotoxin each (PHCC 4, mycophenolic acid: 4.28 μg.kg^{-1}; PHCC 8, sterigmatocystin: 3.80 μg.kg^{-1}).

Table 7. Fungal prevalence in azole-supplemented media from settled dust (CFU.g^{-1}) and vacuuming bag (CFU.m^{-2}) samples.

	PHCC	Species	SAB n (CFU.g^{-1}); %	ITRA n (CFU.g^{-1}); %	VORI n (CFU.g^{-1}); %	POSA n (CFU.g^{-1}); %
Settled dust samples	1	*Penicillium* sp.	1; 50		1;100	
		Cladosporium sp.	1; 50			
	2	*Penicillium* sp.	26; 87	1; 100	2;100	
		Cladosporium sp.	4; 13			1; 100
	3	*Alternaria* sp.	3; 43			
		C. sitophila	2; 29			
		Cladosporium sp.	2; 28			
	4	*Chrysosporium* sp.	4; 80			
		Penicillium sp.	1; 20	1; 100	1; 50	
		Alternaria sp.			1; 50	
	5	*Penicillium* sp.	16; 94	2; 0		
		C. sitophila	1; 6	500; 100		
	6	*Penicillium* sp.	10; 100			
	7	*Chrysosporium* sp.	8; 61			
		Cladosporium sp.	3; 23			
		Penicillium sp.	1; 8	6; 100		
		Stemphilium sp.	1; 8			
	8	*Penicillium* sp.	9; 90			
		Chrysosporium sp.	1; 10	1; 100		
	9	*Aspergillus section Fumigati*	3; 100			
	10	*Penicillium* sp.	12; 2		2; 67	
		C. sitophila	500; 97			
		Alternaria sp.	1; 0			
		Stemphilium sp.	1; 0			
		Chrysosporium sp.			1; 33	
Vacuuming bag (all PHCC)		*Penicillium* sp.	1000; 100	6500; 93	1500; 33	
		Chrysosporium sp.		500; 7		
		Rhizopus sp.			3000; 67	
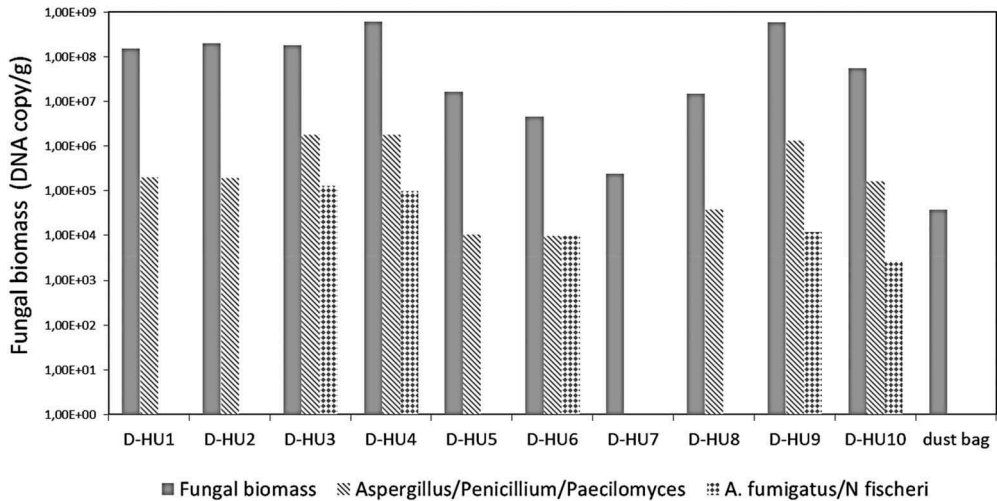
Figure 2. Fungal biomass, *Aspergillus/Penicillliium/Paecilomyces* and *Aspergillus* section *Fumigati* (*A. fumigatus* and *N. fischeri*) levels analyzed in the settled dust and the vacuuming dust bag samples.

Discussion

Bioburden in settled dust can become airborne (Chen and Hildemann 2009). When airborne, bioburden tends to aggregate into particles of different sizes depending on several variables such as source, microbial species, relative humidity and the aerosolization mechanism (Gralton et al. 2011).

Table 8. Results of the Spearman correlation coefficient.

Correlation Coefficient (p)	Fungal biomass levels	Bacteria counts	Gram − counts	Fungal counts on MEA	Fungal counts on DG18
Dust weight used for biomass	−0.600 (0.051)	−0.316 (0.344)	0.047 (0.892)	−0.326 (0.327)	0.539 (0.087)
Fungal biomass levels		0.046 (0.894)	0.117 (0.733)	0.266 (0.429)	−0.384 (0.244)
Bacteria counts			0.094 (0.784)	0.458 (0.157)	−0.106 (0.757)
Gram − counts				−0.105 (0.758)	0.262 (0.436)
Fungal counts on MEA					0.164 (0.630)

Previous studies suggested that the composition of bioburden indoors depends on activities of human occupants (Täubel et al. 2009; Lax et al. 2014). Similarly, there is evidence that mycobiota and bacteriota found indoors can be influenced by ventilation, building design, environmental characteristics (Meadow et al. 2014) or water infiltrations and damage (Emerson et al. 2015), and by the geographical location (Barberán et al. 2015). With so many factors influencing bioburden indoors, passive methods are likely more reliable than active methods to characterize bioburden since they can collect contamination from a longer period, thus, covering all the expected variations (Reponen 2017; Viegas 2018).

Settled dust reservoir has been described as able to predict bioburden levels in indoor air, being more reproducible than active methods (Leppänen et al. 2018), as it replicates the bioburden present in the indoor air. Several authors have reported the monitoring of bioaerosol by settled dust in hospitals as more representative for the evaluation of a long time exposure, because it includes the change in indoor activities and ventilation (Frankel et al. 2012), and it can be used as a quality control measure (Srikanth et al. 2008). Thus, sampling approach performed in indoor environments need to rely on more than one type of sample (Viegas et al. 2017; Leppänen et al. 2018) and settled dust should be included in sampling protocols to assess the exposure to bioburden.

Dust constitutes a microbial source in indoor environments (Osman et al. 2018), including the hospital environment (Sarıca et al. 2002), and the resuspension of settled dust particles in the air is responsible to increase airborne bacterial load (Fröhlich-Nowoisky et al. 2009). Our results for bacterial levels in settled dust samples from PHCC were lower than those concerning other environments, namely, in domestic and specific occupational environments, such as homes with a reported average of 16×10^5 CFU.g^{-1} (Horak et al. 1996) and 2.9×10^5–4.9×10^7 CFU.g^{-1} (Frankel et al. 2012), and different agricultural environments, with bacterial loads up to 19–10^9 CFU.g^{-1} (Shen et al. 1990). This can be due to the disinfection practices carried out in the assessed PHCC to achieve a proper surface disinfection, as a measure of preventing and controlling bacterial contamination, as suggested by all international guidelines for preventing infections (WHO 2002). However, fungi are more resistant to biocides than bacteria and biocides activity against fungi is not as well reported as their activity against bacteria (Sandle et al. 2014). Thus, although bacteria are less present in this indoor environment, visible fungal growth was observed.

The characterization of fungal distribution may indicate that a single genera dominates settled dust samples (Hicks et al. 2005). The dominant fungal species in this study (representing 50% or more of the organisms cultured) was *Penicillium* sp. on MEA and *Penicillium* sp. and *Cladosporium* sp. on DG18, besides *C. sitophila* (fast growing fungi). These findings follow the same trend observed in a previous study performed in a different occupational environment (Viegas et al. 2018a), in which DG18 revealed a higher prevalence of *Cladosporium* sp., similar to other studies (Hicks et al. 2005), whereas MEA revealed a more diverse mycobiota. Indeed, DG18 restricts the colony size of fast growing genera (Bergwall and Stehn 2002), such as *C. sitophila*, therefore, enabling fungal growth of different species (Viegas et al. 2018c).

Aspergillus fumigatus sensu stricto and cryptic species, many resistant to antifungal drugs, are the most frequently observed in invasive aspergillosis (Latge 1999). Thus, it is of upmost

importance to determine the prevalence and susceptibility to antifungal drugs of airborne fungi in different environments. Indeed, due the emergence of isolates of *Aspergillus* section *Fumigati* resistant to azoles, the European Center for Disease Control recommends the improvement of epidemiological surveillance and the investigation of the environmental origin through environmental and laboratory assessments, besides agricultural environments (Kleinkauf et al. 2013).

Fungal growth was observed for *Chrysosporium* sp. and *Penicillium* sp. in the different antifungal-supplemented media, and for *Rhizopus* sp. in voriconazole. *Aspergillus* section *Fumigati* growth was observed in Sabouraud but not in azole-supplemented media. Cryptic *Aspergillus* species might be underestimated, due to the presence of fast growing species, as abovementioned (Bergwall and Stehn 2002). Of note, in 3 out of the 10 assessed PHCC, fungal growth of *Penicillium* genera, comprising potential toxigenic species, was observed in both itraconazole- and voriconazole- supplemented media.

Whereas *Rhizopus* growth in voriconazole constitutes no surprise, as Mucorales are intrinsically resistant to *Candida-* and *Aspergillus*-active antifungal azole drugs, such as voriconazole (Macedo et al. 2018), *Chrysosporium* sp. and *Penicillium* sp. growth in azole-media can be due to the development of secondary resistance to azole-based antifungals (Eliopoulos et al. 2002). This is a significant clinical concern, as fungal infections with drug-resistant strains are difficult to treat. In high-risk patients (e.g. immunocompromised), the exclusion of azole-based antifungals due to resistance can dramatically limit drug choices for prophylaxis and first-line treatment of fungal invasive diseases and increase mortality rates for patients.

The antifungal susceptibility testing of strains that might be implicated in human infections, and also of toxigenic mycotoxin-producer strains, is recommended to evaluate the overall incidence of these species in clinical settings, and for a more accurate exposure risk characterization. Initial therapy should be guided not only by testing clinical isolates, but also by the knowledge of the environmental prevalence of azole-resistant strains in specific geographical regions and settings. Therefore, more environmental and clinical screening studies are necessary to generate the local epidemiologic data if such measures are to be implemented on a sound basis. Molecular methods should be used to evaluate isolates from screening studies, especially in the absence of cultured isolates.

Fungal biomass was effectively measured by the biomass assay, as in other studies (Marchand et al. 2017). All the obtained biomass levels were above the limit of detection, and were similar to the highest levels (2.79×10^7 spore.g-1)) described in a previous study conducted in deposited dust from ventilation systems in several work environments (Marchand et al. 2017). No correlation was found between data obtained from the fungal biomass assay and the culture based-methods. This might be due to an underestimation of fungal counts that seems to be the trend when applying culture-based methods (Degois et al. 2017), since they are dependent on fungal viability. Although viable part constitute only between 1 to 25% of total bioburden (Heikkila et al. 1988), culture-base methods are the gold standard and should always be applied to obtain information on cultivable and infectious organisms (Samson et al. 2000; Eduard and Halstensen 2009), being crucial in exposure assessment studies.

The use of the two different assays (culture based-methods and molecular tools) has given a more accurate scenario regarding fungal contamination. Indeed, dd-PCR analysis successfully detected *Aspergillus* section *Fumigati* in 4 sampling sites where culture base-methods could not identify this section. By qPCR we have detected *Aspergillus* section *Versicolores* in one sampling site, consistently with the results obtained from the culture-bases methods. However, none of the remaining species were detected by qPCR probably due to inhibition of PCR amplification as previously reported (Schrader et al. 2012) or even to incomplete DNA extraction. Noteworthy, fungi with reported toxigenic potential (Varga et al. 2015) were identified in settled dust samples and in vacuuming bag by culture based-methods: *Penicillium* sp. and *Aspergillus* sections *Fumigati, Nigri* and *Versicolore*s, thus, corroborating the most common scenario of co-exposure

to more than one risk factor – bioburden and metabolites (e.g. mycotoxins) (Viegas et al. 2018e) also in the clinical environment.

The presence of mycotoxins in clinical environments is not desirable since it represents an additional health risk factor for patients and workers. Unfortunately, mycotoxins are not commonly analyzed in samples from health care facilities and only one previous report has already reported mycotoxins data (Heutte et al. 2017). No correlations were found between mycotoxins contamination and specific cleaning routines, or other aspects able to explain the findings, since these were similar among all the assessed PHCC. In addition, no correlation was observed between total fungal load and mycotoxins levels. Similar to our findings, sterigmatocystin was already detected in a health care facility in 3 samples of bioaerosols at levels of 0.31, 0.32, and 1.45 µg per m^3 (Heutte et al. 2017). Although in a different matrix the values found were lower than the one found in our study. According to IARC classification, sterigmatocystin is a possible human carcinogen (2B) and show immunotoxic and immunomodulatory activity, together with mutagenic effects (McConnell and Garner 1994; Liu et al. 2014; Gao et al. 2015; Viegas et al. 2018d). The conclusion made by EFSA Panel on Contaminants in the Food Chain (CONTAM) (2013) was that the genotoxicity of sterigmatocystin is based on the formation of DNA adducts that, if unrepaired, increase the likelihood of mutation fixation (Viegas et al. 2018d).

The other mycotoxins detected in our study were never reported in health care facilities until now. Mycophenolic acid, detected in 2 samples, was the mycotoxin measured at higher concentration (4.28 µg.kg^{-1}) and is well known its immunosuppressive properties (Dasgupta 2016). This fungal secondary metabolite is produced by *Penicillium* species, being one of the most reported in settled dust samples (Egbuta et al. 2017). Also produced by *Penicillium*, roquefortine C and griseofulvin were also detected, although in lower amounts, being representative of the ability of a specific fungal species to produce many different mycotoxins (Frisvad et al. 2004).

Additional health risks may be observed as a result of co-exposure to multiple mycotoxins, as observed in 1 sample where 3 different mycotoxins were detected, corresponding to the most common exposure scenario: the simultaneous exposure to several mycotoxins that are often present in indoor environments (Assunção et al. 2015; Martins et al. 2018; Viegas et al. 2018e). These findings support the evidence that the indoor presence of a specific fungal species does not necessary imply the exposure to its specific mycotoxins, under specific environmental conditions (nutrients, temperature, humidity and others) fungi can indeed produce mycotoxins. Therefore, actions should be developed to prevent high levels of fungal contamination, particularly in clinical indoor settings where, besides health workers, immunocompromised patients can be present. Overall, the results obtained regarding mycotoxins presence allowed to recognize the importance to assess mycotoxins in health care facilities.

Air sampling, without the use in parallel of passive sampling, may be insufficient to adequately characterize the exposure to bioburden. This was already described in previous studies comparing settled dust and air samples, with different results for bioburden distribution (qualitative assessment) in settled dust and air samples (Cox et al. 2017), whereas in a different study significant quantitative correlations between airborne load and in settled dust were found (Frankel et al. 2012). If settled dust samples reveal very high concentrations of uncommon fungal genera (e.g. >106 CFU.g^{-1} of toxigenic fungal species) as the dominant (more than 50% of prevalence) (Hicks et al. 2005), the collection of a large number of samples may not be necessary and harmful fungal contamination should be considered (Hicks et al. 2005). This would simplify the exposure assessment indoors, as settled dust is generally easier-to-use and less time-consuming to collect than airborne dust (Frankel et al. 2012).

In light of the results, it can be put in perspective that epidemiological registers regarding settled dust assessments, together with air sampling, will be valuable for comparison purposes in scenarios of high levels of bioburden contamination or even when nosocomial infections are reported. This collected information will support the Infection Control and Risk Assessment Committees to propose and implement the needed control measures in each PHCC, which can implicate more suitable cleaning procedures, altering the disinfection products, changing the frequency of cleaning

or even the duration that a specific cleaning product is kept in contact with the surfaces to guarantee efficacy (Ruiz-Camps et al. 2011). Thus, a standardized settled dust collection should be included in protocols to assess indoor clinical environments, specifically PHCC.

Conclusions

Settling dust sampling in a routine way might provide useful information about bioburden exposure. This study also supports the importance of applying culture based-methods and molecular tools for the assessment of fungal burden. Settled dust represents the exposure over a longer period of time, representing the variation of a working day in indoor activities, being a reliable sampling method to achieve an accurate risk characterization regarding to bioburden exposure.

Our results emphasize the need to implement corrective measures to avoid the bioburden and mycotoxins contamination, and highlight the need for further studies addressing the same environmental parameters (bioburden and mycotoxins) in clinical environments. Eventually, the need to implement monitoring programs should be evaluated, as it will allow assessing the impact of the applied preventive measures.

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Conflict of Interest

None.
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