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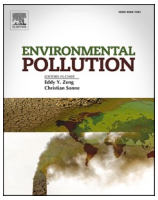

Assessment of Portuguese fitness centers: Bridging the knowledge gap on harmful microbial contamination with focus on fungi☆

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ABSTRACT

The lack of knowledge regarding the extent of microbial contamination in Portuguese fitness centers (FC) puts attendees and athletes at risk for bioaerosol exposure. This study intends to characterize microbial contamination in Portuguese FC by passive sampling methods: electrostatic dust collectors (EDC) (N = 39), settled dust (N = 8), vacuum filters (N = 8), and used cleaning mops (N = 12). The obtained extracts were plated in selective culture media for fungi and bacteria. Filters, EDC, and mop samples' extracts were also screened for antifungal resistance and used for the molecular detection of the selected *Aspergillus* sections. The detection of mycotoxins was conducted using a high-performance liquid chromatograph (HPLC) system and to determine the cytotoxicity of microbial contaminants recovered by passive sampling, HepG2 (human liver carcinoma) and A549 (human alveolar epithelial) cells were employed. The results reinforce the use of passive sampling methods to identify the most critical areas and identify environmental factors that influence microbial contamination, namely having a swimming pool. The cardio fitness area presented the highest median value of total bacteria (TSA: 9.69×10^2 CFU m^{-2}.day^{-1}) and Gram-negative bacteria (VRBA: 1.23 CFU m^{-2}.day^{-1}), while for fungi it was the open space area, with 1.86×10^1 CFU m^{-2}.day^{-1}. *Aspergillus* sp. was present in EDC and in filters used to collect settled dust. Reduced azole susceptibility was observed in filters and EDC (on ICZ and VCZ), and in mops (on ICZ). Fumonisin B2 was the only mycotoxin detected and it was present in all sampling matrixes except settled dust. High and moderate cytotoxicity was obtained, suggesting that A549 cells were more sensitive to samples' contaminants. The observed widespread of critical toxigenic fungal species with clinical relevance, such as *Aspergillus* section *Fumigati*, as well as Fumonisin B2 emphasizes the importance of frequent and effective cleaning procedures while using shared mops appeared as a vehicle of cross-contamination.

1. Introduction

Fitness centers are specific indoor environments where suitable conditions for microbiological proliferation are present. Moisture due to water condensation and human perspiration caused by attendees' physical activities are predominant in this type of setting (Ramos et al., 2016). Furthermore, increased physical activity promotes the resuspension of dust and close contact between the occupants and surfaces (floor and fitness equipment) are conditions that can boost microbial growth (Ramos et al., 2016). Exposure to bioaerosols can potentiate infection, inflammation and/or allergy in susceptible individuals, or be toxigenic, by producing mycotoxins, some of which are carcinogenic

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(Viegas et al., 2020d). However, the contribution of bioaerosols in the transmission and/or exacerbation of disease requires further research if the health implications of bioaerosol exposure is to be clarified.

Fitness center attendees, and most of the athletes working out either indoors or outdoors, present an increased risk of bioaerosol exposure since the ventilation rates could augment the quantity of inhaled microorganisms; during physical activity part of the air is inhaled through the mouth (depending on the attendee's physical condition), avoiding the normal nasal filtration mechanisms of larger particles and; the increased airflow velocity would transport microorganisms deeper into the respiratory airways (Carlisle and Sharp, 2001). Nevertheless, regardless of the importance of healthy air in sports fitness centers, indoor air quality (IAQ) studies have been mostly dedicated to schools (Cabovská et al., 2022; Pegas et al., 2011; Zhang et al., 2006, elderly care centers (Mata et al., 2022; Viegas et al., 2014), dwellings, and other indoor environments (Degois et al., 2021; Mannan and Al-Ghamdi, 2021). In what concerns fitness centers studies, few have been published relying mostly on air sampling or surface swabs and only applying one sampling method (Andersson et al., 2023; Boonrattanakij et al., 2021; Dalman et al., 2019; Goldhammer et al., 2006; Mukherjee et al., 2014; Ramos et al., 2016; Viegas et al., 2011, 2021a; Onchang and Panyakapo, 2014; Szulc et al., 2023) and fewer dedicated to fungal assessment.

Gram-negative bacteria such as *E. coli, Klebsiella pneumoniae,* or *Pseudomonas aeruginosa* are known to be associated with human disease and antimicrobial resistance, most critical for vulnerable populations, representing a public health threat (**Holmes et al., 2021**). *Staphylococcus aureus* and Methicillin-resistant *Staphylococcus aureus* (MRSA) were already isolated from different surfaces in fitness centers (Dalman et al., 2019) and, besides *S.aureus*, were also found in surfaces other pathogenic or potential pathogenic bacterial genera including *Salmonella*, *Klebsiella*, and *Micrococcus* (Mukherjee et al., 2014). The main source of bacteria present on the surface of fitness facilities is people who come to exercise (Ramos et al., 2016), being critical to recommend hygienic measures to be implemented by attendees and fitness centers' staff. Previously, the presence of viruses (mostly rhinoviruses) on surfaces was also reported (Goldhammer et al., 2006). Some international guidelines set a specific quantitative threshold for microbial contamination. European Agency for Safety and Health at Work (Bioaerosols, 2012) establishes limits for non-industrial settings reasonable values of exposure for total bacteria as 1.0×10^3 to 7.0×10^3 CFU m^{-3} and for Gram-negative bacteria as 1.0×10^3 to 2.0×10^4 CFU m^{-3}. In the case of fungal exposure, EU-OSHA recommends reasonable values of exposure in non-industrial workplaces ranging from 1.0×10^1 to 1.0×10^4 CFU m^{-3}, while World health Organization (WHO, 2009; Portaria nº 138-G/2021) recommends an exposure limit for fungi of 150 CFU/m^3. In Portugal, the legal framework in place established limit values for microbiological contamination in indoor environments (Portaria nº 138-G/2021Portaria nº 138-G/2021). Presently, the legal compliance is different concerning bacteria and fungi. Regarding bacterial assessment only a quantitative cut of is given with a differentiation between total bacteria and Gram-negative. Total bacteria should not exceed the outdoor concentration of 350 CFU m^{-3}. Whenever it does, but the CO_2 concentration is lower than 1800 mg/m^3, the assessed environment complies, but the ratio between Gram-negative bacteria and total bacteria should be inferior to 0.5.

Concerning fungi, a quantitative cut off is also employed, but a qualitative assessment with species identification is, in most cases, also needed. Thus, indoor concentrations should be inferior to outdoor concentrations. Whenever this criterion is not in compliance, or if the fungal growth is visible in the assessed environment, a qualitative assessment should be performed to identify fungal species and their mixture through the identification of selected species (Portaria nº 138-G/2021Portaria nº 138-G/2021).

Although air sampling (active sampling methods – pump requiring equipment's) is useful to verify compliance with the legal framework in place, passive sampling methods rely on settled dust collection onto an electrostatic dust cloth (EDC), agar plates, filters, or swabs. These sampling methods are simple to apply, economical, and less obtrusive (Haig et al., 2016). Passive sampling permits sampling for longer periods, allowing the accumulation of dust and, thus, to have insights into a prolonged exposure window. These samples need to be extracted through a liquid solution and can be used both in culture-based methods and molecular tools for microbial assessment (Viegas, 2018; Whitby et al., 2022).

There are no previous studies on the presence of mycotoxins in fitness centers that provide data on the contamination by mycotoxins. However, some studies already mentioned the need of considering mycotoxins presence mainly due to fungal contamination (Ramos et al., 2016).

This study aimed to fill the information gap concerning microbial contamination, focusing in fungal contamination, in fitness centers by using passive sampling methods, namely electrostatic dust cloths (EDC) and settled dust and cleaning materials (mops) and applying culture-based methods and molecular tools. Furthermore, it was designed also to describe the fungal azole resistance profile and mycotoxin contamination, as well as to disclose possible related health effects by cytotoxicity assays.

2. Materials and methods

2.1. Study design and sampling strategy

This study was conducted from November 2021 to February 2022 at 7 fitness centers (FC1-7) in the Northern part of Portugal, in the Porto district. It is part of an enlarged financed study aiming to characterize the air quality in sports facilities and to determine potential public health risks. The 7 fitness centers were selected as the most representative of those included in the enlarged study (Peixoto et al., 2023) and preference was given to FC with larger number of indoor studios in order to obtain representative number of samples for a microbiological characterization. Fitness centers were characterized in the first visit regarding attendees, workers, ventilation conditions, and hygienic procedures in place by interviewing in each fitness center the respective staff (typically gym manager, personal trainers and cleaning staff) (Table 1).

Temperature and relative humidity were controlled by a multiparametric probe GrayWolf Sensing Solutions (model TG 502; GrayWolf Sensing Solutions, Shelton, USA) and were all complying with Portuguese legislation specific for gymnasiums (Temperature: 16 °C to 21 °C Winter and 18 °C to 25 °C Summer; relative hHumidity 55%–75%) (Presidência do Conselho de Ministros, 2023). Prior to the study the equipment was calibrated at the manufacturer. Temperature and humidity were controlled complying with Portuguese legislation specific for gymnasiums (Temperature: 16 °C to 21 °C Winter and 18 °C to 25 °C Summer; Humidity 55%–75%) (Presidência do Conselho de Ministros, 2023).

To determine the levels of exposure to microorganisms, electrostatic dust collectors (EDC) (N = 39), settled dust (N = 8), vacuum filters (N = 8), and used cleaning mops (N = 12) were collected as long-term passive sampling methods (Fig. 1). Both the EDC and the filters for collecting settled dust were exposed to germicidal UV light (30W, UV-C, HNS) at a distance of 20 cm over 20 min before being used for sampling. The EDC were located in the sampling sites at 1.5 m height for 30 days (Viegas et al., 2022a,b). In view of the long passive sampling duration, in each space the EDC were placed in more remote room area (approximately 20–30 cm from wall) in order to maintain the safe use of the rooms for the respective occupants. On the last day of sampling, at the end of the day and/or at each fitness center's peak hours, a common vacuum cleaner with a filter was used to collect settled dust, and analyzed as a composite sample, as previously reported (Viegas et al., 2022b). A thread from each mop was also collected after cleaning use and a 2 cm^2

Table 1
Contextual information retrieved from the fitness centers assessed.

	FC1	FC2	FC3	FC4	FC5	FC6	FC7
Year of constructions	2021	2018	2018	2019	2019	1995	2020
Description	Located at street level of the buildings, direct entrance from the street.	Located on the top floor of a shopping center; With a direct connection to the restaurant area; Unusual architectural layout; Functional zones directly connected to the restaurant areas.	Located on the ground floor of a shopping center; Indoor area connected without any physical barrier.	Located sub-level of a building (car park level); Without barriers, directly connected to the road.	Located in a large shopping center.	Located at the ground level of the building.	Located in an urban/housing environment; Much traffic in the surrounding area; Located on the outskirts of the city of Porto.
Ventilation	HVAC[a]	HVAC +2 ceiling fans + shopping center ventilation	HVAC	HVAC	HVAC	HVAC + natural ventilation	HVAC
Occupancy (clients/day)	~400 - 500	~200 - 210	~400 - 450	~300	~500 - 530	~300 - 350	~2500 - 3000
Peak occupancy	19:00–21:30	11:30–13:00 19:00–21:30	18:30–21:30	10:30–12:30 19:00–21:30	12:00–13:30 18:30–22:00	18:30–21:30	12:00–14:00 18:00–22:00
Swimming pool	Yes	No	No	No	No	Yes	No
Bar	Yes	No	Yes	Yes	Yes	No	Yes
Cardio fitness and bodybuilding area							
Area (m^2):	377	186	700/186	503	307/56	289	275
Height (m):	2.6	4.4	7.2/3.9	3.2	8.8/3.4	3.2	3.2
Studios for group classes							
nº:	1	1	2	2	2	1	1
Area (m^2):	72	119	102/80	128/260	114/67	145	104
Height (m):	3.2	4.4	2.9/2.9	4.1/4.1	6.6/3.4	2.7	3.0
Studio for cycling							
nº:	1	1	1	1	1	1	1
Area (m^2):	93	61	83	63	69	61	98
Height (m):	2.6	4.4	2.9	5.6	3.4	2.9	5.7
Sampling locations							
Swimming pool	×					×	
Bar	×		×	×	×		×
Cardio fitness area		×	×	×	×	×	
Bodybuilding area	×	×	×		×	×	×
Open space		×					
Studio 1	×	×		×	×	×	×
Studio 2			×	×	×		
Studio Cycling		×	×		×	×	×
Changing room	×	×	×	×	×	×	
Sampling methods (nº)							
Electrostatic dust cloths (EDC)	5	6	6	5	7	6	4
Settled Dust	1	1	1	1	2	1	1
Mops	2	2	2	2	2	2	–

[a] HVAC - Heating, Ventilating and Air Conditioning.

of each was used for further analyses. The information regarding the maintenance and use of mops and cleaning accessories was always confirmed with cleaning staff of each FC; in general mops were replaced 4–5 months (or as necessary in case needed). Between each use, the material was typically cleaned only with water and let air-dried.

All collected samples were kept refrigerated (0–4 °C) in sterilized bags, until 4 days, before further analyses (Viegas et al., 2022b). Filters, EDC, and mops were used to characterize bacterial and fungal contamination, and antifungal resistance and evaluate cytotoxicity through *in vitro* tools. Mycotoxin contamination was determined in all matrices (Fig. 1).

2.2. Characterization of microorganisms by culture-based and molecular methods

All samples were extracted in the laboratory, as previously described (Viegas et al., 2022b). Briefly, they were washed with 0.1% Tween 80 saline (0.9%NaCl) solution (250 rpm, 30 min), as follows: 10 mL solution for settled dust filter (2 cm^2) and mops; 20 mL solution for each EDC; 9.1 mL solution for 1 g of a composite settled dust sample from all fitness centers. The obtained extracts were plated (150 μl) in selective culture media for fungi, namely: Malt extract agar (MEA) supplemented with chloramphenicol (0.05%), dichloran-glycerol agar (DG18), and bacteria: tryptic soy agar (TSA) supplemented with nystatin (0.2%), and Violet Red bile agar (VRBA) and incubated at optimal temperature and time conditions for fungi and bacteria. Quantification of the microbial contamination followed the procedures previous published (Viegas et al., 2021b). Fungal species/sections were identified microscopically through macro and microscopic characteristics as noted by De Hoog et al. (2000).

Filters, EDC, and mop samples' extracts were also screened for antifungal resistance towards one concentration of three different antifungals supplemented in Sabouraud agar medium following EUCAST MICs E.Def 10.2 (EUCAST, n.d.). This method is validated for the screening of azole resistance by phenotypic detection of *A. fumigatus* isolates, based on the use of plates containing agars supplemented with

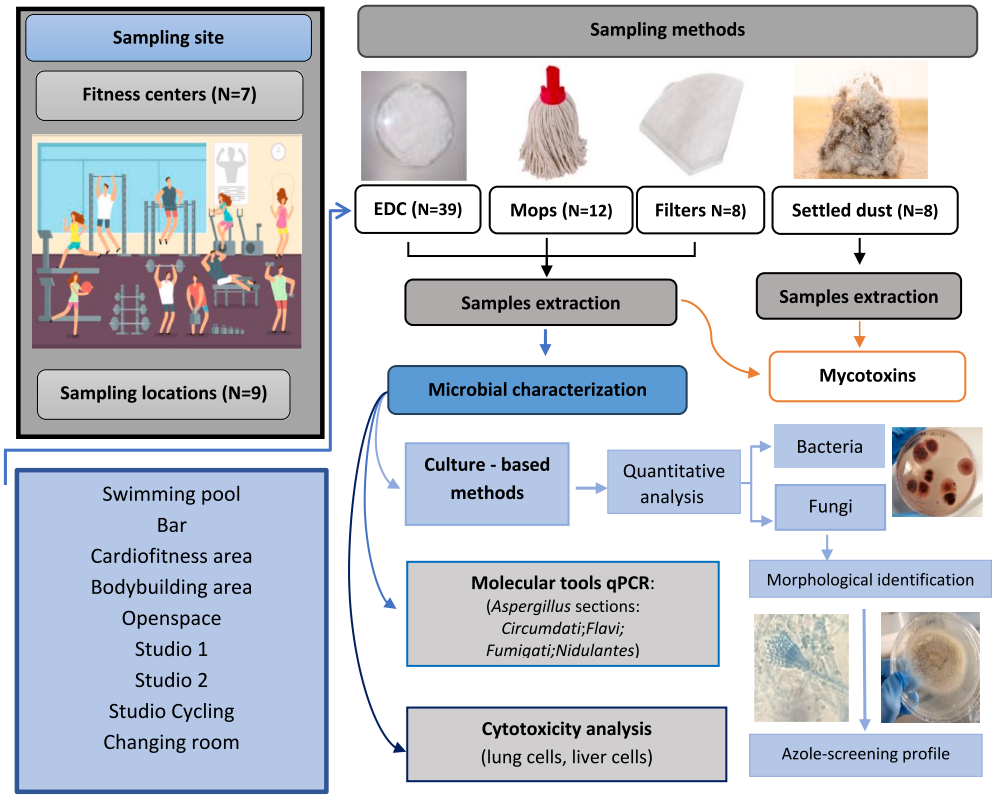

Fig. 1. Sampling methods and assays employed.

itraconazole (ICZ) (4 mg/L), voriconazole (VCZ) (2 mg/L) and posaconazole (PCZ) (0.5 mg/L, optional), and a drug-free agar control plate. Briefly, 150 µl of sample wash suspension were inoculated in Petri dishes containing the azole-supplemented media and were incubated at 27 °C for 48 h, as previously reported (Viegas et al., 2022c). Fungal species/sections were identified microscopically through macro and microscopic characteristics as noted by De Hoog et al. (2000).

The same extracts were also used for the molecular detection of the selected fungal sections (*Aspergillus* sections *Circumdati*, *Flavi, Fumigati,* and *Nidulantes*) following the previously published procedures (Viegas et al., 2022c). Fungal DNA was extracted using the ZR Fungal/Bacterial DNA MiniPrep Kit (Zymo Research, Irvine, USA) following the producer's instructions, and molecular identification was accomplished by Real-Time PCR (qPCR) using the CFX-Connect PCR System (Bio-Rad). For each amplified gene, a non-template control and a positive control (DNA obtained from reference strains kindly provided by the Mycology laboratory of the National Institute of Health Dr. Ricardo Jorge) were employed (Table S1 – Supplementary material).

2.3. Mycotoxin analysis

The sample preparation and chromatographic analysis of mycotoxins followed the procedure outlined in Viegas et al. (2020c). In summary, 0.10 g of settled dust and mop thread samples were subjected to vigorous shaking for 60 min, using 3.0 mL of an acetonitrile/water/-acetic acid mixture (79/20/1; v/v/v). After 5-min centrifugation at 5000 rpm, 2 mL of the extract was evaporated to dryness under a stream of nitrogen and then reconstituted in a 400 µl of methanol/water mixture (2/8; v/v), and centrifuged again for 30 min at 14,500 rpm. Thus, the sample dilution factor was 6.

The detection of mycotoxins was conducted using a high-performance liquid chromatograph (HPLC) system, specifically the Nexera model from Shimadzu (Kyoto, Japan), coupled with a mass spectrometry detector, the 5500 QTrap from Sciex (Foster City, USA). Mycotoxins were separated by chromatography on a Gemini C18 column (150 × 4.6 mm, 5 µm) manufactured by Phenomenex in Torrance, CA, USA. The flow rate was set at 1 mL/min, and a 5 µL injection volume was employed.

Two distinct mobile phases were utilized: Phase A, comprising methanol/water/acetic acid in a ratio of 10/89/1 (v/v/v), and Phase B, consisting of methanol/water/acetic acid in a ratio of 97/2/1 (v/v/v). Both mobile phases were supplemented with 5 mmol/L of ammonium acetate. The chromatographic gradient proceeded as follows: initial elution with 0% B up to 2.0 min, followed by a linear increase to 50% B from 2.0 to 5.0 min, further ramping up to 100% B from 5.0 to 14.0 min, maintaining 100% B until 18.0 min, and ultimately returning to the initial 0% B composition by 22.5 min.

Tandem mass spectrometry analysis was conducted in the scheduled multiple reaction monitoring (sMRM) mode for both negative and positive polarities within a single chromatographic run. The electrospray ionization (ESI) source parameters were set as follows: a curtain gas at 30 psi, collision gas at a medium level, ion spray voltage at −4500 V (negative polarity) and 5500 V (positive polarity), ion source temperature maintained at 550 °C, ion source gas1 at 80 psi, and ion source gas2 at 80 psi. Table S2 (Supplementary material) shows the instrument settings optimized for product ions of each compound. The Analyst 1.6.2 software (Sciex, Foster City, CA) was used for data acquisition and processing.

2.4. Cytotoxicity analysis

To determine the cytotoxicity of microbial contaminants recovered by passive sampling, HepG2 (human liver carcinoma) and A549 (human alveolar epithelial) cells were grown at 37 °C in MEM (Eagle's Minimum Essential Medium) supplemented with 10,000 units penicillin, 10 mg/mL streptomycin and fetal calf serum (FCS, Sigma-Aldrich, USA). Cells were detached from culture flasks by trypsinization with 0.25% (w/v) Trypsin 0.53 mM EDTA and resuspended in culture medium to give a

cell number of 2.0 × 10^5 and 3.0 × 10^5 cells/ml (Scepter™ 2.0 Cell Counter, Merck) for HepG2 and A549, respectively. 100 μl of the cell suspension were dispensed per well in a 96-well plate, and incubated with serial 1:2 dilutions of samples' extracts (prepared as described in section 2.2) for about 48 h at 37 °C in an atmosphere of 5% CO_2 and humid atmosphere. After decanting the cell culture medium, the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)-cell culture assay was performed as previously described (Hanelt et al., 1994), to determine cell proliferation and survival. The optical density of each well was measured spectrophotometrically with an ELISA reader (ELISA LEDETECT 96, Biomed Dr. Wieser GmbH; MikroWin 2013SC software) at a wavelength of 510 nm. The lowest concentration dropping absorption to <50% of cell metabolic activity (IC50) was defined as the threshold toxicity level.

2.5. Statistical analysis

The normality of data, tested by a Shapiro-Wilk test, was not verified. Spearman's correlation coefficient was used to study how bacterial contamination (TSA, VRBA), fungal contamination (MEA, DG18), antifungal resistance (SDA, PCZ, ICZ and VCZ), and cytotoxicity (A549, HepG2) correlated. These variables were also analyzed among fitness centers' locations and occupancy rate (using the Kruskal-Wallis test), and among ventilation types and the presence or absence of a swimming pool (using the Mann-Whitney *U* test). Comparison analysis focused on EDC data only (dataset with the greatest expression). To assess fungal species' diversity, Simpson and Shannon indices, given by **Shannon Index** $(\mathbf{H}) = -\sum_{i=1}^{s} p_i \ln(p_i)$ and **Simpson Index** $(\mathbf{D}) = \frac{1}{\sum_{i=1}^{s} p_i^2}$, were used, where p_i is the proportion (n_i/n) of isolates of one particular species found (n_i) divided by the total number of isolates found (n). All analyses were conducted in SPSS statistical software for Windows, version 27.0. The results were considered significant at the 5% significance level.

3. Results

3.1. Microbial contamination

Concerning EDC, the cardio fitness area presented the highest median value of total bacteria (TSA: 9.69 × 10^2 CFU m^{-2}.day^{-1}) and Gram-negative bacteria (VRBA: 1.23 CFU m^{-2}.day^{-1}). Similar values of total bacteria were obtained in studio 3 (cycle), open space, changing room, and bodybuilding area (TSA: 1.02 × 10^2 CFU m^{-2}.day^{-1} respectively), while Gram-negative bacteria were found in the changing room area (VRBA: 0.325 CFU m^{-2}.day^{-1}). (Fig. 2).

Concerning sampling methods, mops presented the highest bacterial counts (TSA: 2.83 × 10^6 CFU m^{-2}; VRBA: 2.21 × 10^6 CFU m^{-2}), followed by settled dust vacuum filters (TSA: 3.27 × 10^5 CFU m^{-2}; VRBA: 1.00 × 10^3 CFU m^{-2}) and EDC (TSA: 1.31 × 10^4 CFU m^{-2}; VRBA: 4.46 × 10^1 CFU m^{-2}.day^{-1}).

The highest fungal concentrations were taken with EDC at the open space area, with 1.86 × 10^1 CFU m^{-2}.day^{-1} (median value) on MEA and DG18 media. Cardio fitness median values were 6.53 CFU m^{-2}.day^{-1} on MEA and 7.76 CFU m^{-2}.day^{-1} on DG18. Similar values were obtained at the changing room (4.47 CFU m^{-2}.day^{-1} on MEA and DG18), bodybuilding (4.38 CFU m^{-2}.day^{-1} on MEA, and 4.47 CFU m^{-2}.day^{-1} on DG18) and bar (4.44 CFU m^{-2}.day^{-1} on MEA, and 9.99 CFU m^{-2}.day^{-1} on DG18). Lower fungal concentrations were found at studio 1 (2.19 CFU m^{-2}.day^{-1} on MEA, and 1.09 CFU m^{-2}.day^{-1} on DG18), studio 2 (1.12 CFU m^{-2}.day^{-1} on MEA and DG18) and studio 3 (cycle) (1.65 CFU m^{-2}.day^{-1} on MEA, and 1.09 CFU m^{-2}.day^{-1} on DG18. Fungal contamination in the pool area was only found on DG18 medium (1.09 CFU m^{-2}.day^{-1}) (Fig. 3).

As with bacteria, the highest fungal counts were also observed in mops (2.30 × 10^5 CFU m^{-2} on MEA; 5.05 × 10^4 CFU m^{-2} on DG8), followed by filters used during settled dust sampling (1.15 × 10^5 CFU m^{-2} on MEA; 5.45 × 10^4 CFU m^{-2} on DG18), and EDC (6.37 × 10^2 CFU m^{-2}.day^{-1} on MEA; 5.31 × 10^2 CFU m^{-2}.day^{-1} on DG18).

Penicillium sp. was the most prevalent fungal genera in mops (96.95% on MEA; 96.04% on DG18) and in filters (93.04% on MEA; 95.41% on DG18), and *Cladosporium* sp. was dominant in EDC (62.94% on MEA; 53.81% on DG18). *Aspergillus* sp. was present in EDC (5.30% on MEA; 12.32% on DG18) and, to a lower extent, in filters (0.43% on MEA) (Table 2, Fig. S1 – Supplementary material).

Regarding fungal diversity, it was higher on DG18 for EDC (Shanon index = 1.20, Simpson index = 2.52), and on MEA for filters (Shanon index = 0.28, Simpson index = 1.15) and mops (Shanon index = 0.37, Simpson index = 2.40) (Table S3 – Supplementary material), being higher in EDC among all sampling devices.

Six *Aspergillus* sections were identified on DG18 with EDC samples while on MEA 4 sections were observed (Fig. 4). *Aspergillus* section *Nigri* was dominant (100%) in filters inoculated on MEA. *Nidulantes* was the only *Aspergillus* section detected (out of the 4 sections targeted by qPCR), namely, in mops from 3 different fitness centers (50%; 3 out of 6 samples) (Table 2).

Among the different areas from the fitness centers, the highest prevalence of *Aspergillus* sp. was found in the bodybuilding area (21.9%), followed by cardio fitness (14.8%), changing room (11.2%), and bar (10.2%). The lowest prevalence of the genera was found in studio 1 (3.3%), open space (2.9%), and studio 3 (cycle) (1.1%). The genus was not found in pool and studio 2 areas (Fig. S2 – Supplementary material).

From all the targeted *Aspergillus* sections, only *Nidulantes* was detected in mops samples from 3 different fitness centers (50%; 3 out of

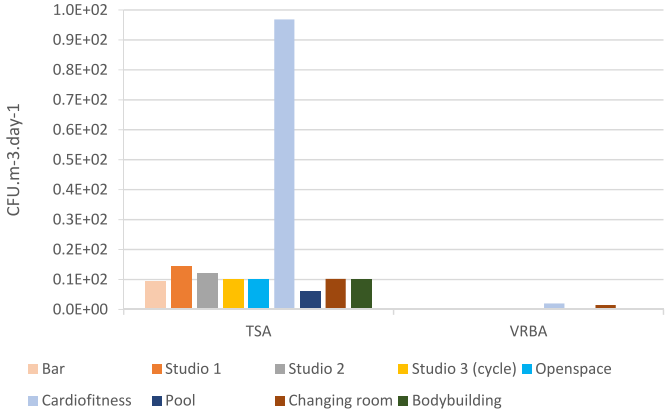

Fig. 2. Total bacteria (TSA) and Gram-negative (VRBA) median values from EDC (CFU.m^{-2}.day^{-1}).

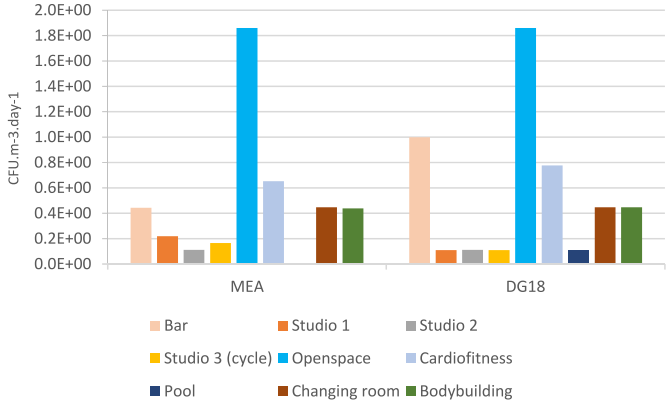

Fig. 3. Fungal median values from EDC samples (CFU.m^{-2}.day^{-1}) inoculated in MEA and DG18.

Table 2
Fungal distribution on MEA and DG18 from EDC, filters, and mops samples collected in different areas from the 7 fitness centers.

Samples	MEA			DG18		
	Genera	CFU.m^{-2}.day^{-1}/m^{-2}	%	Genera	CFU.m^{-2}.day^{-1}/m^{-2}	%
EDC	*Cladosporium* sp.	4.01E+02	62.94	*Cladosporium* sp.	2.86E+02	53.81
	Penicillium sp.	1.80E+02	28.30	*Penicillium* sp.	1.65E+02	31.14
	Aspergillus sp.	3.37E+01	5.30	*Aspergillus* sp.	6.54E+01	12.32
	Others	2.20E+01	3.46	Others	1.45E+01	2.73
	TOTAL	6.37E+02	100.00	TOTAL	5.31 + 02	100.00
Filters	*Penicillium* sp.	1.07 + 05	93.04	*Penicillium* sp.	5.20E+04	95.41
	Cladosporium sp.	7.00E+03	6.09	*Cladosporium* sp.	2.50E+03	4.59
	Aspergillus sp.	5.00E+02	0.43			
	Others	5.00E+02	0.43			
	TOTAL	1.15E+05	100.00	TOTAL	5.545E+04	100.00
Mops	*Penicillium* sp.	2.23E+05	96.95	*Penicillium* sp.	4.85 + 04	96.04
	Cladosporium sp.	5.00E+03	2.18	*Cladosporium* sp.	2.00E+03	3.96
	Rhizopus sp.	2.00E+03	0.87			
	TOTAL	2.30E+05	100.00	TOTAL	5.05 + 04	100.00

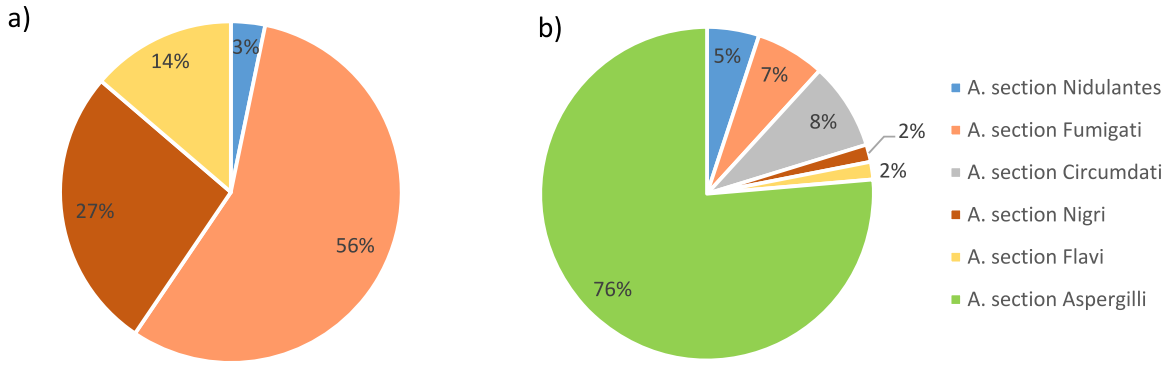

Fig. 4. *Aspergillus* sections distribution on a) MEA and b) DG18 from EDC collected in different areas from the 7 fitness centers.

6 samples) (Table S4 – Supplementary material).

3.2. Antifungal resistance

Reduced azole susceptibility was observed in filters and EDC (on ICZ and VCZ), and in mops (on ICZ only), the most expressive in filters with reduced susceptibility to VCZ. No fungi were observed in PCZ (Fig. S3 – Supplementary material). Regarding fungal diversity in EDC (Table 3), *C. sitophila* and *Rhizopus* sp. were the most prevalent (35.64% each) on SDA, followed by *Cladosporium* sp. on azole-supplemented media (92.37% on ICZ; 79.17% on VCZ).

Penicillium sp. was the most prevalent in mops (96.91% on SDA; 100.00% on ICZ) and filters (96.91% on SDA; 83.33% on VCZ), followed by *Cladosporium* sp. in filters (100.00 % on ICZ). *Aspergillus* sp. was also

Table 3
Fungal diversity in azole screening per sampling type. Filters (CFU.m^{-3}), mops (CFU.m^{-2}), EDC (CFU.m^{-2}.day^{-1}).

		SDA	%	ICZ	%	VCZ	%	PCZ	%
EDC (CFU.m^{-2}.day^{-1})	*Aspergillus* sp.	6.37E+02	0.43						
	Aureobasidium sp	1.06E+02	0.07						
	C. sitophila	5.31E+04	35.64	3.18E+02	2.54	1.06E+02	2.08		
	Chrysosporium sp.	1.06E+02	0.07						
	Cladosporium sp.	2.59E+04	17.39	1.16E+04	92.37	4.03E+03	79.17		
	Fusarium verticilloides	1.06E+02	0.07						
	Mucor sp.	1.06E+02	0.07						
	Penicillium sp.	1.56E+04	10.48	6.37E+02	5.08	7.43E+02	14.58		
	Rhizopus sp.	5.31E+04	35.64			2.12E+02	4.17		
	Trichoderma sp.	2.12E+02	0.14						
	TOTAL	1.49E+05	100.00	1.25E+04	100.00	5.10E+03	100.00	0.00E+00	0.00
Mops (CFU.m^{-2})	*Alternaria* sp.	5.00E+02	0.78						
	Cladosporium sp.	1.15E+04	17.83						
	Penicillium sp.	5.25E+04	81.40	2.50E+03	100.00				
	TOTAL	6.45E+04	100.00	2.50E+03	100.00	0.00E+00	0.00	0.00E+00	0.00
Filters (CFU.m^{-3})	*Aureobasidium* sp.	5.00E+02	0.10						
	Cladosporium sp.	6.50E+03	1.26	1.00E+03	100.00	2.00E+03	16.67		
	Penicillium sp.	8.50E+03	1.64						
	Rhizopus sp.	5.02E+05	96.91			1.00E+04	83.33		
	Syncephalastrum racemosum	5.00E+02	0.10						
	TOTAL	5.18E+05	100.00	1.00E+03	100.00	1.20E+04	100.00	0.00E+00	0.00

CFU, colony forming unit; EDC, Electrostatic dust collector; SDA, Sabouraud dextrose agar; ICZ, itraconazole-supplemented SDA; VCZ, voriconazole-supplemented SDA; PCZ, posaconazole-supplemented SDA.

observed in EDC (0.43% on SDA), with 4 sections identified (Fig. 5).

3.3. Mycotoxins contamination

Fumonisin B2 was the only mycotoxin detected. It was present in all sampling matrixes except settled dust, as follows: in 25% of the filter samples (LOQ = 12 ng/g), in 5.1% of the EDC samples (LOQ = 9 ng/g), and in 25% of the mops samples (LOQ = 20 ng/g).

3.4. Cytotoxicity

Five serial 1:2 dilutions of samples' extracts were tested towards two cell lines. Different cellular responses (measured at 510 nm) were observed among cells (Table S5 – Supplementary material). High and moderate cytotoxicity was obtained, respectively, with 11.86% and 27.12% samples in A549 cells, compared to 0.00% and 5.08% samples in HepG2 cells, suggesting that A549 cells were more sensitive to samples' contaminants.

3.5. Correlation and comparison analyses

Table 4 depicts the Spearman correlation results for different variables assessed in EDC, filters, and mops. In EDC, significant positive correlations, of intensity that varied between weak to strong, were found among:

i. bacteria contamination on TSA and VRBA (p = 0.002);
ii. bacteria contamination on TSA and fungal counts on MEA (p = 0.002), DG18 (p = 0.011), SDA (p = 0.012), and ICZ (p = 0.035);
iii. bacteria contamination on VRBA and fungal counts on MEA (p = 0.009), DG18 (p = 0.009) and SDA (p = 0.005);
iv. fungal contamination on MEA and DG18 (p = 0.000), SDA (p = 0.000), ICZ (p = 0.000) and VCZ (p = 0.000);
v. fungal contamination on DG18 and SDA (p = 0.000), ICZ (p = 0.000) and VCZ (p = 0.000);
vi. fungal contamination on SDA and ICZ (p = 0.000) and VCZ (p = 0.000);
vii. fungal contamination on ICZ and VCZ (p = 0.000).

In filters, significant positive correlations, with strong intensity, were found between bacteria contamination on VRBA and fungal counts on SDA (p = 0.046) and between fungal contamination on DG18 and cytotoxicity in A549 cells (p = 0.005).

In EDC and filters negative correlations of intensity that varied between weak to strong were found in EDC, between fungal counts on MEA and DG18 with cytotoxicity in A549 cells (p = 0.036, and p = 0.036, respectively) and in filters, between fungal contamination on MEA and ICZ (p = 0.037).

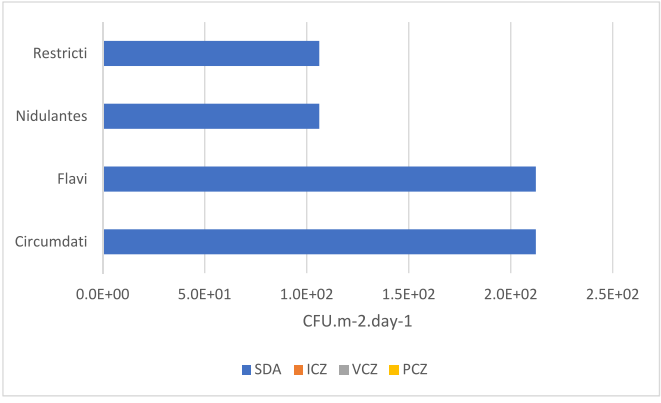

Fig. 5. *Aspergillus* sections prevalence in EDC samples (CFU.m-2.day-1) collected in different areas from the fitness centers. CFU, colony forming unit; SDA, Sabouraud dextrose agar; ICZ, itraconazole-supplemented SDA; VCZ, voriconazole-supplemented SDA; PCZ, posaconazole-supplemented SDA.

Among the locations of the fitness centers, statistically significant differences were detected regarding: i) bacterial contamination ($\chi^2_{K-W}(5) = 16.699, p = 0.005$), with the fitness centers located at the ground level of the building showing greater contamination, followed by those located on the top floor in a shopping center, those located in an urban/housing environment with a lot of traffic in the surrounding area and those located on the ground floor of a shopping center; ii) fungal contamination in both MEA ($\chi^2_{K-W}(5) = 17.870, p = 0.003$) and DG18 ($\chi^2_{K-W}(5) = 17.297, p = 0.004$), with the same trend as bacterial contamination being observed; iii) fungal counts in SDA ($\chi^2_{K-W}(5) = 14.590, p = 0.012$), with the same trend observed previously; iv) cytotoxicity in A549 cells ($\chi^2_{K-W}(5) = 14.590, p = 0.012$), with fitness centers located at building sub-level presenting higher values, followed by those located at street level of the buildings, direct entrance from street and those located in an urban/housing environment with a lot of traffic in the surrounding area (Table S6 – Supplementary material).

Between the ventilation types (HVAC/HVAC and natural ventilation, suspended simultaneously), statistically significant differences were detected regarding fungal contamination in MEA (U = 32.00, p = 0.009), DG18 (U = 26.00, p = 0.004) and SDA (U = 30.00, p = 0.007). In any of the situations, the ventilation mode HVAC and natural ventilation (used simultaneously) presented the highest values (Table S7 – Supplementary material).

No statistically significant differences were detected between the different numbers of customers per day, regarding bacterial contamination, fungal contamination, fungal resistance, and cytotoxicity (p > 0.05).

Between fitness centers that had a swimming pool and those that did not, statistically significant differences were detected only about fungal contamination in DG18 (U = 86.00, p = 0.033), with greater contamination being found in fitness centers that had a swimming pool.

4. Discussion

The comprehensive sampling approach applied, employing EDC, mops, filters, and settled dust, allowed us to obtain different results and more accurate information regarding microbial contamination. Microbial dispersion indoors is different depending on different variables, both from the microorganisms (e.g. size and type of spores) and the environment (e.g. building construction, building materials, ventilation conditions, cleaning practices, type and number of occupants) leading to different contamination patterns (Ramos et al., 2016; Saini et al., 2020). Indeed, the same trend was observed in previous studies developed in different indoor environments (Viegas et al., 2021a, 2022b; 2022c, 2020c). Additionally, the use of different sampling methods allowed for overcoming the limitations of each ensuring an enriched risk characterization (Viegas, 2018). Since EDCs are designed to capture suspended particles such as bioaerosols through their electrostatic charges, complementary sampling with settled dust collection and mops corroborates the presence of microbial contaminants either in suspension in indoor environments or on surfaces (Adams et al., 2021; Whitby et al., 2022).

Higher fungal diversity was found in EDC. These results corroborate EDC sampling suitability to better characterize the risk regarding exposure to critical fungal contamination (Adams et al., 2021; Viegas et al., 2022a). One reason might be that EDC sampling covers a wider period. Positive and negative correlations were found between bacteria and fungi either regarding the same sampling matrix or among different sampling matrices. This data follows the same patterns observed in other indoor environments (Viegas et al., 2022a, 2022b, 2022c, suggesting that several parameters in the assessment methods influence microbial contamination (e.g. culture media or sampling matrix).

Across all sampling methods, Gram-negative bacteria were identified (mops > settled dust filters > EDC) from VRBA agar (a selective agar

Table 4
Study of the relationship between bacterial counts, fungal counts, fungal resistance, and cytotoxicity in each sampling method. Spearman correlation results.

			Bacteria	Fungi			Fungal resistance			Cytotoxicity	
			VRBA	MEA	DG18	SDA	ICZ	VCZ	PSZ	A549 cells	HepG2 cells
EDC	Bacteria	TSA	0.487**	0.473**	0.401*	0.399*	0.339*	0.175		-0.225	-0.416
		VRBA		0.418**	0.419**	0.448**	0.206	0.087		-0.027	-0.271
	Fungi	MEA			0.812**	0.812**	0.592**	0.599**		-0.342*	-0.228
		DG18				0.734**	0.608**	0.562**		-0.413**	-0.464
	Fungal resistance	SDA					0.700**	0.588**		-0.245	-0.164
		ICZ						0.724**		-0.123	-0.195
		VCZ								-0.186	-0.105
		PSZ									
	Cytotoxicity	A549 cells									0.435
Filter	Bacteria	TSA	0.000	0.054	0.321	0.469	-0.267	-0.415		0.060	
		VRBA		0.063	0.000	0.715**	-0.330	0.145		-0.529	
	Fungi	MEA			-0.127	0.506	-0.737**	-0.207		-0.060	
		DG18				0.143	0.331	0.195		0.905**	
	Fungal resistance	SDA					-0.611	0.021		-0.274	
		ICZ						0.189		0.373	
		VCZ								0.333	
		PSZ									
	Cytotoxicity	A549 cells									
MOP	Bacteria	TSA	0.574	0.072	0.145	0.318	-0.056			0.866	
		VRBA		0.178	0.049	0.114	-0.104			-0.500	
	Fungi	MEA			0.011	-0.080	-0.065			0.866	
		DG18				0.242	0.014			0.866	
	Fungal resistance	SDA					-0.220				
		ICZ								-0.500	
		VCZ									
		PSZ									
	Cytotoxicity	A549 cells									

*. Correlation is significant at the 0.05 level (2-tailed). **. Correlation is significant at the 0.01 level (2-tailed). Blue cells represent negative correlations. Pink cells represent positive correlations. The stronger the colour, the stronger the intensity of the relationship.

medium mainly targeting lactose-fermenting Gram-negative bacteria). Total bacteria and Gram-negative bacteria exposure levels for fitness center occupants varied among areas (cardio fitness > studios, open space, changing room, and bodybuilding), likely due to contaminated surfaces from athletic equipment (Wood et al., 2015), the presence of attendees, and activities performed (Małecka-Adamowicz et al., 2019). Although without statistically significant differences in our study, previous studies corroborated the strong influence of attendees' presence in the bacterial contamination (Boonrattanakij et al., 2021; Ramos et al., 2016; 2016).

It was also possible to determine the concentration of potential fungal pathogens (WHO, 2022) using passive sampling and several culture media (culturomics). Different results were obtained applying different culture media (MEA and DG18), since DG18 restricts the growth of fungi with fast-growing rates (e.g. Mucorales and *C. sitophila*), favoring others to growth with toxicological and clinical relevance (e.g. *Aspergillus* sections) (Viegas et al., 2022b, 2022c; 2020c). The assessment of the viability of pathogenic microorganisms indoors is deeply needed to ponder about potential health effects for fitness centers' attendees and, consequently, to achieve a precise risk assessment and identify the most relevant risk management measures to put in place (Madsen et al., 2022). *Aspergillus* section *Fumigati*, listed by WHO as of critical priority and suggested as an indicator of harmful fungal contamination in different indoor environments (Salambanga et al., 2022; Viegas et al., 2021a, 2022b; 2022c), was widespread among all the facilities being most prevalent in EDC on MEA (Bodybuilding > cardio fitness > changing room > bar > studio 1> openspace > studio 3). *Aspergillus* section *Fumigati* is known to cause serious invasive infections, especially within the immunocompromised (Arastehfar et al., 2021; Viegas et al., 2021a,b,d). It is also associated with antifungal resistance, posing a threat to the successful clinical use of azoles in the treatment of invasive aspergillosis (Jeanvoine et al., 2020; Verweij et al., 2016; WHO, 2022). In this study, we tested complete samples' extracts (not isolates) for antifungal resistance screening towards 3 different azoles and found a low incidence of fungal resistance, with no *Aspergillus* section *Fumigati* observed. Other *Aspergillus* sections (*Restricti, Nidulantes Flavi,* and *Circumdati*) were only observed in SDA.

Aspergillus sections *Circumdati, Nidulantes, Flavi, and Nigri*, found in this study, should be highlighted due to their toxigenic potential. Mucorales (*Mucor*, *Rhizopus,* and *Syncephalastrum* genera), as well as *Fusarium verticilloides* (*Fusarium* sp. listed as of high priority by WHO), were also identified in this study. Indeed, the presence of *Fusarium verticilloides* can explain the detection of fumonisin B2 in most of the sample matrixes considered showing the toxigenic potential of this species. Fumonisin is a highly toxic low molecular weight fusarium mycotoxin and fumonisin B1, fumonisin B2 and fumonisin B3 are the main forms. Although with still limited studies on the toxicity of fumonisin B2 and fumonisin B3, the studies available point to lower toxicity when compared with fumonisin B1 (Yu et al., 2020).

The identification and/or molecular detection (*Nidulantes* section

was also detected by qPCR) of these potentially pathogenic/toxigenic fungal species by passive sampling justify an intervention in the fitness centers since they can be resuspended and aerosolized due to the physical activities performed indoors, thus, increasing exposure by inhalation (Viegas et al., 2020a, 2020b).

Aspergillus sections *Fumigati* and *Nigri* are known to have high cytotoxic potentials and might cause health problems to exposed individuals (Gniadek et al., 2017; Lu et al., 2021). Our results revealed high to moderate cytotoxicity in A549 lung cells for 38.98% of samples, and moderate cytotoxicity in HepG2 hepatocytes for 5.08% of samples. Similar studies conducted in healthcare environments also describe a moderate to high cytotoxic effect of *Aspergillus* section *Fumigati*, with cytotoxicity being significantly higher in A549 lung epithelial cells (Viegas et al., 2021e). *A. fumigatus* cytotoxicity has been related to produced mycotoxins, such as gliotoxin and trypacidin (Watanabe et al., 2004; Gauthier et al., 2012). Although the effect of particulate matter and/or volatile organic compounds (not assessed in this study) cannot be excluded, these preliminary results suggest that fungal inhalation may induce harmful cellular effects in the lungs.

As it was suggested in a previous study performed in ambulances (Viegas et al., 2021c), mops can potentiate cross-contamination. The use of a shared mop in different spaces from a single fitness center can promote cross-contamination between areas. This should be highlighted, since the mops were the sampling matrix with higher microbial contamination and even mycotoxins, since fumonisin was also detected in mops. Although mycotoxins are not volatile they can be carried to the attendee's respiratory system by dust or fungal spores if resuspended or aerosolized (Viegas et al., 2020d, 2018b). To avoid the cross contamination between spaces, different mops and other cleaning materials should be distributed for the different places and tagged (to avoid shared mops between spaces) and a disinfection and replacement plane of these materials should be in place. To ensure these measures implementation, the cleaning staff should be aware through a routine education plan specifically dedicated to the hygienic conditions to be observed.

Although a strong positive correlation was found in filters between fungal contamination on DG18 (a selective agar medium for xerophilic fungi, such as *Aspergillus* sp.) and cytotoxicity in A549 cells ($rS = 0.905$, $p = 0.005$), no *Aspergillus* sp. were detected on DG18 in filters. These observations suggest a contribution of other fungal genera, such as *Penicillium* sp., for the observed cytotoxicity in lung cells, probably due to the mycotoxins produced (Skrzydlewski et al., 2022). Interestingly, in EDC, fungal counts on MEA (1log inferior to filter fungal counts) and on DG18 were negatively correlated (although with lower correlation strength) with cytotoxicity in A549 cells (MEA: $r_S = -0.342$, $p = 0.036$); DG18: $r_S = -0.4$, $p = 0.036$). Bacterial contamination in EDC was also 1-log inferior to bacterial counts in filters, thus, suggesting the contribution of contaminants of a distinct nature (e.g. chemicals, particles) in the observed biological effect.

Bacterial contamination is highly influenced by its occupant's presence (Ramos et al., 2016), and environmental factors (Boonrattanakij et al., 2021; Griffith, 2016; Małecka-Adamowicz et al., 2019). Since ground-level fitness centers have direct access to the outdoors, cross-contamination from outdoor sources may also occur. Sources such as soil, dust, and water are often responsible for the contamination indoors and can be brought in by carriers through their shoes (Rashid et al., 2016). Nevertheless, proper sanitation (e.g. washing followed by disinfection) and ventilation are essential to minimize cross-contamination sources and prevent indoor bacterial contamination (Onchang and Panyakapo, 2014; Ramos et al., 2016).

The presence of fungi in construction materials can greatly affect the quality of indoor air by emitting biological particles, such as allergens and mycotoxins, into the surrounding environment (Al Hallak et al., 2023). Fungal contamination is often higher in basement-level buildings due to a combination of factors (Niculita-Hirzel et al., 2020). Basements typically have higher humidity levels, limited ventilation, and reduced exposure to sunlight, creating an optimal environment for fungal growth (Niculita-Hirzel et al., 2020).According to the Institute of Medicine (US) Committee on Damp Indoor Spaces and Health (Institute of Medicine, 2004), water seepage through the foundation or poor drainage can result in damp conditions, which further promote the growth of fungi. These factors collectively contribute to the elevated risk of fungal contamination in basement-level structures.

In agreement with Dacarro et al. (2003), the highest levels of fungal contamination (Table S6) were observed in fitness centers that used (apart from mechanical) natural ventilation. Previous studies highlighted the significance of outdoor fungal sources (e.g., from plants, soil, etc.) for the indoor environment of sports facilities (Boonrattanakij et al., 2021). Similarly, other authors (Andersson et al., 2023; Szulc et al., 2023; Viegas et al., 2021a) reported higher fungus prevalence in the ambient air compared to the indoor air of fitness centers. Apart from the ventilation systems, it is important to acknowledge that human transportation can introduce fungal contamination to the indoor environment of sports facilities (Boonrattanakij et al., 2021). It should be though noted that various environmental factors, including air recirculation and movement, or temperature and relative humidity can further contribute to the observed differences in microbial contamination levels at the examined fitness (Blocken et al., 2020; Onchang and Panyakapo, 2014; Szulc et al., 2023).

Previous studies focused on fungal contamination in swimming pools (Brandi et al., 2007; Viegas et al., 2011) reported the presence of clinically relevant fungi of the genera *Aspergillus* and *Fusarium* (Ekowati et al., 2017; WHO, 2022). Swimming pools are appropriate places for the growth of pathogenic fungi if they are not surveyed and controlled (Rabi et al., 2007). The fact that most of the fungal species need moisture levels above 75% to grow, which is the reason for their development in kitchens and toilets (Adams et al., 2013), justifies also the higher fungal counts in fitness centers that had a swimming pool. Heat, and humidity levels, promoted in the swimming pool areas can enhance the fungal dissemination (Sarmadian et al., 2020). In addition, also swimming pool attendees may carry, on their own body (commensal flora) or clothing, a great diversity of fungal species (Tabatabaei, et al., 2020).

Despite the comprehensive sampling approach followed, one study limitation that can be mentioned is the fact that active air sampling was not performed, hence, hindering the possibility to verify compliance with the Portuguese legal framework. Furthermore, understanding of the ventilation rates and correlation with particulate matter and microbiological data would provide a deeper insight into the analyzed spaces. However, in previous studies correlation was not observed most likely due the fact that only a small portion of microbial contamination found in air is culturable, claiming attention for the underestimation of the results obtained (Whitby et al., 2022). A deeper assessment to the bacterial contamination, beyond the legal criteria, will be of upmost importance to fill the information gap concerning microbiological contamination in this indoor setting.

5. Conclusions

The present study sheds light on the microbial contamination of fitness centers. It suggests that passive sampling can be useful in identifying the most contaminated areas and determining the environmental factors that affect microbial contamination. For instance, having a swimming pool in the facilities was found to be associated with higher levels of microbial contamination. These findings highlight the need for different cleaning procedures in different areas (such as in swimming pool) with a profound revision of all cleaning protocols in place and discourage the use of shared mops and other cleaning materials to prevent cross-contamination. This study also reports the prevalence of toxigenic fungal species with clinical relevance, such as *Aspergillus* section *Fumigati*, in all the assessed facilities. This emphasizes the importance of frequent and effective cleaning procedures, and using reliable cleaning agents, to protect the health of both fitness centers' workers and attendees. Further studies should encompass a higher number of

fitness centers with similar characteristics presented in Table 1 allowing wider and more robust conclusions. In addition, research efforts should be focused in the assessment of the efficacy of different practices and cleaning methods to implement the most suitable protocol.

CRediT authorship contribution statement

Carla Viegas: Writing – review & editing, Writing – original draft, Supervision, Resources, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization. **Cátia Peixoto:** Writing – review & editing, Writing – original draft, Methodology, Formal analysis, Conceptualization. **Bianca Gomes:** Writing – original draft, Formal analysis. **Marta Dias:** Writing – original draft, Formal analysis. **Renata Cervantes:** Writing – original draft, Formal analysis. **Pedro Pena:** Writing – original draft, Formal analysis. **Klara Slezakova:** Writing – original draft, Formal analysis. **Maria do Carmo Pereira:** Writing – original draft, Formal analysis. **Simone Morais:** Writing – original draft, Formal analysis. **Elisabete Carolino:** Writing – original draft, Formal analysis. **Magdalena Twarużek:** Writing – original draft, Formal analysis. **Susana Viegas:** Writing – review & editing, Writing – original draft, Formal analysis. **Liliana Aranha Caetano:** Writing – review & editing, Writing – original draft, Methodology, Formal analysis.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envpol.2024.123976.

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