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# Microbial contamination in grocery stores from Portugal and Spain — The neglected indoor environment to be tackled in the scope of the One Health approach

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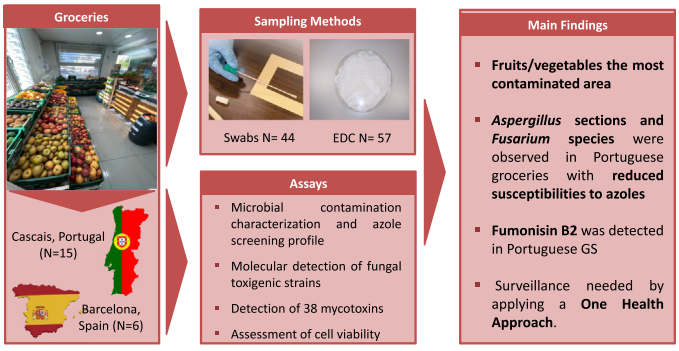
## HIGHLIGHTS

- The sampling approach applied was useful to screen the most critical workstations.
- The most contaminated area in grocery stores was the fruits/vegetables area.
- It was observed opportunistic fungal species with reduced susceptibilities to azoles.
- Fumonisin B2 was detected in Portuguese grocery stores.
- The One Health approach will be the best approach to implement in this setting.

## GRAPHICAL ABSTRACT

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## ABSTRACT

Microbial contamination in grocery shops (GS) should be evaluated since food commodities are commonly handled by workers and customers increasing the risk of food contamination and disease transmission. The aim of this study was to evaluate the microbial contamination in Portuguese and Spanish GS with a multi-approach protocol using passive (electrostatic dust cloths and surface swabs) sampling methods. The molecular detection of *Aspergillus* sections, mycotoxin analysis, screening of azole resistance as well as cytotoxicity measurement were conducted to better estimate the potential health risks of exposure and to identify possible relations between the risk factors studied.
Fruits/vegetables sampling location was the one identified has being the most contaminated (bacteria and fungi) area in GS from both countries. *Aspergillus* section *Fumigati* and *Fusarium* species were observed in samples from Portuguese groceries with reduced susceptibilities to azoles commonly used in the clinical treatment of fungal infections. Fumonisin B2 was detected in Portuguese GS possible unveiling this emergent threat concerning occupational exposure and food safety. Overall, the results obtained raise concerns regarding human health and food safety and must be surveilled applying a One Health approach.

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## 1. Introduction

Indoor microbiological contamination is often neglected as a potential risk factor for workers and occupants (Domingo and Martí Nadal, 2009). However, it is required for employers to assess and prevent occupational exposure to all risk factors (including microbiological risks) in all European countries (Directive 89/391/EEC, 1989). In fact, it was already extensively reported that personal indoor exposure levels are ten- to a hundredfold larger than outdoor exposure (Rasmussen et al., 2021). Furthermore, exposure to microbial contamination, such as bacteria and fungi, can lead to a wide range of health effects with predominance of respiratory diseases (Baxi et al., 2016; Górny, 2020; Lugauskas et al., 2004; Rusca et al., 2008; Sabino et al., 2019; Skorge et al., 2005).

When deposited on surfaces, microorganisms can preserve their viability for a prolonged period, representing a potential threat to workers' and all occupants' health. Nevertheless, their survival decreases under specific environmental conditions, such as the scarcity of nutrients or variations in humidity and/or temperature which do not predispose their growth or dissemination (Górny, 2020). Employees working indoors are commonly exposed to microbiological agents (Shan et al., 2019), bacteria and fungi among others, which under suitable conditions may grow and proliferate (Leppänen et al., 2017).

Microbial contamination in grocery shops should be of concern because food handling and the lack hygiene measures may increase the risk of disease transmission (Paulin et al., 2017). In addition to other common indoor environmental variables, that can potentiate the proliferation and dissemination of microorganisms (such as human occupancy and their activities, humidity levels, ventilation, environmental characteristics, water infiltrations, building and decoration materials and outdoor air) (APA, 2010; WHO, 2009), other factors should be considered. Personal hygiene of workers and customers is a very important issue as it is well recognized that contaminated hands may spread infections (Chung et al., 2008; Paulin et al., 2017). Bacteria from customer's hands, that may contain between 10,000 and 10 million on each hand (Rasmussen et al., 2021), can be transferred to and remain on a product (Paulin et al., 2017). Hence, the manipulation of fruits and vegetables can be an important indoor source of microbial contamination and increase the chances of foodborne illnesses. For instance, fungi are commonly associated with the spoilage of these food commodities (Moss, 2008). Fruits and vegetables may get contaminated at any stage of the production and supply chain, through direct contact with wastewater irrigation or the use of animal manure as crop fertilizer (Chee-Sanford et al., 2009; He et al., 2020; Rahman et al., 2022). Noteworthy, fruits and vegetables, particularly raw leafy greens, are recognized as key vehicles for the spread of human infections previously associated with animal-derived diets (Rahman et al., 2022).

Although the use of preservatives and aseptic processing measures mitigate the spoilage, certain fungi developed resistance to these treatments and are able to persist in storage and distribution environments (Davenport, 1998), some of which being mycotoxin producers and/or opportunistic infectious agents (Benedict et al., 2016; WHO, 2018). In addition, the emergence of new pathogens and increased prevalence of fungicide resistant strains, in the actual context of climate change and spread of fungal phytopathogens into warming climates, represent an additional challenge regarding food security (Avery et al., 2019). Moreover, the development of antifungal resistance, as a consequence of fungicide use in agricultural ecosystems, may enhance the risk for transfer of antifungal resistance to humans, thus, jeopardizing the efficacy of antifungal therapies, currently mostly based on the use of azoles, echinocandins and polyenes (Sharma and Chowdhary, 2017; Verweij et al., 2016).

Given the relevance of fungal contaminants in food commodities, due to both economic and public health reasons, this study considered the screening of azole resistance in the surveyed grocery shops. Moreover, as reported in previous studies dedicated to settings where microorganism's contamination and human exposure can occur (Viegas et al., 2022a, 2022c), mycotoxins contamination was studied to provide more accurate information on human exposure to microbial contamination and to better estimate the related health effects.

Since the One Health approach supports global health by fostering coordination, collaboration and communication between different sectors to tackle shared health threats such as antimicrobial resistance (AMR), food safety, zoonotic diseases, occupational health and several others (Viegas et al., 2022b, 2022c), it is crucial to consider the different exposure scenarios when focusing on microbial contamination of grocery shops, such as through: (i) food consumption being a food safety issue, (ii) the occupancy of an indoor space by the customers, being an indoor air quality issue and, (iii) the grocery workers exposure being an occupational exposure issue. Identifying the variables that may impact the microbial contamination of groceries' environment is of utmost importance to identify risk management measures that can prevent exposure to several risk factors simultaneously, applying a One Health approach.

Despite this extreme importance and relevance of microbial contamination to human health its assessment on this specific indoor environment was not performed so far. The aim of this study was to evaluate the microbial contamination in Portuguese and Spanish groceries with a multi-approach protocol using passive (electrostatic dust cloths and surface swabs) sampling methods. The molecular detection of *Aspergillus* sections, mycotoxin analysis, screening of azole resistance as well as cytotoxicity measurement were conducted to better estimate the health risks of exposure and to identify possible relations between the risk factors.

## 2. Materials and methods

This study was conducted in 15 grocery stores (GS) in Cascais, Portugal between November and December 2021, and in 6 GS in Barcelona, Spain during February and April 2022. All the assessed GS from Portugal and Spain were located in an urban environment and were opened for 10 to 12 h.

### 2.1. Grocery shops' characterization

During the recruitment process, a walkthrough survey (cleaning procedure, ventilation type, etc.) was carried out in each GS (Table 1) and the common sampling locations (checkout, fruits/vegetables area, and warehouse/dispenser) were selected beforehand (Table 1).

### 2.2. Sampling

Two passive sampling methods were used in Portugal (Electrostatic Dust Cloths — EDC) and surface swabs) and one in Spain (EDC) (Fig. 1). Surface samples (N = 44, Portugal) from each sampling site were collected by swabbing the floor using a 10 cm × 10 cm square stencil disinfected with a 70 % alcohol solution (ISO 18593, 2004).

Settled dust was assessed with EDC (Normand et al., 2009; Noss et al., 2008; Viegas et al., 2022b). Each EDC (N = 39 from Portugal; N = 18 from Spain) had a surface exposure area of 0.0014 $m^2$ and 0.0064 $m^2$ respectively, and were placed in each sampling site for 30 days (Fig. 1).

### 2.3. Sample preparation

Swabs were extracted with 1 mL of 0.1 % Tween™ 80 saline solution (0.9 % NaCl) (Frilabo, Maia, Portugal) for 30 min at 250 rpm on an orbital shaker and seeded onto the selected media.

Each EDC cloth (Procter & Gamble Company, Lisbon, Portugal) was washed with 20 mL 0.9 % NaCl with 0.05 % Tween™ 80 by orbital shaking (250 rpm, 30 min, at room temperature) and seeded onto the selected media, in order to assess and characterize the microbial contamination (Viegas et al., 2022b). EDC extracts were also used for the molecular detection of selected *Aspergillus* sections.

**Table 1**
Type of ventilation and cleaning procedures in the GS assessed.

| GS | Country | Type of ventilation* | Cleaning procedures | Observations |
|---|---|---|---|---|
| 1 | Portugal | Natural and mechanical | Daily at closing | Professional cleaning products |
| 2 | Portugal | Natural | Daily at closing | Household cleaning products |
| 3 | Portugal | Natural | Twice a day | Household cleaning products |
| 4 | Portugal | Natural and mechanical | Daily at closing | Only with organic products without chemicals |
| 5 | Portugal | Natural | Daily at closing | Household cleaning products |
| 6 | Portugal | Natural and mechanical | Twice a day | Professional cleaning products |
| 7 | Portugal | Natural and mechanical | Daily at closing | Household cleaning products |
| 8 | Portugal | Natural and mechanical | Two to three times a day | Professional cleaning products |
| 9 | Portugal | Natural | Two to three times a day | Household cleaning products |
| 10 | Portugal | Natural and mechanical | Two to three times a day | Professional cleaning products |
| 11 | Portugal | Natural and mechanical | Daily at closing | Household cleaning products |
| 12 | Portugal | Natural and mechanical | Daily in the morning (1 h before opening by an external company) | Only with organic products without chemicals |
| 13 | Portugal | Natural | Daily at closing | Professional cleaning products |
| 14 | Portugal | Natural | Daily at closing | Household cleaning products |
| 15 | Portugal | Natural and mechanical | Two to three times a day | Professional cleaning products |
| 16 | Spain | Natural | Daily at closing | Only with organic products without chemicals |
| 17 | Spain | Natural | Daily at closing | Household cleaning products |
| 18 | Spain | Natural and mechanical | Twice a day | Only with organic products without chemicals |
| 19 | Spain | Natural | Daily at closing | Household cleaning products |
| 20 | Spain | Natural | Daily at closing | Household cleaning products |
| 21 | Spain | Natural and mechanical | Daily at closing | Household cleaning products |

* Natural ventilation is guaranteed by open windows and mechanical by a ventilation system that promotes air renovation (indoor-outdoor).

### 2.4. Determination of the concentration and diversity of bacteria and fungi

In order to determine the concentration and species composition of bacteria and fungi, 150 μL of the swabs and EDC samples' extracts were seeded in four different culture media, selected in order to enhance the selectivity for bacterial and fungal growth, as follows: 2 % malt extract agar (MEA) (Frilabo, Maia, Portugal) with 0.05 g $L^{-1}$ chloramphenicol media, and dichloran glycerol (DG18) (Frilabo, Maia, Portugal) agar-based media, for fungal characterization; tryptic soy agar (TSA) (Frilabo, Maia, Portugal) with 0.2 % nystatin, for total bacteria assessment; and Violet Red Bile Agar (VRBA) (Frilabo, Maia, Portugal), for Gram-negative bacteria.

For fungal resistance screening, 150 μL of the EDC samples' extracts (N = 39 from Portugal; N = 18 from Spain) were seeded on Sabouraud dextrose agar (SDA) (Frilabo, Maia, Portugal) supplemented with 4 mg $L^{-1}$ itraconazole (ITZ) (Frilabo, Maia, Portugal), 2 mg $L^{-1}$ voriconazole (VCZ) (Frilabo, Maia, Portugal), or 0.5 mg $L^{-1}$ posaconazole (PCZ) (Frilabo, Maia, Portugal), and a non-supplemented SDA media as control, adapted from EUCAST guidelines (Arendrup et al., 2013; EUCAST, 2020).

After incubation of MEA and DG18 at 27 °C for 5 to 7 days for fungi, TSA and VRBA at 30 and 35 °C for 7 days for mesophilic bacteria and coliforms (Gram-negative bacteria), respectively, and azole-supplemented SDA media at 27 °C for 3 to 4 days, bioburden densities (colony-forming units, CFU·$m^{-3}$, CFU·$g^{-1}$ of dust, CFU·$m^{-2}$, CFU·$m^{-2}$·$day^{-1}$) 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). Negative controls were performed to ensure non-existence of background contamination, namely all culture media and extracts of control samples (non-used swabs and EDC) were subjected to the same assays.

### 2.5. Detection of Aspergillus sections

Prior to molecular detection of selected *Aspergillus* sections, 8.8 mL of EDC samples' extracts were used for fungal DNA extraction using the ZR Fungal/Bacterial DNA MiniPrep Kit (Zymo Research, Irvine, USA). Real-time PCR (qPCR), with the CFX-Connect PCR System (Bio-Rad), was used

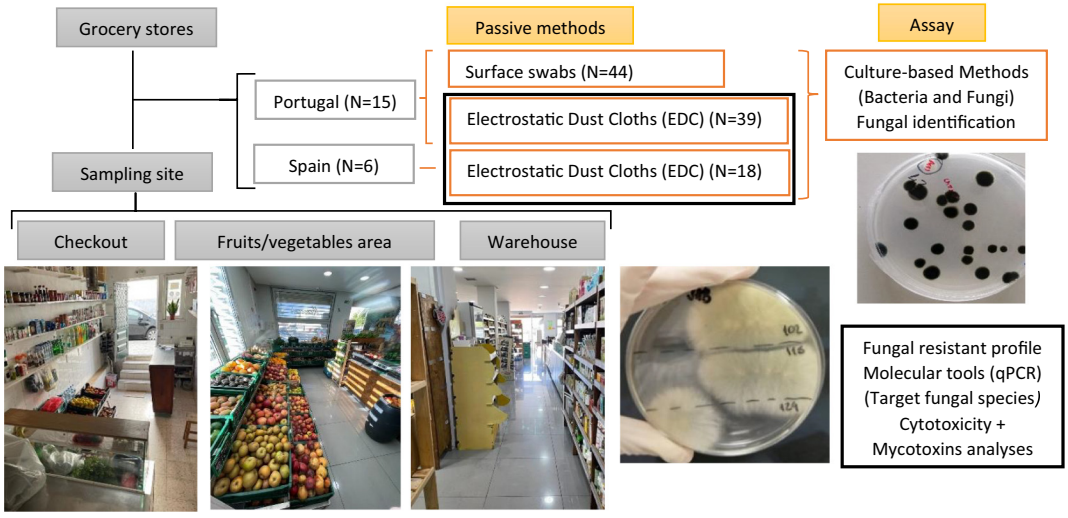


**Fig. 1.** Sampling approach applied in the groceries assessed.

for molecular detection, as follows: reactions included 1 × iQ Supermix (Bio-Rad, Portugal), 0.5 μM of each primer, and 0.375 μM of TaqMan probe in a total volume of 20 μL; amplification followed a three-step PCR: 40 cycles with denaturation at 95 °C for 30 s, annealing at 52 °C for 30 s, and extension at 72 °C for 30 s.

As controls, a negative control and a positive control (DNA from references *Aspergillus* strains) were used. These strains were sequenced for ITS, B-tubulin, and Calmodulin (Table S1 — Supplementary material).

### 2.6. Detection of mycotoxins

EDC samples (N = 39 from Portugal; N = 18 from Spain) were extracted and processed as previously described (Viegas et al., 2021) to a final analyte dilution factor of 10.

Instrumental analysis and ESI–MS/MS were performed by multiple reaction monitoring (MRM) in positive and negative ion modes. The specific transitions of precursor and product ions are presented in Table S2 – Supplementary material. Recovery values (in triplicate), limits of detection (LOD) and limits of quantification (LOQ) for each mycotoxin were calculated from the mycotoxin standards added to a sample free of tested compounds. The LOD and LOQ values were calculated based on signal-to-noise (S/N) ratios of 3:1 and 10:1, respectively (Table S3 – Supplementary material).

### 2.7. Assessment of cell viability

To assess the effect of EDC's contaminants on cell viability, human lung epithelial cells (A549) and human liver carcinoma (HepG2) cells were used, as models for inhalation and oral exposure, respectively (regarding the liver as target for hepatotoxic mycotoxins) (Milićević et al., 2010). Cells were incubated with EDC extracts (N = 39, Portugal; N = 18, Spain) to assess the effect of grocery stores' contaminants on cell viability.

Cells were initially maintained in Eagle's Minimum Essential Medium (MEM) supplemented with 10,000 units of penicillin and 10 mg $mL^{-1}$ streptomycin in 0.9 % NaCl and fetal bovine serum (Sigma-Aldrich, USA). Cell detachment was achieved with 0.25 % (w/v) 0.53 mM Trypsin EDTA). Then, 100 μL of cell suspensions were transferred to a 96-well plate in series of five dilutions (D1:2), with the following cell densities (Scepter™ 2.0 Cell Counter, Merck): $4.0 \times 10^5$ and $4.5 \times 10^5$ cells $mL^{-1}$ for A549 and HepG2 cells, respectively, for EDC from Portugal; $3.0 \times 10^5$ and $2.0 \times 10^5$ cells $mL^{-1}$ for A549 and HepG2 cells, respectively, for EDC from Spain.

Cells' incubation with EDC extracts occurred for 48 h at 5 % $CO_2$, 37 °C in a humidified atmosphere. Cell viability was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay at 510 nm (LEDETECT 96 ELISA, biomedical Dr. Wieser GmbH; MikroWin 2013SC software), as described previously (Hanelt et al., 1994). As negative control, the medium used to apportion the dilutions of the test samples was used. Threshold toxicity level was considered the lowest concentration, decreasing absorption to <50 % of cellular metabolic activity (IC50).

### 2.8. Statistical analysis

Data were analyzed using SPSS statistical software, version 27.0 for Windows. The results were considered significant at the 5 % significance level. To test the normality of the data, the Shapiro-Wilk test was used. For the comparison of bacterial contamination, fungal contamination, fungal resistance profile and mycotoxins contamination between the collection sites, the Kruskal-Wallis test was used, since the normality assumption was not verified. For the comparison of microbial contamination (bacteria and fungi) and fungal resistance profile between the two countries, in each collection site, the Mann-Whitney-*U* test was used, since the normality assumption was not verified. To study the relationship between microbial contamination, fungal resistance profile and mycotoxins contamination, Spearman's correlation coefficient was used, since the assumption of normality was not verified. To assess species diversity, Simpson and Shannon indices, given by Shannon Index $(H) = -\Sigma_{i=1}^{s} p_i \ln(p_i)$ and Simpson Index $(D) = \frac{1}{\Sigma_{i=1}^{s} p_i^2}$, were used, where $p_i$ is the proportion ($n_i$/n) of individuals of one particular species found ($n_i$) divided by the total number of individuals found (n).

## 3. Results

### 3.1. Viable bacterial contamination

In Portuguese GS, the highest median value for total bacteria contamination (TSA) was observed in surface swabs samples with $9.50 \times 10^3$ CFU·$m^{-2}$ in checkout, fruits/vegetables area and warehouse, while only $1.95 \times 10^2$ CFU·$m^{-2}$·$day^{-1}$ were detected in EDC samples (Fig. 2). The highest CFU of Gram-negative bacteria (VRBA) were also found in surface samples, with the highest median value observed in checkout ($6.35 \times 10^5$ CFU·$m^{-2}$), followed by fruits/vegetables area ($9.50 \times 10^4$ CFU·$m^{-2}$) and warehouse ($2.00 \times 10^4$ CFU·$m^{-2}$). EDC samples showed a similar trend, the highest median values were obtained in checkout ($2.65 \times 10^1$ CFU·$m^{-2}$·$day^{-1}$), followed by fruits/vegetables area ($2.27 \times 10^1$ CFU·$m^{-2}$·$day^{-1}$) and the lowest in warehouse samples (1 CFU·$m^{-2}$·$day^{-1}$).

In Spanish GS, total bacteria (TSA) had the highest median value in EDC from fruits/vegetables area (4.65 CFU·$m^{-2}$·$day^{-1}$), while checkout and warehouse had similar median values (3.79 CFU·$m^{-2}$·$day^{-1}$). The highest median value of Gram-negative bacteria (VRBA) was observed in checkout and warehouse (1 CFU·$m^{-2}$·$day^{-1}$), as no Gram-negative bacteria were found in fruits/vegetables area (Fig. 2). Of interest, the total median number of bacterial counts was lower in EDC from Spain samples in comparison to Portuguese samples. The 1st and 3rd quartiles as well as the minimum and maximum levels as presented in Table S7.

### 3.2. Viable fungal contamination

Among all the collected samples from Portuguese GS, higher median values for fungal contamination were observed in surface swabs than in EDC samples, same as with bacterial contamination. Fruits/vegetables area had the highest median values (MEA: $4.00 \times 10^4$ CFU·$m^{-2}$; DG18: $5.00 \times 10^4$ CFU·$m^{-2}$), followed by warehouse (MEA: $2.00 \times 10^4$ CFU·$m^{-2}$; DG18: $9.00 \times 10^4$ CFU·$m^{-2}$) and checkout (MEA: $2.00 \times 10^4$ CFU·$m^{-2}$; DG18: $3.00 \times 10^4$ CFU·$m^{-2}$). Slightly higher number of CFU in EDC samples were obtained from checkout (MEA: $2.27 \times 10^1$ CFU·$m^{-2}$·$day^{-1}$; DG18: 7.58 CFU·$m^{-2}$·$day^{-1}$), then in fruits/vegetables area (MEA: $2.27 \times 10^1$ CFU·$m^{-2}$·$day^{-1}$; DG18: $2.20 \times 10^1$ CFU·$m^{-2}$·$day^{-1}$) and warehouse (MEA: $1.52 \times 10^1$ CFU·$m^{-2}$·$day^{-1}$; DG18: $3.79 \times 10^1$ CFU·$m^{-2}$·$day^{-1}$).

The highest median fungal CFU in EDC from Spanish GS were detected in checkout area (MEA: $1.90 \times 10^2$ CFU·$m^{-2}$·$day^{-1}$; DG18: $3.79 \times 10^1$ CFU·$m^{-2}$·$day^{-1}$), followed by the warehouse (MEA: $6.82 \times 10^1$ CFU·$m^{-2}$·$day^{-1}$; DG18: 7.58 CFU·$m^{-2}$·$day^{-1}$) and fruits/vegetables area (MEA: $5.31 \times 10^1$ CFU·$m^{-2}$·$day^{-1}$; DG18: $1.52 \times 10^{-1}$ CFU·$m^{-2}$·$day^{-1}$) (Fig. 3). The 1st and 3rd quartiles as well as the minimum and maximum levels as presented in Table S8.

In Portugal, higher diversity of fungal species was detected in EDC (Shannon Index (H) = 1.56, Simpson Index (D) = 3.38) compared to swabs (Shannon Index (H) = 1.13, Simpson Index (D) = 2.50). When analyzing fungal diversity in EDC between Portugal and Spain, greater fungal diversity (H = 1.56, D = 3.38) was detected in Portugal than Spain (H = 1.23, D = 2.80) (Table S4 – Supplementary material).

Samples from surface swabs evidenced *Cladosporium* sp. as the dominant genera in checkout (56.25 % MEA; 52.38 % DG18), warehouse (51.35 % MEA; 49.02 % DG18) and in fruits/vegetables area in MEA (52.47 %), but not in DG18 for this sampling site. In contrast, *Penicillium* sp. was prevalent on DG18 (50.56 %). The most common genera in EDC from Portuguese GS was *Penicillium* sp. in checkout (72.18 % MEA; 85.33 % DG18) and fruits/vegetables area (69.60 % MEA; 43.19 %

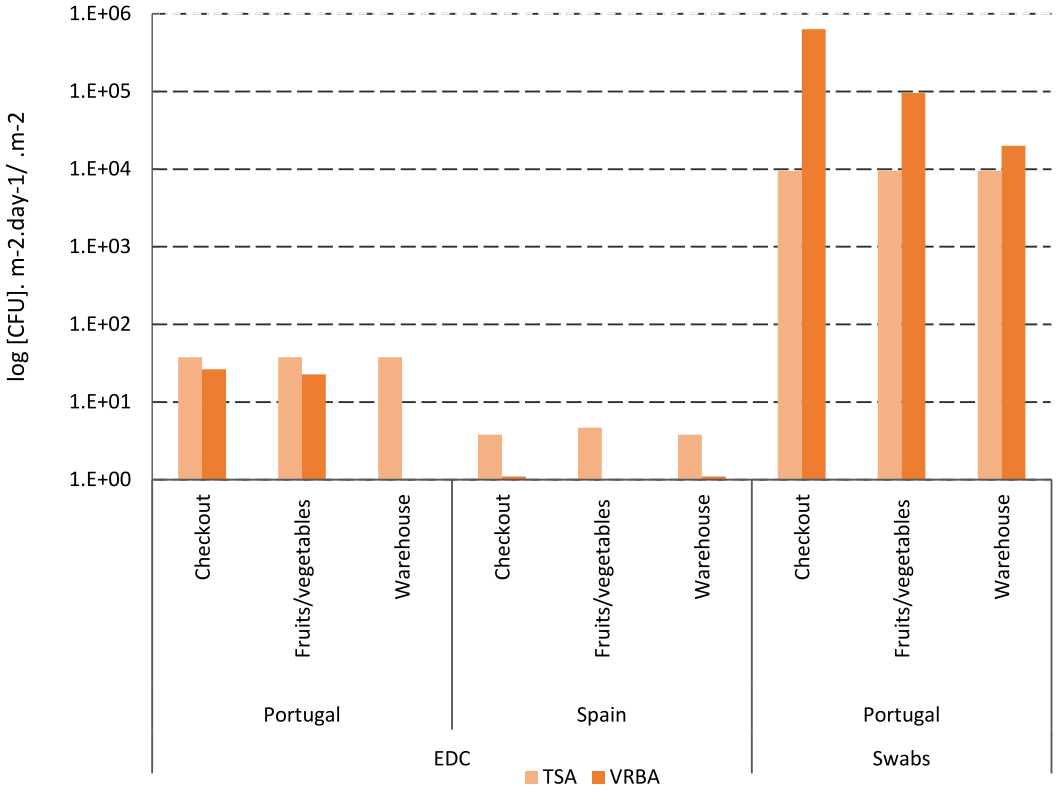


**Fig. 2.** Bacterial (TSA; VRBA) distribution among the sampled matrices and sites (EDC: log [CFU·m$^{-2}$·day$^{-1}$]; Surface swabs: log [CFU·m$^{-2}$]) in grocery stores from Portugal (Surface swabs: checkout n = 15; fruits/vegetables n = 15; warehouse n = 14; EDC: checkout n = 12; fruits/vegetables n = 15; warehouse n = 14) and Spain (EDC: checkout n = 6; fruits/vegetables n = 6; warehouse n = 6).

DG18). In the warehouse *Penicillium* sp. was frequent on MEA (48.45 %), while on DG18 *Cladosporium* sp. was most commonly found (54.71 %). In Spanish GS, *Penicillium* sp. was dominant in all the sampling sites: fruits/vegetables area (62.56 % MEA; 65.84 % DG18), warehouse (53.58 % MEA; 76.17 % DG18) and checkout (43.63 % MEA; 45.32 % DG18) (Table 2).

*Aspergillus* sp. abundance from surface swabs were similar in warehouse and checkout sampling sites on MEA (9.46 % and 9.38 %, respectively),

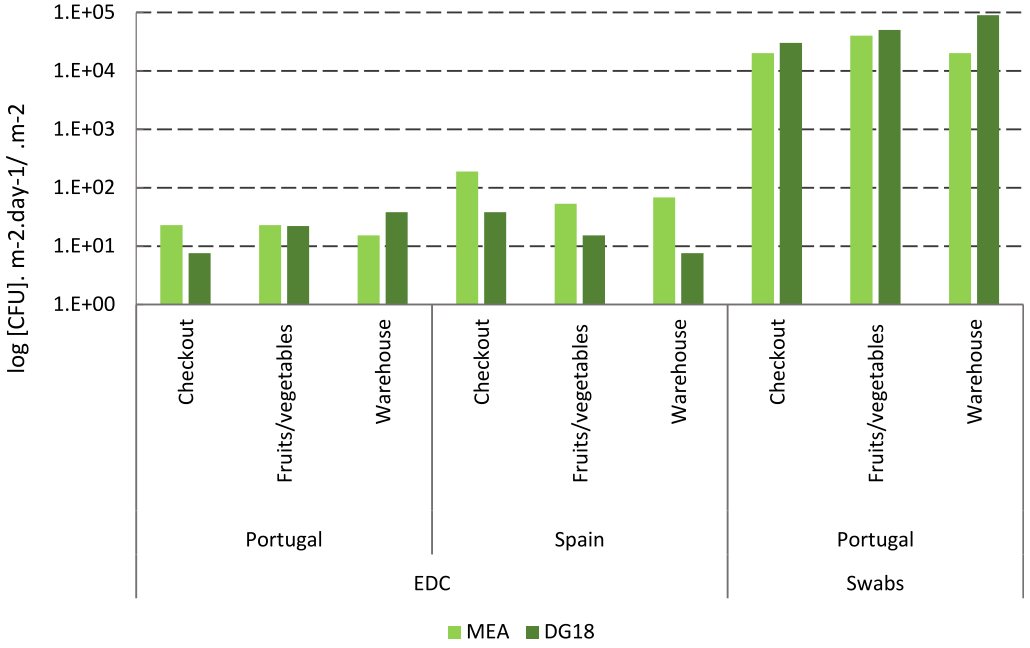


**Fig. 3.** Fungal (MEA; DG18) distribution among the sampled sites and matrices (EDC: log [CFU·m$^{-2}$·day$^{-1}$]; Surface swabs: log [CFU·m$^{-2}$]) in grocery stores from Portugal (Surface swabs: checkout n = 15; fruits/vegetables n = 15; warehouse n = 14; EDC: checkout n = 12; fruits/vegetables n = 15; warehouse n = 14) and Spain (EDC: checkout n = 6; fruits/vegetables n = 6; warehouse n = 6).

**Table 2**
Fungal distribution on MEA and DG18 in the different environmental samples from Portuguese and Spanish grocery stores (EDC: log [CFU·m$^{-2}$·day$^{-1}$]; surface swabs: log [CFU·m$^{-2}$]).

| EDC | | MEA | | | DG18 | | |
|---|---|---|---|---|---|---|---|
| Sampling site | Country | Fungi | CFU·m$^{-2}$·day$^{-1}$ | % | Fungi | CFU·m$^{-2}$·day$^{-1}$ | % |
| Checkout | Portugal | *Penicillium* sp. | 1.36 × 10$^3$ | 72.18 | *Penicillium* sp. | 1.46 × 10$^3$ | 85.33 |
| | | *Cladosporium* sp. | 2.20 × 10$^2$ | 11.69 | *Aspergillus* section *Circumdati* | 1.97 × 10$^2$ | 11.56 |
| | | *Aspergillus* section *Nigri* | 9.10 × 10$^1$ | 4.84 | | | |
| | | Other species | 2.12 × 10$^2$ | 11.29 | *Cladosporium* sp. | 3.79 × 10$^1$ | 2.22 |
| | | | | | *Aspergillus* section *Aspergilli* | 1.52 × 10$^1$ | 0.89 |
| | Spain | | | | *Cladosporium* sp. | 1.08 × 10$^2$ | 45.32 |
| | | *Penicillium* sp. | 1.23 × 10$^2$ | 43.63 | *Penicillium* sp. | 1.08 × 10$^2$ | 45.32 |
| | | *Cladosporium* sp. | 1.11 × 10$^2$ | 39.46 | *Aspergillus* section *Circumdati* | 2.00 × 10$^2$ | 8.37 |
| | | *Aspergillus* section *Nigri* | 2.55 × 10$^1$ | 9.07 | | | |
| | | Other species | 2.21 × 10$^1$ | 7.84 | *Aspergillus* section *Aspergilli* | 1.38 × 10 | 0.58 |
| | | | | | Other species | 1 × 10 | 0.42 |
| Fruits/vegetables | Portugal | *Penicillium* sp. | 2.40 × 10$^3$ | 69.60 | *Penicillium* sp. | 1.42 × 10$^3$ | 43.19 |
| | | *Cladosporium* sp. | 4.47 × 10$^2$ | 12.88 | *Aspergillus* section *Circumdati* | 1.10 × 10$^3$ | 33.35 |
| | | *Aspergillus* section *Nigri* | 4.25 × 10$^2$ | 12.23 | *Aspergillus* section *Aspergilli* | 1.59 × 10$^2$ | 4.83 |
| | | Other species | 2.05 × 10$^2$ | 5.90 | Aspergillus section *Fumigati* | 4.55 × 10$^1$ | 1.38 |
| | | | | | Other species | 5.69 × 10$^2$ | 17.25 |
| | Spain | *Penicillium* sp. | 1.96 × 10$^2$ | 62.56 | *Penicillium* sp. | 1.28 × 10$^2$ | 65.84 |
| | | *Cladosporium* sp. | 6.41 × 10$^1$ | 20.48 | *Cladosporium* sp. | 3.38 × 10$^1$ | 17.44 |
| | | *Aspergillus* section *Nigri* | 3.31 × 10$^1$ | 10.57 | *Aspergillus* section *Circumdati* | 1.72 × 10$^1$ | 8.90 |
| | | Other species | 2.00 × 10$^1$ | 6.39 | *Aspergillus* section *Nigri* | 1.38 × 10 | 0.71 |
| | | | | | Other species | 1.38 × 10$^1$ | 7.12 |
| Warehouse | Portugal | *Penicillium* sp. | 7.13 × 10$^2$ | 48.45 | *Cladosporium* sp. | 9.25 × 10$^2$ | 54.71 |
| | | *Cladosporium* sp. | 2.96 × 10$^2$ | 20.10 | *Penicillium* sp. | 6.22 × 10$^2$ | 36.77 |
| | | *Fusarium poae* | 1.21 × 10$^2$ | 8.25 | *Aspergillus* section *Circumdati* | 1.44 × 10$^2$ | 8.52 |
| | | *Aspergillus* section *Nigri* | 8.34 × 10$^1$ | 5.67 | | | |
| | | Other species | 2.58 × 10$^2$ | 17.53 | | | |
| | Spain | *Penicillium* sp. | 1.39 × 10$^2$ | 53.58 | *Penicillium* sp. | 1.12 × 10$^2$ | 76.01 |
| | | *Cladosporium* sp. | 1.09 × 10$^2$ | 41.91 | *Cladosporium* sp. | 2.48 × 10$^2$ | 16.79 |
| | | *Aspergillus* section *Nigri* | 5.51 × 10 | 2.12 | *Aspergillus* section *Circumdati* | 9.65 × 10$^1$ | 6.53 |
| | | Other species | 6.20 × 10 | 2.39 | *Aspergillus* section *Aspergilli* | 1 × 10 | 0.68 |

| SWABS | | MEA | | | DG18 | | |
|---|---|---|---|---|---|---|---|
| Sampling site | Country | Fungi | CFU·m$^{-2}$ | % | Fungi | CFU·m$^{-2}$ | % |
| Checkout | Portugal | *Cladosporium* sp. | 3.60 × 10$^5$ | 56.25 | | | |
| | | *Penicillium* sp. | 1.90 × 10$^5$ | 29.69 | *Cladosporium* sp. | 1.10 × 10$^5$ | 52.38 |
| | | *Aspergillus* section *Circumdati* | 3.00 × 10$^4$ | 4.69 | *Penicillium* | 8.00 × 10$^4$ | 38.10 |
| | | *Aspergillus* section *Nigri* | 3.00 × 10$^4$ | 4.69 | *Aspergillus* section *Circumdati* | 2.00 × 10$^4$ | 9.52 |
| | | Other species | 3.00 × 10$^4$ | 4.69 | | | |
| Fruits/vegetables | Portugal | *Cladosporium* sp. | 2.34 × 10$^6$ | 52.47 | *Penicillium* sp. | 9.00 × 10$^5$ | 50.56 |
| | | *Penicillium* sp. | 1.53 × 10$^6$ | 34.30 | *Cladosporium* sp. | 6.20 × 10$^5$ | 34.83 |
| | | *Paecilomyces* sp. | 3.50 × 10$^5$ | 7.85 | *Aspergillus* section *Circumdati* | 1.80 × 10$^5$ | 10.11 |
| | | *Aspergillus* section *Nigri* | 9.00 × 10$^4$ | 2.02 | *Aspergillus* section *Aspergilli* | 1.00 × 10$^4$ | 0.56 |
| | | Other species | 1.50 × 10$^5$ | 3.36 | Other species | 7.00 × 10$^4$ | 3.93 |
| Warehouse | Portugal | *Cladosporium* sp. | 3.80 × 10$^5$ | 51.35 | *Cladosporium* sp. | 2.50 × 10$^5$ | 49.02 |
| | | *Penicillium* sp. | 1.90 × 10$^5$ | 25.68 | *Penicillium* sp. | 1.30 × 10$^5$ | 25.49 |
| | | *Geotrichum* sp. | 9.00 × 10$^4$ | 12.16 | *Aspergillus* section *Circumdati* | 8.00 × 10$^4$ | 15.69 |
| | | *Aspergillus* section *Candidi* | 4.00 × 10$^4$ | 5.41 | *Aspergillus* section *Aspergilli* | 5.00 × 10$^4$ | 9.80 |
| | | *Aspergillus* section *Nigri* | 3.00 × 10$^4$ | 4.05 | | | |
| | | Other species | 1.00 × 10$^4$ | 1.35 | | | |

while a lower abundance was detected from fruits/vegetables area (2.02 %). However, higher *Aspergillus* sp. growth on DG18 from the warehouse site (25.49 %) was observed when compared to the fruits/vegetables area (10.67 %) and checkout (9.52 %).

Surprisingly, slightly different results were obtained from EDC samples. Both in Portugal and Spain the most contaminated site with *Aspergillus* sp. was the fruits/vegetables area (12.23 % MEA; 39.56 % DG18 in Portugal; 10.57 % MEA; 9.51 % DG18 in Spain). Discrepancy was observed in remaining sampling sites, as in Portugal *Aspergillus* sp. was more abundant in the warehouse (5.67 % MEA; 8.52 % DG18) rather than in the checkout area (4.84 % MEA; 12.44 % DG18) in contrast to Spanish GS: checkout area (9.07 % MEA; 8.96 % DG18) and warehouse (2.12 % MEA; 7.22 % DG18). Regarding *Aspergillus* sections distribution on EDC samples from Portuguese GS, section *Nigri* was the only section (100 %) found on MEA in all the sampling sites. In DG18, 3 sections were identified on fruits/vegetables area (84.30 % *Circumdati*; 12.21 %; *Aspergilli*; 3.49 % *Fumigati*), and 2 sections on checkout (92.86 % *Circumdati*; 7.14 % *Aspergilli*), while on the warehouse, section *Circumdati* was prevalent (100 %).

In surface swabs collected in Portuguese GS, 2 sections were found on MEA in checkout (50 % *Circumdati*; 50 % *Nigri*) and warehouse areas (57.14 % *Candidi*; 42.86 % *Nigri*), whereas 2 sections (61.54 % *Circumdati*; 38.46 % *Aspergilli*) from the warehouse and 1 section (100 % *Circumdati*) from checkout were cultured on DG18. Section *Nigri* was dominant in the fruits/vegetables area (100 %) when cultured on MEA, while 2 sections growth on DG18 media (94.74 % *Circumdati*; 5.26 % *Aspergilli*) (Fig. 4).

Regarding Spanish GS, *Aspergillus* section *Nigri* was prevalent on MEA in all sampling sites (100 %, same as in Portuguese GS), while on DG18, 2 sections were found on the checkout (93.55 % *Circumdati*; 6.45 % *Aspergilli*), warehouse (93.55 % *Circumdati*; 6.67 % *Aspergilli*) and fruits/vegetables areas (92.59 % *Circumdati*; 7.41 % *Nigri*). *Aspergillus* section *Fumigati* was not detected in EDC samples from Spanish GS (Fig. 5).

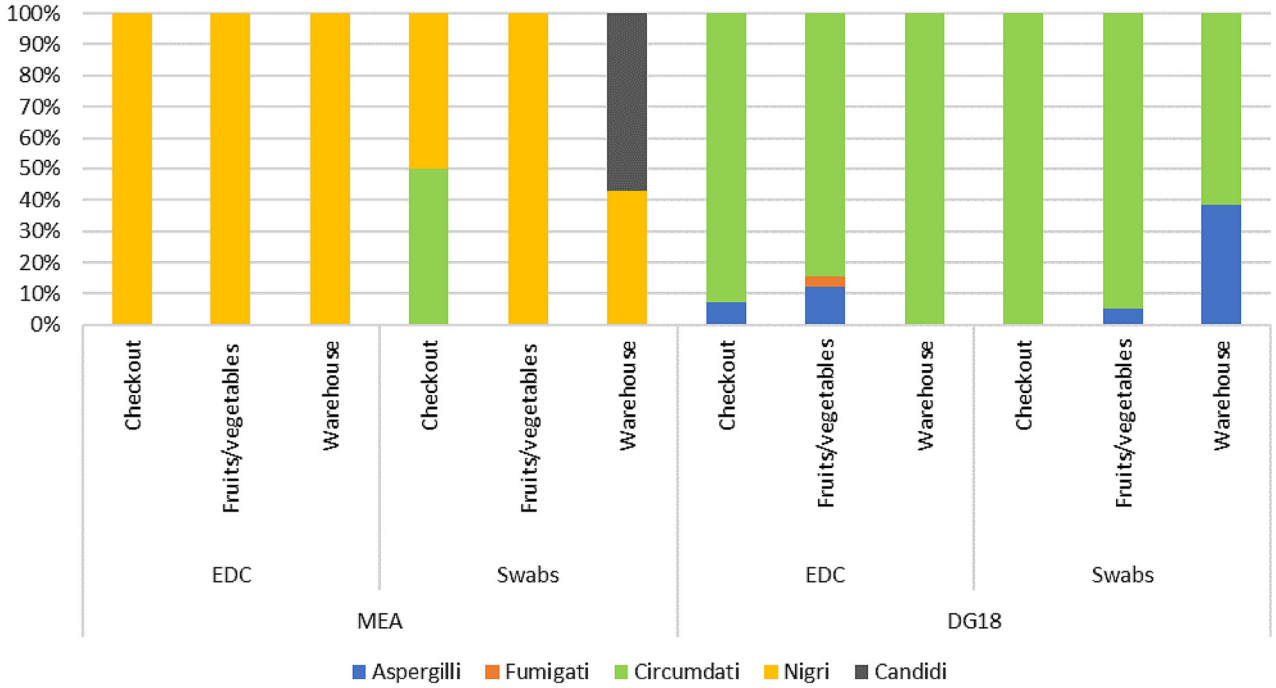


**Fig. 4.** *Aspergillus* sections distribution in MEA and DG18 in Portuguese GS (EDC: log [CFU·m$^{-2}$·day$^{-1}$]; surface swabs: log [CFU·m$^{-2}$]).

### 3.3. Fungal growth in azole-supplemented media

EDC from grocery stores in Portugal and Spain were screened for azole resistance. The results are presented in Fig. S1 – Supplementary material. In all cases, fungal burden was lower in azole-supplemented SDA media compared to SDA. Fungal burden was also lower in most EDC from Spain, the exception being EDC from the warehouse of Spanish grocery stores in itraconazole-supplemented SDA media.

Fungal diversity is depicted in Table 3. *Penicillium* sp. and *Cladosporium* sp. were the most prevalent species. *Penicillium* sp. was observed in posaconazole-supplemented SDA media in all sampling sites in Portuguese grocery stores (suggesting some reduced sensitivity to this antifungal agent), but not in EDC from Spanish grocery stores. *Cladosporium* sp. was observed in all azoles at all sampling sites in both countries. *Aspergillus* sp. was observed (in SDA only) at all sampling sites from both countries. *Fusarium* sp. (including *F. graminearum* and *F. verticillioides*) was observed in the "checkout" and "fruits/vegetables" areas in Portuguese grocery stores all azole-supplemented media. Two opportunistic human pathogens, *Aspergillus* sections *Fumigati* and *Circumdati*, were detected in the "fruits/vegetables" area in Portuguese grocery stores in voriconazole-supplemented SDA media. *Aureobasidium* sp. and *Mucor* sp. were observed in azole-supplemented media (ICZ and VCZ, respectively) in Spanish grocery stores only.

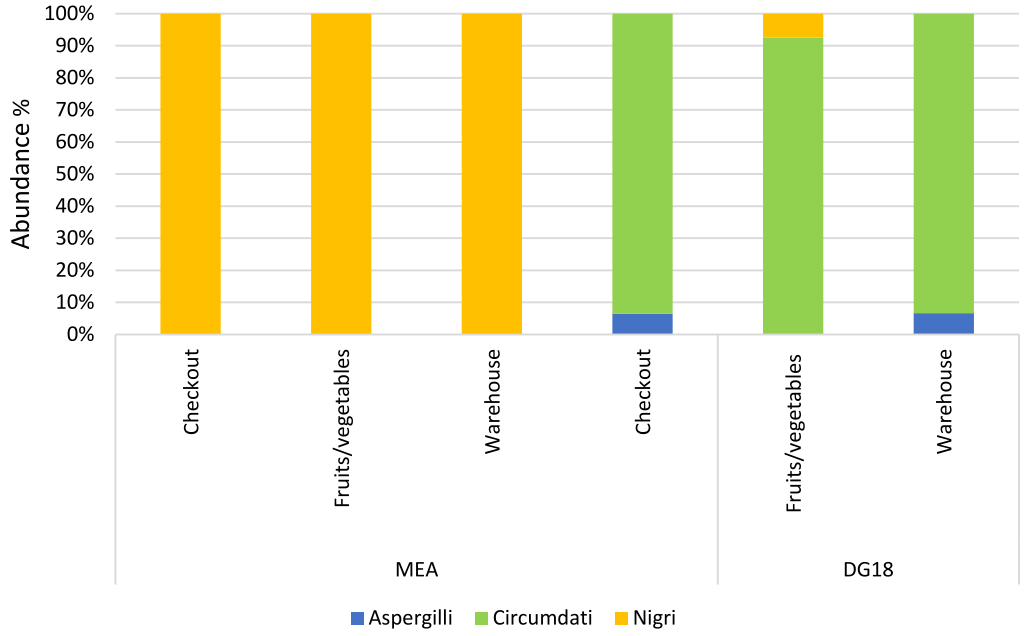


**Fig. 5.** *Aspergillus* sections distribution in MEA and DG18 in Spanish GS (EDC: log [CFU·m$^{-2}$·day$^{-1}$]).

**Table 3**
Fungal distribution on azole-supplemented media in the different environmental samples from Portuguese and Spanish grocery stores (EDC: log [CFU·m$^{-2}$·day$^{-1}$]).

| Sampling site | SDA | | | ITZ | | | VCZ | | | PCZ | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Fungi | CFU·m$^{-2}$·day$^{-1}$ | % | Fungi | CFU·m$^{-2}$·day$^{-1}$ | % | Fungi | CFU·m$^{-2}$·day$^{-1}$ | % | Fungi | CFU·m$^{-}$2·day$^{-1}$ | % |
| Checkout (Portugal) | *Penicillium* sp. | 1.44E + 03 | 60.70 | *Penicillium* sp. | 3.34E + 02 | 49.44 | *Penicillium* sp. | 1.60E + 03 | 86.83 | *Cladosporium* sp. | 8.14E + 01 | 46.00 |
| | *Paecilomyces* sp. | 4.09E + 02 | 17.25 | *Cladosporium* sp. | 2.50E + 02 | 37.08 | *Cladosporium* sp. | 2.20E + 02 | 0.82 | *Penicillium* sp. | 7.78E + 01 | 44.00 |
| | *Cladosporium* sp. | 3.64E + 02 | 15.34 | *Paecilomyces* sp. | 8.34E + 01 | 12.36 | *Fusarium graminearum* | 1.52E + 01 | 11.93 | *Chrysonilia sitophila* | 1.77E + 01 | 10.00 |
| | *A.* section *Terrei* | 1.52E + 01 | 0.64 | Other species | 7.58E + 00 | 1.12 | Other species | 7.58E + 00 | 0.41 | | | |
| | *A.* section *Fumigati* | 1.52E + 01 | 0.64 | | | | | | | | | |
| | *A.* section *Circumdati* | 7.58E + 00 | 0.32 | | | | | | | | | |
| | *A.* section *Nigri* | 1.14E + 02 | 4.79 | | | | | | | | | |
| | Other species | 7.58E + 00 | 0.32 | | | | | | | | | |
| Checkout (Spain) | *Penicillium* sp. | 8.48E + 01 | 48.62 | *Cladosporium* sp. | 1.01E + 02 | 62.93 | *Cladosporium* sp. | 4.20E + 01 | 48.80 | *Cladosporium* sp. | 6.20E + 00 | 100.00 |
| | *Cladosporium* sp. | 6.62E + 01 | 37.94 | *Paecilomyces* sp. | 4.20E + 01 | 26.29 | *Penicillium* sp. | 4.00E + 01 | 46.40 | | | |
| | *A.* section *Nigri* | 2.07E + 01 | 11.86 | *Penicillium* sp. | 1.72E + 01 | 10.78 | Other species | 4.14E + 00 | 4.80 | | | |
| | *A.* section *Circumdati* | 6.89E-01 | 0.40 | | | | | | | | | |
| | Other species | 2.07E + 00 | 1.19 | | | | | | | | | |
| Fruits/vegetables (Portugal) | *Penicillium* sp. | 2.93E + 03 | 63.40 | *Penicillium* sp. | 5.30E + 02 | 28.32 | *Penicillium* sp. | 1.62E + 03 | 50.68 | *Cladosporium* sp. | 4.65E + 02 | 42.71 |
| | *Cladosporium* sp. | 8.19E + 02 | 17.73 | *Cladosporium* sp. | 6.22E + 02 | 33.21 | *Cladosporium* sp. | 8.27E + 02 | 25.92 | *Penicillium* sp. | 4.62E + 02 | 42.39 |
| | *A.* section *Nigri* | 2.35E + 02 | 5.08 | *Fusarium verticillioides* | 1.29E + 02 | 6.88 | *Paecilomyces* sp. | 6.45E + 02 | 20.21 | *Fusarium verticillioides* | 1.21E + 02 | 11.10 |
| | *A.* section *Fumigati* | 1.59E + 02 | 3.45 | Other species | 5.91E + 02 | 31.59 | *A.* section *Circumdati* | 6.82E + 01 | 2.14 | Other species | 4.15E + 01 | 3.81 |
| | A. section *Circumdati* | 6.82E + 01 | 1.48 | | | | *A.* section *Fumigati* | 7.58E + 00 | 0.24 | | | |
| | Other species | 4.09E + 02 | 8.87 | | | | Other species | 2.58E + 01 | 0.81 | | | |
| Fruits/vegetables (Spain) | *Penicillium* sp. | 1.77E + 02 | 84.26 | *Cladosporium* sp. | 2.62E + 01 | 31.15 | *Penicillium* sp. | 8.27E + 01 | 71.86 | *Cladosporium* sp. | 6.89E-01 | 100.00 |
| | *A.* section *Nigri* | 2.07E + 01 | 9.84 | *Aureobasidium* sp. | 2.21E + 01 | 26.23 | *Cladosporium* sp. | 3.24E + 01 | 28.14 | | | |
| | *Cladosporium* sp. | 8.96E + 00 | 4.26 | *Penicillium* sp. | 1.93E + 01 | 22.95 | | | | | | |
| | *A.* section *Circumdati* | 3.45E + 00 | 1.64 | Other species | 1.65E + 01 | 19.67 | | | | | | |
| Warehouse (Portugal) | *Penicillium* sp. | 6.82E + 02 | 38.79 | *Cladosporium* sp. | 1.29E + 02 | 39.53 | *Penicillium* sp. | 5.61E + 02 | 73.27 | *Cladosporium* sp. | 1.59E + 02 | 70.00 |
| | *Paecilomyces* sp. | 4.55E + 02 | 25.86 | *Penicillium* sp. | 7.58E + 01 | 23.26 | *Cladosporium* sp. | 1.82E + 02 | 23.76 | *Chrysonilia sitophila* | 4.55E + 01 | 20.00 |
| | *Cladosporium* sp. | 2.58E + 02 | 14.66 | *Paecilomyces* sp. | 1.14E + 02 | 34.88 | *Chrysonilia sitophila* | 7.58E + 00 | 0.99 | *Penicillium* sp. | 2.27E + 01 | 10.00 |
| | *A.* section *Fumigati* | 8.34E + 01 | 4.74 | Other species | 7.58E + 00 | 2.33 | Other species | 1.52E + 01 | 1.98 | | | |
| | *A.* section *Nigri* | 1.67E + 02 | 9.48 | | | | | | | | | |
| | Other species | 1.14E + 02 | 6.47 | | | | | | | | | |
| Warehouse (Spain) | *Penicillium* sp. | 1.15E + 03 | 87.17 | *Cladosporium* sp. | 9.41E + 02 | 78.70 | *Cladosporium* sp. | 1.31E + 02 | 44.05 | *Cladosporium* sp. | 1.17E + 02 | 100.00 |
| | *Cladosporium* sp. | 9.91E + 01 | 7.49 | *Penicillium* sp. | 2.55E + 02 | 21.30 | *Penicillium* sp. | 1.20E + 02 | 40.48 | | | |
| | *A.* section *Nigri* | 3.18E + 01 | 2.41 | | | | *Mucor* sp. | 2.48E + 01 | 8.33 | | | |
| | Other species | 3.89E + 01 | 2.94 | | | | Other species | 2.12E + 01 | 7.14 | | | |

### 3.4. Molecular detection of Aspergillus sections

From all four targeted *Aspergillus* sections only *Nidulantes* was detected in GS from both countries, being detected in 5 EDC from Portuguese GS (12.8 %; 5 out of 39 samples) and in 15 EDC from Spanish GS (83.3 %; 15 out of 18 samples) (Table S5 – Supplementary material).

### 3.5. Mycotoxins contamination

From the 39 Portuguese GS EDC samples analyzed only fumonisin B2 was detected in 2 samples (5 %), and both values were below the limit of quantification (LOQ). In the 18 EDC samples analyzed from Spanish groceries none showed contamination by mycotoxins.

### 3.6. Effect on human cell viability

Based on their ability to decrease cell metabolic activity, a total of 8 (14.8 %) EDC exhibited some level of cytotoxicity. Two EDC from Portuguese grocery stores with low cytotoxicity were from "fruits/vegetables" area (A549 cells) and from "warehouse" (HepG2 cells). Regarding Spanish grocery stores, 6 EDC exhibited low to moderate cytotoxicity, being 3 from the "checkout" area (A549 and HepG2 cells), 2 from "fruits/vegetables" area (A549 and HepG2 cells) and 1 from the "warehouse" (A549 and HepG2 cells) (Fig. 6).

### 3.7. Comparison and correlation analyses

In the EDC samples, considering both countries, statistically significant differences were detected between the sampling sites as following: i) fungal counts in MEA ($\chi^2_{K\_W}(3) = 6.348, p = 0.042$), with the fruits/vegetables area showing the highest values; ii) fungal contamination in ITZ supplemented media ($\chi^2_{K\_W}(3) = 5.995, p = 0.050$), with again the vegetables/fruits area showing the highest contamination. Regarding other fungal resistances, no statistically significant differences were detected between the sampling sites (Fig. 7).

In the surface swabs, only statistically significant differences between the sampling sites were detected regarding the fungal counts in DG18 ($\chi^2_{K\_W}(3) = 9.924, p = 0.007$), being the fruits/vegetables area and the warehouse the ones that showed the highest fungal contamination (Fig. 8).

The two countries differ significantly in most parameters evaluated in the checkout and fruits/vegetables sampling sites ($p' < 0.05$). However, in the warehouse, significant differences were observed only for bacterial growth on TSA ($U = 6.000, p = 0.003$) and fungal abundance on PSZ ($U = 13.500, p = 0.021$). Portugal has the highest values, since it has the highest mean ranks (Table 4).

In EDC samples, the results revealed that higher bacterial counts in TSA are related to higher fungal counts in MEA ($r_S = 0.408, p = 0.002$) and in DG18 ($r_S = 0.444, p = 0.001$), with higher counts in SDA ($r_S = 0.418, p = 0.001$), in VCZ ($r_S = 0.394, p = 0.002$) and in PSZ ($r_S = 0.452, p = 0.000$). Higher fungal counts in MEA are related with higher fungal counts in DG18 ($r_S = 0.845. p = 0.000$), higher counts in SDA ($r_S = 0.886, p = 0.000$), in ITZ ($r_S = 0.713, p = 0.000$), in VCZ ($r_S = 0.812, p = 0.000$) and in PSZ ($r_S = 0.696, p = 0.000$). Higher counts in DG18 are related with higher counts in SDA ($r_S = 0.792, p = 0.000$), in ITZ ($r_S = 0.635, p = 0.000$), in VCZ ($r_S = 0.782, p = 0.000$) and in PSZ ($r_S = 0.651, p = 0.000$). Higher counts in SDA are related with higher values in ITZ ($r_S = 0.712, p = 0.000$), in VCZ ($r_S = 0.847, p = 0.000$) and in PSZ ($r_S = 0.730, p = 0.000$). Higher counts in ITZ are related with higher counts in VCZ ($r_S = 0.810, p = 0.000$) and in PSZ ($r_S = 0.609, p = 0.000$). Higher counts in VCZ are related with higher counts in PSZ ($r_S = 0.723, p = 0.000$) (Table 4).

In surface swabs, the results revealed that higher counts in VRBA are related with fungal counts in MEA ($r_S = 0.369, p = 0.014$) and higher fungal counts in MEA are related with fungal counts in DG18 ($r_S = 0.766, p = 0.000$) (Table 4).

## 4. Discussion

For the characterization of indoor bioaerosols in GS two different sampling approaches, EDC and surface swabs were implemented. EDC have been widely used to assess microbiological contamination in studies developed in indoor and occupational environments (Viegas et al., 2022b), and also to identify the microorganisms associated with building moisture damage and assess their impact on human health (Adams et al., 2021). Surface swabs were useful to identify and corroborate potential sources of contamination (Viegas et al., 2021), as well as to provide a more complete snapshot of microbial contamination. In the present study, we detected higher bacterial and fungal abundance in swabs than in EDC collected in Portuguese GC. Besides being different sampling methods they also have different extraction protocols that can influence the microbial counts and diversity obtained. Additionally, higher bacterial counts in surface swabs may be because of drastic bacterial sensitivity to different environmental conditions (Cox, 1989). Thus, methods with shorter sampling times will be more suitable to assess the bacterial contamination. However, a higher fungal diversity was observed in EDC. Furthermore, previous studies suggested that the use of various sampling approaches in parallel will allow overcoming the limitations of one or another technique and providing a comprehensive and accurate characterization of bioaerosols and, consequently, a more representative scenario of indoor exposure (Viegas et al., 2022a; Whitby et al., 2022). Unfortunately, it was not possible to apply both sampling methods in Spanish GS.

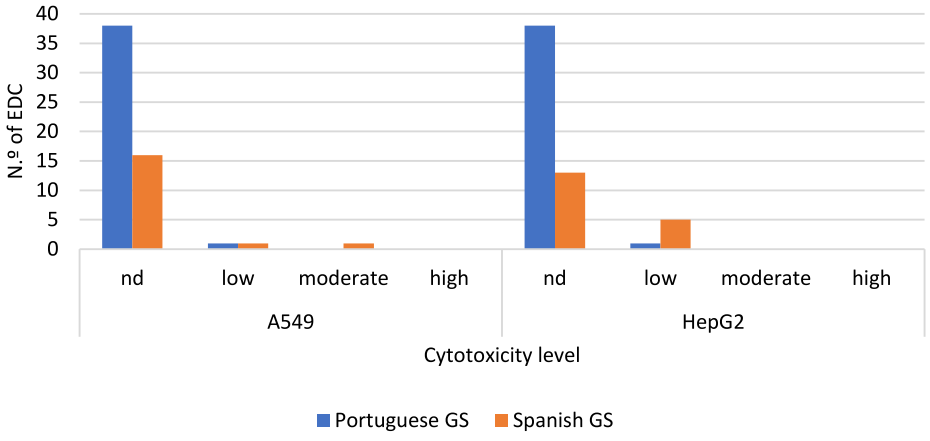


**Fig. 6.** Cytotoxicity level of EDC in A549 and HepG2 cells (based on the results of the MTT assay for determination of cell viability). GS, grocery stores; Nd, not detected; low, IC50 at 1:2 dilution of EDC's extracts; moderate, IC50 at 1:4 dilution of EDC's extracts; high, IC50 above 1:4 dilution of EDC's extracts.

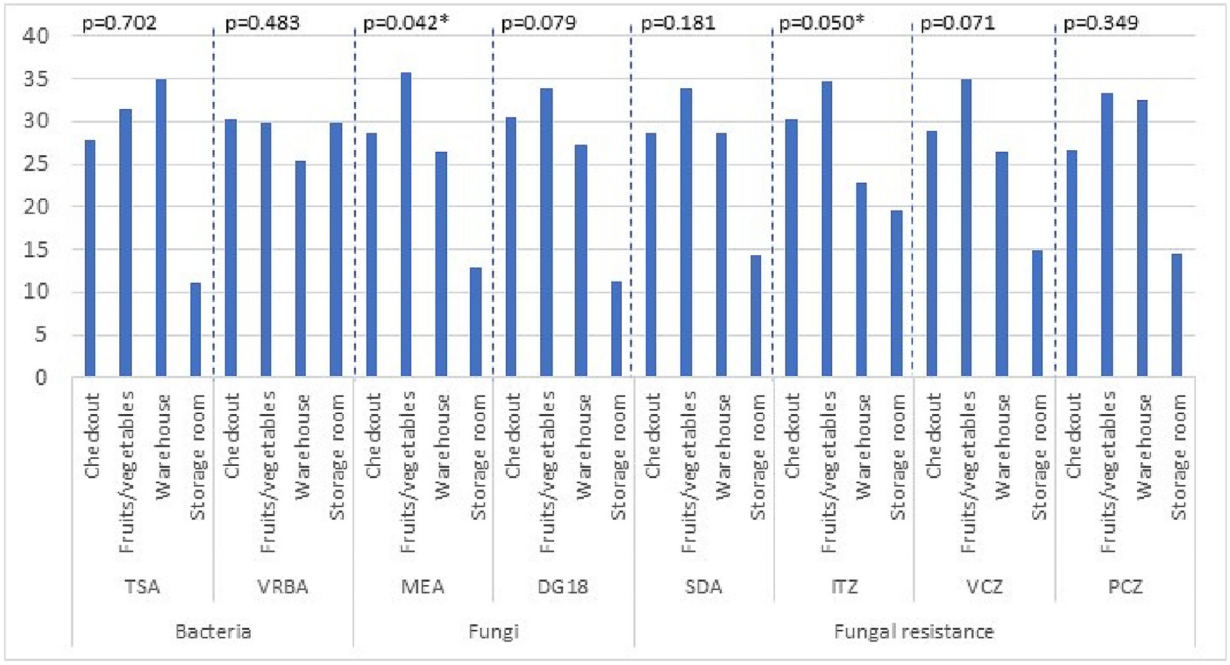


**Fig. 7.** Comparison of microbial contamination and fungal resistance profile found in EDC between sampling sites from both countries. Kruskal-Wallis test results. *Statistically significant differences at 5 % significance level.

The most fungal contaminated areas in GS from both countries were fruits/vegetables and checkout sites. Higher microbial counts at those areas may be due to poor hygienic practices from customers and workers or because of the steady flow of products and people (Paulin et al., 2017), particularly at the checkout. Previously, the raw materials or the main products sold (fruits/vegetables) were already identified as a potential source of microbial indoor contamination in the food industry environment (Mritunjay and Kumar, 2017). In fact, also in bakeries or even in the feed industry, the flour and the cereals grains were shown to be the main sources of indoor contamination (Viegas et al., 2020a), which can also be the case for fruits and vegetables in GS.

Intriguingly, significantly higher bacterial and fungal contamination was detected in Portuguese GS than in Spanish ones. This may be explained by different environmental variables that can influence microbial contamination, such as ventilation conditions, cleaning practices or less crowded indoor environment in Spanish GS (APA, 2010; Chung et al., 2008; Paulin et al., 2017; WHO, 2009). In fact, in what concerns the hygiene status of Portuguese GS, some of them had visible filth and surrounding areas that required cleaning. Furthermore, decreased diversity in microbial contamination may be a consequence of the exaggerated use of pesticides in Spain, one of the main concerns not only within the country but in a global scale (González et al., 2021). This discrepancy should be further studied in future studies dedicated to the same indoor setting. Additionally, besides being in different geographical regions from Peninsula Iberian, the studies were developed in different seasons (Portuguese GS in Winter, while in Spanish GS was held during Winter and Spring) and both discrepancies can influence not only microbial contamination (Karlsson et al., 2020), but also ventilation practices inside GS (Stamp et al., 2022).

*Penicillium* and *Cladosporium* genera were the most abundant in GS from both countries. However, the different sampling methods and culture media applied provided different fungal profiles. In fact, different *Aspergillus* sp. were collected by the two sampling methods and cultured in distinct media with more species obtained from EDC in DG18 (Portuguese and Spanish GS). As previous reported, the growth media can significantly

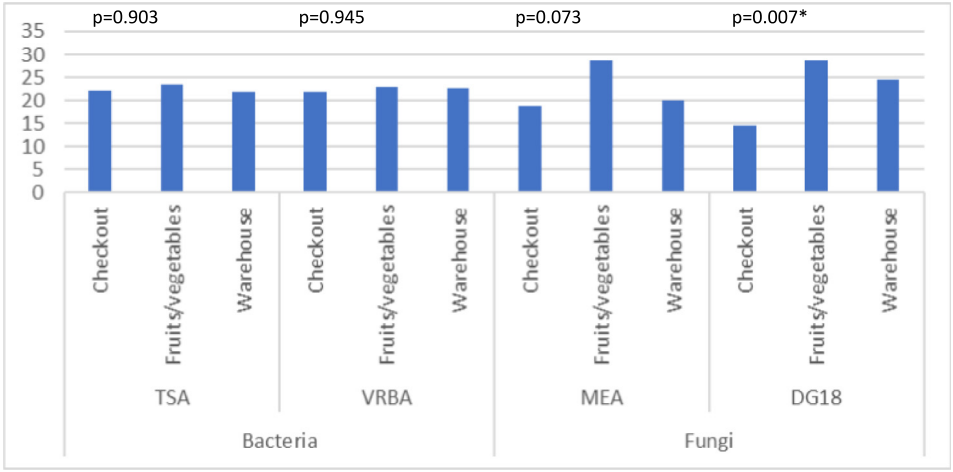


**Fig. 8.** Comparison of microbial contamination on surface swabs between sampling sites. Kruskal-Wallis test results. *Statistically significant differences at 5 % significance level.

**Table 4**
Study of the relationship between bacterial contamination (TSA and VRBA), fungal contamination (MEA and DG18) and fungal resistance profile (SDA, ITZ, VCZ and PSZ). Results of the Spearman correlation coefficient.

| | | | Bacteria | Fungi | | Fungal resistance | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | VRBA | MEA | DG18 | SDA | ITZ | VCZ | PSZ |
| EDC | Bacteria | TSA | −0.045 | 0.408** | 0.444** | 0.418** | 0.208 | 0.394** | 0.452** |
| | | VRBA | | 0.068 | 0.046 | 0.034 | 0.149 | 0.068 | −0.046 |
| | Fungi | MEA | | | 0.845** | 0.886** | 0.713** | 0.812** | 0.696** |
| | | DG18 | | | | 0.791** | 0.635** | 0.782** | 0.651** |
| | Fungal resistance | SDA | | | | | 0.712** | 0.847** | 0.730** |
| | | ITZ | | | | | | 0.810** | 0.609** |
| | | VCZ | | | | | | | 0.723** |
| | | PSZ | | | | | | | |
| SWABS | Bacteria | TSA | 0.053 | 0.207 | 0.088 | | | | |
| | | VRBA | | 0.369* | 0.255 | | | | |
| | Fungi | MEA | | | 0.766** | | | | |

* Statistically significant differences at 5 % significance level.
** Statistically significant differences at 1 % significance level.

contribute to variability of cultured species. For instance, MEA has high sugar amount and water activity, thus potentiating fast growing fungal species, while DG18, that has a limited water availability, allows more varied fungal flora to thrive (Chao et al., 2002; Mensah-Attipoe and Täubel, 2017; Wu et al., 2000). It was already described that fungal species, and in particular from *Aspergillus* genera, are sensitive to both collection approach as well as culture media used (Chao et al., 2002; Wu et al., 2000; Viegas et al., 2021).

*Aspergillus* section *Fumigati* observed in EDC samples from Portuguese GS and observed in supplement media (VCZ) should be highlighted, since in the recently published WHO fungal priority list to guide research, development and public health action (WHO, 2022), this section was listed in the critical priority group due to specific criteria, related with clinical relevance such as antifungal resistance, followed by deaths, evidence-based treatment, access to diagnostics, annual incidence and complications and sequelae (WHO, 2022). Furthermore, also *Fusarium* species, listed by the same WHO list in the high priority group, were observed in all the 3 supplemented media (VCZ, PCZ and ITZ) corroborating the need to intervene in this specific indoor setting. However, the concern regarding the toxigenic potential of specific fungal species and strains was disregarded in the WHO list hamper a more precise intervention in different research fields, such as occupational health, and food safety and security.

In none of the samples where the *A.* section *Nidulantes* was detected by qPCR it was cultured, corroborating the utmost importance of using culture based-methods and molecular approaches side-by-side to obtain a broader and more accurate exposure assessment (Mbareche, n.d.; Viegas et al., 2021, 2022b). However, the other targeted sections were not detected although observed in culture and this can be due to several variables that can impair the molecular tools efficiency, such as material loss during sample processing and assay specific bias (Unterwurzacher et al., 2018). Although more refined tools are being used in microbiologic agent's occupational exposure assessments (Degois et al., 2017, 2021; O'Brien et al., 2016; Pan et al., 2021), they are not able to assess microbial viability in single measurements (Cangelosi and Meschke, 2014), which is critical to ponder the potential health effects on workers (Madsen et al., 2020). However, we should be aware that only a small fraction of species can be cultured, thus underestimating the microbial diversity (Degois et al., 2021; Mensah-Attipoe and Täubel, 2017; Montalvo et al., 2014; Tomic-Canic et al., 2014; Viegas et al., 2021). Because of all these limitations the comprehensive characterization of fungal contamination that might impact human health remains incomplete.

Interestingly, not only higher fungal contamination was detected in Portuguese GS but more resistant taxa were detected in comparison to Spanish GS. In fact, fungal growth in azole-supplemented media was higher in Portugal, with significant differences among sampling sites, as follows: for VCZ and PCZ in the checkout; for all azoles in the fruits/vegetables area; and for PCZ in the warehouse (Mann-Whitney test). Considering all sampling sites in both countries, fungal growth was significantly higher in the fruits/vegetables for ITZ (Kruskal-Wallis test). This finding is of interest and requires further investigation, namely, considering the fruits/vegetables area present at each grocery store and differences among countries. The rising development of fungal resistance against azoles is due to the use of azole fungicides in agriculture to protect a wide range of crops, including cereals and fruits, from the devastating effects of fungal plant pathogens, which are costly to the agricultural economy and threaten food security. Therefore, it is important to understand how these fungicides are used and how and where resistance arises in order to fully address this issue. The use of fungicides is also expected to increase due to the climate change scenario (EFSA, 2020; Viegas, 2021). Indeed, climate change may have a significant impact on food safety at various levels (Tirado et al., 2010), particularly in Portugal and Spain which are highly susceptible to it due to their geographical location at the extreme southwest of Europe (Campos et al., 2017). In fact, besides chemical risks increased exposure, microbiological risks are also predicted to influence food and feed safety due to climate change (Misiou and Koutsoumanis, 2022). Especially mycotoxins contamination in food and feed is of great concern (Duchenne-Moutien and Neetoo, 2021; Stroka and Gonçalves, 2019). In Portugal, different studies have already reported mycotoxins contamination in different occupational and indoor settings related to food and feed production chain and intensive animal production such as swine and poultry (Viegas et al., 2019, 2020b) and in specific food commodities sold in the GS, such as coffee and tea (Assunção et al., 2021; Viegas et al., 2022b, 2022c). Mycotoxins analyses showed that Portuguese GS seem to present some contamination when comparing with Spanish GS. Fumonisin B2, produced by *Fusarium moniliforme*, was the only detected mycotoxin. Out of the six known fumonisins, only fumonisins B1, B2 and B3 (FB1, FB2, FB3) are produced in substantial amounts by cultured *Fusarium moniliforme* and are responsible for many toxic effects such as acute renal and liver toxins in rats, and tumor promoting activity (Mohan et al., 2022; Norred et al., 1996; Shier et al., 1991; Yu et al., 2020). FB1 and FB2 have been classified by the International Agency for Research on Cancer as class 2B carcinogens, indicating potential toxicity to humans (IARC, 2022).

To evaluate EDC's cytotoxicity, the MTT assay was used. The incubation time to determine the impact of contaminants varies from 24 to 72 h. Considering the method and sampling sites, we presumed samples to have low toxicity level. As such, the incubation time of 48 h was chosen. As expected, cytotoxicity was observed in only 14.8 % EDC, ranging from low to moderate cytotoxicity in both cell lines. The observed cytotoxicity might be related to EDC's contamination with microbial (bacteria, fungi, mycotoxins), chemicals or particles (not assessed). For instance, *Fusarium* sp. produces specific mycotoxins (fumonisins) that induce cellular toxicity via mitochondrial stress and mitophagy (Mohan et al., 2022). Epidemiological data has also suggested an association between ingestion of corn contaminated with *F. verticillioides* and human esophageal cancer (Sydenham et al., 1991). Traditionally, chemical pollutants are more emphasized in in vitro toxicology, with microbial contaminants being assessed in a more

exploratory fashion (Aslantürk, 2018; Ochiai et al., 2020). We propose an incremental use of in vitro toxicology tools, using relevant cell lines, for a better assessment of biological effects and risk characterization in different environments. Further research on the relation between azole resistance and mycotoxigenic fungi in food commodities is required for broader conclusions regarding food safety and occupational and public health risks.

## 5. Conclusions

Overall, the sampling approach applied was useful to screen the most critical workstations in what concerns microbial contamination and occupational exposure in this setting. It was possible to observe that GS indoors is a rich environmental habitat that may host diverse microbial communities, some of which have a direct relevance to human health, as several opportunistic pathogens were detected. Indeed, the approach used allowed to identify the most contaminated area in GS, which was confirmed to be the same for both studied countries — the fruits/vegetables area. The presence of *Aspergillus* section *Fumigati* and *Fusarium* species in samples from Portuguese GS with reduced susceptibilities to azoles commonly used in the clinical treatment of fungal infections raises concerns regarding human health and food safety and must be surveilled. Furthermore, we have detected Fumonisin B2 in Portuguese GS, possible unveiling this emergent threat concerning occupational exposure and food safety.

Considering the multiple risk factors present and associated with microbiological contamination, the One Health approach will be the best approach when defining preventive measures that could tackle most of these risk factors. In fact, the findings obtained support the need to reinforce and improve the cleaning procedures (e.g. frequency) and increase the ventilation resources, particularly in the fruits/vegetables area.

## CRediT authorship contribution statement

Carla Viegas: Conceptualization; Funding acquisition; Methodology; Supervision; Validation; Roles/Writing - original draft; Writing - review & editing.

Bianca Gomes, Renata Cervantes, Sílvia Moreira, Marta Dias, Pedro Pena, Elisabete Carolino, Magdalena Twarużek, Robert Kosicki, Ewelina Soszczyńska: Formal analyses; Methodology.

Liliana Caetano, Lídia Cañas, Sofya Pozdniakova, Sílvia Borràs, Susana Viegas: Roles/Writing - original draft; Writing - review & editing.

## Data availability

Data will be made available on request.

## Declaration of competing interest

None. Authors have full control of all primary data and permission is given to the journal to review the data if requested.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2023.162602.

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