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Poultry farms as reservoirs of azole-resistant fungi: Occupational health risks in agricultural facilities

Bianca Gomes [a,b,*], Marta Dias [b,c], Renata Cervantes [b,c], Pedro Pena [b,c], Elisabete Carolino [a], Liliana Aranha Caetano [b,d], Susana Viegas [c], Carla Viegas [b,c]

[a] CE3C—Center for Ecology, Evolution and Environmental Change, Faculdade de Ciências, Universidade de Lisboa, 1749–016 Lisbon, Portugal
[b] H&TRC – Health & Technology Research Center, ESSL– Escola Superior de Saúde de Lisboa, Instituto Politécnico de Lisboa, Portugal
[c] NOVA National School of Public Health, Public Health Research Centre, Comprehensive Health Research Center, CHRC, REAL, CCAL, NOVA University Lisbon, Lisbon, Portugal
[d] Research Institute for Medicines (iMed.uLisboa), Faculty of Pharmacy, University of Lisbon, 1649–003 Lisbon, Portugal

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ABSTRACT

The intensification of poultry farming has raises concerns regarding microbial contamination and antifungal resistance, particularly involving *Aspergillus fumigatus*, a key pathogen presenting clinical relevance. This study aims to present the first in-depth evaluation of environmental conditions, airborne particles, microbial contamination, and fungal resistance to azole drugs in poultry farm environments. A multi-approach sampling strategy (passive and active sampling methods) was conducted throughout the poultry production cycle. Microbial characterization was performed, combining culture-dependent methods and molecular techniques with a focus on fungal diversity and azole resistance. Bacteria and fungal loads indoors exceed the outdoor levels in 80.85 % ($n = 38/47$) and 78.72 % ($n = 37/47$) of air samples, respectively. Toxigenic fungal species (*Aspergillus* spp. and *Penicilium* spp.) were widespread (air, swabs, electrostatic dust cloths,feed, bed). Fungi resistant to at least one antifungal (itraconazole, voriconazole, posaconazole) was found in 53.3 % of the total samples collected inside poultry pavilions ($n = 45/126$), including potentially azole-resistant *Aspergillus* species. Particulate matter acts as a carrier of microorganisms, enhancing workers respiratory exposure risks. Electrostatic dust cloths proved to be a valuable sampling method for exposure assessment to potential pathogenic and resistant fungi. Our findings identify poultry farms as potential hotspots for toxigenic and azole-resistant fungi with implications for occupational health. These results highlight the urgent need for targeted biosafety strategies to mitigate not only microbial contamination on the workplace, but also the spread of antifungal-resistant fungi in poultry facilities and surroundings

1. Introduction

While poultry production is one of the most resource-efficient livestock systems for meat production, it still poses notable concerns for microbial exposure and occupational health [1].

In poultry facilities, pathogens can spread through various pathways (e.g. airborne dust, feed, poultry litter) [1]. In this matter, poultry dust is a relevant concern as it contains poultry residues, feed and feathers and also provides a suitable habitat for the growth of harmful microorganisms (bacteria, fungi, viruses) that produce toxins and allergens (endotoxins, mycotoxins, and (1–3)- β-D-glucan) [2–5].

Dust includes particulate matter (PM), which is classified into various fractions. Particles of particular concern for human health include PM_{10} (aerodynamic diameter ≤ 10 μm) and $PM_{2.5}$ (aerodynamic diameter ≤ 2.5 μm). PM_{10} refers to thoracic particles that can reach the respiratory tract (e.g. tracheobronchial region), while $PM_{2.5}$ comprises fine particulate matter that can penetrate the gas-exchange region [6,7].

Microorganisms can be found in aggregates or on larger dust particles comprising organic dust [3]. In poultry farms, these particles can be released into the air, forming bioaerosols [6], with PM acting as a vector for microbial suspension and dispersion [7,8]. Consequently, PM composition, size distribution, and density have a direct influence on workers health through differential aerosolization and bioaerosol

* Corresponding author: ESTeSL – Escola Superior de Tecnologia da Saúde de 15 Lisboa, Av. D. João II, Lote 4.69.01, 1990–096 Lisbon, Portugal.
E-mail address: bianca.gomes@estesl.ipl.pt (B. Gomes).

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contamination [5].

For poultry workers, the main health risk is most likely posed by exposure to bioaerosols [9]. Prolonged exposure to highly contaminated biological environments further exacerbates respiratory risks [4,9].

Currently, the main European legislation for workers' protection against biological agents is the Directive 2000/54/EC [10], based on what is recognized as occupational hazards [9]. Portugal has implemented these regulations by Decree-Law No. 84/97, which covers hazardous microorganisms and containment protocols [11]. Still, a critical regulatory gap persists, as there are no European standards or legal limits for acceptable concentrations of microorganisms in indoor environments [8,12]. Some scientific proposed limits are often used as guidelines, such as the Indoor/Outdoor (I/O) ratio, which compares fungal concentrations in indoor and outdoor environments. Where an I/O ratio exceeding 1 ($I/O > 1$) suggests the presence of an indoor fungal source [8,13]

Poultry production environments have been considered reservoirs for antibiotic-resistant bacteria [14,15]. While fungal studies remain limited, species from *Aspergillus* genus, belonging to WHO critical priority group [16], were already recovered from these environments [17–19]. Moreover, clinically relevant toxigenic fungal species, not considered in the WHO pathogen priority list, have been identified [1,20].

Regarding poultry facilities, microbial composition, concentration and viability is influenced by several factors and the diversity of literature results can be justified by the sampling methods performed, building features, microclimate conditions (e.g. temperature, humidity) and the outdoor environment [8,20].

This study aimed to fill the research gap regarding microbial contamination in poultry farms by using a multi-approach sampling strategy (active and passive sampling methods) and applying culture-based methods and molecular tools. As the first integrative study, it also considers environmental parameters and particulate matter monitoring and describes azole susceptibility patterns throughout the poultry production cycle.

2. Material and methods

2.1. Poultry farms characterization

This study was carried out on poultry farms ($n = 5$) located in Madeira Island, Portugal. All farms were dedicated to broiler production. Briefly, day-old chicks are transferred from hatcheries to the poultry pavilions (PP), where wood shavings were used as bedding material and maintained throughout the growth cycle (approximately 35 days). During that time 3 types of feed are used, starting feed in granulated powder (F1), and pellets used during 2nd week (F2) and 3rd week (F3). At the end of the cycle, the manure is loaded onto trucks to be transported to the fields where it is used as fertilizer. The PP are cleaned and disinfected and must remain empty for a minimum of 10 days (sanitary control) (DGAV, 2021). Poultry workers regularly monitor bird health, modify feed and water equipment, and administer vaccines. Among the requirements for uniforms and personal protective equipment (PPE) contained in the Biosafety Manual for Poultry Farms [21], limited PPE was used, mainly boots.

Initially, a walkthrough survey was conducted at each poultry farm to collect information regarding PP features, cleaning practices, workers' routine, and other factors that might impact microbial exposure (Table S1- Supplementary material, similarly to the checklist used by C.Viegas and team members [22]. In general, two workers operated per farm, spending 6 h/day inside facilities. In most PP, only natural ventilation was used during sampling. During sanitary control, a bactericidal, fungicidal, virucidal and sporicidal solution is applied to surfaces and water tanks. The cleaning frequency is approximately every 40 days.

2.2. Sampling approach and strategy

The sampling campaign occurred in 14 PP, where a multi-approach sampling protocol comprising active sampling methods (MAS-100) and passive sampling methods (electrostatic dust collectors (EDC), surface swabs, and material collection (feed and animal bedding) was conducted. Similarly to previously published methodologies [8,22]. For microbial characterization, a total of 260 samples (47 air samples, 47 EDC, 47 feed, 47 bed, 47 swabs) were collected indoors PP. Outdoor air samples were also collected as reference ($n = 20$) [8,19]. These samples were obtained during 4 stages of the production cycle as follows: stage 0 - sanitary control period (clean and disinfected empty pavilion) and during poultry growth cycle - stages 1,2,3 (1st, 2nd, 3rd weeks respectively). The number of samples per stage is provided in Table S2 – Supplementary material. For environmental parameters evaluation, PM levels ($PM_{0.3}$, $PM_{0.5}$, PM_1, $PM_{2.5}$, PM_5, and PM_{10}) temperature (T°C) and relative humidity (HR%) were monitored. Measurements, 1 per PP, were taken 1.5 m above ground level to simulate the human breathing zone [9]. A total of 47 indoor and 20 outdoor measurements were collected from all PP. Following sampling, samples were kept refrigerated (0-4 °C) in sterilized bags and transported to the lab for various analyses [23].

A schematic representation of the measurements, samples collected, and laboratory analyses carried out is shown in Fig. 1.

2.2.1. Sampling of culturable microorganisms

Indoor air samples were collected at a central location of each PP. Outdoor samples were also collected in the vicinity of PP (1 m) as this location is designated as reference site [19,24,25]. Samples were collected in each stage of the production cycle at a height of 1.5 m [9,14,26]. To reduce CFU over-load caused by high sample flow rate, the impactor MAS-100 was set at 100 L/min, for 5 min [27]. Air volume ($v = 500$ L) was selected based on the expected contamination [28]. Polycarbonate filters (0.2 µm pore; 47 mm; Merck Millipore Ltd., Cork, Ireland) were applied to the plates to capture bioaerosols [29] and 150 µL of sterile glycerol 10 % [*v*/v] was added to minimize particle bounce [30].

Regarding passive sampling methods, a sterilized EDC was placed in a previously sanitized horizontal hanging plate in the centre of PP, 1.5 m above the ground. The EDC was replaced weekly [31]. Surface swabs were performed on PP walls by swabbing one 10x10cm area per week and by using a square stencil, disinfected between each sampling with a 70 % alcohol solution [19]. A composite sample (weight = 10 g) of animal bedding was collected from 5 random locations in each PP [32]. Also, a composite sample of feed (weight = 10 g) was collected from 5 random feeders inside PP (F1;F2;F3) during the 1st; 2nd and 3rd week, respectively [33]. Three types of feed were used during the poultry growth cycle. Sealed feed samples, provided directly from the feed industry (weight = 10 g), were used as control samples (CF1;CF2;CF3).

Microbiota characterization

2.2.2. Culture dependent methodologies

For microbial evaluation, polycarbonate filters (PC) were washed in 5.0 mL of 0.1 % Tween 80 saline (0.9 % NaCl) by orbital shaking (500 rpm, for 15 min)[34]. A similar procedure was conducted in swabs and EDC, where samples were washed with 1 mL and 20 mL, respectively, on the orbital shaker (250 rpm, 30 min) [22]. Also, 10 g of feed and bed samples were diluted in 90 mL 0.1 % Tween 80 saline (0.9 % NaCl) as reported before [33,35].

Following extraction, 150 uL aliquots from the suspensions were spread on three different culture media. Dichloran-glycerol agar (DG18) was used for fungi, while Tryptic soy agar (TSA) supplemented with nystatin (0.2 %) and MacConkey agar (MAC) were used to cultivate total bacteria and gram-negative bacteria, respectively. After inoculation, DG18 plates were incubated at 27 °C, 5–7 days for fungi; TSA, 30 °C, 7 days for mesophilic bacteria, and MAC, 37 °C, 7 days for gram-

negative bacteria.

Air, EDC, feed and bed matrices were tested for microorganisms' susceptibility to azole drugs, by inoculating 150 uL of the obtained extracts onto sabouraud dextrose agar (SDA) enriched with 4 mg/L itraconazole (ITR), 2 mg/L voriconazole (VOR), or 0.5 mg/L posaconazole (POS) and incubated for 48 h at 27 °C. This procedure was adapted from the EUCAST guidelines [36] and reported elsewhere [22].

Microbial densities, colony-forming units (CFU), were calculated for air (CFU/m^3), surface swabs (CFU/m^2), EDC (CFU/m^2/day), feed and bed (CFU/g). Fungal identification was performed through macro and microscopic characteristics [37].

2.2.3. Molecular detection

Molecular detection of *Aspergillus* sections *Fumigati, Nidulantes* and *Circumdati* was carried on MAS-100 filters, EDC, swabs, feed and bed samples extracts. Firstly, DNA was extracted from the washed samples (5 mL) according to a pre-established methodology [19,22,38]. The ZR Fungal/Bacterial DNA MiniPrep Kit (Zymo Research, Irvine, USA) was used for DNA extraction and molecular detection was performed by real-time PCR (qPCR) on the Bio-Rad CFX-Connect PCR System. A total amount of 20 µl was used for the reactions, which contained 1× iQ Supermix (Bio-Rad, Portugal), 0.5 µM of each primer, and 0.375 µM of TaqMan probe (Table.S3- Supplementary material). A three-step PCR was used for amplification, consisting of 40 cycles of denaturation at 95 °C for 30 s, annealing at 52 °C for 30 s, and extension at 72 °C for 30 s each as previously performed [2,39,40].

Controls included non-template and positive control consisting of DNA extracted from reference specimens. The reference specimens were obtained from the culture collection of the Reference Unit for Parasitic and Fungal Infections, Department of Infectious Diseases of the National Institute of Health, Dr. Ricardo Jorge and from the fungal collection of the University of Minho, Braga, Portugal. These strains were sequenced for ITS, B-tubulin, and Calmodulin.

2.3. Particle matter measurements

Lighthouse Handheld Particle Counter HH3016-IAQ was used to assess particle matter, temperature and humidity. Particle concentrations were measured at six distinct sizes ($PM_{0.3}$, $PM_{0.5}$, PM_1; $PM_{2.5}$, PM_5, and PM_{10}) at a flow rate of 2.83 L/min during 5 min, as performed in occupational health studies [2,5]. Differentiation between size fractions was performed due to their importance in health studies, to estimate dust penetration within the respiratory system and, as a result, their potential health effect [8,25].

2.4. Statistical analysis

Data were analysed using RStudio 2023 [41] statistical software for Windows, version 12.1, and corrplot package [42]. Results were considered significant at the 5 % significance level. To test the normality of the data, the Shapiro-Wilk test was used. For qualitative data, frequency analysis (n, %) was conducted, and for quantitative data, minimum, maximum, mean, and standard deviation were used if normality was verified; otherwise, median and interquartile range were used. To compare fungal and bacterial contamination and the concentration of particulate matter between stages, the ANOVA or Kruskal-Wallis test was used, depending on whether the assumption of normality has been verified. To study the relationship between fungal and bacterial contamination and the concentration of particulate matter, Spearman's correlation coefficient was used, since the assumption of normality was not verified. To assess species diversity, Simpson and Shannon indices, given by eq. (1) Shannon Index $(H) = -\sum_{i=1}^{s} p_i \ln(p_i)$ and eq. (2) Simpson Index $(D) = \frac{1}{\sum_{i=1}^{s} p_i^2}$, were used, where pi is the proportion (ni/n) of individuals of one particular species found (ni) divided by the total number of individuals found (n).

3. Results

3.1. Microbial load indoor and outdoor

Total bacteria median values indoors (TSA culture medium) increased throughout the poultry growth cycle as follows: 1.80×10^1 CFU/m^3 sanitary control; 6.43×10^2 CFU/m^3 1st week; 1.03×10^3 CFU/m^3 2nd week; 2.63×10^3 CFU/m^3 3rd week). In the outdoors the highest median value was obtained during the 3rd week, as follows: 2.00×10^2 CFU/m^3 sanitary control; 4.00×10^2 CFU/m^3 1st week; 2.00×10^2 CFU/m^3 2nd week; 1.00×10^3 CFU/m^3 3rd week (Fig. 2a).

Regarding fungi, median values indoors increased after poultry introduction (2.40×10^1 CFU/m^3 sanitary control, 5.90×10^1 CFU/m^3 1st week). Lower median values were obtained in the following weeks (4.90×10^1 CFU/m^3 2nd week; 1.90×10^1 CFU/m^3 3rd week). This was also observed in the outdoors, where fungal median values were higher on the 1st week when considering poultry growth cycle (2 CFU/m^3 sanitary control; 8 CFU/m^3 1st week; 6 CFU/m^3 2nd week; 4 CFU/m^3 3rd week) (Fig. 2b).

3.2. Emission sources based on indoor/outdoor ratio

Considering the ratio indoor/outdoor from all air samples collected inside PP ($n = 47$) fungi and total bacteria loads indoors exceed the outdoors in 80.85 % ($n = 38$) and 78.72 % ($n = 37$) of samples, respectively. This indicates that increased concentrations indoors are caused by indoor contamination sources.

3.3. Microbial quantification: Sanitary control and growth stages

3.3.1. Bacteria

Bacteria contamination varied significantly across poultry growth stages, depending on the matrix. Air samples evidence a significant progressive increase (MAS-100) $(\chi^2_{K-W}(3) = 17.223, p = 0.001)$, with bacteria median values rising from 1.80×10^1 CFU/m^3 during sanitary control to 2.63×10^3 CFU/m^3 in the 3rd week. EDC evidence a significant variation $(\chi^2_{K-W}(3) = 11.205, p = 0.011)$, with a progressive increase from the sanitary control (2.05×10^2 CFU/m^2/day) to the 2nd week, where the highest value was obtained (8.18×10^2 CFU/m^2/day). Significant variations were observed in bed $(\chi^2_{K-W}(3) = 25.319, p = 0.000)$. Surprisingly, the highest median value was obtained during the sanitary control (1.17×10^4 CFU/g), following by a gradual increase till the 3rd week (6.98×10^3 CFU/g).Bacteria contamination in feed samples collected directly from the feeders also evidence significant variations $(\chi^2_{K-W}(3) = 10.496, p = 0.005)$ and the highest median values were obtained in the feed collected during the 1st and 3rd weeks (1.78×10^3 CFU/g and 1.71×10^3 CFU/g respectively). Similarly, higher bacteria contamination was obtained in control feed samples (CF1/CF2/CF3), obtained directly from the manufacturer, in 1st week feed (1.78×10^3 CFU/g) and 3rd week feed (1.71×10^3 CFU/g) even though no significant differences were found. In swabs, no significant differences were found between stages. However, bacteria median value was highest during sanitary control (8.00×10^5 CFU/g) evidencing persistent surface colonization (Table 1).

Regarding gram-negative bacteria contamination, among all matrices, EDC, bed and feed exhibit statistically significant differences between the stages $(\text{EDC}: \chi^2_{K-W}(3) = 15.208, p = 0.002; \text{Bed}: \chi^2_{K-W}(3) = 24.580, p = 0.000; \text{feed}\ (3) = 13.881, p = 0.003$ respectively). Gram-negative bacteria values increase weekly, and the highest median value was found during the 2nd week in EDC (4.08×10^3 CFU/m^2/day), while on bed was on the 3rd week (8.09×10^3 CFU/g). In contrast to the feed samples collected, where the highest median contamination value was found during the 1st week (2.37×10^3 CFU/g).

In air samples, gram-negative bacteria contamination remained consistently low throughout the stages (1–3) and the highest median

value was obtained during the 2nd week (4 CFU/m^3). This was also observed in swabs, where no significant variation of gram-negative bacteria contamination was found. Still, the highest median value was observed during the 3rd week (1.05×10^4 CFU/m^2). In control feed samples, the highest median value was observed in 3rd week feed (CF3: 1.37×10^3 CFU/g) (Table 1). (See Figs. 1–4.)

3.3.2. Fungi

Concerning fungal contamination, only significant statistically differences were detected between stages in feed samples collected directly from the feeders ($\chi^2_{K-W}(3) = 10.344, p = 0.016$), and the highest median values was found during the 2nd week (1.50×10^2 CFU/g^1). Fungal contamination in the other matrices (Air,EDC,bed,swabs,control feed) remained relatively constant over time. Yet, in air samples the highest median values occurred during the 1st week (5.90×10^1 CFU/m^3) followed by a gradual decline. In EDC and swabs fungal contamination was highest during the 2nd week (EDC: 2.24×10^2 CFU/m^2/day; swabs: 1.15×10^2 CFU/m^2). In bed samples, fungal median values increased progressively through stages (0–3), and the highest median value was obtained in the 3rd week (2.80×10^2 CFU/g). In control feed samples (CF1/CF2/CF3), fungal contamination was highest on 1st week feed (2.00×10^1 CFU/g) (Table 1).

3.3.3. Fungal biodiversity and prevalent species

The Shannon (H) and Simpson (D) indices are standard metrics to characterize both the richness and evenness of microbial communities [43]. Notably, EDCs evidence the highest fungal diversity and values increase weekly (sanitary control: H = 1.63;D = 4.25; 1st week: H = 1.62;D = 3.76; 2nd week: H = 1.94;D = 6.13; 3rd week: H = 1.99; D = 5.85).Overall, fungal communities were richer (H) and taxonomically diverse (D) in all matrices during the 3rd week (Table.S4- Supplementary material).

Fungal species varied across matrices and *Penicillium* spp. and *Aspergillus* spp. were widespread (Fig. 3). *Penicillum* spp. was dominant in air (76.74 %), bed (85.10 %) and feed samples (69.15 %), while *Aspergillus* spp. was prevalent on EDC (62.01 %). Swabs evidence a different composition, with *Cladosporium* spp. being the most frequent (44.86 %). In feed samples, *Paecilo*myces spp. contributed notably to the composition (14.10 %) (Fig. 3).

3.4. Fungal susceptibility to azoles

The results of the antifungal susceptibility are depicted in Table 2. Of note, high prevalence of *Penicillium* spp. with reduced susceptibility to VOR in air, EDC and bed, and to POS in air samples. *Aspergillus* spp. reduced susceptibility to ITR was observed in EDC. *Mucor* spp. reduced susceptibility to ITR was observed in air, feed, and bed. Similar results were observed in POS in EDC. *Paecilomyces* spp. and *Rhizopus* spp., included in 'other species' due to their low prevalence, evidence reduced susceptibility to VOR and POS in EDC. *P*aecilomyces spp. reduced susceptibility to VOR was observed in feed samples. (See Table 3.)

Considering the environmental matrices, EDC exhibited the highest number of samples where fungal resistance to at least one antifungal agent was observed (78.7 %, n = 37/47) (Fig. 4a).

Regarding the production cycle, fungi resistant to at least one antifungal was found in 53.3 % of sample ($n = 8/15$) during sanitary control and in 35.7 %, ($n = 45/126$) throughout poultry growth cycle (Fig. 4b). No statistically significant differences were detected between the stages (p's > 0.05), which was expected, considering the reduced number identified in the different culture media (SDA, ITR, VOR, POS).

3.5. Detection of Aspergillus sections

Regarding *Aspergillus* sections (*Fumigati*, *Nidulantes* and *Circumdati*): *Fumigati was* recurrent in EDC (53.19 %, 25 out of 47), feed (29.63 %,16 out of 54) and bed (23.40 %, 11 out of 47) matrices. Lowest values were obtained in swabs (6.38 %, 3 out of 47). *Nidulantes* was frequent in EDC (42.55 %, 20 out of 47), swabs (25.53 %, 12 out of 47), bed (12.77 %, 6 out of 47) and feed (17.02 %, 8 out of 54) matrices. *Circumdati* was not detected in any of the environmental samples (Table S5- supplementary material). Molecular detection enabled the identification of *A*. section *Fumigati* in 28 % of samples ($n = 67/242$), and *A*. section *Nidulantes* in 14 % of samples ($n = 29/242$) that had not been identified using culture based- methods.

3.6. Particulate matter assessment

In terms of mass concentration, particles with bigger sizes were prevalent throughout the poultry growth cycle. Regarding PM concentrations indoors, significant variations were observed for particle sizes: PM_1 ($p = 0.005$), $PM_{2.5}$ ($p = 0.012$), PM_5 ($p = 0.007$), and PM_{10} (p = 0.012). In general, the highest median values were obtained in the

Table 1
Comparison of median microbial values in air (CFU/m^3), bed/feed (CFU/g), EDC (CFU/m^2/day), swabs (CFU/m^2) during poultry growth cycle. Kruskal-Wallis test results.[a, *]

Microorganism	Matrice	Poultry growth cycle				
		Sanitary control	1st week	2nd week	3rd week	*p*-value
Bacteria	AIR	1.80×10^1	6.43×10^2	1.03×10^3	2.63×10^3	0.001*
	EDC	2.05×10^2	4.89×10^2	8.18×10^2	7.31×10^2	0.011*
	BED	1.17×10^4	3.87×10^3	5.80×10^3	6.98×10^3	0.000*
	SWAB	8.00×10^5	4.17×10^5	6.45×10^5	2.82×10^5	0.090
	FEED	–	1.78×10^3	9.50×10^2	1.71×10^3	0.030*
	CF1/CF2/CF3[a]	–	1.78×10^3	1.70×10^2	2.41×10^3	0.108
Gram-negative bacteria	AIR	<1	2.00	5.00	4.00	0.898
	EDC	7.58×10^1	8.64×10^2	4.08×10^3	3.29×10^3	0.002*
	BED	3.52×10^3	4.17×10^3	6.23×10^3	8.41×10^3	0.000*
	SWAB	<1	1.00×10^3	1.50×10^3	1.05×10^4	0.100
	FEED	–	2.37×10^3	2.17×10^3	2.15×10^3	0.003*
	CF1/CF2/CF3[a]	–	<1	1.70×10^1	1.37×10^3	0.434
Fungi	AIR	2.40×10^1	5.90×10^1	4.90×10^1	1.90×10^1	0.119
	EDC	3.79×10^1	1.88×10^2	2.24×10^2	2.08×10^2	0.330
	BED	7.00×10^1	1.95×10^2	1.75×10^2	2.80×10^2	0.921
	SWAB	7.00×10^4	5.50×10^4	1.15×10^5	9.50×10^4	0.745
	FEED	–	8.00×10^1	1.50×10^2	9.00×10^1	0.016*
	CF1/CF2/CF3[a]	–	2.00×10^1	<1	1.50×10^1	0.533

[a] Control feed samples used during 1st week (CF1); 2nd week (CF2); 3rd week (CF3).
* Statistically significant differences at 5 % significance level.

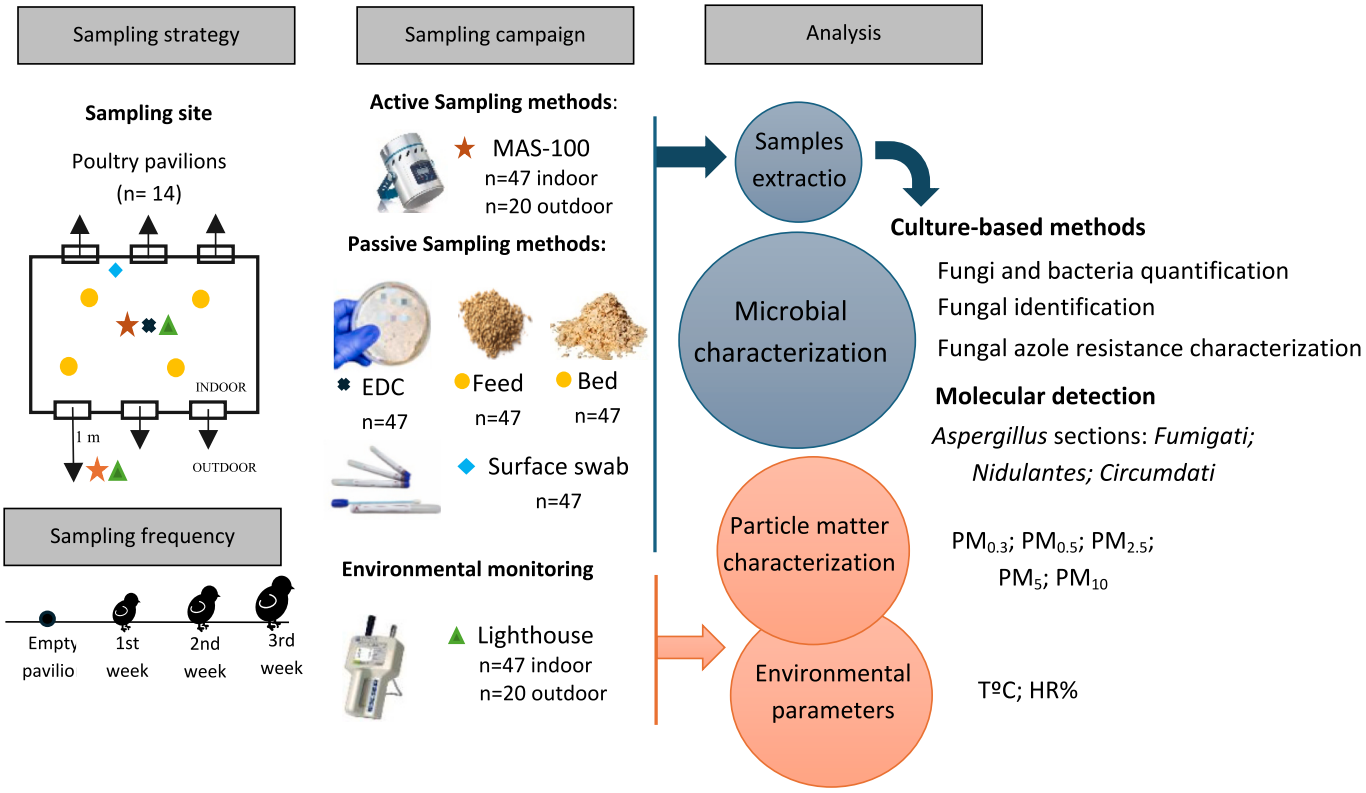

Fig. 1. Schematic representation of the measures and samples collected in poultry farms and subsequent analysis.

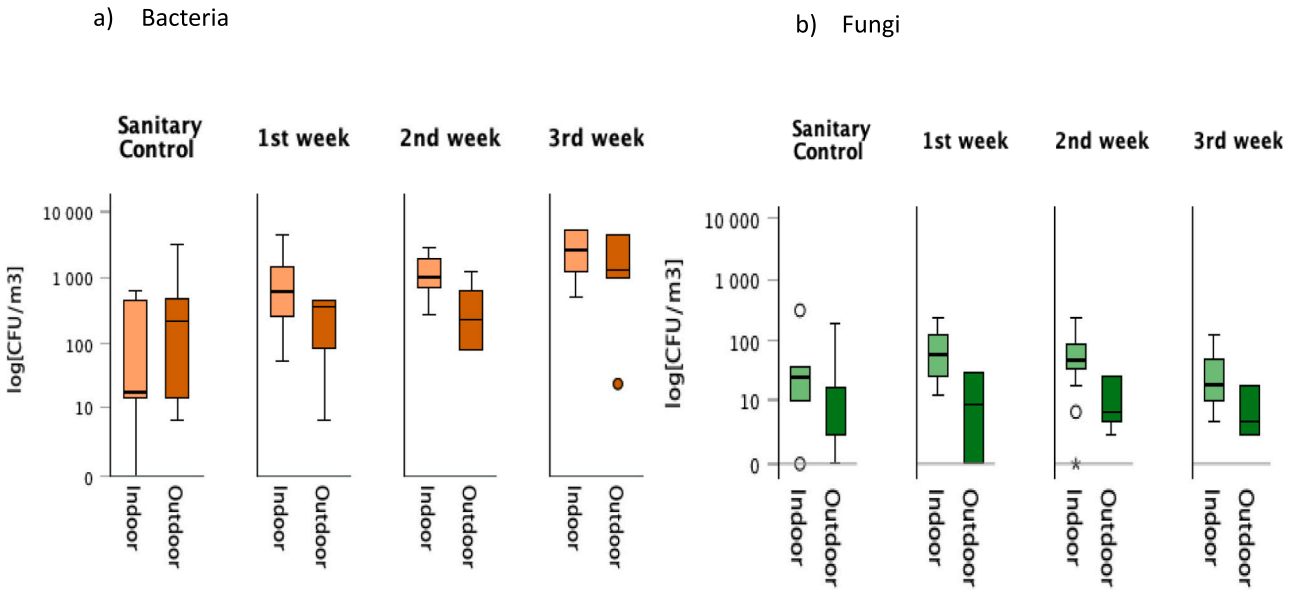

Fig. 2. Box-plots of: a) bacteria median values (CFU/m^3) b) fungi median values (CFU/m^3) indoor and outdoor pavilions through poultry growth cycle (Sanitary control; 1st week; 2nd week; 3rd week). Error bars represent the variability among replicates. Wide error bars indicate higher variability, whereas the absence of error bars indicates negligible variability.

3rd week, particularly for $PM_{2.5}$ (2.95×10^2 µgr/m^3), PM_5 ($6.61x10^2$µgr/m^3) and PM_{10} (2.09×10^3 µgr/m^3) (Table 3). Whereas in the outdoor, significant temporal variations were also observed across particle fractions: $PM_{0.5}$ ($p = 0.000$), PM_1 ($p = 0.002$), $PM_{2.5}$ ($p = 0.000$), PM_5 ($p = 0.000$) and PM_{10} ($p = 0.003$). Notably, the highest median concentration outdoors were recorded during the 1st week for most particle sizes ($PM_{0.5}$: 3.10×10^2 µgr/m^3; $PM_{2.5}$: 1.80×10^2 µgr/m^3, PM_5: 1.80×10^2 µgr/m^3; PM_{10}: 9.23×10^1 µgr/m^3). Considering environmental parameters, temperature varied significantly indoors ($p = 0.000$) and outdoors ($p = 0.012$) and the highest median value was obtained during the 1st week (indoor: 25.3 °C; outdoor:15.9 °C). Relative humidity remained consistently high throughout the poultry growth cycle (>85 % indoors and outdoor).

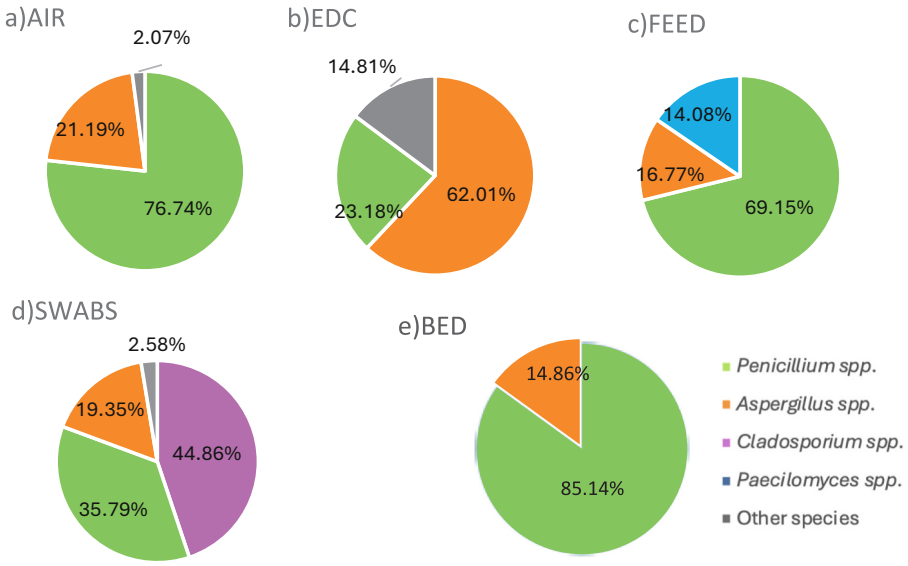

Fig. 3. Relative abundance (%) of fungal species in environmental samples.

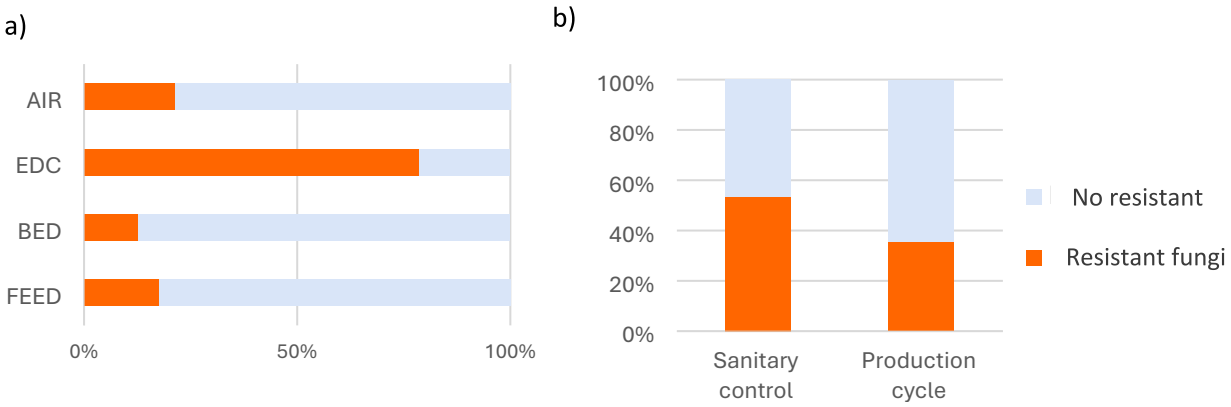

Fig. 4. Fungal resistance to at least on antifungal (itraconazole,voriconazole, posaconazole) among a) matrices (air; electrostatic dust cloths (EDC), bed, feed); b) production cycle (sanitary control(0); poultry growth cycle (1st, 2nd, 3rd weeks).

Table 2
Prevalence (%) of antifungal susceptibility on sabouraud dextrose agar (SDA) azole-supplemented media (Itraconazole (ITR); Voriconazole (VOR); posaconazole (POS)).

Matrice	Fungi	% Prevalence			
		SDA	ITR	VOR	POS
AIR	*Penicillium* spp.	99.01	0.00	97.09	100.00
	Mucor spp.	0.99	100.00	2.91	0.00
EDC	*Aspergillus* spp.	68.08	90.29	1.62	13.44
	Penicillium spp.	7.93	2.58	83.96	14.67
	Mucor spp.	20.10	1.97	8.83	49.19
	Other species	3.89	5.16	5.60	22.70
FEED	*Paecilomyces* spp.	47.70	0.00	95.40	0.00
	Penicillium spp.	43.39	0.00	0.00	0.00
	Mucor spp.	7.76	100.00	4.60	0.00
	Aspergillus spp.	0.86	0.00	0.00	0.00
	Other species	0.29	0.00	0.00	0.00
BED	*Penicillium* spp.	76.07	0.00	6.67	0.00
	Geotrichum spp.	13.50	0.00	0.00	42.86
	F.verticilloides	10.43	0.00	0.00	0.00
	Mucor	0.00	100.00	93.33	57.14

3.7. Correlation analysis

3.7.1. Indoor-outdoor association in microbial contamination and particle matter fractions

The following correlations were detected between indoor and outdoor: a) Greater fungal contamination indoors relates to greater fungal contamination outdoors ($r_S = 0.585$. $p < 0.001$). Similar results were obtained for airborne bacteria ($r_S = 0.557$. $p < 0.001$) and gram-negative bacteria ($r_S = 0.407$. $p = 0.005$). Regarding PM, higher concentrations of $PM_{0.5}$ outdoors relates to higher concentration of $PM_{0.5}$ ($r_S = 0.528$. $p < 0.001$) and PM_{10} ($r_S = 0.306$. $p = 0.037$) indoors (Fig. S4 Supplementary material).

3.7.2. Influence of environmental parameters on microbial contamination and PM

Several correlations were obtained and are displayed in(Fig. S5-Supplementary material). Of note higher animal density related to: g) higher temperature ($r_S = 0.607$, $p < 0.001$); h) higher bacterial contamination in air ($r_S = 0.327$, $p = 0.025$) and feed ($r_S = 0.543$, $p < 0.001$); i) higher gram-negative bacteria contamination in bed ($r_S = 0.446$, $p = 0.002$); j) lower bacterial contamination in bed ($r_S = -$

Table 3
Median values of particle fractions concentration (μgr/m^3) and environmental parameters: temperature (TºC); relative humidity (HR%), indoor and outdoor poultry pavilions through poultry growth cycle. Kruskal-Wallis test results*

Poultry growth cycle		Sanitary control	1st week	2nd week	3rd week	p-value
		Indoor				
Particle fraction (μgr/m^3)	$PM_{0.3}$	1.30×10^1	1.08	1.07	1.67	0.063
	$PM_{0.5}$	1.34	1.79	1.66	1.85	0.424
	PM_1	5.98	2.73×10^1	2.14×10^1	2.33×10^1	0.005*
	$PM_{2.5}$	2.68×10^1	2.42×10^2	2.30×10^2	2.95×10^2	0.012*
	PM_5	3.63×10^1	5.46×10^2	6.14×10^2	6.61×10^2	0.007*
	PM_{10}	5.55×10^1	1.14×10^3	1.60×10^3	2.09×10^3	0.012*
Environmental parameters	TºC	15.9	25.3	23.8	21.9	0.000*
	HR%	99.0	89.8	93.8	96.9	0.156
		Outdoor				
Particle fraction (μgr/m^3)	$PM_{0.3}$	1.31	1.06	1.20	7.10×10^{-1}	0.050
	$PM_{0.5}$	1.14	3.10×10^2	1.95	9.90×10^{-1}	0.000*
	PM_1	7.80	5.29×10^1	7.83	9.64×10^1	0.002*
	$PM_{2.5}$	3.40×10^1	1.80×10^2	2.57×10^1	5.80×10^1	0.000*
	PM_5	3.40×10^1	1.80×10^2	2.57×10^1	5.80×10^1	0.000*
	PM_{10}	1.91×10^1	9.23×10^1	9.95	5.48×10^1	0.003*
Environmental parameters	TºC	12.8	15.9	12.8	9.6	0.012*
	HR%	99.0	99.0	99.0	99.0	0.171

* Statistically significant differences at 5 % significance level.

0.204, $p = 0.035$); k) higher fungal contamination in EDC ($r_S = 0.466$, $p < 0.001$);

Increased relative humidity related to: l) higher gram-negative bacterial contamination in EDC ($r_S = 0.360$, $p = 0.01$); m) higher $PM_{0.3}$ concentration ($r_S = 0.735$, $p < 0.001$); n) lower fungal contamination in air ($r_S = -0.496$, $p < 0.001$).

Higher temperature related to: o) higher bacteria and gram-negative bacteria contamination in feed ($r_S = 0.598$, $p < 0.001$; $r_S = 0.315$, $p = 0.035$ respectively); p) higher gram-negative contamination in air ($r_S = 0.315$, $p = 0.031$); q) lower gram-negative bacteria contamination in EDC ($r_S = -0.440$, $p = 0.002$); r) lower bacteria contamination in bed ($r_S = -0.409$, $p = 0.004$).

Elevated levels of airborne bacteria related to: s) higher gram-negative bacterial contamination in air ($r_S = 0.294$, $p = 0.045$), bed ($r_S = 0.374$, $p = 0.010$), swabs ($r_S = 0.364$, $p = 0.012$) and feed ($r_S = 0.342$, $p = 0.022$); t)higher concentrations of PM_1($r_S = 0.292$, $p = 0.049$), $PM_{2.5}$ ($r_S = 0.351$, $p = 0.016$), PM_5 ($r_S = 0.292$, $p = 0.049$).

4. Discussion

4.1. Airborne microorganisms and particulate matter dynamics

The proposed indoor/outdoor limit ratio was applied [13], and results indicate higher microbial levels indoors, corroborating the idea that the PP are likely the source of microbial pollutants to the surrounding environment [25,44]. Underscoring the need for research not only on the human health effects but also on environmental consequences [1,45].

Airborne microorganisms are influenced by building features, ventilation systems, and microclimatic conditions (e.g. temperature, humidity and housing density - kg/m^2) [46,47]. Among these factors, the positive association between animal density and airborne bacteria confirms stocking density as a determinant of bacterial proliferation, has evidence before [48]. Also, high temperature, together with sufficient moisture content indoors creates favorable conditions for the growth of specific bacteria [43]. This was observed by the positive association between temperature and airborne gram-negative bacteria. These findings warrant particular attention, as these bacteria are often associated to antimicrobial resistance, underscoring implications for both animal welfare and occupational exposure [49].

Regarding fungi, the feathers of day-old chicks can retain fungal spores from the incubation facilities [34,46]. This may have contributed to the spread of fungi in the initial stage [1].

Bioaerosol monitoring revealed higher bacterial loads when compared to fungal loads indoors, as observed by some studies [43,45,50]. Unlike bacteria, fungal dispersion depends on environmental parameters (TºC;HR%), fungal features, spores production and release [51]. Whereas bacteria are usually aerodynamically smaller. Thus, the period they remain airborne is longer when compared to fungal conidia [52]. Notably, the positive association observed between elevated levels of airborne bacteria and higher concentrations of PM_1, $PM_20_{.5}$, PM_5, supports the hypothesis that PM plays a crucial role in the dissemination of microorganisms [8,46].

Considering animals and workers health, PM results indicate that particulates can reach the respiratory tract (particulates $\leq PM_{10}$) and deepen into the gas exchange zone of the lung (particulates $<PM_5$) [8,53]. This might imply, besides local effects, systematic effects depending of PM composition and what they are carrying [54].

4.2. Microbial contamination sources indoors

When evaluating microbiological contamination indoors, it is essential to recognize that air samples represent snapshots of a highly dynamic environment and variations in microbial contamination are to be expected [35,46,55]. The use of passive sampling enables the characterization of contamination from a longer period, overcoming active sampling methods limitations (related to collection time and microbial dissection) [1,8,22,56]. The benefits of combining both methodologies was already highlighted [8,22,49]. Therefore, to obtain the most accurate exposure scenario a multi-approach sampling campaign was conducted.

Field observations during sanitary control (empty, cleaned and disinfected pavilion), indicate concerning deviations from biosafety protocols, as some poultry farmers reported shortening the minimum sanitary control period from 10 days to just 5 days. These changes likely contributed to the highest microbial contamination obtained in surface swabs during the sanitary control period, suggesting improper cleaning and disinfection [57].

Poultry bedding can be made of several materials, among them, wood shavings are commonly used [58]. Despite its common utilization, wood shavings have been identified as a reservoir of pathogenic microorganisms [8,20]. Also, the use of moist wood shavings by some

farmers creates optimal conditions for microbial proliferation, contributing to the initial contamination indoors PP [5,59].

Regarding feed samples, in general those collected from the feeders exhibited higher contamination compared to those obtained directly from the feed industry (control). This result was expected, as during the production cycle, poultry feces and bedding material may contaminate the feeders, contributing to the observed values [20,60].

4.3. Fungal pathogens and health hazards

From the viewpoint of the health status of both workers and animals, it is important to evaluate the viable microbiota, since viability impacts biological processes (e.g. inflammatory, cytotoxic reactions) [61,62]. This information is retrieved only by culture-based methods, enabling susceptibility testing and improving data findings for more detailed and accurate risk characterization [8,22,59].

In general, more research has focused on bacteria than on fungi and viruses [63]. Thus, mycobiota composition was further characterized. The most abundant genera were *Penicillium* spp. and *Aspergillus* spp., which is consistent with prior research [19,35,46,64,65]. These genera include opportunistic human and animal pathogens [46]. Additionally, they can produce mycotoxins [66], which pose serious health risks, due to their carcinogenic, mutagenic, teratogenic and estrogenic effects on humans and animals [52,67].

Another concern relies on the frequent occurrence of fungi in animal feedstuff [33,67]. Such contamination, which includes fungi and mycotoxins, occurs both during preharvest/postharvest of raw material and processing stages (e.g. storage, transport) [67,68]. Animal feed provides a significant source of mycotoxins. In case of consumption (contaminated feed), mycotoxins can carry-over into animal-derived products (e.g. meat,eggs), ultimately threatening humans health [67,68]. To minimize risks, strict feed quality control, proper storage, and regular mycotoxin monitoring are essential throughout the feed and food supply chain [67,68].

This study prioritized *Aspergillus* spp. due to their significant pathogenic potential, clinical relevance, and documented risk of azole resistance. Consequently, molecular detection was applied specifically to key *Aspergillus* sections [18,69]. Other fungal genera were identified through culture-based methods to characterize overall fungal diversity. Also, the focused approach on *Aspergillus* spp. aligns with WHO recommendations [16], and is justified by its high prevalence in our samples, allowing for targeted analysis of the most critical species.

Molecular detection enabled the identification of *Aspergillus* section *Fumigati* species across all matrices. These findings are of particular concern given that *Aspergillus fumigatus* is categorized as a risk group 2 pathogen [70], it is also recognized by WHO (2022) for its infectious potential [71,72]. In poultry, this pathogen can cause dyspnea, pneumonia and chronic aspergillosis [17,73]. Whereas on poultry workers, exposure may lead to allergic bronchopulmonary mycoses, fungal sinusitis and severe asthma with fungal sensitization [5].

Regarding workers health, biomonitoring (BM) is a method used in occupational health risk assessment to evaluate workers' exposure to hazardous chemicals and their metabolites, including mycotoxins, through biological samples like urine or hair. Compared to environmental monitoring, BM offers a more accurate assessment of the real human exposure [74,75]. In fact, Portuguese poultry workers have already shown quantifiable human exposure to aflatoxin B1 (AFB1), underscoring the importance of biomonitoring in this industry [76]. While not used in this study, BM and environmental samples should be combined in future studies to improve risk assessment, provide a comprehensive understanding of occupational health hazards and guide successful preventative actions, [74,75].

4.4. Azole resistance and one health implications

Azoles are an important class of chemicals widely used for control fungal growth in agriculture, medical and veterinary applications, and material conservation. Among them, itraconazole, voriconazole and posaconazole are key therapeutic options [77], particularly for prophylaxis and treatment of aspergillosis both in humans and animals. The development of antifungal drugs that lack side effects remains challenging. Consequently, the current treatment options for mycosis are limited [78].

The growing threat of antifungal resistance has prompted shifts in study priorities [77,78]. Recently, a joined report from five EU health and environment agencies (EFSA, ECDC, ECHA, EEA, EMA) [79] associated the widespread application of azole fungicides in agriculture to the development of azole-resistant *Aspergillus* spp., a non-target pathogen [69]. The risk may extend to other sectors where azole-treated wood are used such as woodworking facilities [8] and poultry farms [20]. It can also impact the environment through the application of animals bedding material as organic fertilizer [20,80]. The complexity of azole resistance reinforces the need for more investigations through a One Health approach [49,77].

This study evidence reduced azole susceptibility among *Aspergillus* spp., indicating that antifungal resistance is an emergent challenge in farm environments, emphasizing additional occupational health risks [81]. The primary mechanism of azole resistance in *A.fumigatus* involves mutations in the ergosterol biosynthesis pathway, particularly in the cyp51A gene. This gene encodes the cytochrome P450 14α-lanosterol demethylase, the primary target of azole antifungals [82]. As molecular characterization of resistance mechanisms was not performed in this study, we acknowledge this as a limitation. Future research should prioritize azole-resistance screening of *Aspergillus* section *Fumigati* isolates, identify resistance-associated mutations and conduct antifungal susceptibility testing [83]. Notably, EDC evidence the highest fungal biodiversity, *Aspergillus* spp. prevalence and the highest proportion of azole-resistant fungi. These findings support the use of EDC as a reliable sampling method for integrative surveillance of potentially harmful and resistant fungi in animal production facilities [62].

5. Conclusion

This study integrates environmental parameters, particulate matter, microbial contamination and fungal susceptibility to azole drugs in poultry farms. The findings reveal important links between production practices, bioaerosol dynamics, and contamination sources (e.g. moist wood shavings). Our results underscore the significance of poultry farms not only as reservoirs of toxigenic mycobiota (*Aspergillus* spp., *Penicillium* spp.) but also as potential hotspots for antifungal resistance with implications for animal and occupational health. Particulate matter (particles $<PM_{10}$) facilitates the spread of microorganisms, with capacity of penetrating the respiratory tract of exposed individuals. A limitation of this study was the short duration of air and PM sampling, which may not reflect temporal variations as well as the limited number of farms included. Therefore, future studies should include several farms and cover different periods of the year to better understand common patterns and temporal dynamics. EDC proved to be an effective sampling method for *Aspergillus* spp. and azole-resistant isolates in poultry farms, and may be particularly useful in longitudinal studies for monitoring temporal changes.

Given the growing concern over fungal diseases and emerging antifungal resistance, future research should focus on mycotoxins exposure assessment, occupational and animal health risks from microbial exposure and a target evaluation of *Aspergillus* genera and potential azole resistance due to its widespread distribution in poultry facilities and interconnected risks to workers and animal health.

CRediT authorship contribution statement

Bianca Gomes: Writing – original draft, Visualization, Methodology, Investigation, Data curation, Conceptualization. **Marta Dias:**

Methodology. **Renata Cervantes:** Methodology. **Pedro Pena:** Methodology. **Elisabete Carolino:** Writing – review & editing, Validation, Software. **Liliana Aranha Caetano:** Writing – review & editing. **Susana Viegas:** Writing – review & editing, Validation. **Carla Viegas:** Writing – review & editing, Validation, Supervision, Resources, Funding acquisition.

Declaration of competing interest

None. We 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.onehlt.2025.101230.

Data availability

Data will be made available on request.

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