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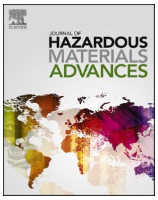

The need of a one health approach to tackle microbiological contamination in animal and dairy production - the case of Portuguese feedlots farms and dairies

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ABSTRACT

With the global population rising, there is increasing demand for agricultural productivity, particularly for animal-derived proteins and products. This trend places additional pressure on natural resources and the workforce in the animal production sector. This study aims to address environmental and health factors influenced by animal production within the One Health framework, encompassing animal welfare, food security, food safety, workeŕs health and climate change. In this context, three feedlots' farms and two dairies from the North of Portugal, representing the worst-case exposure scenario, were engaged in this study. Samples were collected using Electrostatic Dust Cloths (EDC) placed on farm surfaces and attached to work clothing (EDCT). Additional samples were obtained from feed, used bedding material, and surface swabs from feeders, drinkers, milking parlors, and frequently touched areas in social spaces (e.g., offices and changing rooms). Microbial characterization and azole-resistance screening were performed using diverse culture media, complemented by molecular assays (qPCR) targeting toxigenic fungal species. Thirty-eight mycotoxins were analyzed across the sampled matrices. This comprehensive approach identified critical sources of microbial and mycotoxin contamination: bedding material showed the highest bacterial contamination (TSA; 5.40 X 10^3 CFU.g-1), while swabs (MEA; 2.5 X 10^4 CFU.m-2 to 9.00 X 10^4 CFU.m-2) and feed (MEA; ranged from 1.33 X 10^2 CFU.g-1 to 8.00 X 10^2 CFU.g-1) exhibited the greatest fungal contamination. Feed was identified as the main source of mycotoxin exposure for both animals and workers, since all 16 feed samples tested positive for mycotoxin contamination. Results revealed widespread distribution of *Aspergillus* sp. across environmental matrices, highlighting the need for targeted interventions. Azole-resistance screening and mycotoxin profiling further emphasize the importance of implement targeted interventions to prevent, monitor, and remediate environmental contamination by fungi and mycotoxins across different contexts (food safety, animal health, public and occupational health) underscoring the value of a One Health approach.

1. Introduction

With the growth of population worldwide, there is an associated increasing demand to boost agricultural productivity and an enlarged demand for animal protein and products (Ortiz et al., 2021; Tilman et al., 2011; Zhang et al., 2024). This will lead to increased pressure on natural resources and an augmented workforce dedicated to the animal production industry.

Between 2015 and 2017, the European Agency for Safety and Health at Work (EU-OSHA) 2017 carried out a project to address the lack of knowledge on and awareness of exposures to biological agents and the related health problems in animal-related occupations concluding that these occupations are clearly at risk of infection due to unintentional exposure to bacteria, fungi, parasites, prions, organic dust (which is a mixture of (products of) biological agents), and allergenic agents, namely animal-derived antigens and toxins/pathogens (European

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Agency for Safety and Health at Work (EU-OSHA) 2017). In what concerns workers from livestock, the exposure to microorganisms can be related to different contamination sources, such as animal hair and dander, animal feed and litter (European Agency for Safety and Health at Work (EU-OSHA) 2017). On livestock farms, which often have a small number of people working and a high number of self-employed workers, exposure to microorganisms is more difficult to assess and to control than in larger animal facilities. These family farms are less prepared in what concerns working conditions and animals handling. Therefore, workers in a family business, such as most of the Portuguese farms, are considered a vulnerable group (European Agency for Safety and Health at Work (EU-OSHA) 2017). Regarding cattle production distribution in Portugal, higher number of feedlots farms are found in the North of the country (13,307 out of 25 492 farms) and dairies in the center (1088 out of 4250 dairies) (DGAV, 2021). This distribution is dependent of cultural and geographical conditions that varied through all the territory Portugal mainland.

Multidrug-resistant bacteria, mostly due to the higher use of antibiotics in farming, are reported as an emerging risk, affecting many people, including workers. Since multidrugresistant bacteria can be present in both animals and humans, the increased use of antibiotics is critical for both (European Agency for Safety and Health at Work (EU-OSHA) 2017). Furthermore, the increasing environmental temperatures due to climate change (CC), might provide conditions for the fungal stress adaptation to high-temperature environments, promoting their pathogenicity in humans and also animals (Seidel et al. 2024). In fact, the CC threat to food security and safety has promoted adaptive agricultural practices (including increased use of fungicides for farming) (Rhodes and McCarl 2020). Thus, acquired azole resistance in *Aspergillus fumigatus,* driven also by the use of environmental fungicides, is leading to increased cases of aspergillosis in humans difficult to manage (Arastehfar et al., 2020). Actions to tackle antimicrobial resistance (AMR), and specifically fungal resistance, since is less studied compared with multidrug-resistant bacteria (Gomes et al., 2023), are critical urgent and need attention and coordination from decision and policy makers from all sectors in the different One health domains (Seidel et al., 2024). In addition, as mentioned earlier, most Portuguese farms are small family businesses and are also less prepared to address antimicrobial resistance; therefore, coordinated and urgent actions are of utmost importance. Mycotoxins have been reported as one of the most important contaminants in the raw materials of feed (Binder et al., 2007; Gomes et al., 2025; Tima et al. 2016; Viegas et al., 2019b,c) also triggered by CC (Viegas, 2021). Mycotoxins can cause wide array of adverse health effects' posing health threats to both humans and livestock. These health effects range from acute poisoning to long-term effects such as cancer and immune deficiency (Assunção and Viegas, 2020; Berrington de González et al., 2024; WHO, 2024). In fact, feed can be a contamination source of mycotoxins inside the farms affecting human and animals' health (Gomes et al., 2025; Viegas et al., 2019b,c), and a food safety concern, since the milk and the meat can be contaminated with mycotoxins due to feed contamination (EFSA, 2011; Gomes et al., 2025). In a previous study developed in a Portuguese dairy, all the samples analyzed presented contamination by at least two mycotoxins and up to a maximum of 13 mycotoxins in the same sample. The most common found were Zearalenone (ZEA), deoxynivalenol (DON) and ochratoxin A (OTA), but in one sample Aflatoxin B1 (AFB1) was also detected (Viegas et al. 2019c). In addition, the same study also highlighted the potential impact of feed contamination on workers' exposure to mycotoxins (Viegas et al. 2019c). In fact, occupational exposure may occur via inhalation or dermal contact, leading to systemic absorption or unintentional ingestion. Although mycotoxins are non-volatile, they can be carried to the respiratory tract through airborne dust, fungal spores, and microbial fragments (Martins et al., 2025; Viegas et al. 2018; Viegas et al., 2020a,b). In dairy farm settings, dust generated during feed handling is therefore a significant contributor to exposure by inhalation (Viegas et al., 2019c). Overall, CC can boost crops contamination by toxigenic fungal species, increasing human and animal exposure to mycotoxins, besides promoting the acquired azole resistance leading to the occurrence of life-threatening infections (Viegas et al., 2021).

To our knowledge, this is the first study in Portugal assessing feedlot farms and encompassing a wide range of passive sampling methods and assays. This study investigates environmental and health factors related to animal production through a One Health lens, with the aim of producing knowledge to support coordinated interventions addressing animal welfare, food security and safety, workers' health, and climate change. In this context, three feedlots' farms and two dairies from the North of Portugal were engaged in this study.

2. Materials and methods

2.1. Farms characterization

This study was performed in three feedlots farms and two dairies from the North of Portugal (Fig. 1). These five properties, the oldest in northern Portugal and dedicated exclusively to cattle farming and the production of food for human consumption (meat and milk), were selected to characterize a worst-case scenario regarding the facilities and equipment used in both settings.

A walkthrough survey list was performed specifically to collect contextual information from the farms covering the variables that have been reported as influencing microbial contamination, namely: area allocated, number of animals and ventilation conditions (Table 1).

2.2. Sampling approach

The samples were collected using Electrostatic Dust Cloths (EDCs; Procter & Gamble Company, Lisbon, Portugal), including standard EDCs placed on farm surfaces and EDCs attached to work clothing (EDCT). Additional samples were obtained from feed, bedding material being used (straw was the common material), and surface swabs taken from various surfaces such as feeders, drinkers, milking parlors, and frequently touched areas in social spaces (e.g. door handles, faucets and locker doors from offices and changing rooms) (Fig. 2). Apart from the EDCs, which remained for 14 to 15 days, all other samples were collected during the routine daily activities of each farm.

After collection, the EDC was closed in the Petri dish with a lid, and the exact number of days of sampling was recorded. The EDCTs were placed (one from V1 and other from V4 farms) in workers' jackets (restricted use only in the farms), using a pin disinfected with 70% alcohol, on the coats of the main workers responsible for feeding, cleaning spaces, animal health and animal welfare, preparation and administration of feed, during a work shift (approximately 7/8 h) (Fig. 2; Table S1 - Supplementary material). At the end of the sampling time, the EDCT was placed in a sterilized bag. The surface swabs were performed through sterilized swabs moistened in sterilized water and using 10 x 10 cm square of stainless steel disinfected with 70% alcohol between samples. Samples of bedding and feed with approximately 5 g were collected with sterile gloves into a sterile bag (Fig. 2).

2.3. Microbial distribution

Upon arrival at the laboratory, all samples were processed as previously described (Viegas et al. 2024). Samples collected from the farms were washed with 0.05% Tween 80 in 0.9% NaCl saline solution (shaken at 250 rpm for 30 min), following the bellow procedures: For every 1 g of feed, 9.1 mL of the saline solution was used for extraction; EDCs and EDCTs were placed into 50 mL Falcon tubes with 10 mL and 25 mL of solution, respectively; Each swab was cut and placed into a 2 mL Eppendorf tube containing 1 mL of the same solution.

Aliquots of the resulting extracts (150 µL) were plated onto selective media for fungal and bacterial growth. Fungal cultures were grown on malt extract agar (MEA) supplemented with chloramphenicol (0.05%)

Fig. 1. Geographical distribution of the feedlots assessed.

Table 1
Characterization of the assessed farms.

ID	Total area (m^2)	Indoor area (m^2)	N° of animals	Ventilation
V1	19,000	1400	170	Natural
V2	14,000	500	150	Natural
V3	8100	135	20	Natural
V4	6000	1700	5	Natural
V5	3310	145	9	Natural

(Frilabo, Maia, Portugal) and dichloran glycerol agar (DG18) (Frilabo, Maia, Portugal), while bacterial cultures were grown on tryptic soy agar (TSA) (Frilabo, Maia, Portugal) supplemented with nystatin (0.2%) for mesophilic bacteria, violet red bile agar (VRBA), for coliforms (Frilabo, Maia, Portugal), and MacConkey agar (MAC), for lactose-fermenting and non-lactose-fermenting Gram-Negative bacteria (Frilabo, Maia, Portugal). Incubation conditions were optimized for each medium: 30 °C for TSA. 27 and 37 °C for DG18. 27 °C for MEA. and 37 °C for VRBA and MAC.

The same extracts were used to screen for azole resistance by inoculating 150 µL of the samples onto Sabouraud dextrose agar (SDA) supplemented with 4 mg/L itraconazole (ITR), 2 mg/L voriconazole (VOR), 0.5 mg/L Posaconazole (POS), or SDA alone (as control) (adapted from the (EUCAST, 2017). A reference strain *A. fumigatus* ATCC 204,305 resistant to azoles was used as positive control (kindly provided by Reference Unit for Parasitic and Fungal Infections. Department of Infectious Diseases of the National Institute of Health. from Dr. Ricardo Jorge).

Quantification of microbial contamination was performed according to procedures previously published by (Viegas et al. 2024). Fungal isolates were identified by macroscopic and microscopic morphology using tease mount or Scotch tape mount and lactophenol cotton blue mount procedures (de Hoog et al., 2016). Whenever colony overgrowth was observed due to fungi with fast growing rates (*Chrysonilia sitophila, Trichoderma* sp. and Mucorales order), a quantitative cut off of 500 isolates (CFU) was applied and the median of the results obtained from each environmental matrix was used for passive sampling (Viegas et al., 2019a).

From the EDCT extract, 100 µL aliquots were inoculated onto CHROMagar™ MRSA (Frilabo, Maia, Portugal) alongside control strains. The positive control was a known MRSA isolate from the laboratory collection, and the negative control was *Staphylococcus aureus*

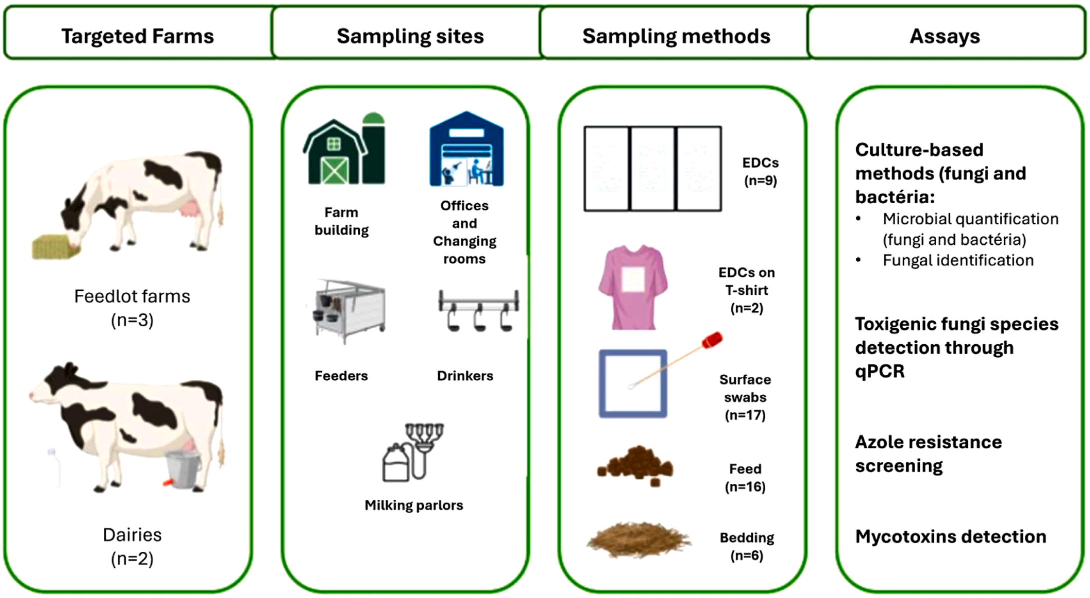

Fig. 2. Sampling strategy and assays employed.

ATCC 25,923. All plates were incubated at 37 °C for 24 to 48 h following previous reported procedures (Oliveira et al., 2022; Viegas et al., 2021). Colonies with pink/red coloration, considered presumptive MRSA, were collected and suspended in 1x PBS containing 250 μL of glycerol before being stored at −80 °C.

2.4. Molecular detection of Aspergillus section Fumigati and MRSA

For molecular identification of specific *Aspergillus* sections, fungal DNA was extracted from 8.8 mL of each passive sample extract using the ZR Fungal/Bacterial DNA MiniPrep Kit (Zymo Research, Irvine, USA). Quantitative real-time PCR (qPCR) was conducted on a CFX-Connect PCR System (Bio-Rad), following a standardized protocol. Each PCR reaction contained 1× iQ Supermix (Bio-Rad, Portugal), 0.5 μM of each primer, and 0.375 μM of a TaqMan probe, with a total reaction volume of 20 μL. The thermal cycling conditions involved 40 cycles comprising denaturation at 95 °C for 30 s. annealing at 52 °C for 30 s, and extension at 72 °C for 30 s. Each run included both negative controls (no template) and positive controls (Viegas et al., 2024). The positive controls were composed of DNA from reference *Aspergillus* strains, supplied by the Reference Unit for Parasitic and Fungal Infections. Department of Infectious Diseases, National Health Institute, Dr. Ricardo Jorge, IP. These strains had been previously characterized through sequencing of the ITS, β-tubulin, and calmodulin gene regions (Table S2 - Supplementary material).

For the molecular confirmation of MRSA, preserved isolates were first subcultured onto Blood Agar (5% sheep blood; Frilabo, Maia, Portugal) to ensure purity and viability. Following a 24-hour incubation, pure colonies were harvested and suspended in 1 mL of Milli-Q water. The bacterial suspension was then heated at 95 °C for 15 min to lyse the cells and centrifuged at 20,870 × g for 1 min. The resulting clear supernatant was used directly as the DNA template in subsequent PCR assays. The multiplex PCR targeted the S. aureus-specific nuc gene and the methicillin resistance mecA gene using the following primers: NUC1 (5′-GCG ATT GAT GGT GAT ACG GTT-3′) and NUC2 (5′-AGC CAA GCC TTG ACG AAC TAA AGC-3′) for nuc, and MECA1 (5′-GCA ATC GCT AAA GAA CTA AG-3′) and MECA2 (5′-GGG ACC AAC ATA ACC TAA TA-3′) for mecA (Fang and Hedin 2003). Each 20 μL reaction contained 10 μL of NZYSupreme qPCR Green Master Mix (2x; NZYtech, Portugal), 1 μL of each forward and reverse primer (0.5 μM final concentration each), 4 μL of DNA template, and 4 μL of nuclease-free water. The amplification was performed under the following conditions: initial denaturation at 95 °C for 5 min; 40 cycles of denaturation at 95 °C for 5 s and a combined annealing/extension at 60 °C for 15 s. A melt curve analysis was subsequently conducted by heating the products from 58 °C to 95 °C to verify amplification specificity. Isolates were considered positive for mecA or nuc if their melting temperatures (Tm) were within ±0.8 °C or ±0.5 °C of the positive control's Tm, respectively (Fang and Hedin, 2003).

2.5. Mycotoxin detection

A total of 33 samples were thoroughly screened to evaluate the occurrence of 38 different mycotoxins in the feedlot farms and dairies. The sampling included 6 bedding material samples of straw, 16 feed samples, 9 EDCs and 2 EDCTs. Mycotoxin analysis was performed using high-performance liquid chromatography (HPLC) on a Nexera system (Shimadzu, Tokyo, Japan), connected to a triple quadrupole-linear ion trap mass spectrometer (5500 Qtrap; Sciex. Foster City. CA. United States), following procedures already reported (Viegas et al., 2020a).

2.6. Statistical analysis

The data were analyzed using R-Studio software, version 4.3.3, for Windows. The results were considered significant at the 5% significance level. The Shapiro-Wilk test (shapiro.test() command) was used to test data normality. The Mann-Whitney test (wilcox_test() command) from library(coin) was used to compare fungal and bacterial contamination and azole resistance between the two farm types, since the normality assumption was not met. To study the relationship between fungal contamination, bacterial contamination, and azole resistance values (in the different culture media), the Spearman's rank correlation coefficient was used (library(corrplot), library(Hmisc), library(dplyr), rcorr(, type = "spearman"), and corrplot( ) commands). To better understand the effects of farm type, sampling methods, and environmental variables on microbial community structure and contamination levels, multivariate analyses were also performed. Microbial counts were transformed into log(x + 1) prior to multivariate analyses to reduce skewness and the influence of extreme values while preserving quantitative differences in contamination levels. To compare fungal and bacterial contamination and azole resistance between farm type and sampling methods, PERMANOVA (Permutational Multivariate Analysis of Variance) based on Bray–Curtis distances was used (commands vegdist(data, method = "bray") and adonis2(dist ∼ group, data = metadata, permutations = 999)). To test the internal variability of the groups, the PERMDISP test (Permutational Analysis of Multivariate Dispersions - command betadisper(dist, group)) was used. To identify multivariate patterns in the microbiological data, PCA with VARIMAX rotation and Keiser normalization was used (commands eigen(cor(var))$values, sum(ev > 1) and pca.var <- principal(var.log, nfactors = 4, rotate = "varimax", scale = TRUE)). After the PCA, PERMANOVA was performed again on the PCA scores, based on Euclidean distances. PERMANOVA Pairwaise comparisons were used when justified (comand pairwise_permanova ()). To assess species diversity, Shannon and Simpson indices, given by $\mathit{Shannon\,Index}\,(H) = -\sum_{i=1}^{s} p_i \ln(p_i)$ and $\mathit{Simpson\ Index}\,(D) = \frac{1}{\sum_{i=1}^{s} p_i^2}$, respectively, 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. Microbial quantification and identification

3.1.1. Viable bacterial contamination quantification

Fig. 3 shows the bacterial contamination in TSA, VRBA and MAC in EDC on the five cattle farms (V1 to V5). The bacterial contamination in TSA, VRBA and MAC in EDC (A) was higher on dairy farms (V1 and V4). For TSA the values ranged between 1.91 X 10^2 CFU.m-2.day-1 and 1.17 X 10^3 CFU.m-2.day-1, for the V2 and V1, respectively. For VRBA the higer value was obteined in V3 and V1 (5.66 X 10^2 CFU.m-2.day-1). For MAC, the higestvalue was onbteined in V5 (1.81 X 10^3 CFU.m-2.day-1). In TSA, contamination values are higher in V1, while VRBA and MAC are in V4 and V5 (Fig. 3A).

The EDCTs present equal values in TSA (2.20 X 10^4 CFU.m-2.day-1) for one working day (8 h) in the V1 and V4 dairies. In VRBA the values vary between 1.00 X 10^3 CFU.m-2.day-1 and 2.00 X 10^3 CFU.m-2.day-1 (Fig. 3A). No growth in MAC was observed.

Fig. 3B shows the bacterial contamination of bedding and feeding in TSA, VRBA and MAC on dairy farms V1 to V4. In feedlot farm V5, no bedding was collected for safety reasons at the time of sample collection. In TSA, the values were 5.40 X 10^3 CFU.g-1 on the four properties where bedding samples were taken. In VRBA, the values ranged from 1.53 X 10^3 CFU.g-1 to 5.70 X 10^3 CFU.g-1 on V4 and V1, respectively. The values in MAC ranged from 1.93 X 10^3 CFU.g-1 to 9.17 X 10^3 CFU.g-1 on dairy farm V4 and V1, respectively. Feedlot V2 stands out for its MAC value (4.36 X 10^4 CFU.g-1). In VRBA, contamination values were higher in V1, while in MAC they were higher in V2.

In the feed (Fig. 3B), in MAC, the values were 3.60 X 10^3 CFU.g-1 in the V1, V4 and V5. While in TSA, the values ranged between 5.80 X 10^3 CFU.g-1 (V1 to V3 and V5) and 1.41 X 10^4 CFU.g-1 (V4). The values in VRBA ranged between 2.67 X 10^2 CFU.g-1 (V2) and 2.40 X 10^3 CFU.g-1

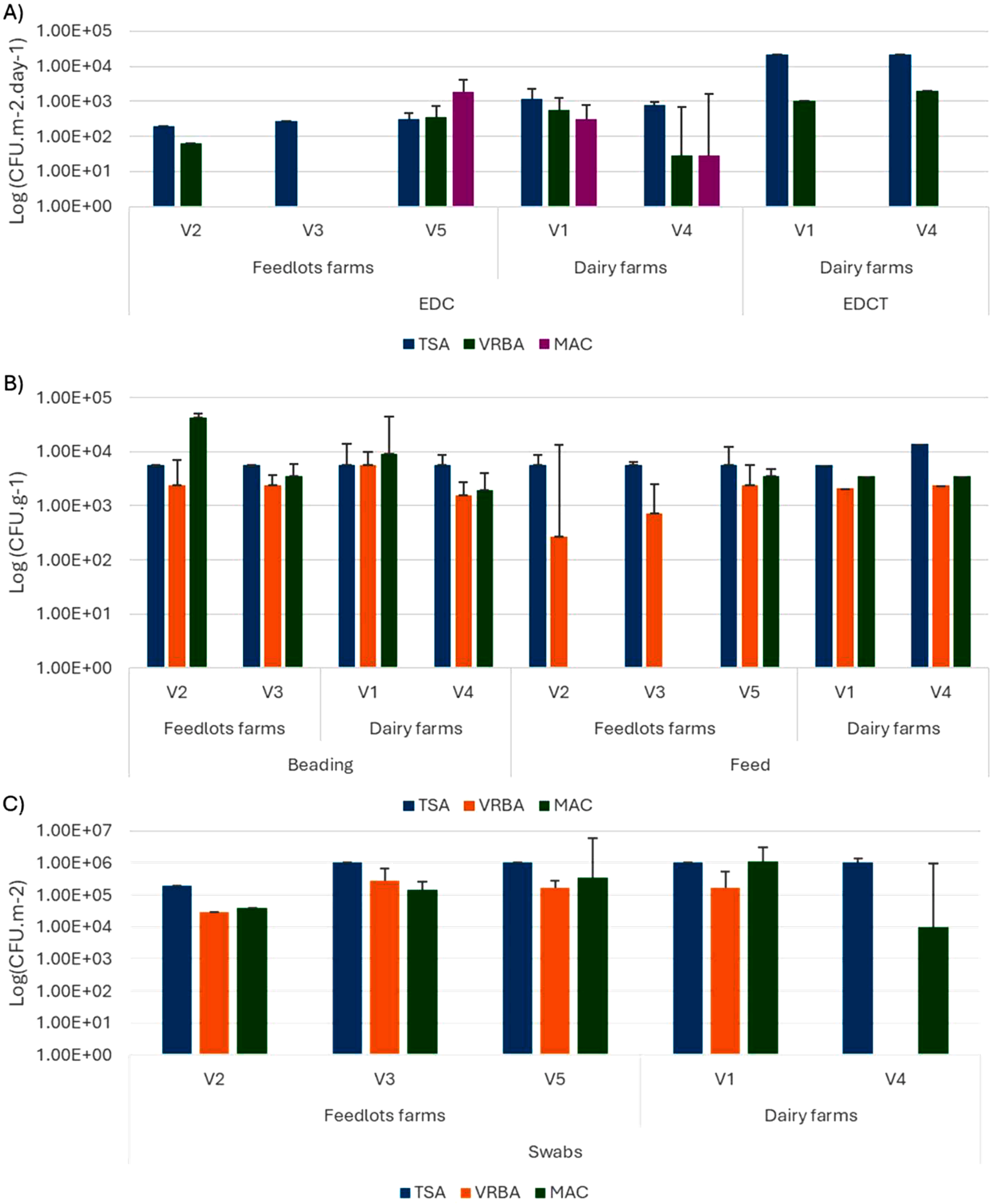

Fig. 3. . Median bacterial contamination across sample types and production systems. (A) Settled dust (EDC and EDCT) expressed as CFU.m-2.day-1. (B) Bedding and feed expressed as CFU.g-1. (C) Surface swabs expressed as CFU.m-2. Data are presented as medians; boxes represent the interquartile range (IQR), and whiskers extend to maximum values.

(V5 and V4). Contamination values were higher in V4. in all the culture media.

Fig. 3C represents the contamination values of surface swabs in the five farms. For TSA, the median values ranged from 2.00 X 10^5 CFU.m-2 to 1.20 X 10^6 CFU.m-2 in the V2 and V4 farms, respectively. In VRBA, the values ranged from 3.00 X 10^4 CFU.m-2 to 2.75 X 10^5 CFU.m-2 in the V2 and V3 feedlot farms, respectively. The values in MAC varied between 1.00 X 10^4 CFU.m-2 day and 1.09 X 10^6 CFU.m-2 in the V4 and V1 dairy farms, respectively. In TSA the contamination values were higher in V1, V3 to V5, while in VRBA and in MAC they were in V1.

3.1.2. *Viable fungal contamination quantification*

Regarding the fungal contamination in EDC placed on outdoor surfaces in the five dairy farms, in MEA, the values ranged from 5.26 X 10^3

CFU.m-2.day-1 in V2, to 1.38 X 10^3 CFU.m-2.day-1 in V4 (Fig. 4A). In DG18 at 27 °C, the highest value was detected in V2, 7.43 X 10^4 CFU.m-2.day-1, and the lowest value was detected in V1. 7.51 X 10^2 CFU.m-2.day-1. In DG18 at 37 °C, the contamination values ranged from no contamination in V3 to 2.50 x 10^2 CFU.m-2.day-1 in V2 (Fig. 4A).

In the EDCTs there was no fungal growth in DG18 at 27 °C. In MEA, fungal contamination was 1.25 X 10^3 in V1 and V4 and 1.00 x 10^3 CFU.m-2.day-1 on DG18 at 37 °C in V4 (Fig. 4A).

In bedding samples (Fig. 4B), in MEA the median fungal contamination values ranged from 3.33 X 10^1 CFU.g-1 in V4 to 6.67 X 10^2 CFU.g-1 in V1. In DG18 at 27 °C the counts ranged from 2.33 X 10^2 CFU.g-1 to 2.67 X 10^3 CFU.g-1 in V2. In DG18 at 37 °C the counts ranged from 3.33 X 10^1 CFU.g-1 in V1 to 2.67 X 10^2 CFU.g-1 in V2.

In the feed samples (Fig. 4B), in MEA, the median fungal contamination values ranged from 1.33 X 10^2 CFU.g-1 in V5 to 8.00 X 10^2 CFU.g-1 in V2. In DG18 at 27 °C, the counts ranged from 6.67 X 10^1 CFU.g-1 in V5 to 5.33 X 10^2 CFU.g-1 in V2. In DG18 at 37 °C, the counts varied between 1.67 X 10^1 CFU.g-1 in V2 and V1, to 1.33 X 10^2 CFU.g-1 in V5.

In the swab samples (Fig. 4C) in MEA, the average values of fungal contamination varied from 2.5 X 10^4 CFU.m-2 in V3 to 9.00 X 10^4 CFU.m-2 in V5. In DG18 at 27 °C, the contamination varied from 1.00 X 10^4 CFU.m-2 to 9.00 X 10^4 CFU.m-2 in V5. In DG18 at 37 °C, the contamination varied from 2.50 X 10^4 CFU.m-2 to 7.50 X 10^4 CFU.m-2 in V5 (Fig. 4C).

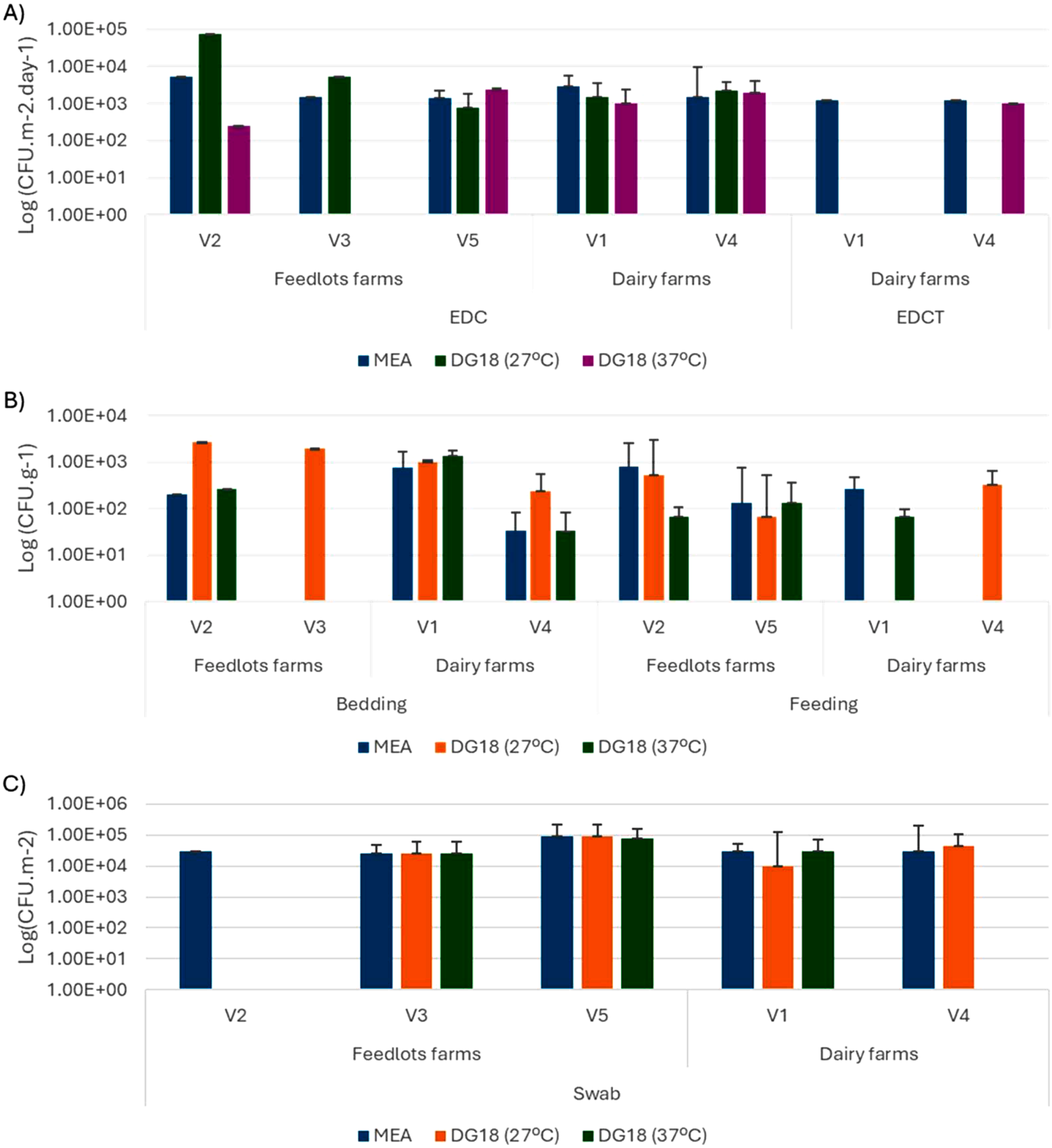

Fig. 4. Median fungal contamination across sample types and culture conditions. A) Settled dust (EDC and EDCT) expressed as CFU.m-2.day-1. B) Bedding and feed expressed as CFU.g-1. C) Surface swabs expressed as CFU.m-2. Fungi were cultured on MEA (27 °C) and DG18 (27 °C and 37 °C). Data are presented as medians with IQR; whiskers denote max values.

3.1.3. Fungal identification

In Supplementary Table S3 presents the prevalence and fungal characterization of environmental samples. In dairy farms in the EDC, *Penicillium* sp. was dominant in MEA (74% in V1; 67% in V4), while DG18 at 27 °C showed high prevalence of *Penicillium* sp. in V1 (92%) and *Aspergillus* sp. in V4 (40%). At 37 °C, *Mucor* sp. dominated in V1 (63%), whereas *Fusarium verticilloides* was most frequent in V4 (62%). For EDCT, *Penicillium* sp. prevailed in MEA (80% in V1; 60% in V4), and *Aspergillus* sp. was exclusive to DG18 at 37 °C (100% in V4), with no growth observed at 27 °C. Bedding samples revealed co-dominance of *Alternaria* sp. (41%) and *Aspergillus* sp. (41%) in MEA (V1), while *Aspergillus* sp. was consistently dominant in V4 (100% across MEA, DG18 at 27 °C, and 37 °C). In feed, *Penicillium* sp. dominated MEA in V1 (69%), contrasting with *Aspergillus* sp. in V4 (100%); DG18 at 27 °C favored *Aspergillus* sp. in V1 (62%) and *Mucor* sp. in V4 (63%), while 37 °C showed equal *Aspergillus* sp. and *Mucor* sp. in V1 (50% each). Swabs exhibited *Penicillium* sp. dominance in MEA (V1: 69%; V4: *Cladosporium* sp. at 68%), with *Aspergillus* sp. prevalent in DG18 at 37 °C (V1: 94%; V4: 84%).

In feedlot farms, EDC results highlighted *Aspergillus* sp. dominance in MEA (V2: 90%; V5: 55%) and DG18 at 27 °C (V2: *Cladosporium* sp. at 82%; V5: 50% *Aspergillus* sp./50% *Mucor* sp.). At 37 °C, *Penicillium* sp. dominated in V2 (100%), while *Aspergillus* sp. prevailed in V5 (63%). Bedding material samples showed exclusive *Aspergillus* sp. in V2 (MEA. DG18 at 27 °C/37 °C: 100%) and *Penicillium* in V3 (DG18 at 27 °C: 100%). Feed samples were dominated by *Aspergillus* sp. in MEA (V2: 95%; V5: 89%) and DG18 at 27 °C (V2: 95%; V5: 92%), while *Fusarium verticilloides* dominated at 37 °C (100% in both V2 and V5). Swabs revealed *Aspergillus* sp. dominance in MEA (V2: 67%; V5: 91%) and DG18 at 37 °C (V3: 100%; V5: 65%), with *Penicillium* sp. being exclusive in DG18 at 27 °C (V3: 100%) (Fig. 5).

Regarding *Aspergillus* sections distribution on dairy farms, from EDC on MEA cultures, V1 showed 75% *Flavi* and 25% *Fumigati* prevalence, V4 exhibited 5.88% *Flavi* and 94.12% *Fumigati* in MEA. For DG18 at 27 °C, V4 presented equal prevalence of *Aspergilli* (50%) and *Fumigati* (50%). At 37 °C, V4 showed exclusive *Fumigati* prevalence (100%). V1 EDCT in MEA demonstrated 100% *Nidulantes* prevalence. V4 EDCT showed 100% *Aspergilli* in DG18 at 37 °C and 100% *Aspergilli* in MEA. For the bedding of dairy farms samples, contamination on MEA revealed 100% *Flavi* in V4 and 99.79% *Aspergilli* with 0.21% *Nigri* in V1. DG18 at 27 °C showed 66.67% *Aspergilli* and 33.33% *Fumigati* in V4, while V1 contained 61.11% *Aspergilli* and 38.89% *Nigri*. At 37 °C, V1 bedding exhibited 37.5% *Aspergilli*, 20.83% *Fumigati*, and 41.67% *Restricti*. From Feed samples, MEA cultures showed 100% *Aspergilli* in V1 and 100% *Nigri* in V4. DG18 at 27 °C maintained 100% *Aspergilli* in V1 and 100% *Nigri* in V4. At 37 °C, V1 feed contained 100% *Restricti*. From swab samples, MEA demonstrated 98.68% *Fumigati* and 1.32% *Nigri* in V1, and 83.33% *Fumigati* with 16.67% *Nigri* in V4. DG18 at 27 °C showed 66.67% *Flavi* and 33.33% *Fumigati* in V1, while V4 exhibited 90.48% *Fumigati* and 9.52% *Aspergilli*. At 37 °C, V1 swabs presented 93.75% *Fumigati* and 6.25% *Nigri*, and V4 showed 100% *Fumigati* (Fig. 5).

From Feedlot Farms (V2, V3, V5) on EDC samples, MEA cultures showed 84.21% *Fumigati* and 15.79% *Nigri* in V2, and 100% *Fumigati* in V5. DG18 at 27 °C revealed 44.83% *Aspergilli* and 55.17% *Flavi* in V2, and 100% *Fumigati* in V5. At 37 °C, V5 EDC contained 25% *Flavi*, 25% *Fumigati*, and 50% *Restricti*. From bedding, MEA and DG18 at 27 °C both showed 100% *Nigri* in V2. At 37 °C, V2 bedding exhibited equal prevalence of *Nigri* and *Restricti* (50% each). From Feed, MEA demonstrated 80% *Flavi* and 20% *Fumigati* in V2, and 100% *Fumigati* in V5. DG18 at 27 °C showed varied distribution in V2: 60.81% *Nigri*, 27.03% *Aspergilli*, 6.76% *Fumigati*, and 5.41% *Nidulantes*. At 37 °C, V5 feed showed 100% *Fumigati*. From Swabs MEA revealed 100% *Fumigati* in V2 and V5. DG18 at 27 °C showed 90.63% *Fumigati* and 9.38% *Nidulantes* in V5. At 37 °C, V3 swabs presented 80% *Fumigati* and 20% *Nigr,* while V5 showed 100% *Fumigati* (Fig. 5).

3.2. Azole resistance screening

Antifungal resistance profile in feedlot farms showed variable contamination levels across antifungal agents. On feedlot farms, EDC samples exhibited contamination on SDA (V2: 5.01×10^{2} CFU.m-2.day-1; V3: 2.50×10^{2} CFU.m-2.day-1; V5: 2.13×10^{3} CFU.m-2.day-1) and ITZ (V5: 2.50×10^{2} CFU.m-2.day-1). On bedding samples, contamination was observed on VOZ (V2: 3.33×10^{2} CFU.g-1), while feed samples displayed low-level contamination on SDA (V3: 6.67×10^{1} CFU.g-1; V5: 6.67×10^{1} CFU.g-1), and VOZ (V5: 9.33×10^{2} CFU.g-1).

On dairy farms, EDC samples demonstrated consistent on SDA (V1: 5.01×10^{2} CFU.m-2.day-1; V4: 5.01×10^{2} CFU.m-2.day-1). EDCT samples mirrored this pattern. Bedding material analysis revealed distinct patterns: V1 showed no SDA growth, but VOZ contamination (5.67×10^{2} CFU.g-1) and POZ contamination (3.33×10^{1} CFU.g-1). While V4 exhibited exclusively contamination on SDA (1.67×10^{2} CFU.g-1).

Regarding the prevalence of fungal genera on dairy farms, EDC samples revelead on SDA *Mucor* sp. (V1: 100%), and *Penicillium* sp. (V4: 100%). On EDCT samples presented only *Aspergillus* sp. (V4: 100%). Bedding materials presented on SDA *Geotrichum* sp. (V4: 80%) and *Rhizopus* sp. (V4: 20%), while presenting resistance profiles on both VOZ and POZ with *Geotrichum* sp. (V1: 100%). Feeding was exclusively

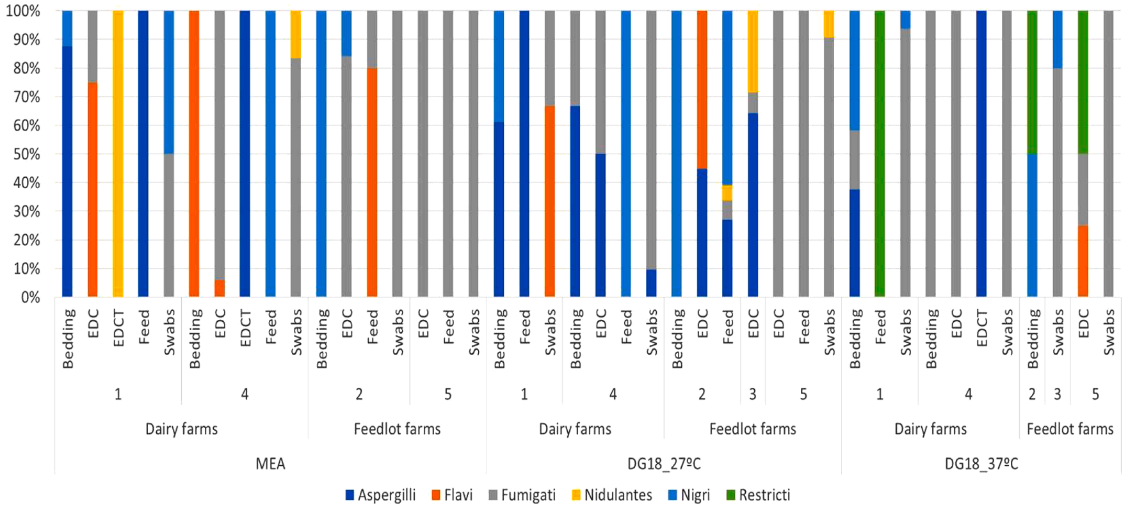

Fig. 5. Relative distribution of *Aspergillus* sections across sample types and culture conditions. Data are shown as the percentage of isolates belonging to each section, identified morphologically. Samples include settled dust (EDC, EDCT), bedding, feed, and surface swabs from five cattle farms.

contaminated by *C. sitophila* (100% in V4) on SDA (Supplementary material - Table S4).

On the feedlot farms, EDC samples presented on SDA *Mucor* sp. (V2: 100%), V3 *Aspergillus* sp. (V3: 100%; V5: 65%), *C. sitophila* (V5: 35%), and only *Mucor* sp. (V5: 100%) on ITZ. On bedding, only *Mucor* sp. was present in VOZ (V2: 100%). Feed samples presented on SDA *Mucor* sp. (V2: 100%), and *C. sitophila* (V3: 100%; V5: 100%), with growth on V5 on VOZ: *Mucor* sp., (26 %), *C. sitophila* (19%), and *Geotrichum* sp. (55%) (Supplementary material - Table S4).

3.3. Molecular detection

Among the 34 samples analyzed via qPCR, fungal DNA from *Aspergillus* section *Fumigati,* was identified across the various sampling matrices, including feed, bedding, EDCs and EDCTs. Multiple samples yielded quantifiable Cq values, confirming the presence of fungal DNA. *Aspergillus* section *Fumigati* was detected in 9 out of 17 feed samples (52.9%), 1 out of 5 bedding samples (20.0%), 8 out of 9 EDCs (88.9%), and 2 out of 2 EDCTs (100%) (Table S5 – Supplementary material). Overall, it was possible detecting in 20 out of 34 where it was not possible to identify.

Concurrent MRSA screening of the EDCT extracts identified two suspicious colonies. Subsequent qPCR analysis confirmed that neither isolate was positive for the mecA and nuc genes, and therefore, no MRSA was detected.

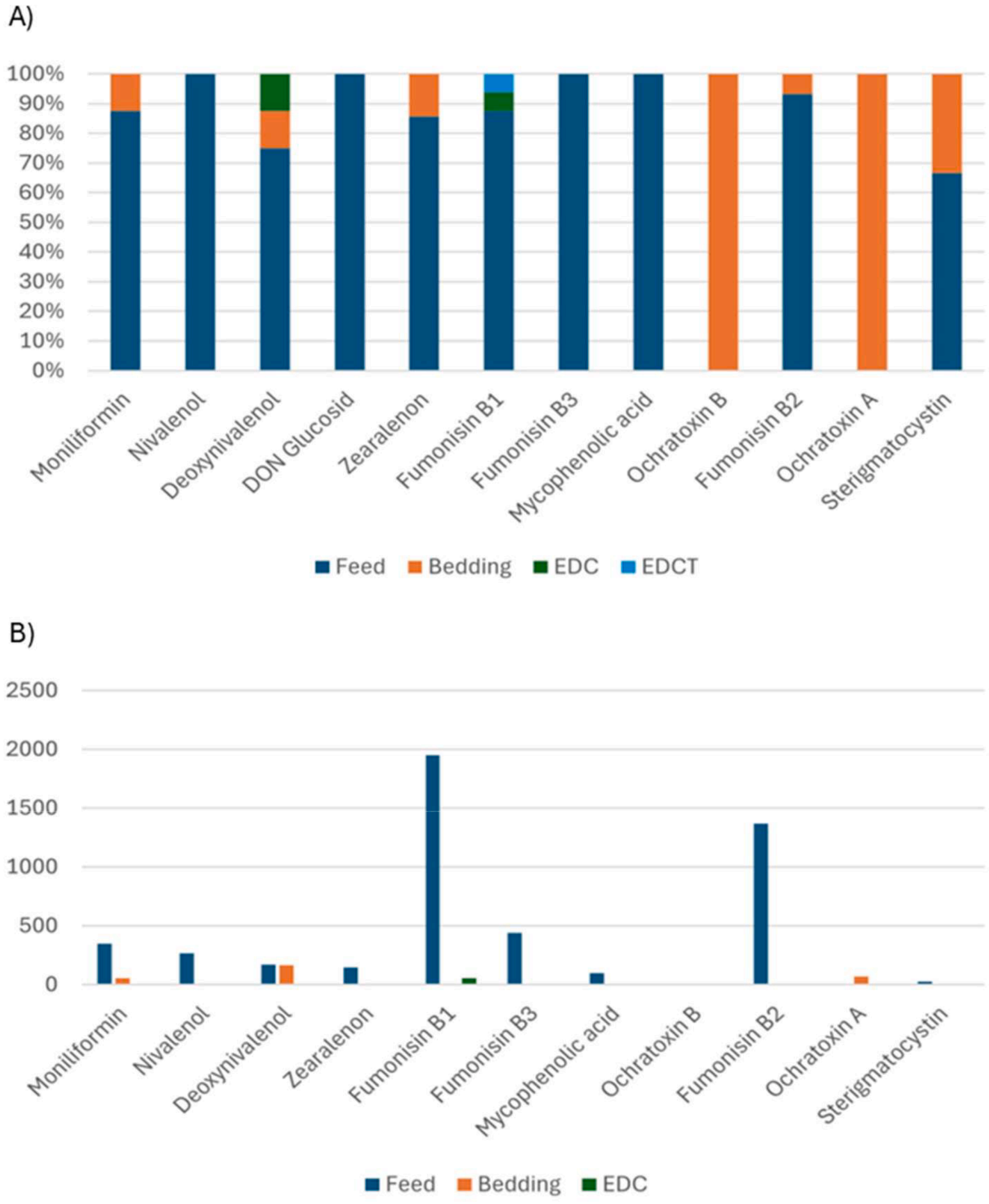

Fig. 6. (A) (A) Distribution of mycotoxin-positive samples across matrices. Bars represent the proportion (%) of total positive detections for each mycotoxin that originated from feed, bedding, or settled dust (EDC/EDCT). (B) Maximum detected concentration (μg/kg) of each mycotoxin per matrix.

3.4. Mycotoxins detection

Fumonisin B1 (FB1) was detected in feedlot and dairy farm samples, presented in 45.5% (15/33) of all samples analyzed (LOQ = 40 and 46 μg/kg). It was detected in feed and EDC's, with the highest occurrence in feed (87.5%, 14/16 samples), where concentrations reached up to 1950 μg/kg. It was also detected in environmental dust (EDC/EDCT 18.2%. 2/11 samples; up to 53 μg/kg). Fumonisin B2 (FB2) was also identified in 45.5% (15/33) of the samples (LOQ = 38 and 25 μg/kg), mainly in feed (87.5%, 14/16), with a maximum concentration of 1370 μg/kg, and in bedding samples (16.7%, 1/6). Zearalenone (ZEA) (LOQ = 3.2 and 2.7 μg/kg) were detected in 42.4% (14/33) of samples, detected mostly in feed samples (75%, 12/16) and in bedding samples (33.3%, 2/6) with a maximum concentration of 147 μg/kg obtained in a feed sample. Moniliformin (MON) (LOQ = 30 and 27 μg/kg) was detected in feed (87.5%, 14/16), with a maximum concentration of 347 μg/kg, and in bedding samples (33.3%, 2/6) with the maximum concentration of 54.1μg/kg. Sterigmatocystin (STERI) was also detected in feed and bedding samples but in lower frequency, 12.5% and 16.7%, respectively. Nivalenol (NIV), Deoxynivalenol (DON) and Mycophenolic acid (MYCO) were only detected in feed samples, 43.8%, 31.3% and 18.8%, respectively. Ochratoxin A (OTA) and Ochratoxin B (OTB) were both detected in the same bedding sample, with concentrations of 69.3 and 6.27, respectively.

All 16 feed samples tested positive for mycotoxin contamination. Only one sample contained a single mycotoxin, while the remaining samples showed co-contamination with between two and eight different mycotoxins (Fig. 6).

Detailed information regarding the specific mycotoxins assessed and their respective limits of detection (LOD) is provided in (Supplementary material - Table S6).

3.5. Comparison and correlation analysis

Among the dairy and feedlot farms only statistically significant differences regarding bacterial contamination in TSA (U = 180.0. p = 0.005) were detected, with greater contamination in the dairy farms, with a relevant magnitude effect (Effect size = 0.48) (Table 2).

It was found that greater fungal contamination in MEA is related to higher fungal contamination in DG18 ($r_S = 0.472$, $p < 0.001$), to higher contamination in DG18 at 37 °C ($r_S = 0.398$, $p = 0.004$), to higher bacterial contamination in TSA ($r_s = 0.585$, $p < 0.001$) and in VRBA ($r_s = 0.293$, $p = 0.037$). Higher fungal contamination in DG18 is related to greater accounting in DG18 at 37 °C ($r_S = 0.320$, $p = 0.022$). Greater bacterial contamination in TSA is related to higher bacterial contamination in VRBA ($r_S = 0.540$, $p < 0.001$) and in MAC ($r_S = 0.437$, $p = 0.001$) and with lower azole resistance values in SDA ($r_S = -0.406$, $p = 0.017$). Higher bacterial contamination in VRBA is related to greater contamination in MAC ($r_s = 0.796$, $p < 0.001$), to higher azole resistance values in VCZ ($r_S = 0.449$, $p = 0.008$). Higher bacterial contamination in MAC is related to higher azole resistance values in VCZ ($r_S = 0.447$, $p = 0.008$). Greater azole resistance values in VCZ are related to higher values in PSZ ($r_S = 0.491$, $p = 0.003$) (Fig. 7; Supplementary material Table S7).

No significant correlation was detected between bacterial and fungal contamination and azole resistance with total area (m^2), indoor area and number of animals (p's>0.05) (Supplementary material - Table S7). Although statistically significant, correlations of moderate to weak strength should be interpreted with caution. Their significance indicates that they are unlikely to be due to chance.

PCA revealed four major components explaining 71.6% of the total variance (Table 3), corresponding to gradients of bacterial counts, fungal contamination, and antifungal resistance, highlighting distinct but complementary microbiological processes structuring the dataset, as explained below (Table 3). The components are: 1) component 1 (PC1), which explains 21.3% of the overall variation, consisting of VRBA, MAC and TSA (bacterial contamination gradient). High RC1 values reflect samples with a higher overall bacterial contamination. ii) Component 2 (PC2), which explains 19.7% of the global variation and consists of MEA, DG18, and DG18 37 °C (fungal contamination); iii) Component 3 (PC3),

Table 2
Comparison of bacterial and fungal contamination and azole resistance between the two types of contexts. Mann-Whitney test results.

	Culture media	Context	N	Ranks		Test Statistics		Effect size
				Mean Rank	Sum of Ranks	Mann-Whitney U	p	
Bacteria	TSA	Dairy farms	24	32.00	768.00	180.000	0.005	0.48
		Feedlot farms	27	20.67	558.00			
		Total	51					
	VRBA	Dairy farms	24	27.42	658.00	290.000	0.519	0.11
		Feedlot farms	27	24.74	668.00			
		Total	51					
	MAC	Dairy farms	24	27.06	649.50	298.500	0.623	0.08
		Feedlot farms	27	25.06	676.50			
		Total	51					
Fungi	MEA	Dairy farms	24	26.67	640.00	308.000	0.761	0.05
		Feedlot farms	27	25.41	686.00			
		Total	51					
	DG18	Dairy farms	24	25.58	614.00	314.000	0.846	0.03
		Feedlot farms	27	26.37	712.00			
		Total	51					
	DG18 (37 °C)	Dairy farms	24	25.25	606.00	306.000	0.726	0.06
		Feedlot farms	27	26.67	720.00			
		Total	51					
Azole resistance	SDA	Dairy farms	14	15.25	213.50	108.500	0.223	0.21
		Feedlot farms	20	19.08	381.50			
		Total	34					
	ITZ	Dairy farms	14	17.00	238.00	133.000	0.403	0.14
		Feedlot farms	20	17.85	357.00			
		Total	34					
	VCZ	Dairy farms	14	16.75	234.50	129.500	0.511	0.11
		Feedlot farms	20	18.03	360.50			
		Total	34					
	PSZ	Dairy farms	14	18.21	255.00	130.000	0.232	0.20
		Feedlot farms	20	17.00	340.00			
		Total	34					

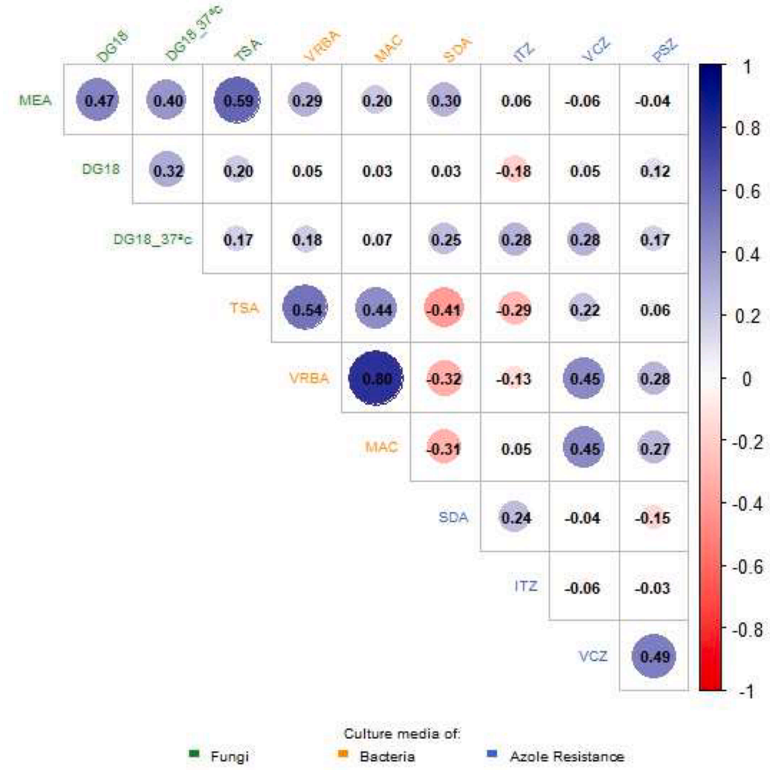

Fig. 7. Correlation matrix between fungal, bacterial and azole resistance contamination in different culture media. Spearman correlation coefficient results.

Table 3
Principal component analysis (PCA*).

	PC1	PC2	PC3	PC4
TSA	0.518			
VRBA	0.880			
MAX	0.907			
MEA		0.797		
DG18		0.726		
DG18 at 37 °C		0.743		
SDA				0.657
ITZ				0.830
VCZ			0.856	
PSZ			0.836	
Eighenvalue	2.125	1.968	1.578	1.491
Proportion of Variance	0.213	0.197	0.158	0.149
Cumulative Variance	0.213	0.409	0.567	0.716

*PCA with VARIMAX rotation and Keiser normalization. Eighenvalues greater than 1. Facyor loadings greater than 0.5.

which explains 15.8% of the global variability, consisting of VCZ and PSZ, which corresponds to the fungal resistance profile. iv) Component 4 (PC4), which explains 14.9% and consists of ITZ and SDA, corresponding to selective environments.

PERMANOVA revealed a statistically significant difference in overall microbial profiles between groups ($F = 3.40$, $R^2 = 0.065$, $p = 0.015$). However, PERMDISP indicated substantial heterogeneity in multivariate dispersion ($F = 16.19$, $p < 0.001$), suggesting that the observed effect is primarily driven by differences in within-group variability rather than by shifts in centroid location. As shown in Fig. 8, variability is considerably greater in feedlot farms than in dairy farms (Fig. 8).

Consistently, PERMANOVA performed on PCA scores showed no significant separation between groups ($p = 0.326$). Observation of the boxplots (Fig. 9) indicates that the central tendency of the PCA scores is very similar across farm types.

PERMANOVA on PCA scores revealed significant differences among sample types ($F = 7.37$, $R^2 = 0.39$, $p = 0.001$), with homogeneous variances confirmed by PERMDISP ($F = 0.54$, $p = 0.707$). Swab samples were the most distinct, differing from feed ($R^2 = 0.41$, $p\_adj = 0.002$), EDC ($R^2 = 0.40$, $p\_adj = 0.002$), and bedding ($R^2 = 0.26$, $p\_adj = 0.003$). Feed and EDC also differed ($R^2 = 0.18$, $p\_adj = 0.003$), while EDCT overlapped with bedding and feed ($R^2 \leq 0.11$, $p\_adj > 0.05$), suggesting an intermediate microbial profile (Supplementary material – Table S8).

The components that differed between sampling types were: i) PC1 (total bacterial contamination), between swabs and EDC ($p < 0.001$) and feed ($p < 0.001$); ii) PC3 (fungal resistance profile) between EDC and Feed ($p = 0.039$). These differences reflect different bacterial contamination as well as highlight distinct selective regimes shaping fungal communities. In both cases, EDC shows negative scores and less variability (Fig. 10), suggesting lower bacterial contamination and a lower fungal resistance profile.

4. Discussion

4.1. Sampling methods, assays employed and data analyses performed

A comprehensive field sampling campaign was carried out to provide a more accurate and realistic characterization of the microbial contamination worst-case exposure scenario (AIHA, 2005; Eduard, 2009), following the multi-method protocols demonstrated by Viegas and colleagues in waste sorting facilities, fitness centers and bakeries (Viegas et al., 2020a, 2023, 2024). Passive sampling methods combined with material recovery were selected for logistical reasons (facilitating extraction and shipment) and to enable extended sampling periods, thereby capturing temporal variations in environmental conditions (AIHA, 2005; Dias et al., 2025; Macher, 2001; Manibusan et al., 2022; Noss et al., 2008; Viegas et al., 2024; Vincent-Hubert et al., 2021). Indeed, these approaches allow for longer sampling durations by promoting dust accumulation over days or weeks, thus providing a more integrated representation of fungal contamination (Cervantes et al., 2025), in contrast to active sampling methods (air samples), which

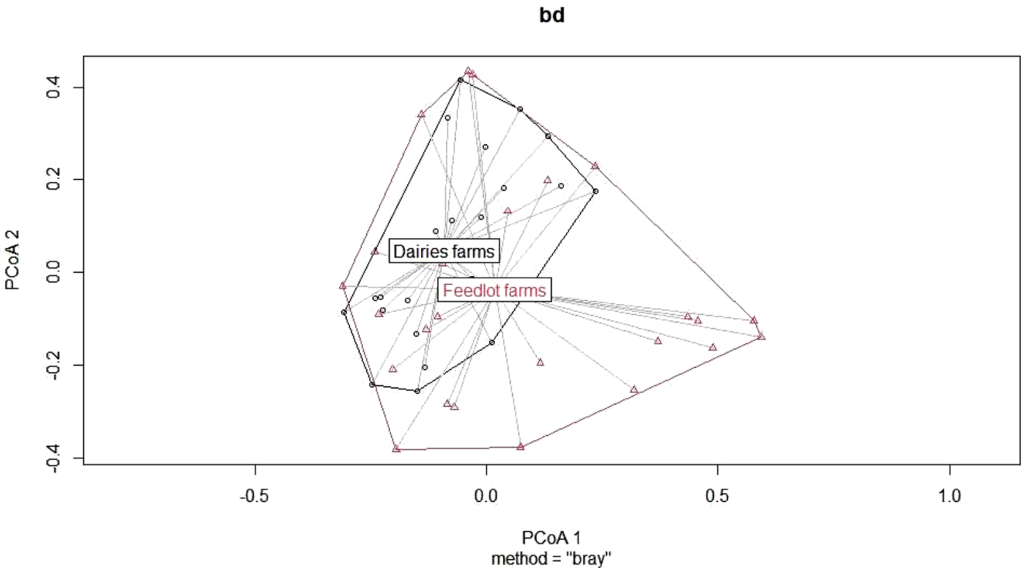

Fig. 8. Dispersion by farm type. bd - betadispersion.

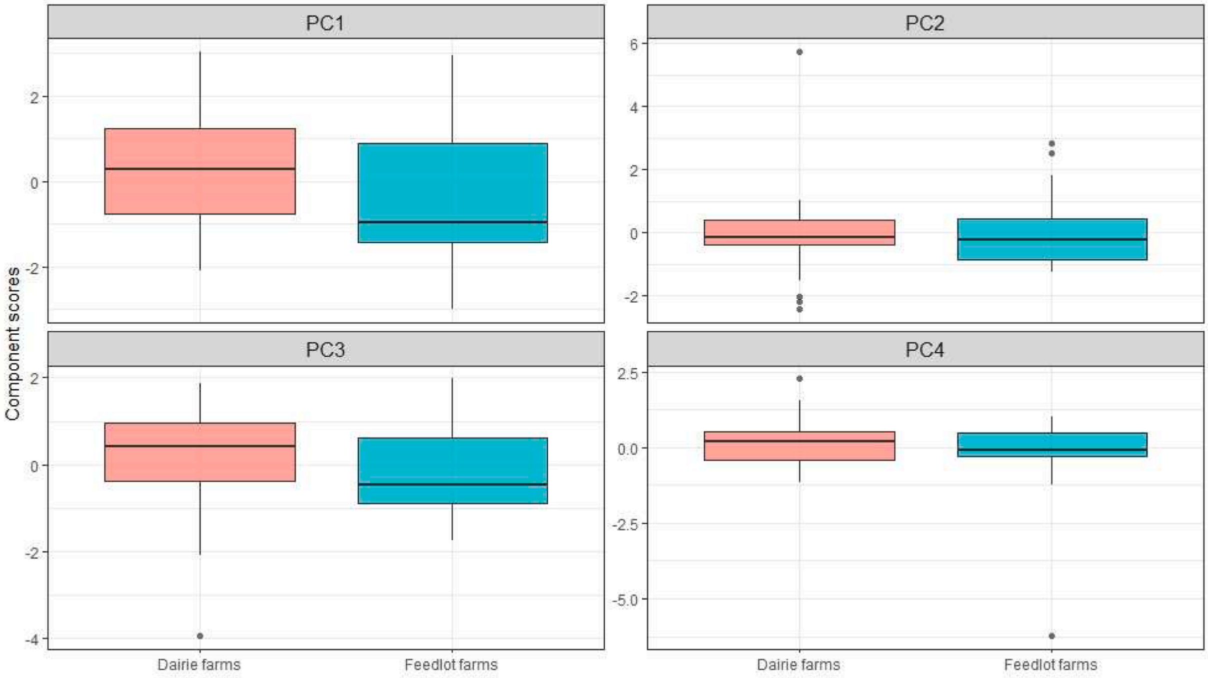

Fig. 9. Comparison of PCA scores between farm types.

collect air over only a few minutes and therefore reflect short-term conditions.

The value of a multi-sampling approach was evident: microbial communities differed by sample type, with swabs being the most distinct, feed and EDC showing moderate differences, and EDCT exhibiting an intermediate profile. This strategy also revealed greater microbial diversity in feedlot farms compared with dairy farms and lower variability in EDC samples relative to other methods, although the wider use of this sampling method in different occupational studies (Viegas et al., 2024; Dias et al., 2025).

In addition, applying culturomics using several culture media enabled a broader characterization of the microbial diversity present in the environments under evaluation (Dias et al. 2024, 2025; Viegas et al., 2024), as it was corroborated with PCA results showing that the dataset is structured by different microbial components, each contributing in a distinct but complementary way. Furthermore, the combined use of both culture-based and molecular assays (qPCR) helps to overcome the limitations inherent to each method (Whitby et al., 2022) as demonstrated in this study. The correlation analyses revealed an elevated risk of bacterial contamination in dairy farms. Furthermore, the trends observed across different culture media were consistent with those reported in previous studies conducted in various settings (Dias et al., 2025; Viegas et al., 2020a,b), whereas the lack of correlation between microbial counts and animal density observed in other studies (de Rooij et al. 2019; Gladding et al. 2020) was not evident in the obtained results.

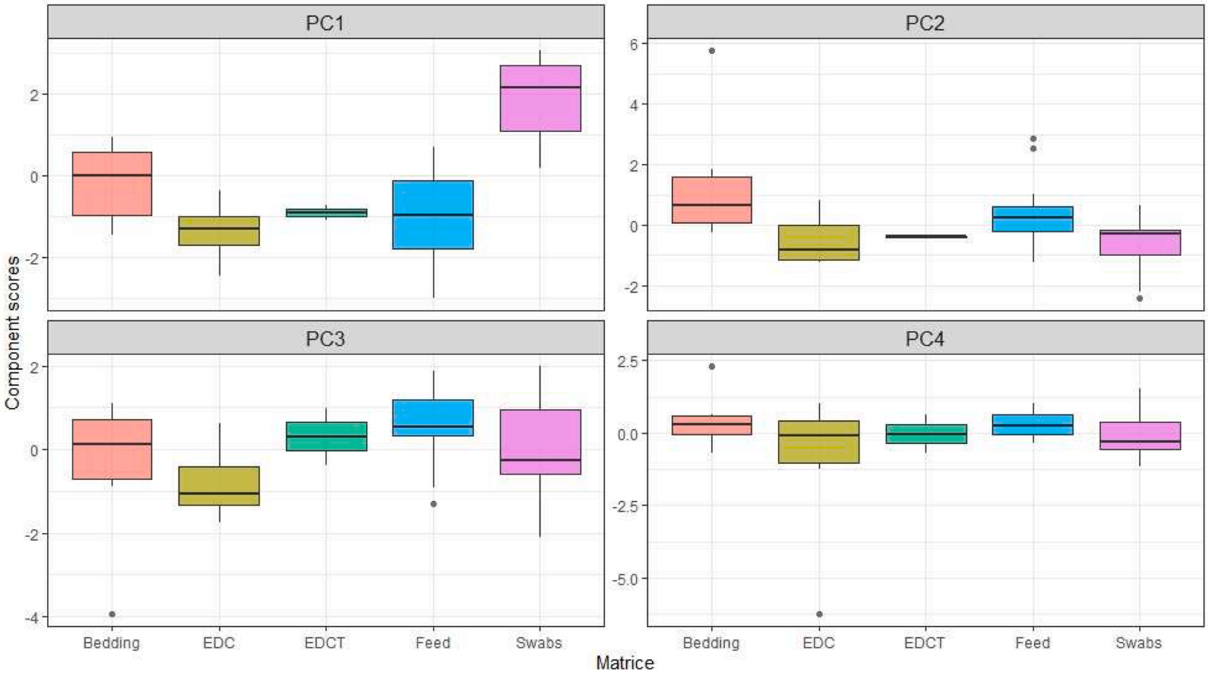

Fig. 10. Comparison of PCA scores between sampling typesRegarding species biodiversity by matrix, in bedding, the greatest diversity was detected in dairy farms in DG18 (27 °C) (H = 1.55, D = 4.15). In EDCs, the greatest diversity was detected in feedlot farms in DG18 (37 °C) (H = 1.69, D = 5.00). In feed, the greatest diversity was detected in dairy farms in DG18 (27 °C) (H = 1.60, D = 3.98). Finally, in swabs, the greatest diversity was detected in dairy farms in DG18 (27 °C) (H = 1.20, D = 2.80) (Supplementary material – Table S9).

4.2. *Bacterial contamination*

Bacterial contamination across the five cattle farms revealed significant influences of the production system on microbial contamination. Dairy farms (V1, V4) consistently exhibit higher counts across multiple sample types (EDCs, bedding, feed, and surfaces). Dairy farms are typically confined spaces with high animal density, leading to elevated humidity levels (Quintana et al., 2020). These conditions promote the spread and proliferation of microbes on surfaces, in feed and bedding, and throughout the air (Sanz et al., 2015). Consequently, the lower microbial variability observed in dairy farms likely reflects their more confined environment compared with feedlot farms.

The results from the EDCs confirm that the air in the dairy farms, particularly V1 and V4, acts as a continuous and significant reservoir of bacterial contamination. Bacterial contamination on TSA, VRBA, and MAC media confirms the presence of airborne contaminants with a high general aerobic population and Gram-negative bacteria and fecal coliforms, corroborating EDCs as a useful method to assess bacterial contamination in animal production environment (Luiken et al., 2022; Rittscher et al., 2023).

Although with a low number of samples, the pioneering EDCT personal samplers (Pena et al., 2025; Madsen et al., 2022) revealed that the microbial exposure for a worker over a single 8-hour shift (e.g., 2.20 x 10^4 CFU.m-2.day-1 on TSA) was ten times higher than the cumulative environmental deposition measured by the EDC. Since EDCTs are in close contact with contaminated clothing, the combination of high respirable and clothing-borne contamination makes it imperative to establish and enforce strict hygiene protocols (Kóvacs et al., 2025). This includes dedicated decontamination (Duchaine, 2016) of work clothing to prevent employees from carrying this significant microbial burden home, thereby mitigating the risk of disseminating potential pathogens beyond the farm environment and protecting both worker and public health (Kanwar et al., 2018).

Despite the widespread and high overall bacterial contamination, targeted surveillance for a key antimicrobial-resistant pathogen yielded a negative result, as Methicillin-resistant *Staphylococcus aureus (*MRSA) was not detected in the EDCT samples. The negative finding demonstrates the utility of this sampling matrix for effective screening of specific pathogens like MRSA in occupational settings. Nevertheless, the continued application of such targeted screening remains of utmost importance for the early detection and prevention of community spreading of resistant pathogens (Kanwar et al., 2018).

Bedding material seems to be the highest contaminated matrix, particularly with Gram-negative, as in V2. VRBA indicates the level of recent fecal contamination by selecting coliforms, confirming that the bedding is a primary reservoir for manure-derived bacteria like *Escherichia coli* (Leclercq et al., 2002; Ferraz et al., 2020). In contrast, MacConkey Agar provides a broader assessment of the Gram-negative population, including both fecal coliforms and environmental bacteria that thrive in the bedding (Leclercq et al., 2002). This creates a significant animal health risk, as the bedding acts as a constant source of exposure to pathogens that can cause infections like environmental mastitis (Patel et al., 2019), directly linking these microbiological results to critical issues of sanitation and herd management on the farm (Rowbotham and Ruegg, 2016).

The feed analysis revealed substantial bacterial contamination, indicating a high aerobic mesophilic population that thrives on the nutrient-rich substrate. The consistent growth on specific media suggests a significant portion of these microbes are Gram-negative bacteria, including fecal coliforms, which could indicate a sanitary compromise during handling, storage, or from contaminated ingredients (Sanderson et al., 2005). The significant contamination levels identified at V4 align with evidence that feed commodities act as a primary source and amplifier of microbial burden in dairy environments (Sanderson et al., 2005), highlighting it as a potential critical control point for intervention.

The surface swabs results reveal high bacterial contamination, with median values reaching up to 1.20 x 10^6 CFU.m-2 for general aerobes (TSA) and 1.09 x 10^6 CFU.m-2 for coliforms (MAC), demonstrate that critical surfaces are heavily contaminated. This is particularly evident in dairy farms V1 and V4, which consistently showed elevated counts. The substantial recovery of bacteria on VRBA and MAC confirms that this surface contamination includes Gram-negative organisms and fecal coliforms, posing a potential direct and ongoing risk for cross-contamination to animals and the broader environment (Luyckx et al., 2015).

4.3. Fungal contamination

The patterns of fungal contamination observed in all the examined cattle farms revealed a great level of variability which depended on production system (feedlots or dairies), location, and type of collected sample. The fungal profiles of feedlots and dairies were different, and dairy farms had a higher percentage of fungal contamination in most matrices, especially the swabs and feed samples, although feedlots presented more microbial diversity in general. These results are consistent with the literature that indicates that cramped and damp conditions support fungal growth (Gomes et al., 2025; Viegas et al., 2016). DG18 medium at 27 °C supported the highest colony counts, revealing the predominance of mesophilic fungi, while growth at 37 °C demonstrated the potential of thermotolerant or opportunistic pathogenic species, including *Aspergillus* and *Fusarium* (Dias et al., 2022). The elevated fungal counts in swab samples suggest that surfaces act as reservoirs, facilitating cross-contamination (Salaheen et al., 2015). The bedding material presented detectable fungal contamination, suggesting that the organic residues present can act as a stable substrate supporting spore persistence and serving as a potential reservoir of particles that can be re-suspended into the air through animal movement and bedding handling (Pusz et al., 2015; Gomes et al., 2022). The results support the use of multi-medium culturomics approaches (MEA and DG18) to achieve a comprehensive characterization of viable fungi under different environmental stress conditions (Viegas et al., 2021; Gomes et al., 2022). The observed variability between farms reinforces the impact of bedding management on fungal burden, emphasizing the need for integrated environmental monitoring to mitigate exposure for both animals and workers. The fungal community structure identified in both dairy and feedlot farms revealed a dominance of *Aspergillus, Penicillium, Cladosporium*, and *Mucor* genera, consistent with previously reported profiles in agricultural and livestock environments (Viegas et al., 2019b, 2019c). These genera are common airborne and feed-associated contaminants with implications for air quality, animal health, and mycotoxin production (Szulc et al., 2020) The distribution patterns suggest that feed and bedding act as key reservoirs for *Aspergillus* spp., particularly sections *Fumigati* and *Flavi*, which are associated with toxigenic and clinical relevance (Cervantes et al., 2025; Gomes et al., 2022). Their detection across multiple matrices—including EDCs, bedding, and feed—demonstrates the wide spreading and adaptability of these fungi. The high frequency of *Penicillium* spp. observed may be associated with contamination originating from stored materials and feedstocks, a relationship that has been consistently reported in recent studies (Topi et al., 2024; Viegas et al., 2019b, 2019c). The dominance of thermotolerant species such as *Aspergillus* sections *Fumigati* and *Nigri* at 37 °C raises occupational and veterinary health concerns due to their role in respiratory mycoses and invasive infections under immunocompromised conditions (Walsh et al., 2008). The identification of *Fusarium verticillioides* in these matrices supports the connection between environmental fungal reservoirs and mycotoxin contamination pathways, underscoring the interconnection between fungal ecology and food safety within a One Health framework (EFSA, 2018).

4.4. Azole resistance screening

The azole resistance screening revealed the environmental occurrence of fungi able to grow under azole-supplemented conditions, particularly in bedding and EDC samples. Although growth was limited, the detection of colonies on ITZ, VOZ, and POZ plates indicates the presence of tolerant or potentially resistant strains within the farming environment. This is consistent with global trends linking agricultural azole use to the selection of resistant *Aspergillus fumigatus* strains (Chowdhary et al., 2013; Gonçalves et al., 2021). The identification of *Mucor* spp., *Geotrichum* spp. and *C. sitophila* in azole-supplemented media suggests that non-*Aspergillus* genera also contribute to environmental persistence under antifungal pressure (Caetano et al., 2018; Viegas et al., 2022). Feedlots farms had higher resistance diversity than dairies, which could be associated with the difference in exposure history of fungicides, feed composition, or substrate management (EFSA, 2025) and the fact they less enclosed than dairy farms. Their presence in rural settings is also interesting though the general resistance levels were low as their spread could be promoted by the growth of dust and feed-handling operations (Pusz et al. 2015). The presence of both the resistant and susceptible isolates in a single microenvironment indicates a continuous process of environmental selection that could be in a result of using fungicides in crop production or storing the feed (Hui et al., 2024). Furthermore, due to the fact that feed is prepared manually on-site, the grinding, mixing, and handling process may contribute to fungal further growth and the development or propagation of resistant strains even further, and it can be concluded that the sources of contamination are likely to be even more extensive than storage conditions to include farm-level feed preparation practices as well (Pusz et al., 2015). Continuous surveillance combining culture-based azole screening and molecular detection of resistance markers (e.g., *cyp51A* mutations) is recommended to monitor the evolution of environmental resistance reservoirs (Burks et al., 2021). In fact, in a previous study where a Portuguese dairy farm was also assessed, an isolate obtained from an air sample presented the mutation TR_{34}/L98H in the *cyp51A* gene, corroborating the need of targeted surveillance in this specific setting (Gonçalves et al., 2021). These data reinforce the need of a One Health approach when addressing antifungal resistance, since not only clinical contexts but also in agricultural settings this could be observed, with environmental sources contributing to human and animal exposure in both contexts (Burks et al., 2021).

4.5. Mycotoxins

FB1 and FB2 were the most prevalent mycotoxins detected across all sample types (feed, environmental dust, and bedding) collected from both feedlot and dairy farms, with the highest concentrations consistently found in feed samples. This pattern was also observed for most of the other mycotoxins detected in our study, with the only exception being ochratoxin A and B, which were detected in just one bedding sample (straw). Straw is commonly used for bedding but data available on straw contamination is still scarce with some previous reports showing that ochratoxin can be present, but with low frequency of detection, in combination with other mycotoxins more frequently detected such as DON, ZEA, enniatins, aflatoxins and much more (Drakopoulos et al., 2021; Sinha et al., 2001). In opposite, the presence of mycotoxins in animal feed is well documented, and extensive global surveys have shown that mycotoxin co-contamination is common, often exhibiting regional trends influenced by climate and weather conditions (EFSA 2014; Gruber-Dorninger et al., 2019; Muñoz-Solano and González-Peñas, 2023). Our findings align with these reports, as only one feed sample in our study contained a single mycotoxin, while all others presented multiple mycotoxins. This is particularly relevant, as EU maximum limits defined for mycotoxins in feed are established based on single-compound exposure, as in most of other regulatory frameworks addressing chemical safety, and do not account for the frequent co-contamination that results in exposure to mycotoxin mixtures. In addition, not all the mycotoxins measured commonly in feed (e.g. STER, measured in 12.5% in our feed samples) have maximum levels defined in the EU regulation (EU, 2019).

Furthermore, climate change is expected to exacerbate the risk and severity of feed contamination by promoting fungal growth, increasing the frequency of extreme weather events, and causing crop stress. In our study, we did not try to find a correlation between fungal and mycotoxin contamination, since mycotoxins can be present in the environment long after fungi have been eliminated due to adverse environmental conditions. Also, not all the fungi produce mycotoxins (Halstensen, 2008).

Previously, in a study developed in a Portuguese dairy farm also feed showed frequent and multiple contamination (Viegas et al., 2019c). In contrast, bedding and environmental dust samples showed less frequent and diverse contamination demonstrating that feed is the most relevant mycotoxins contamination source of this workplace environment and representing also a food safety issue due to animals' exposure through feed ingestion. In the same way, our study identified fungal and mycotoxin contamination across different environmental matrices, including surfaces, EDCs, feed, and bedding, that interact with animals and workers. These findings indicate that different exposure routes exist (e.g. ingestion in case of the animals when eating the feed; inhalation for workers when handling the feed), with potential health impacts for both animals and workers, and may also indirectly affect the safety of dairy products, representing a food safety concern. This represents an important challenge to be addressed since the actual scenario of climate change will continue to bring difficulties when aiming to prevent contamination of food and feed by mycotoxins (EEA, 2025).

4.6. Study limitations

Despite the comprehensive sampling approach adopted, one limitation of the study was the absence of active air sampling, which prevented direct comparison with other investigations that employed this method and providing CFU.m-3 results. However, passive sampling methods, such as EDCs, EDCTs and settled dust, are effective in identifying long-term fungal reservoirs on surfaces and in dust (Cervantes et al., 2025; Whitby et al., 2022). Additionally, correlating particulate matter levels with microbiological data could have provided deeper insights into the environmental conditions across the different farms. However, previous studies have reported a lack of correlation between these parameters, likely because only a small fraction of airborne microorganisms is culturable, thereby highlighting the potential underestimation of results obtained through culture-based methods (Whitby et al., 2022). A more detailed assessment of bacterial contamination would therefore be crucial to address the existing knowledge gap regarding microbiological exposure in this occupational setting and for future studies, the integration of high-throughput sequencing could be considered to explore the broader microbiome and resistance gene diversity, which may provide complementary ecological insights beyond the scope of occupational exposure risk assessment (Whitby et al., 2022).

The limited number of participating farms may have reduced the extent to which the findings represent the sector in Portugal. Despite this limitation, our primary goal was to demonstrate the importance of adopting a One Health approach to effectively address microbiological contamination in animal and dairy production systems.

5. Conclusions

Using a comprehensive sampling approach enabled the identification of the matrice most critical for microbial and mycotoxin contamination, thereby highlighting potential contamination sources within farms that represent the worst-case exposure scenario in Northern Portugal.For bacterial contamination, the bedding material was the matrix with the highest levels, whereas for fungal contamination the swabs and feed showed the greatest burden. In the case of mycotoxins contamination, feed is the main source of workplace environment contamination creating conditions for animals and workers exposure. Moreover, combining culture-based methods (culturomics) with molecular tools (qPCR) provided added value by improving accuracy in risk characterization. The obtained results highlight the widespread distribution of *Aspergillus* across diverse environmental matrices in the studied settings and underscore the need for comprehensive and integrated intervention.

The azole-resistance screening and mycotoxins results further highlight the importance of monitoring how environmental resistance reservoirs and mycotoxins contamination evolve in a climate change scenario and underscore the need to apply a One Health approach to define interventions that tackle, simultaneously, both public health concerns. An example of an intervention that can reduce both animals and workers exposure to mycotoxins could be a stricter selection of feed suppliers, choosing the ones with lowest contamination, including low diversity of the mycotoxins present and low levels of contamination, of regulated and not regulated mycotoxins. Considering climate change, monitoring the geographical location of feed suppliers could serve as an additional criterion, given that environmental conditions and contamination risks may shift from year to year.

CRediT authorship contribution statement

Carla Viegas: Writing – original draft. **Ana Filipa Gouveia:** Writing – original draft, Formal analysis. **Renata Cervantes:** Writing – original draft, Formal analysis. **Pedro Pena:** Writing – original draft, Formal analysis. **Elisabete Carolino:** Writing – original draft, Formal analysis. **Magdalena Twarużek:** Writing – original draft, Formal analysis. **Susana Viegas:** Writing – original draft, Supervision, Funding acquisition, Formal analysis, Conceptualization.

Declaration of competing interest

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

I have full control of all primary data and permission is given to the journal to review the data if requested.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.hazadv.2026.101230.

Data availability

Data will be made available on request.

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