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DIVERSITY OF FEEDSTUFF MYCOBIOME IS DETERMINED BY FEEDSTUFF COMPOSITION AND CULTURING CONDITIONS

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ABSTRACT

Fungal contamination of feedstuffs is a serious threat impacting domestic animals via the production of mycotoxins. This study surveys the mycobiome diversity of some Egyptian feedstuffs in relation to the type of feed and some culturing conditions viz. type of nutrient medium and incubation temperature. Poultry, rabbit and cattle feeds from the Nile Delta, Egypt were assayed for nutritional components and fungal contamination. Feed inoculants were incubated on potato dextrose agar (PDA) and malt extract agar (MEA) at 25 °C and 30 °C to specify the appropriate culturing conditions for each feedstuff mycobiome. Poultry feed was relatively rich in protein, insoluble sugars and fat; cattle feed was rich in soluble sugars, fiber, moisture and ash; while rabbit feed had moderate composition. PDA rather than MEA favored maximal species richness, species diversity and germ load of feedstuffs. Despite its low fungal count, rabbit feed has high species diversity and species richness but cattle feed exhibited the opposite pattern. The most dominant fungal division was the Ascomycota, particularly *Aspergillus* spp. and *Monascus ruber*, followed by Zygomycota with a rarity of Basidiomycota. Some fungal species were confined to certain feedstuffs. The three fungal divisions exhibited different preferences for incubation temperature and nutrient medium. The low moisture content and fungal load refer to the hygienic nature of feedstuffs. Nevertheless, the prevalence of *Aspergillus* species points to a potential mycotoxin production. Each feedstuff has a unique fungal community, which is further screened by the culturing conditions. The species diversity and species richness of a feedstuff might contrast its fungal count. This study pays attention towards the consequences of seeping of mycotoxins, produced by the feed-born fungi, into the food chain from domestic animals to the humans.

Keywords: Animal feed, Fungal diversity, Species richness, Incubation temperature, Culture media

INTRODUCTION

Currently, feed production is an established industry subjected to rigorous criteria to produce stuff formulae appropriate for each type of domestic animals. Recently, feedstuffs are manufactured by blending different ingredients in the right proportions to ensure the highest quality to enhance the health of domestic animals and increase their productivity of meat, milk and eggs. A balanced feed formula must contain adequate supply of carbohydrates, lipids and proteins, in addition to the essential vitamins and dietary minerals and fibers (**Damerow, 2012**). Animal feeds can be classified into three main types: fodder (usually the dried plant foliage and roughages), forage (the palatable and grazable whole plant parts) and compound feeds, which are usually produced in the form of pellets and crumbles amended with nutrients and vitamin supplements (**Karangiya** ***et al.*****, 2016; Martin** ***et al.*****, 2017; Kim** ***et al.*****, 2020**).
Unfortunately, contamination of food- and feed-stuffs with saprophytic fungi is widespread and represents a serious health problem, particularly in the tropics where the warm and humid climate provides optimum conditions for the rapid proliferation of many opportunistic fungal species. The problem is impressive in light of the fact that the ease of fungal contamination of foods and feeds in the developing countries of the tropics is associated with low public concern towards the problem of contamination and the hygienic measures of handling and storage of food- and feed-stuffs. Saprophytic fungi are ubiquitous in nature; they are armed with extensive machinery of enzymes that enable them to colonize diverse substrates (**Gow** ***et al*****., 2002**). Food and feed commodities are usually contaminated with a multitude of fungal species that can invade them either during crop growth, at harvesting or during postharvest storage. The occurrence of fungal propagules and the probable subtle fungal growth on foods and feeds have adverse consequences on human and animal health since deterioration of the nutritional quality of the agricultural produce and production of mycotoxins can probably precede detection of any visible fungal growth (**Cegielska-Radziejewska** ***et al*****., 2013**).
The microbiome diversity of a feedstuff depends on several factors, among which is the feed moisture content, pH, oxygen tension and the feed nutritional composition (**Gherbawy** ***et al*****., 2020**). Regarding the nutritional factor, it has been claimed that fungi, upon invading a suitable substrate can consume its nutrient resources (**Okoli** ***et al*****., 2006**) with probable production of mycotoxins, thus leading to serious economic losses and health hazards (**Greco** ***et al*****., 2014**). According to their ability to produce toxins, molds that contaminate food- and feed-stuffs can be sorted as mycotoxigenic or non-mycotoxigenic. The mycotoxigenic fungi, under favorable conditions, may lead to mycotoxin buildup in the feedstuff to injurious levels for farm animals and human health. Secondary metabolites produced by species of *Fusarium*, *Aspergillus* and *Penicillium* are of high toxicity to humans and domesticated animals (**Kumari** ***et al*****., 2021).** Therefore, the occurrence of fungi or their spores in food- and feed-stuffs can be taken as an indicator of potential presence of mycotoxins (**Ariyo** ***et al*****., 2013**). Among the frequent and most potent mycotoxins are aflatoxins, zearalenone, fumonisins, ochratoxins and trichothecenes (**Njumbe Ediage** ***et al*****., 2011**). Mycotoxins taken up by the animal upon feeding on a polluted feed can cause a multitude of disorders including malnutrition and disturbed metabolism, disturbance of the endocrine and exocrine systems and suppression of the immune system. These consequences ultimately result in loss of appetite, poor weight gain, decrease in egg and milk production and mortality. Further, the metabolized mycotoxins can linger in milk, meat, and eggs where they can contribute to human consumption of mycotoxins as carry-over effects from contaminated feed (**Kumari** ***et al*****., 2021**).
Therefore, the identification and characterization of spoiling molds is essential for the control of food and feed contamination by these microorganisms and for awareness of the potential mycotoxin hazard in feedstuffs. The present study aims to monitor the incidence of fungal contamination in different types of feedstuffs of widespread use in the Nile Delta, Egypt. The subtropical climate of Egypt - characterized by high humidity and warm temperature - can aggravate the problem of spoilage of agricultural crops as a result of contamination with many toxic and non-toxic fungi (**Kana** ***et al*****., 2013; Dubale** ***et al*****., 2014**). The potential fungal community of feedstuffs might depend on the type of feed as well as on the culturing conditions such as nutritional composition, pH of the culture medium and incubation temperature. The question is: are certain fungal groups confined to certain feedstuffs? In addition, it is essential to identify the culturing conditions favorite for the development of the maximal fungal load of a given feedstuff in the laboratory.

MATERIALS AND METHODS

Feedstuffs

Three feedstuffs of wide use in Egypt (cattle feed, rabbit feed and poultry feed) were collected, each from four different localities within the Nile Delta, Egypt during the period from October 2018 to March 2019; each locality produces its own brand of feedstuff. The feedstuffs were stored in airtight bags at -10 °C until used for chemical analysis as well as for development of fungal community on growth media. Detailed information on type, brand and site of collection of feedstuffs are presented in Table 1.

Culture media

Potato dextrose agar (PDA) medium was prepared by mixing 200 g potato extract, 20 g glucose and 20 g agar in 1 l distilled water. Malt extract agar (MEA) medium was prepared by mixing 20 g malt extract, 6 g peptone, 20 g glucose and 20 g agar in 1 l distilled water. Yeast extract sucrose agar (YES) medium was prepared by mixing 20 g yeast extract, 150 g sucrose, 0.5 g $MgSO_4.7H_2O$, 0.01 g $ZnSO_4.7H_2O$, 5 mg $CuSO_4.5H_2O$ and 20 g agar in 1 l distilled water. Czapek yeast autolysate agar (CYA) medium was prepared by mixing 1 g K_2HPO_4, 10 ml Czapek concentrate, 5 g yeast extract, 30 g sucrose and 20 g agar in 1 l distilled water. Czapek concentrate was prepared by mixing 30 g $NaNO_3$, 5 g KCl, 5 g $MgSO_4.7H_2O$, 0.1 g $FeSO_4.7H_2O$, 0.1 g $ZnSO_4.7\ H_2O$ and 50 mg $CuSO_4.5H_2O$ in 1 l distilled water. Streptomycin and chloramphenicol, each at a dose of 50 mg l^{-1}, were added to PDA and MEA media as bacteriostatic agents. Culture media were autoclaved at 15 psi (0.1 MPa) and 121 °C for 20 minutes, and dispended in 12-cm Petri dishes before inoculation with feed suspensions.

Estimation of moisture content

Before grinding and quickly after collection of feedstuffs, an aliquot of about 3 g of the native feed was dried in an air-forced oven at 100 °C for 24 h till constant weight and moisture content was calculated by expressing weight loss upon drying as % of the original weight (**AOAC, 1997**).

Chemical analysis of feedstuffs

The feed pellets were collected form stores in clean bags, then ground into a fine powder by using a clean sterilized mill under aseptic laboratory conditions to avoid incidence of air-born contaminants, and the resulting powders were stored in airtight containers for use in chemical analysis and monitoring of fungal community of feedstuffs.

Table 1 Type, brand and site of collection of feedstuffs used in the study

Type of feedstuff	Site	Brand
Poultry feed	1. Damietta	Hodyhed company
	2. Menoufia	Holman feed company
	3. Menoufia	Herman feed-RONT VITA company
	4. Dakahlia	Cooperative association
Rabbit feed	1. Gharbiya	Hefnawi rabbit farms
	2. Sharqia	Extra animal feed company
	3. Alexandria	Alexandria company
	4. Damietta	Al-Ghanim company
Cattle feed	1. Damietta	El-Shennawy factory
	2. Damietta	Brawn-Mabrouka company
	3. Cairo	Cairo company
	4. Dakahlia	New Bermbal feed factory

Estimation of protein content

Protein content of feedstuffs was extracted by vortexing 0.1 g of the powdered feed in 5 ml of 0.15 N NaOH for 5 minutes, followed by steeping at 4 °C for 24 hours. The slurry was centrifuged at 10,000 rpm for 10 minutes. Protein content was assayed by adding 0.1 ml of the supernatant to 0.9 ml of 0.15 N NaOH, followed by the addition of 5 ml of the Coomassie brilliant blue (G250) reagent and incubation of the mixture at room temperature for 30 minutes. Absorbance was measured at 595 nm, and protein content was calculated with reference to bovine serum albumin (BSA) calibration curve in the range of 0–100 µg ml^{-1} (**Bradford 1976**).

Estimation of carbohydrate fractions

For extraction of soluble sugars, about 0.01 g of the powdered feed was soaked in 1 ml of 80% ethanol for 24 hours at room temperature (**Schortemeyer *et al*., 1997**). The debris was removed by centrifugation at 10,000 rpm for 10 minutes, and the extract was evaporated on water bath at 35–40 °C. The resulting residue was re-dissolved in 1 ml distilled water for determination of total soluble sugars (TSS). The debris was re-suspended in 1.6 M perchloric acid on a water bath at 70 °C for 2 h, the slurry was centrifuged at 10,000 rpm for 10 minutes, and insoluble sugars (IS) were assayed in the supernatant. Both soluble and insoluble sugars were estimated by the anthrone method adopted by **Schlüter and Crawford (2001)**. An aliquot of 0.1 ml of the aqueous feed extract was carefully mixed with 3 ml anthrone reagent (8.6 mM anthrone in 80% v/v H_2SO_4) and the mixture was heated for 10 minutes, cooled on an ice bath for 30 minutes and absorbance was recorded at 625 nm. Sugar content was calculated from a glucose calibration curve in the range of 0–100 µg. ml^{-1}.

Estimation of crude fats

Crude fats were extracted by steeping 3 g of the powdered feed in petroleum ether (40–60) in a Soxhlet apparatus for 8 hours. The debris was discarded, and the solvent was evaporated. The extract was completely dried at 105 °C for 30 minutes, and weight of the extracted lipids was recorded after cooling in a desiccator (**AOAC, 1997**).

Estimation of crude fibers and ash

An aliquot of 3 g fat-free powdered feed was mixed with 200 ml of 1.25% H_2SO_4, and the mixture was boiled for 30 minutes, filtered and washed 3–4 times with boiled distilled water. The washings were discarded, and 200 ml of 1.25% NaOH were added to the washed residue and boiled for 30 minutes. The mixture was filtered, and the residue was washed 3–4 times with boiled distilled water. Again, the washings were discarded, the washed residue was dried in an air-forced oven at 100 °C for 3–4 hours until constant weight and the oven-dry weight was recorded. The obtained dry matter was ignited in a muffle furnace at 550 °C for 4 hours (until a grey ash was obtained), then cooled in a desiccator and re-weighed to obtain the ash content (**AOAC, 1997**). The difference between the oven-dry weight and ash weight (loss-on-ignition weight) was expressed as a percentage of the oven-dry weight to estimate the % crude fiber content.

Isolation and purification of fungi from feedstuffs

Fungal isolation from feedstuffs was carried out using the dilute plate technique (**Pitt and Hocking, 2009**). For each type of feed, an aliquot of 5 g powder from each site of collection was dispersed in 45 ml distilled water with shaking for 30–60 minutes at room temperature in a rotary shaker at 150 rpm to homogenize the fungal propagules throughout the suspension. Serial dilutions of 10, 100 and 1000 folds were made, and 1 ml aliquot of each dilution was inoculated, in duplicates, on the surface of solidified PDA and MEA media in 12-cm Petri dishes. The cultures were incubated at two temperatures: 25 °C and 30 °C for 5–7 days. Fungal populations were identified to the level of species, and numbers of colonies of each species developed from the feed samples at the four sites of feed collection were summed to give the total number of colony-forming units (CFU g^{-1} feed dry weight). Subsamples of fungal colonies were cultured on PDA slants and incubated at 25 °C for 7 days, then an aliquot was stored at 4 °C and another aliquot was stored in 10% glycerol at -80 °C (**El-Gharabawy, 2016**).

Identification of fungal isolates

Fungal isolates were identified according to the morphological characters (both macro- and micro-scopic) according to the standard keys of **Barnett and Hunter (1998), Pitt and Hocking (2009)** and **Simmons (2009)**. The morphological characteristics included color of mycelium, presence of exudates, texture and reverse color; while the microscopic characteristics included shape, size, color and surface of conidia, conidiophores, vesicles, metulae, hyphae and mycelia. In particular, *Penicillium* spp. were additionally cultured on MEA, YES, and CYA for precise identification.

Statistical analysis

Statistical analysis was performed according to **Zar (1999)**. The data of chemical analysis of feedstuffs were processed as nested ANOVA, since each of the three types of feed was collected from four different localities. Mean separation was performed according to the Duncan's multiple range test at $P \leq 0.05$. Means of the three types of feed and those of the four sites of collection for each feedstuff were separated by lower-case and upper-case letters, respectively. The correlation among the different indices of diversity and the different feed components was summarized by employing Principal Component Analysis (PCA) using SPSS version 22. The extent of variability, among the different feed types as well as among the different sites of collection of each feed, was evaluated in terms of the coefficient of variation (CV).

$$CV = \frac{S \times 100}{\bar{Y}}$$

where S is the standard deviation and $\bar{Y}$ is the mean of the different feedstuffs/sites.

The Shannon index of diversity ($\grave{H}$) of fungal species was calculated according to the formula:

$\grave{H} = - \sum_{1}^{s} (ni/n) \log_2 (n_i/n)$

where: n_i is the number of colonies of the ith species, n is the total number of colonies and S is the total number of fungal species.

The evenness index ($\acute{J}$) was calculated according to the formula:

$\grave{J} = \grave{H}/\grave{H}_{max}$

where $\grave{H}_{max}$ is the maximum value for the Shannon diversity index, $\acute{H}_{max} = \log_2 S$.

The species richness and group richness were calculated by counting the total fungal species or fungal groups for the different feed-medium-temperature combinations.

RESULTS

Feed composition

Table 2 reveals greater inter-site variability (lower P values) relative to inter-feed variability in chemical composition of feed. For example, the effect of type of feed was non-significant (P≥0.05) on protein, IS, fat and ash contents, just significant (P<0.05) on moisture and TSS contents with a highly significant effect (P<0.001) only on fiber content. By contrast, the effect of site of collection was consistently very highly significant (P<0.001) on all feed constituents, particularly the fiber and fat contents. Based on the magnitude of CV, the inter-site variability in feed composition was most evident in cattle feed for IS, fat, fiber, moisture and ash contents, in poultry feed for protein and TSS contents but was intermediate for all constituents of rabbit feed (Table 3). The feed content of protein, TSS and moisture exhibited both limited inter-site and inter-feed variability, those of IS and fat exhibited moderate inter-site variability along with low inter-feed variability, while fiber content exhibited moderate inter-site variability but high inter-feed variability and finally ash content exhibited both high inter-site and inter-feed variability (Table 3).

The highest contents of protein and IS (with averages of 6.96% and 12.5%, respectively) were found in poultry feed, followed by rabbit feed (protein) and cattle feed (IS). Also, fat content was higher in poultry and rabbit feeds, with an average of 12%, than in cattle feed. By contrast, the highest content of TSS, fibers and ash (with averages of 8.39, 15.5 and 0.67%, respectively) was found in cattle feed followed by rabbit feed; but the highest moisture content was found in cattle and poultry feeds with an average of 9.4%. Thus, poultry feed was characterized by the highest content of protein, IS and fat, along with fairly high moisture content but the lowest content of TSS, fiber and ash. In turn, cattle feed was characterized by the highest content of TSS, fiber, moisture and ash, along with the lowest content of protein and fat; but rabbit feed was characterized by moderate content of protein, TSS, fat, fiber and ash along with the lowest content of IS and moisture (Table 3).

Table 2 Nested ANOVA showing the effect of groups (type of animal feed) and sub-groups (site of collection) on the chemical composition of feed

Variable and source of variation	DF	SS	MS	F	P
Protein content					
Total	35	321.1			
Among all subgroups	11	262.6			
Groups	2	117.3	58.63	3.630	0.070
Sub-groups	9	145.4	16.15	6.628	0.000
Error	24	58.49	2.437		
Soluble sugars					
Total	35	166.7			
Among all subgroups	11	150.2			
Groups	2	101.8	50.91	9.465	0.006
Sub-groups	9	48.40	5.378	7.835	0.000
Error	24	16.47	0.686		
Insoluble sugars					
Total	35	6723			
Among all subgroups	11	5752			
Groups	2	2444	1222	3.324	0.083
Sub-groups	9	3308	367.6	9.080	0.000
Error	24	971.5	40.48		
Fat content					
Total	35	353.4			
Among all subgroups	11	344.3			
Groups	2	143.2	71.59	3.204	0.089
Sub-groups	9	201.1	22.34	58.59	0.000
Error	24	9.152	0.381		
Fiber content					
Total	35	1631			
Among all subgroups	11	1624			
Groups	2	1266	633.0	15.91	0.001
Sub-groups	9	358.1	39.79	144.7	0.000
Error	24	6.599	0.275		
Moisture content					
Total	35	86.39			
Among all subgroups	11	81.98			
Groups	2	39.83	19.92	4.253	0.050
Sub-groups	9	42.15	4.683	25.49	0.000
Error	24	4.409	0.184		
Ash content					
Total	35	6.037			
Among all subgroups	11	5.697			
Groups	2	2.439	1.219	3.368	0.081
Sub-groups	9	3.258	0.362	25.58	0.000
Error	24	0.340	0.014		

Fungal diversity

Inoculants from the three feedstuffs were incubated on two nutrient media (PDA and MEA) and at two temperatures (25 °C and 30 °C) to identify the culturing conditions appropriate to develop the potential mycobiome load of feedstuffs. The present results reveal a marked discrepancy between the total number of colonies (germ bank), number of species (species richness) and distribution of colonies among species (species diversity) for the different feed × medium × temperature combinations (Table 4). This discrepancy was more evident among the three feedstuffs than among the two nutrient media but was not evident for the two

incubation temperatures. For example, the greatest number of colonies recorded for cattle feed was associated with the lowest species richness and diversity index. Likewise, but to a lesser extent, PDA medium developed a low number of colonies and low species richness but high species diversity relative to MEA medium. By contrast, the low incubation temperature (25 °C) gathered both greater number of colonies, higher species richness and higher species diversity relative to the high temperature (30 °C).

Dominance of fungal groups and species

The fungal load of the experimental feedstuffs is in overall low, being within 10^3–10^4 CFU g^{-1}. The mycobiome community of the feedstuffs counted up to 43 species of 12 families, 10 orders, 8 classes and three divisions (Figure 1). Irrespective of the type of feed, nutrient medium and incubation temperature, the most dominant fungal division was Ascomycota (with hundreds of colonies particularly in cattle feed), followed by Zygomycota (with tens of colonies in rabbit and cattle feeds but almost no occurrence in poultry feed); while Basidiomycota was the rarest group, with occasional occurrence only in poultry feed developed on PDA and absolute absence from the other feed × medium × temperature combinations (Table 5). Distinctly, Ascomycota was the sole fungal division encountered in poultry feed incubated on MEA at 25 °C, and Basidiomycota recorded their occurrence only in poultry feed incubated on PDA at both temperatures, with absence of Zygomycota. The dominance of Ascomycota can be attributed primarily to Eurotiomycetes, which was the most dominant fungal class, followed by Zygomycetes (of division Zygomycota). However, there are some exceptions, e.g. generally in poultry feed as well as in cattle feed, both on MEA at 25 °C, the second dominant class was the ascomycetes Dothideomycetes and Euascomycetes, respectively. On the other hand, some fungal classes recorded rare occurrence only under certain circumstances, e.g. Saccharomycetes in rabbit feed on PDA at 25 °C and Leotiomycetes in rabbit feed on MEA at 30 °C, with single occurrence in both cases.

In turn, the dominance of Eurotiomycetes was primarily attributed to Eurotiales which was the only order of this class and the most dominant order in all feed × medium × temperature combinations. Generally, Eurotiales was followed by Mucorales (of the Zygomycetes) and Capnodiales (of the Dothideomycetes). However, Mucorales was almost absent from poultry feed; and Hypocreales (of Euascomycetes) as well as Trichosphaeriales and Sordariales (of Sordariomycetes) contributed considerable number of colonies in cattle feed and rabbit feed, respectively only at 25 °C. Some fungal orders recorded occasional occurrence only under certain circumstances, e.g. Sordariales only in rabbit feed on MEA at 25 °C, Saccharomycetales only in rabbit feed on PDA at 25 °C, Helotiales (of Leotiomycetes) only in rabbit feed on MEA at 30 °C and Cantharellales (of Agaricomycetes) only in poultry feed on PDA (Table 5).

Table 3 Chemical composition of feedstuffs (poultry, rabbit and cattle feeds) collected from different sites within the Nile Delta (Egypt). Each value is the mean of three replicates ± SE. Means with common letters are non-significantly different at P<0.05. Means of feed × site combinations were separated by small-case letters while major means of feedstuffs were separated by capital letters

Type of feed and site of collection	Protein (% DW)	Soluble sugars (% DW)	Insoluble sugars (% DW)	Fat (% DW)	Fiber (% DW)	Moisture (% FW)	Ash (% DW)
Poultry feed							
Site 1	5.63 ± 0.78^{abc}	3.10 ± 0.24^{a}	46.0 ± 2.40^{fg}	11.6 ± 0.60^{c}	1.64 ± 0.02^{a}	9.09 ± 0.04^{cd}	0.07 ± 0.00^{ab}
Site 2	10.2 ± 0.93^{e}	3.72 ± 0.29^{ab}	43.5 ± 6.33^{f}	11.7 ± 0.28^{c}	1.69 ± 0.04^{ab}	9.51 ± 0.03^{de}	0.05 ± 0.02^{ab}
Site 3	9.15 ± 0.17^{de}	6.20 ± 0.61^{cd}	37.4 ± 1.31^{ef}	12.7 ± 0.22^{d}	1.24 ± 0.06^{a}	9.04 ± 0.15^{cd}	0.05 ± 0.00^{ab}
Site 4	13.7 ± 1.58^{f}	4.14 ± 0.56^{ab}	58.2 ± 2.08^{h}	14.1 ± 0.18^{e}	2.57 ± 0.07^{b}	8.72 ± 0.1^{6c}	0.01 ± 0.00^{a}
Average	**9.69 ± 0.97^{B}**	**4.29 ± 0.40^{A}**	**46.3 ± 2.75^{B}**	**12.5 ± 0.34^{B}**	**1.79 ± 0.15^{A}**	**9.09 ± 0.10^{B}**	**0.05 ± 0.01^{A}**
CV of sites (%)	**34.5**	**31.28**	**18.88**	**9.38**	**31.42**	**3.56**	**51.33**
Rabbit feed							
Site 1	8.10 ± 0.10^{cde}	8.34 ± 0.36^{e}	22.6 ± 1.65^{abc}	13.0 ± 0.09^{d}	10.5 ± 0.54^{c}	7.50 ± 0.02^{b}	0.25 ± 0.02^{bc}
Site 2	6.00 ± 0.57^{abc}	5.00 ± 0.63^{bc}	28.9 ±4.88^{bcde}	11.6 ± 0.27^{c}	12.7 ± 0.37^{de}	7.52 ± 0.31^{b}	0.62 ± 0.04^{e}
Site 3	9.69 ± 1.10^{e}	7.10 ± 0.13^{de}	21.4 ± 4.47^{ab}	10.5 ± 0.16^{b}	10.2 ± 0.30^{c}	6.62 ± 0.49^{a}	0.45 ± 0.02^{de}
Site 4	6.16 ± 0.27^{abc}	6.42 ± 0.31^{d}	32.7 ± 4.59^{cde}	10.9 ± 0.29^{bc}	17.7 ± 0.34^{f}	7.40 ± 0.32^{b}	0.52 ± 0.14^{de}
Average	**7.49 ± 0.53^{A}**	**6.71 ± 0.40^{B}**	**26.4 ± 2.24^{A}**	**11.5 ± 0.30^{AB}**	**12.8 ± 0.93^{B}**	**7.26 ± 0.18^{A}**	**0.46 ± 0.05^{AB}**
CV of sites (%)	**23.42**	**20.69**	**20.26**	**9.44**	**27.33**	**5.92**	**34.89**
Cattle feed							
Site 1	7.02 ± 0.62^{bcd}	7.35 ± 0.24^{de}	36.3 ± 0.93^{def}	13.0 ± 0.83^{d}	13.0 ± 0.23^{de}	10.6 ± 0.38^{f}	0.20 ± 0.01^{abc}
Site 2	3.75 ± 0.73^{a}	8.43 ± 1.00^{e}	16.9 ± 1.63^{a}	10.3 ± 0.36^{b}	23.3 ± 0.31^{g}	9.86 ± 0.13^{ef}	0.61 ± 0.15^{e}
Site 3	4.74 ± 1.49^{ab}	7.60 ± 0.40^{de}	25.6 ± 1.91^{abcd}	4.15 ± 0.01^{a}	13.4 ± 0.01^{e}	6.82 ± 0.23^{ab}	1.51 ± 0.10^{f}
Site 4	5.56 ± 1.01^{abc}	10.2 ± 0.28^{f}	54.5 ± 5.95^{gh}	4.16 ± 0.03^{a}	12.3 ± 0.56^{d}	11.7 ± 0.17^{g}	0.38 ± 0.06^{cd}
Average	**5.27 ± 0.56^{A}**	**8.39 ± 0.41^{B}**	**33.3 ± 4.45^{AB}**	**7.89 ± 1.18^{A}**	**15.5 ± 1.37^{B}**	**9.74 ± 0.56^{B}**	**0.67 ± 0.16^{B}**
CV of sites (%)	**26.23**	**15.29**	**48.62**	**56.40**	**33.68**	**21.51**	**86.00**
CV of feeds (%)	**29.55**	**31.85**	**28.56**	**22.95**	**72.45**	**14.81**	**81.07**

Table 4 Shannon's index of species diversity ($\acute{H}$), relative species diversity ($\acute{J}$), species richness and total number of colony-forming units of fungi developed from three feedstuffs (poultry, rabbit and cattle feeds) incubated on PDA and MEA media at two 25 °C and 30 °C

Type of feed, nutrient medium and temperature	Shannon's index of species diversity	Relative species diversity	Species richness	Total number of colony-forming units (CFU g^{-1} feed)
Poultry feed				
PDA, 25 °C	3.09	0.86	12	39
PDA, 30 °C	2.51	0.76	10	64
MEA, 25 °C	2.34	0.78	8	66
MEA, 30 °C	2.56	0.77	10	68
Rabbit feed				
PDA, 25 °C	3.39	0.76	22	127
PDA, 30 °C	2.39	0.69	11	98
MEA, 25 °C	2.08	0.51	17	255
MEA, 30 °C	2.96	0.74	16	42
Cattle feed				
PDA, 25 °C	2.56	0.67	14	895
PDA, 30 °C	2.00	0.58	11	647
MEA, 25 °C	2.54	0.62	17	701

MEA, 30 °C	2.20	0.56	15	461
Averages				
Poultry feed	3.02	0.70	20	237
Rabbit feed	3.27	0.65	32	522
Cattle feed	2.72	0.65	18	2704
PDA	3.20	0.62	36	1870
MEA	3.48	0.70	31	1593
25 °C	3.36	0.64	38	2083
30 °C	2.76	0.56	31	1380

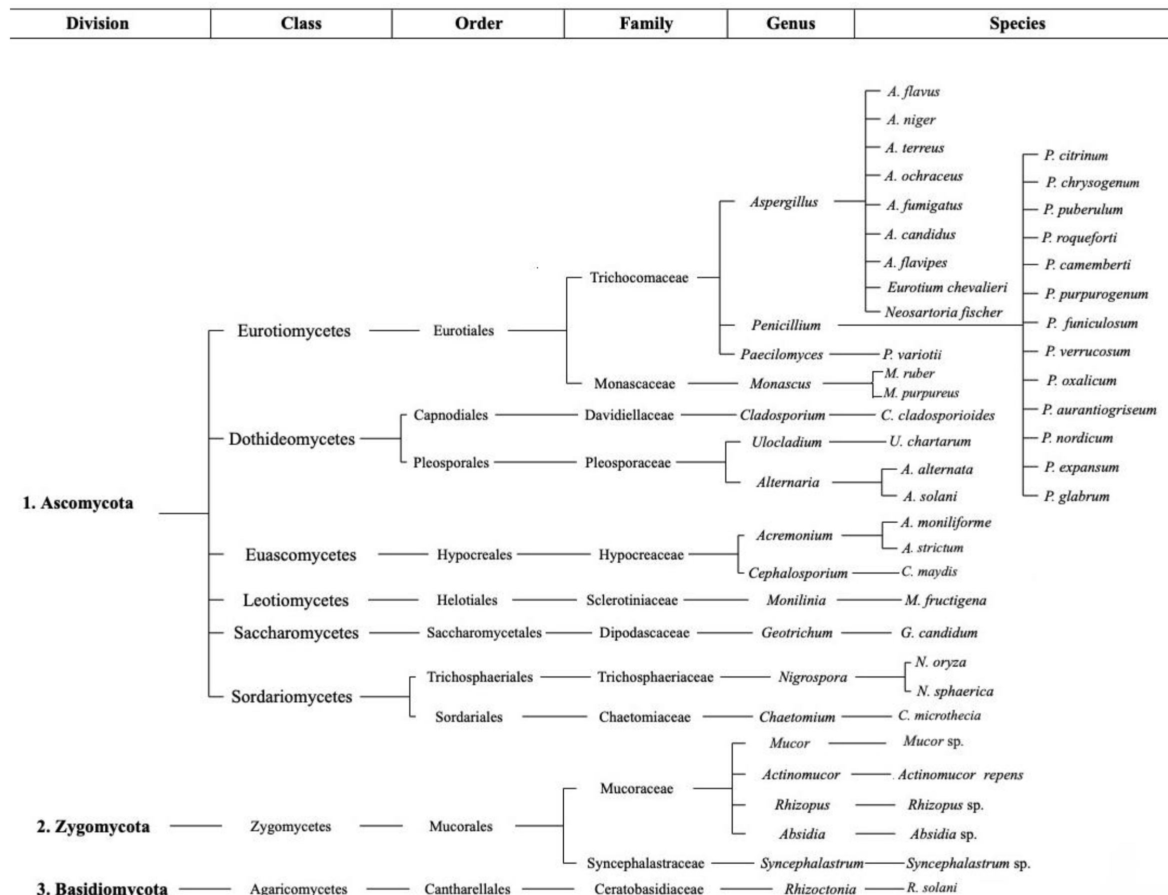

Figure 1 Summary of the different fungal taxa encountered in the present survey showing their taxonomical relationships

Order Eurotiales was represented by two families, among which Trichocomaceae was the most dominant family, followed by Monascaceae, while Mucoraceae (order Mucorales, class Zygomycetes) came third. Furthermore, the occurrence of Monascaceae was restricted mainly to cattle feed with rare occurrence in poultry feed and almost complete absence from rabbit feed. Other examples of patchy and occasional occurrence of some families are: 1) the confined occurrence of Davidiellaceae (order Capnodiales, class Dothideomycetes) to poultry and rabbit feeds only at 25 °C, 2) occurrence of Trichosphaeriaceae (order Trichosphaeriales, class Sordariomycetes) only in rabbit feed on PDA at 25 °C, 3) occurrence of Chaetomiaceae (order Sordariales, class Sordariomycetes) only in rabbit feed on MEA at 25 °C and 4), a single occurrence of Dipodascaceae (order Saccharomycetales, class Saccharomycetes), Sclerotiniaceae (order Helotiales, class Leotiomycetes), Syncephalastraceae (order Mucorales, class Zygomycetes) and Ceratobasidiaceae (order Cantharellales, class Basidiomycetes) in rabbit feed on PDA at 25 °C, rabbit feed on MEA at 30 °C, rabbit feed on PDA at 30 °C and poultry feed on PDA at both temperatures, respectively (Table 5).

Based on the frequency of occurrence across the different feed × medium × temperature combinations in association with the number of colony-forming units, it can be claimed that the most frequent fungal species were the *Aspergillus* spp. (*A. niger*, *A. flavus*, *A. ochraceus* and *A. fumigatus*), followed by *Monascus ruber* and *Eurotium chevalieri*; then came *Mucor* sp. and *Rhizopus* sp. However, there are some distinct feed- as well as medium- and temperature- preferences of some fungal species. Regarding feed preference, both *A. fumigatus* and *Cladosporium cladosporioides* exhibited a bias to poultry feed, despite the ubiquitous occurrence of *A. fumigatus* in the three feedstuffs. Likewise, *A. niger* and *Alternaria alternata* exhibited preference for rabbit feed, while *A. terreus*, *A. ochraceus*, *A. candidus*, *P. chrysogenium*, *Monascus ruber*, *Eurotium chevalieri* and *Paecilomyces variotii* preferred cattle feed. There are some other distinct cases of exclusive occurrence of some fungi in some feedstuffs; for example, the fungi *A. flavipes*, *P. puberulum*, *P. roqueforti*, *P. purpurogenum*, *P. funiculosum*, *P. verrucosum*, *Nigrospora sphaerica*, *Geotrichum candidum*, *Chaetomium microthecia* and *Absidia* sp. were confined only to rabbit feed; while *Monascus purpureus* was confined only to cattle feed, and *Acremonium strictum*, *Rhizoctonia solani* and *Cephalosporium maydis* were confined only to poultry feed. Thus, rabbit feed had the most diverse mycobiome among the three feedstuffs, followed by poultry feed and lastly came cattle feed. However, despite the high number of unique species in rabbit feed, each species occurred with a few numbers of colony-forming units (Table 6). Cultures of some frequent fungal species isolated on PDA medium at 25 °C are presented in Figure 2.

Regarding the temperature-preference of the different fungal taxa, the present results reveal that Ascomycota, in overall, preferred the low temperature (25 °C) over high temperature (30 °C), with no distinct temperature-preference for Zygomycota and Basidiomycota. Within Ascomycota, Eurotiomycetes (except species isolated from poultry feed) and Dothideomycetes preferred 25 °C; with exclusive development only at 25 °C of Sordariomycetes. By contrast, family Monascaceae (of Eurotiomycetes) along with family Mucoraceae (of Zygomycetes) preferred 30 °C, while Sclerotiniaceae (of Leotiomycetes) and Syncephalastraceae (of Zygomycetes) exhibited exclusive development only at 30 °C (Table 5). Regarding temperature-preference of species, it is evident that *A. niger*, *A. candidus*, *P. chrysogenium*, *Alternaria alternata*, *Eurotium chevalieri*,

Cladosporium cladosporioides and *Absidia* sp. preferred 25 °C over 30 °C, while *P. roqueforti*, *P. purpurogenum*, *Neosartorya fischeri*, *Nigrospora sphaerica* and *Chaetomium microthecia* were developed exclusively at 25 °C. On the other hand, *P. funiculosum*, *Monascus ruber*, *Monascus purpureus*, *Mucor* sp., *Rhizopus* sp. and *Paecilomyces variotii* preferred 30 °C, while *P. citrinium* and *Acremoniumstrictum* were developed exclusively at 30 °C. There are three species that is *A. terreus*, *A. ochraceus* and *A. fumigatus* which were temperature-indifferent (Table 6).

Regarding medium preference of fungi, it is evident that *Zygomycota*, generally, preferred PDA over MEA, with exclusive development of *Basidiomycota* only on PDA, while *Ascomycota* exhibited no distinct medium-indifferent. Within the neutral *Ascomycota*, preference of PDA was relatively distinct for *Eurotiomycetes* and *Sordariomycetes*; while *Dothideomycetes* was medium-indifferent. The medium-preference was distinct in order *Eurotiales*, being in favor of PDA against MEA for isolates from cattle feed, while the reverse was true for isolates from poultry feed, with no distinct preference for rabbit feed isolates. An absolute preference of PDA was evident in *Trichosphaeriales*. By contrast, limited preference of MEA was exhibited by *Capnodiales* and *Hypocreales*, and being absolute in *Sordariales*. The preference of PDA was distinct in families *Monascaceae* and *Trichocomaceae* isolated only from cattle feed, being absolute for *Trichosphaeriaceae*. On the other hand, preference of MEA was evident in *Hypocreaceae* and was absolute in *Chaetomiaceae* (Table 5). At the level of species, preference of PDA over MEA was evident in *A. flavus*, *A. niger*, *A. ochraceus*, *A. candidus*, *A. terreus*, *Monascus ruber*, *Monascus purpureus*, *Alternaria alternata*, *Eurotium chevalieri* and *Mucor* sp., particularly in the high-count cattle feed isolates; with absolute PDA preference in *P. camemberti*, *P. purpurogenum*, *P. funiculosum*, *Rhizoctonia solani*., and *Nigrospora sphaerica*. On the other hand, MEA preference was evident in few species, e.g. *Acremonium moniliforme*, *Paecilomyces-variotii* and *Absidia* sp., being absolute in *Chaetomium microthecia* (Table 6).

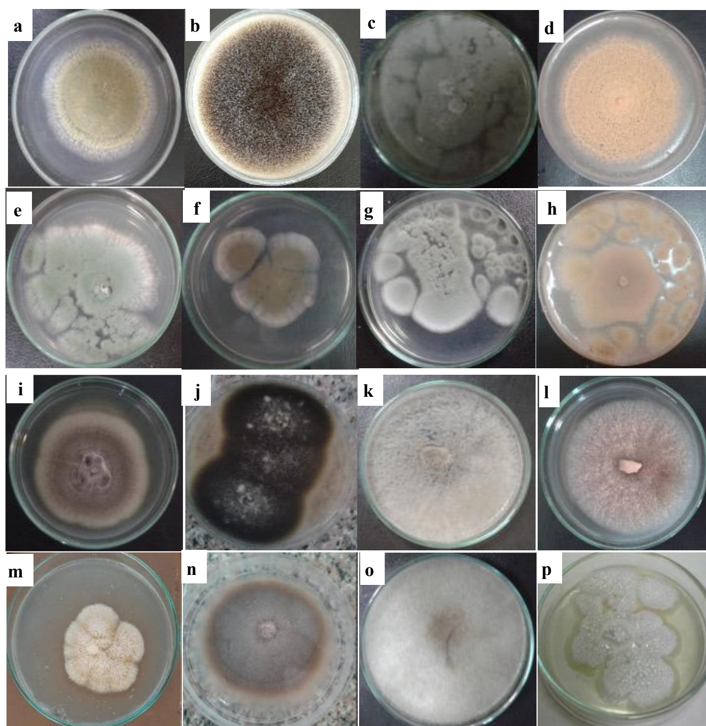

Figure 2 Cultures of some isolated fungal species growing at 25 °C on PDA media for 7 days: a) *Aspergillus flavus*, b) *A. niger*, c) *A. fumigatus*, d) *A. ochraceus*, e) *Penicillium roqueforti*, f) *P. purpurogenum*, g) *P. puberulum*, h) *Paecilomyces variotii*, i) *Alternaria solani*, j) *Ulocladium chartarum*, k) *Nigrospora sphaerica*, l) *Rhizoctonia solani*, m) *Monoascus purpureus*, n) *M. ruber*, o) *Syncephlastrum* sp., p) *Neosartorya fischeri*.

DISCUSSION

Despite the great inter-site variability in the chemical composition of feedstuffs, each type of feed has its distinct composition. It can be claimed that poultry feed is characterized with relatively high contents of protein, IS, fat and moisture, while cattle feed is characterized with high contents of SS, fiber, moisture and ash, versus moderate content of most components in rabbit feed. The grouping of protein, fat and IS contents of the feedstuffs on one hand against SS, fiber and ash contents on the other hand (Figure 3) summarizes this pattern quite explicitly. Nevertheless, with reference to the recommended nutrient levels for rabbit feed (**Owen** *et al.*, **2009; Abo-Eid** *et al.*, **2016**), poultry feed **(Pavlović** *et al.*, **2019)** and cattle feed (**Pinto and Millen, 2018**), it can be claimed that the experimental feedstuffs share a common pattern of low protein and ash contents in favor of high fat content. The generally low moisture content of the experimental feedstuffs, compared with those reported by **Okoli** *et al*. **(2006)** and **Gherbawy** *et al*. **(2020)** refers to the hygienic nature of feeds with low opportunity for fungal proliferation and feed spoilage during storage.

The limited mycobiome community of the feedstuffs–which amounted to 43 species of 12 families, 10 orders, eight classes and three divisions, with overall low fungal load– is in accordance with the overall low moisture content of feedstuffs and refers again to their hygienic nature. According to **Krnjaja** *et al*. **(2010)** a feedstuff can be qualified as good, regular or bad if the fungal count approached $<3\times10^4$, $3–7\times10^4$ or $>7\times10^4$ CFU g^{-1}, respectively. The overall low fungal count of the experimental feedstuffs (within $10^3–10^4$ CFU g^{-1}) along with the rank of germ load in the order: cattle feed > rabbit feed > poultry feed are in line with the overall low moisture content of feedstuffs and with the rank of moisture in the three feedstuffs which was higher in cattle feed than poultry and rabbit feeds. This agreement between germ load and moisture content of feedstuffs emphasizes the role of moisture in fungal proliferation in food- and feed-stuffs proposed by **Gherbawy** *et al*. **(2020), Wei** *et al*. **(2020)** and **Kesho** *et al.* **(2019)**. In this regard, **Okoli** *et al*. **(2006)** claimed that 12% moisture content of a feedstuff is adequate for fungal growth; fortunately, the highest moisture content of the investigated feedstuffs, that is of cattle feed (around 9.7%), is lower than either the limit set by **Okoli** *et al*. **(2006)** or the range reported by **Gherbawy** *et al*. **(2020)** which is 11–14.5%.

The present findings suggest that the development of the potential germ load of feedstuff in terms of the number of species or dominance of a given species over others might depend on the subsequent incubation conditions. It seems that MEA rather than PDA is the proper medium for maximum development of the germ load of poultry and rabbit feeds while the reverse is true for cattle feed. Nevertheless, regarding mycobiome diversity, it seems that PDA rather than MEA is appropriate to explicit the mycobiome diversity of poultry feed (leading to higher species richness) while the reverse seems true for cattle feed but with no media-preference for rabbit feed. Similarly, low temperature (25 °C) seems, in general, more appropriate than high temperature (30 °C) for development of the potential mycobiome of feedstuffs leading to higher species richness and species diversity. Temperature and relative humidity of storage play important roles in fungal infestation of grains of wheat (**Kesho** *et al*., **2019**) and walnut (**Wei** *et al*., **2020**). The report of **Ghiasian and Maghsood (2011)** about greater invasion of concentrated feeds by aflatoxigenic fungi in winter than summer points indirectly to preference of low temperature by this group of fungi, despite the probable contribution of additional confounding factors e.g. relative humidity.

The great germ load recorded in cattle feed was associated with low species diversity that is the great number of colonies was distributed among a limited number of major species with many minor species; on the other hand, the low germ load of rabbit feed was distributed among relatively large number of species (i.e. high species diversity). This pattern signifies a discrepancy among the investigated feedstuffs in the total number of colonies (germ bank) and distribution of colonies among species (species diversity). Also, the low number of colonies developed on MEA relative to that of PDA was accompanied with high species diversity; but the low incubation temperature (25 °C) gathered both a great number of colonies and high species diversity relative to the high temperature (30 °C).

Table 5 Number of colony-forming units (CFU/g feed) belonging to the different fungal taxa isolated from three feedstuffs (poultry, rabbit and cattle feeds) on two PDA and MEA media and incubated at 25 °C and 30 °C

Taxon	Poultry feed				Rabbit feed				Cattle feed			
	PDA		MEA		PDA		MEA		PDA		MEA	
	25 °C	30 °C	25 °C	30 °C	25 °C	30 °C	25 °C	30 °C	25 °C	30 °C	25 °C	30 °C
Division												
Ascomycota	38	36	66	67	115	78	235	36	862	619	688	422
Zygomycota	0	0	0	1	12	20	20	6	33	28	13	39
Basidiomycota	1	1	0	0	0	0	0	0	0	0	0	0
Class												
Eurotiomycetes	23	55	54	63	95	77	220	33	861	619	641	422
Dothideomycetes	11	2	12	0	6	1	6	2	1	0	1	0
Euascomycetes	3	6	0	4	1	0	0	0	0	0	46	0
Sordariomycetes	1	0	0	0	12	0	9	0	0	0	0	0
Saccharomycetes	0	0	0	0	1	0	0	0	0	0	0	0
Leotiomycetes	0	0	0	0	0	0	0	1	0	0	0	0
Zygomycetes	0	0	0	1	12	20	20	6	33	28	13	39
Agaricomycetes	1	1	0	0	0	0	0	0	0	0	0	0
Order												
Eurotiales	23	55	54	63	95	77	223	33	861	619	641	422
Capnodiales	8	0	11	0	3	0	3	1	1	0	0	0
Pleosporales	3	2	1	0	3	1	3	1	0	0	1	0
Hypocreales	3	6	0	4	1	0	0	0	0	0	46	0
Trichosphaeriales	1	0	0	0	12	0	0	0	0	0	0	0
Sordariales	0	0	0	0	0	0	9	0	0	0	0	0
Saccharomycetales	0	0	0	0	1	0	0	0	0	0	0	0
Helotiales	0	0	0	0	0	0	0	1	0	0	0	0
Mucorales	0	0	0	1	12	20	20	6	33	28	13	39
Cantharellales	1	1	0	0	0	0	0	0	0	0	0	0
Family												
Trichocomaceae	22	46	54	44	95	77	220	32	707	293	610	156
Monascaceae	1	9	0	19	0	0	0	1	154	326	31	266
Davidiellaceae	8	0	11	0	3	0	3	1	1	0	0	0
Pleosporaceae	3	2	1	0	3	1	3	1	0	0	1	0
Hypocreaceae	3	6	0	4	1	0	0	0	0	0	46	0
Trichosphaeriaceae	1	0	0	0	12	0	0	0	0	0	0	0
Chaetomiaceae	0	0	0	0	0	0	9	0	0	0	0	0
Dipodascaceae	0	0	0	0	1	0	0	0	0	0	0	0
Sclerotiniaceae	0	0	0	0	0	0	0	1	0	0	0	0
Mucoraceae	0	0	0	1	12	19	20	6	33	28	13	39
Syncephalastraceae	0	0	0	0	0	1	0	0	0	0	0	0
Ceratobasidiaceae	1	1	0	0	0	0	0	0	0	0	0	0

Table 6 Number of colony-forming units (CFU g^{-1} feed) of the different fungal species isolated from the feedstuffs (poultry, rabbit and cattle feeds) on PDA and MEA media and incubated at 25 °C and 30 °C

Species	Poultry feed				Rabbit feed				Cattle feed			
	PDA		MEA		PDA		MEA		PDA		MEA	
	25 °C	30 °C	25 °C	30 °C	25 °C	30 °C	25 °C	30°C	25°C	30 °C	25 °C	30 °C
A. flavus	4	8	6	5	14	39	9	5	21	12	14	10
A. niger	5	8	24	12	48	26	31	19	11	16	9	8
A. terreus	0	0	0	2	1	0	0	2	7	5	4	3
A. ochraceus	0	2	2	1	0	1	2	1	15	30	15	2
A. fumigatus	10	28	18	21	3	0	1	1	12	0	7	4
A. candidus	0	0	0	0	0	0	0	1	287	226	283	115
A. flavipes	0	0	0	0	0	1	2	0	0	0	0	0
Eurotium chevalieri	3	0	2	0	5	0	3	1	268	0	185	2
Neosartorya fischeri	0	0	2	0	0	0	0	0	0	0	0	0
Aspergillus	**22**	**46**	**54**	**41**	**71**	**67**	**48**	**30**	**621**	**289**	**517**	**144**
P. citrinum	0	0	0	2	0	0	0	0	0	0	0	0
P. chrysogenum	0	0	0	0	7	0	2	1	75	0	82	1
P. puberulum	0	0	0	0	0	0	3	0	0	0	0	0
P. roqueforti	0	0	0	0	2	0	2	0	0	0	0	0
P. camemberti	0	0	0	0	1	0	0	0	0	0	0	0
P. purpurogenum	0	0	0	0	2	0	0	0	0	0	0	0
P. funiculosum	0	0	0	0	1	6	0	0	0	0	0	0
P. verrucosum	0	0	0	0	2	0	0	1	0	0	0	0
P. oxalicum	0	0	0	0	0	0	0	0	11	2	9	2
P. aurantiogriseum	0	0	0	1	1	0	165	0	0	0	0	0
P. nordicum	0	0	0	0	0	0	0	0	0	0	1	3
P. expansum	0	0	0	0	5	4	0	0	0	0	0	0
P. glabrum	0	0	0	0	3	0	0	0	0	0	0	0
Penicillium	**0**	**0**	**0**	**3**	**24**	**10**	**172**	**2**	**86**	**2**	**92**	**6**
Paecilomyces variotii	0	0	0	0	0	0	0	0	0	2	1	6
Paecilomyces	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**2**	**1**	**6**
Monascus ruber	1	9	0	19	0	0	0	1	147	298	29	239
Monascus purpureus	0	0	0	0	0	0	0	0	7	28	2	27
Monascus	**1**	**9**	**0**	**19**	**0**	**0**	**0**	**1**	**154**	**326**	**31**	**266**
Cladosporium	8	0	11	0	3	0	3	1	1	0	0	0
Cladosporium	**8**	**0**	**11**	**0**	**3**	**0**	**3**	**1**	**1**	**0**	**0**	**0**

Ulocladium chartarum.	0	1	0	0	0	0	1	1	0	0	0	0
Ulocladium	**0**	**1**	**0**	**0**	**0**	**0**	**1**	**1**	**0**	**0**	**0**	**0**
Alternaria alternata	2	1	1	0	3	1	2	0	0	0	1	0
Alternaria solani	1	0	0	0	0	0	0	0	0	0	0	0
Alternaria	**3**	**1**	**1**	**0**	**3**	**1**	**2**	**0**	**0**	**0**	**1**	**0**
Acremonium moniliforme	1	2	0	0	1	0	0	0	0	0	46	0
Acremonium strictum.	0	4	0	4	0	0	0	0	0	0	0	0
Acremonium	**1**	**6**	**0**	**4**	**1**	**0**	**0**	**0**	**0**	**0**	**46**	**0**
Cephalosporium maydis.	2	0	0	0	0	0	0	0	0	0	0	0
Cephalosporium	**2**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**
Nigrospora oryzae	1	0	0	0	0	0	0	0	0	0	0	0
Nigrospora sphaerica	0	0	0	0	12	0	0	0	0	0	0	0
Nigrospora	**1**	**0**	**0**	**0**	**12**	**0**	**0**	**0**	**0**	**0**	**0**	**0**
Chaetomium microthecia	0	0	0	0	0	0	9	0	0	0	0	0
Chaetomium	**0**	**0**	**0**	**0**	**0**	**0**	**9**	**0**	**0**	**0**	**0**	**0**
Geotrichum candidum	0	0	0	0	1	0	0	0	0	0	0	0
Geotrichum	**0**	**0**	**0**	**0**	**1**	**0**	**0**	**0**	**0**	**0**	**0**	**0**
Monilinia fructigena	0	0	0	0	0	0	0	1	0	0	0	0
Monilinia	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**1**	**0**	**0**	**0**	**0**
Mucor sp.	0	0	0	1	3	2	9	0	26	26	5	20
Mucor	**0**	**0**	**0**	**1**	**3**	**2**	**9**	**0**	**26**	**26**	**5**	**20**
Actinomucor repens	0	0	0	0	0	0	0	1	0	0	0	0
Actinomucor	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**1**	**0**	**0**	**0**	**0**
Rhizopus sp.	0	0	0	0	6	15	2	4	7	2	8	19
Rhizopus	**0**	**0**	**0**	**0**	**6**	**15**	**2**	**4**	**7**	**2**	**8**	**19**
Absidia sp.	0	0	0	0	3	2	9	1	0	0	0	0
Absidia	**0**	**0**	**0**	**0**	**3**	**2**	**9**	**1**	**0**	**0**	**0**	**0**
Syncephalastrum sp.	0	0	0	0	0	1	0	0	0	0	0	0
Syncephalastrum	**0**	**0**	**0**	**0**	**0**	**1**	**0**	**0**	**0**	**0**	**0**	**0**
Rhizoctonia solani	1	1	0	0	0	0	0	0	0	0	0	0
Rhizoctonia	**1**	**1**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**

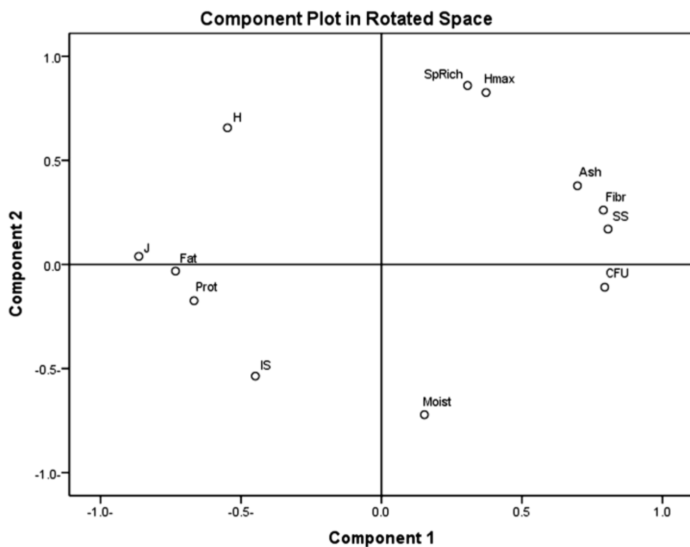

Figure 3 Principal component analysis (PCA) summarizing the relationships between feedstuff composition and the measures of fungal diversity

The dominance of Ascomycota across the investigated feedstuffs is in agreement with the dominance of ascomycetes in several terrestrial microhabitats such as the phyllosphere of terrestrial plants **(Bajpai *et al*., 2019)** and rhizosphere of desert plants **(Noor *et al*., 2021).** In the present investigation, the dominance of Ascomycota can be attributed to Eurotiomycetes; and in turn, the dominance of Eurotiomycetes can be primarily attributed to Eurotiales. The exclusive occurrence of some ascomycete classes, with few counts, under certain circumstances, e.g. of Saccharomycetes and Leotiomycetes only in rabbit feed on PDA at 25 °C and on MEA at 30 °C, respectively, along with the occurrence of only Sordariomycetes in both of poultry feed and rabbit feed on PDA at 25 °C and of Agaricomycetes only in poultry feed on PDA points to the role of culturing conditions in development of the latent mycobiome of a feedstuff. The dominance of Ascomycota in the rhizosphere of five desert plants was attributed to Sordariomycetes and Dothideomycetes (**Noor *et al*., 2021**); nevertheless, these classes were of minor occurrence in the mycobiome of the investigated feedstuffs.

Among the two encountered families of Eurotiales, Trichocomaceae dominated over Monascaceae, then came Mucoraceae (of order Mucorales) as the third dominant family. The uniqueness of rabbit feed with Trichosphaeriaceae, Chaetomiaceae, Dipodascaceae, Sclerotiniaceae and Syncephalastraceae is in accordance with the remarkably high fungal diversity and species richness of rabbit feed despite its relatively low fungal count. In addition, the differential development of these families under certain culturing circumstances of nutrient medium and incubation temperature points again to the role of culturing conditions in the development of the latent mycobiome of a feedstuff. Also, the detection of Ceratobasidiaceae only in poultry feed and of Davidiellaceae in poultry and rabbit feeds with absolute absence from cattle feed is in accordance with the low fungal diversity and species richness of cattle feed despite its high fungal count.

Considering the frequency of occurrence of fungal species in the different feed × medium × temperature combinations, it can be concluded that the most dominant fungal species were the ascomycetes *Aspergillus* sp. and teleomorphs (*A. niger*, *A. flavus*, *A. ochraceus* and *A. fumigatus*), *Monascus ruber* and *Eurotium chevalieri*, followed by the zygomycetes *Mucor* sp. and *Rhizopus* sp. In accordance with the present findings, prevalence of *Aspergillus* sp., *Penicillium* sp., *Mucor* sp. and *Rhizopus* sp. has been reported in poultry feed **(Okoli *et al*., 2006)**, corn silage **(González Pereyra *et al*., 2011)**, lignocellulosic materials **(Boonyuen *et al*., 2014)**, poultry and animal feeds **(Gherbawy *et al*., 2019)**, several foodstuffs **(Aasa, 2021)**, dried fish **(Deng *et al*. 2021)** and silo-bags **(Brito *et al*., 2022)**. It has been claimed that *Penicillium* and *Aspergillus* spp. are the most common fungi in stored feedstuffs (**Agriopoulou *et al*., 2020**). Nevertheless, the higher count of *Aspergillus* sp. in cattle feed relative to rabbit and poultry feeds, observed in the present work, contradicts the higher rates of occurrence in poultry feed than in livestock feed reported by **Minooeianhaghighi *et al*. (2021)**. Anyway, the prevalence of *Aspergillus* species within the investigated feedstuffs, despite their relatively low count, is worrying since several species of *Aspergillus* are potential producers of mycotoxins such as aflatoxins, sterigmatocystin, citrinin and ochratoxin (**Daghir, 2008**).

Some fungal species exhibit distinct feed- as well as medium- and temperature-preferences. For example, despite the widespread occurrence of *A. fumigatus* in the three feedstuffs, it exhibited bias to poultry feed. Similarly, *A. flavus* and *A. niger* were biased to rabbit feed, while *A. terreus*, *A. ochraceus*, *A. candidus*, *P. chrysogenium*, *Monascus ruber*, *Eurotium chevalieri*, *Rhizopus* sp. and *Paecilomyces variotii* seem adherent to cattle feed. In addition, there are some distinct cases of exclusive engagement of some fungi to some feedstuffs; for example, several *Penicillium* sp., *A. flavipes*, *Chaetomium microthecia* and *Absidia* sp. were confined only to rabbit feed, while *Acremonium strictum*, *Nigrospora sphaerica* and *Rhizoctonia solani* were characteristic to poultry feed. However, despite the high fungal count of cattle feed, it exhibited uniqueness with only one species that is *Monascus purpureus*; this is in marked contrast to rabbit feed which although of its low germ load it had the most diverse mycobiome.

Regarding temperature preference, the present results suggest that while Ascomycota seem to prefer low temperature (25 °C), Zygomycota and Basidiomycota seem temperature-indifferent. Within Ascomycota, the orders of classes Sordariomycetes and Saccharomycetes, in addition to *Eurotium chevalieri* and *Cladosporium cladosporioides* exhibited an exclusive preference of 25 °C. Preference of 30 °C was sparingly evident in some families such as *Monascaceae* and *Syncephalastraceae*, as well as some species such as *P. funiculosum*, *Monascus ruber*, *Monascus purpureus*, *Rhizopus* sp., and *Paecilomyces variotii*. However, some *Aspergillus* sp. (*A. terreus*, *A. ochraceus* and *A. fumigatus*) and *Rhizoctonia solani* seem temperature-indifferent. In accordance with the present findings, the ascomycetes *Aspergillus* sp., *Penicillium* sp., as well as the zygomycetes *Mucor* sp., and *Rhizopus* sp. recorded greater occurrence in poultry feed at low temperatures than at high temperatures (**Okoli** ***et al*****., 2006**). Also, contamination of livestock feed and poultry feed with *Aspergillus* sp. was reported to be higher in autumn than in summer (**Minooeianhaghighi** ***et al*****., 2021**).

Preference of PDA over MEA was apparent in Zygomycota and exclusive in Basidiomycota. Media preference of Ascomycota varied according to the type of feed, being in favor of MEA in poultry feed isolates but in favor of PDA in cattle feed isolates with no definite preference in rabbit feed isolates. Within Ascomycota, PDA preference was evident in some *Aspergillus* sp., *Penicillium* sp. and *Monascus* sp., *Alternaria alternata*, *Eurotium chevalieri* as well as in the zygomycete *Mucor* sp. and was absolute in some *Penicillium* sp., *Rhizoctonia solani* and *Nigrospora sphaerica*. On the other hand, MEA preference was evident in *Acremonium moniliforme*, *Cladosporium cladosporioides, Paecilomyces variotii* and *Absidia* sp., being absolute in some *Penicillium* sp., *Neosartorya fischeri* and *Chaetomium microthecia.* However, there are some species with no definite media preference that is *A. fumigatus* and *P. chrysogenium*.

CONCLUSION

The generally low moisture content of feedstuffs along with overall low fungal load refers to the hygienic nature of the experimental feedstuffs. The high germ load of cattle feed can be partially attributed to its high moisture content which emphasizes the role of moisture in fungal proliferation in food- and feed-stuffs. In addition to the uniqueness of each type of feed with its fungal community, the culturing conditions play a role in the development of the latent mycobiome of a feedstuff. The remarkably high fungal diversity, species richness and number of unique species of rabbit feed despite its relatively low fungal count contrast the low fungal diversity and species richness of cattle feed despite it recorded the highest fungal count. Despite the overall low fungal count, and hence the hygienic nature of feedstuffs, the prevalence of *Aspergillus* species is worrying regarding the potential mycotoxin production.

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