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Survey of *Aspergillus* and *Fusarium* species and their mycotoxins in raw materials and poultry feeds from Córdoba, Argentina

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Abstract The aims of the present work were: (1) to determine both mycobiota in raw materials and finisher poultry feed, as well as the ability to produce aflatoxin B_1 by *A. flavus* strains, and (2) to evaluate the natural co-occurrence of aflatoxins (AFs), fumonisins (FBs), gliotoxin, diacetoxyscirpenol (DAS), HT-2 toxin, and T-2 toxin in poultry feed by LC-MS/MS. Nineteen percent of raw materials and 79% of finisher poultry feed samples exceeded the maximum allowed total fungal count (1×10^4 CFU g^{-1}) to ensure hygienic quality. *Aspergillus flavus* was the only species belonging to section *Flavi* which was isolated while *Fusarium verticillioides* was the prevalent species. Forty-seven percent of *A. flavus* strains were aflatoxin B_1 producers and the highest frequency of aflatoxigenic strains was isolated from finisher poultry feeds. Principal component analysis showed that corn grains are closely related with total fungal and *Fusarium* counts. This positive relationship suggests that total fungal and *Fusarium* spp. counts in poultry feed might come mainly from corn grains. Regarding poultry feeds, in ground finisher type, *Aspergillus* spp. counts increased as water activity (a_w) diminished. A positive relationship among a_w, total fungal and *Fusarium* spp. counts was observed in both ground finisher and ground starter feed. Several mycotoxins were monitored in feeds by applying the LC MS/MS technique. One hundred percent of poultry samples were contaminated with FB_1, and the highest levels were detected in pelleted finisher poultry. AFB_1, gliotoxin, DAS, HT-2 toxin, and T-2 toxin were not detected in any poultry feed. The scarcity of available mycotoxicological studies from Argentinean poultry feed using a multitoxin analysis technique enhances the contribution of the findings of this report.

Keywords *Aspergillus flavus* · *Fusarium* spp. · Mycotoxins · Raw materials · Poultry feeds

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Introduction

Argentinian poultry production has been growing considerably in the recent past and is expected to continue expanding in the near future. Poultry feed is one of the most important components in the modern systems of production (Secretaría de Agricultura Ganadería y Pesca 2007). Their composition includes mixtures of different raw materials such as home-grown cereals, mainly corn grains and soybeans, plus other additives. The toxigenic fungal contamination of the raw materials occurs during the pre-harvest and/or the post-harvest periods, and the finished feeds are also exposed during production, processing, transportation, and storage. Temperature and humidity play an important role not only in the development of fungi but also in mycotoxin production (Magan and Aldred 2007). The occurrence of *Aspergillus*

spp. and *Fusarium* spp. is widespread in tropical countries, and they have also been observed in feeds from Argentina (Okoli et al. 2006; Magnoli et al. 2002, 2005). In particular, they have been associated with cereals and animal feed-based cereals, and comprise several species which are mycotoxin producers and therefore have adverse effects on human and animals, resulting in illness and economic losses (Hussein and Brasel 2001).

Aflatoxins (AFs) are by far the most studied group of mycotoxins and their production is attributed to *Aspergillus flavus* and *A. parasiticus*. Aflatoxin B_1 (AFB_1) is the most potent natural carcinogen known and is classified by the International Agency of Research on Cancer as a Group 1 carcinogen (International Agency for Research on Cancer 1993). In poultry, AFs produce hepatic and kidney disorders, suppress immune function, and decrease productive parameters. Pale, enlarged, friable, and fatty livers are characteristic of acute aflatoxicosis in poultry (Council of Agriculture, Science and Technology 2003).

Fumonisins (FBs) are mycotoxins produced mainly by *Fusarium verticillioides* and *F. proliferatum*. Among the naturally occurring FBs, fumonisin B_1 (FB_1) is usually the most abundant and represents about 70% of the total concentration in corn and feeds. The studies of the effects of this toxin in poultry began in the last decade. Its occurrence in corn and poultry feeds has been associated with poor feed conversion, hepatotoxicity, impaired disease immunity, diarrhea, increase in organ weight, total body weight decrease, and increased susceptibility to environmental and microbial agents (Fandohan et al. 2003).

Several species of *Fusarium* are aggressive plant pathogens and have found to be trichothecenes producers. Among them, *F. sporotrichioides* and *F. poae* are the most frequent producers of T-2 toxin and diacetoxyscirpenol (DAS), whereas *F. crookwellense*, *F. culmorum*, *F. graminearum*, and *F. sambucinum* produce DAS and deoxynivalenol (DON or vomitoxin). Oral lesions in poultry are the primary means of diagnosing trichothecenes toxicoses in the field. In addition, asthenia and diarrhea decrease of the growth rate as well as egg production, and immunotoxicity has also been observed (Bennett and Klich 2003; Eriksen and Pettersson 2004; Iheshiulor et al. 2011). The most prevalent mycotoxins of the trichothecene group detected in agricultural products contaminated with *Fusarium* species were T-2 toxin and DON (Labuda et al. 2005; Lincy et al. 2008). Diacetoxyscirpenol was reported as a co-occurring mycotoxin though in trace amounts (Labuda et al. 2005).

Several studies have shown the prevalence of toxigenic mycobiota in poultry feeds from many countries and AFs and FBs as the most frequent mycotoxins (Shetty and Bhat 1997; Siame et al. 1998; Ali et al. 1998; Scudamore et al. 1998; Accensi et al. 2004; Oliveira et al. 2006; Rosa et al. 2006, Osho et al. 2007). On the other hand, previous studies reported that *A. flavus* and *F. verticillioides* isolated from poultry feeds were the main AFs and FBs producers in Argentina, and AFs, FBs, zearalenone (ZEA), DON, and ochratoxin A (OTA) were the prevalent mycotoxins (Dalcero et al. 1997, 1998, 2002; Magnoli et al. 2002, 2005).

In recent years, LC-MS/MS multi-analyte methods have been applied as useful tools to monitor the range of mycotoxins potentially occurring in moldy food and feed products. This technique provides an easy assessment of the risk that these compounds pose to human and animal health. Therefore, the aims of the present work were: (1) to determine the mycobiota present in raw materials and finished poultry feed, as well as the ability of *A. flavus* isolated strains to produce aflatoxin B_1, and (2) to evaluate the natural co-occurrence of AFB_1, FB_1, gliotoxin, DAS, HT-2 toxin, and T-2 toxin in poultry feed by LC-MS/MS over a sampling period of 12 months

Materials and methods

Sampling

Samples were collected in a feed-processing plant located in Río Cuarto, Cordoba Province, Argentina. A total of 148 samples including poultry feed (108 samples of different types of feeds: starter and finisher, either ground or pelleted) and corresponding raw materials (10 samples of each of the following feedstock: corn grains, soy pellets, soybean off and meat meal) were analyzed. Soybean off refers to soybeans that have been treated to deactivate anti-nutritional factors. Poultry feed samples were collected at random every month during a year between August 2008 and July 2009, and raw materials were collected from October 2008 to July 2009

Sampling was performed manually from the silos in transects at three levels (upper, middle and low). At each site, two sub-samples of 2 kg each were collected. In the laboratory, samples were homogenized and quartered to obtain 1-kg primary samples. These samples were finely ground, immediately analyzed for moisture content and fungal contamination, and then stored at 4°C for mycotoxin analyses.

Moisture content of samples

Water activity (a_w) determinations of the samples were carried out with AQUALAB CX2 (Decagon Devices, Pullman, WA, USA) and calibration was performed before sample measurements. Before calibrating, Aqua Lab was located in an area with a stable ambient temperature and the reference humidity standards were at room temperature. New sample cups for the reference humidity standards were

used. Sodium chloride (NaCl) which has 0.760 a_w was used as humidity standard and its a_w was compared with the table in the operator's manual. Readings of the humidity standard were within the stated ranges, and duplicate readings of distilled water were made.

Mycobiota isolation and identification

Quantitative enumeration of fungal propagules was done on solid media using the surface spread method by blending 10 g of each sample with 90 ml of 0.1% peptone water solution for 30 min. Serial dilutions from 10^{-1} to 10^{-3} concentrations were made and 0.1-ml aliquots were inoculated in triplicate on two culture media: Dichloran Rose Bengal Chloranphenicol Agar (DRBC) and Dichloran 18% Glycerol Agar (DG18) (Pitt and Hocking 1997). The plates were incubated in darkness at 25°C for 7 days. On the last day of incubation, only plates containing 10–100 colonies were used for counting, and the results were expressed as colony-forming units (CFU) per gram of sample (King 1992). Each colony of *Aspergillus* section *Flavi* and *Fusarium* genus was transferred on Malt Extract Agar (MEA) and Carnation Leaf Agar (CLA), respectively. Taxonomic identification of *Aspergillus* section *Flavi* species was achieved through macroscopic and microscopic studies following the schemes proposed by Klich (2002). In the case of *Fusarium* species the identification was performed according to Nelson et al. (1983) and Leslie and Summerell (2006). The results were expressed as fungal counts and frequency (percentage of samples in which each species was present).

Aflatoxin production by *Aspergillus flavus* strains

Ninety-seven *A. flavus* strains (37 isolated from raw materials and 60 from poultry feeds) were evaluated for their ability to produce AFs following the methodology described by Geisen (1996). This technique was done by screening of aflatoxigenic capacity of the isolated strains. The strains were grown in MEA at 25°C for 7 days in darkness, after which mycelium and conidia were collected from the agar surface with a sterile brush and transferred to microtubes. Aflatoxins were extracted with chloroform (500 μl) and centrifuged at 896*g* for 10 min. The chloroform phase was transferred to a clean microtube, evaporated to dryness, and stored until AFs analysis. The chloroformic extracts were spotted together with standard solutions of AFB_1, aflatoxin B_2 (AFB_2), aflatoxin G_1 (AFG_1), and aflatoxin G_2 (AFG_2) (Sigma Chemical, St. Louis, MO, USA) and screened by thin layer chromatography (TLC). Silica gel plates without fluorescent indicator (0.25 mm, G60; Merck, Buenos Aires, Argentina), and chloroform:acetone (9:1, v/v) as developing solvent were used. Plates were then examined under long-wave UV light (365 nm) and AFs concentrations were determined throughout visual comparison with standard solutions. The detection limit of the analytical method was 1 μg g^{-1}.

Assay of spiking and recovery of mycotoxins from poultry feed

The mycotoxins AFB_1, FB_1, gliotoxin, DAS, HT-2 toxin, and T-2 toxin were obtained from Sigma-Aldrich Chemical (Dorset, UK). Stock and working solutions were prepared in acetonitrile.

In order to determine the efficacy of the analytical method, recovery studies were performed as follows: poultry feeds samples (0.5 g) were fortified at two spiking levels with 1 mL of a combined mycotoxins working solution. Each spiking level was conducted by triplicate. The samples were subsequently stored for 3 days at 40°C to allow equilibration between the analytes and the matrix and also solvent evaporation. After this period of time, mycotoxin extraction was performed. Matrix blank extraction was also included for comparison

Extraction and detection of mycotoxins from poultry feed

Mycotoxin extraction and detection were performed according to Sulyok et al. (2007). Briefly, 2 mL of extraction solvent (acetonitrile/water/acetic acid 79:20:1, v/v/v) was added to the samples and extracted for 90 min using a GFL 3017 rotary shaker (Vicking model DUBNOFF). Samples were subsequently centrifuged for 2 min at 1,512*g* (Rolco SRL). The extracts (1 mL) were transferred into glass vials using Pasteur pipettes and were diluted with the same volume of dilution solvent (acetonitrile/water/acetic acid 20:79:1, v/v/v). After appropriate mixing, 50 μL of the diluted extracts were injected into the LC-MS/MS system without further treatment. Results were not corrected by recovery. Table 1 shows calibration curve data, contamination and recovery levels, and detection and quantification limits of each mycotoxin.

All LC-MS/MS analyses were performed using a Waters 2695 Alliance HPLC (Waters, Milford, MA, USA) equipped with a Waters Alliance 2685 pump, a Waters Alliance 2695 autosampler, and a diode array detector Waters 2996 PDA interfaced to a Quattro Ultima Platinum tandem quadrupole mass spectrometer with electrospray ionization (ESI) source. An XBridge™ C18 (3.5 μm, 2.1×150 mm) column with a guard column was used. The mobile phase of the chromatographic procedure was a gradient of aqueous 1% acetic acid/5 mM ammonium acetate (solvent A) and 0.1% methanol/1% acetic acid/5 mM ammonium acetate (solvent B). The program gradient is shown in Table 2. The flow rate was 0.2 mL/min. The temperature of column was kept at 22°C. For the sake of sensitivity, the most abundant trace in

Table 1 Validation results in poultry feed samples

Mycotoxin	a	b	r^2	Cal. Range (μg kg^{-1})	Validation levels (μg kg^{-1})	Recovery (%)	RSD (%)	LOQ (μg kg^{-1})	LOD (μg kg^{-1})
Aflatoxin B_1	1×10^6	295.51	0.99	32–480	200 400	56	11.26	5.3	1.6
Fumonisin B_1	1×10^6	295.51	0.99	32–480	20,000 40,000	68	7.60	7.28	2.42
Gliotoxin	6×10^6	5,434.7	0.98	160–2,400	1,040 2,080	82	3.32	133	44
DAS	2×10^7	31,052	0.99	320–4,816	2,000 4,000	54	8.70	40	13
T2 toxin	5×10^6	13,291	0.98	320–4,816	2,000 4,000	89	2.63	40	13
HT-2 toxin	1×10^6	295.51	0.99	32–480	2,000 4,000	68	7.60	40	13

LOQ limit of quantification, *LOD* limit of detection, *RSD* relative standard deviation (n=84)

the multiple reaction monitoring (MRM) mode was used for quantitative analysis. Retention time differences lower than 0.05 min relative to toxin standards together with two precursor/product ion transitions were considered for qualification. The interfaces were operated in a positive ion mode. Nitrogen gas was used for both nebulization and desolvation heated to 150 and 200°C, respectively. The capillary voltage was 3.00 kV. The nitrogen flow was adjusted to 104 and 678 L/h for cone and desolvation gases, respectively. The MRM transitions and experimental conditions are shown in Table 3. Dwell time was set at 0.1 ms for all transitions. Data acquisition and processing were performed using Mass Lynx V.4.1 software (Waters).

Statistical analyses

Data were analyzed with analysis of variance. Means were compared using a linear mixed model and Fisher's protected least significant difference (LSD) test to determine the significant differences among means of water activities, fungal counts, percentage of samples contaminated by *A. flavus*, *Fusarium* species, and presence of mycotoxins in raw materials and poultry feeds (p<0.05). Principal component analysis (PCA) of the data was carried out in order to determine the relationships among water activities, fungal counts, and mycotoxin levels in raw materials and different types of terminated poultry feeds (Quinn and Keough 2002). The analysis was conducted using software InfoStat (2008 version; group InfoStat, National University of Córdoba, Argentina).

Results

Mycobiota contamination

Water activity of the samples throughout the sampling period ranged from 0.534 to 0.694 in raw materials and from 0.541 to 0.637 in poultry feeds, respectively. Statistical analyses demonstrated that lowest levels of a_w (mean 0.550) were obtained from meat meal and pelleted finisher poultry feed samples. No significant differences were observed among the rest of the raw materials and poultry feeds (p<0.05) (data not shown).

Analysis of total fungal counts from raw materials and poultry feed samples did not show significant differences between media. The means of fungal total counts of raw materials ranged from 2×10^3 to 2×10^6 CFU g^{-1}. Highest fungal total counts were observed in corn samples (in the order of 10^6 CFU g^{-1}). No statistical significant differences

Table 2 Gradient program of the LC system

Step	Time (min)	Solvent A[a] (%)	Solvent B[b] (%)	Flow (ml min^{-1})	Curve
1	0.00	10.5	89.5	0.2	1
2	2.00	10.5	89.5	0.2	1
3	14.00	97.5	2.5	0.2	6
4	17.00	97.5	2.5	0.2	6
5	17.10	10.5	89.5	0.2	6
6	22.00	10.5	89.5	0.2	6

[a]Acetic acid (1%)/ ammonium acetate (5 mM)

[b]Methanol (0.1%)/ acetic acid (1%)/ ammonium acetate (5 mM)

Table 3 Overview of all relevant information regarding the investigated mycotoxins: parent and daughter ions, cone (*CV*) and collision (*CE*) voltages, ratios, and retention times

Mycotoxin	Retention time (min)	Precursor ion	Products ions	Ratio	CV	CE
Aflatoxin B_1	14.42	313	241	1.7	76	33
			285		76	33
Fumonisin B_1	16.55	722	334	1.4	91	57
			352		91	55
Gliotoxin	14.61	327	263	10.5	35	15
			241		35	25
Diacetoxiscerpenol	14.47	384	305	1.0	51	17
			105		51	61
T-2 toxin	16.67	484	215	1.1	56	31
			185		56	29
H-T2 toxin	15.88	442	263	2.5	46	21
		447	345		101	27

were observed between meat meal and soy pellets ($p<0.05$). The lowest counts were found in soybean off (in the order of 10^4 CFU g^{-1}) (Fig. 1a). The fungal total counts in poultry feeds varied depending on month of sampling and the special feed type, and the means varied from 10^3 to 10^7 CFU g^{-1}. In general, ground starter poultry feed samples showed higher counts than those observed in finisher feed samples either ground or pelleted. All feeds, except finisher pelleted type collected in August, December and February, exceeded the values of 1×10^4 CFU g^{-1}(Fig. 1b–d).

Aspergillus flavus was the only species belonging to section *Flavi* isolated from raw materials and terminated poultry feeds. Three *Fusarium* species were found: *F. verticilliodes* was the prevalent in all samples, followed by *F. proliferatum* (33.3%) and *F. subglutinans* (16.6%). The last two species were only isolated from poultry feeds. Figure 2 shows the frequency of *A. flavus* and *F. verticillioides* in raw materials. The frequency of *A. flavus* varied from 40 to 100% depending on culture medium. The highest frequencies were found in meat meal and soy pellet samples. From the *Fusarium* genus, *F. verticillioides* was isolated in 100% of the meat meal samples (in DG18), and in corn grains and soy pellets (in both media), while lower frequencies were observed in soybean off samples (25 and 50%).

With regard to finisher feeds, *A. flavus* was isolated only in some months of sampling from starter and finisher ground poultry and the frequency of contaminated samples varied from 20 to 100% and 40 to 100%, respectively. On the contrary, this species was isolated all months of sampling, except to November (2008), June and July (2009), from pelleted finisher poultry; and the frequency of contaminated samples was 100% from December (2008) to May (2009) (Fig. 3).

From the *Fusarium* genus, *F. verticillioides* was isolated from 100% from all poultry feed samples, except in some months of sampling. In August, this species was isolated in 80–90% of the samples in both media from all feeds. In February, the percentages of contaminated samples were 90 and 50% from ground starter and pelleted finisher poultry, respectively. While in July, only 33% of pelleted finisher samples contained this species (data not shown).

Aflatoxin production by *Aspergillus flavus* strains

Table 4 shows the potential aflatoxigenic of *A. flavus* strains in raw materials and poultry feeds. The distribution of AFs producer strains varied according to the type of feed. From 97 tested strains, 46 (47%) were AFB_1 producers with levels ranged from <1.0 to 20.6 μg g^{-1}. The highest frequency of aflatoxigenic strains was isolated from poultry feed samples.

Mycotoxins determination

Clean chromatograms of each mycotoxin naturally contaminated poultry feed samples were obtained. One hundred percent of poultry feed samples were contaminated with FB_1, in levels ranging from 26.30 to 859.18, 37.4 to 825.2, and 41.6 to 217.2 μg kg^{-1} from ground starter, ground finisher, and pelleted finisher feed, respectively. The highest levels of FB_1 were detected in pelleted finisher feed samples ($p<0.05$). Aflatoxin B_1, gliotoxin, DAS, HT-2 toxin, and T-2 toxin were not detected in any poultry feed sample; see limit of detection (LOD) of the particular technique in Table 1.

Multivariate statistical analysis

Figure 4 shows the bi-plot for principal component analysis (PCA) in order to determine the influence of type of raw materials or poultry feed on a_w, total fungal counts, *A. flavus* and *F. verticillioides* counts, and FB_1 levels. As can be observed, corn grains (C) are closely related to total fungal and *Fusarium* counts. This positive relationship suggests that total fungal and *Fusarium* spp. counts in finisher feed

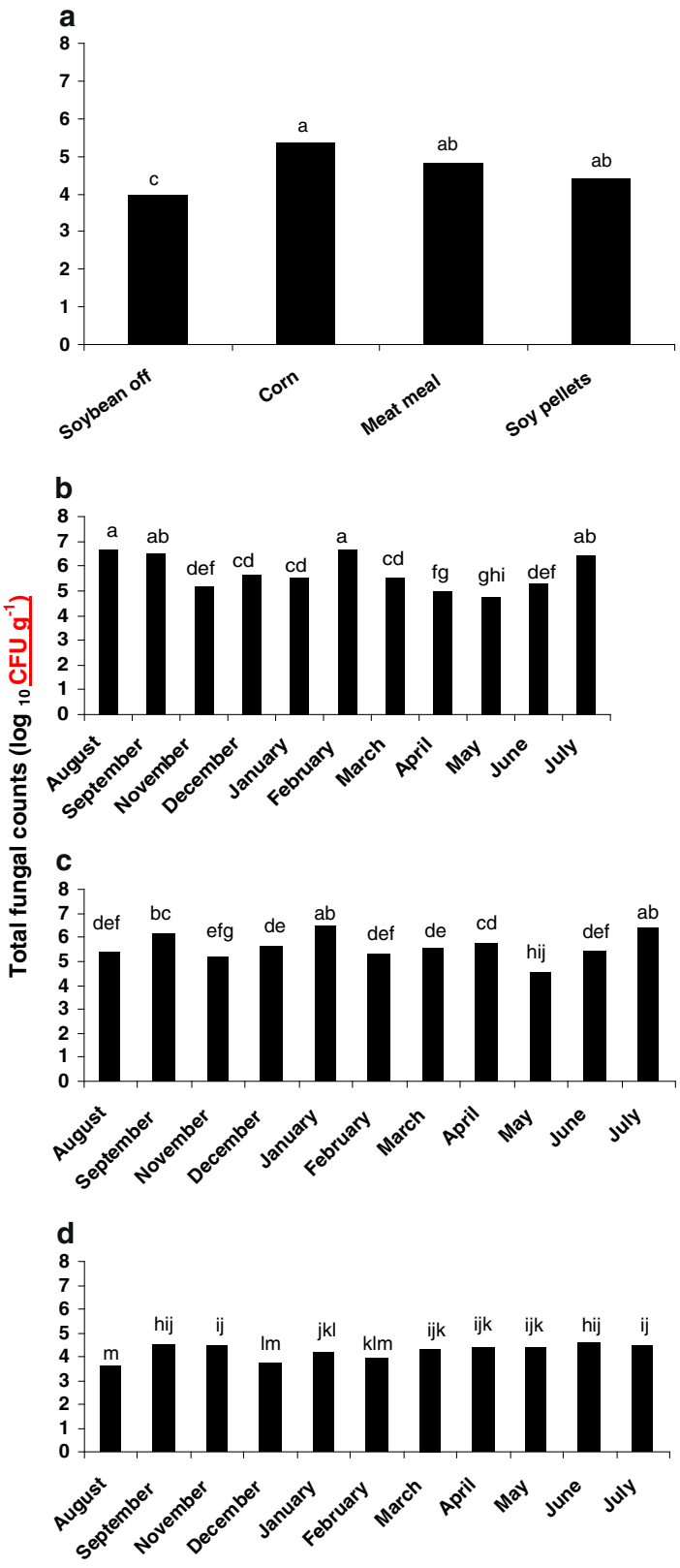

Fig. 1 Total fungal counts in raw materials (**a**), ground starter poultry (**b**), ground finisher poultry (**c**) and pelleted finisher poultry (**d**). Columns with a letter in common are not significantly different according to LSD test ($p<0.05$). $n = 40$ samples of raw materials and 108 samples of poultry feeds

samples might come mainly from corn grains. Regarding meat meal (MM) at high a_w, a closely relationship is then observed between this raw material and *Aspergillus* counts. A defined behavior pattern among the variables assayed in soy pellets and soybean off was not observed (Fig. 4a). Regarding poultry feeds, in both ground starter and finisher

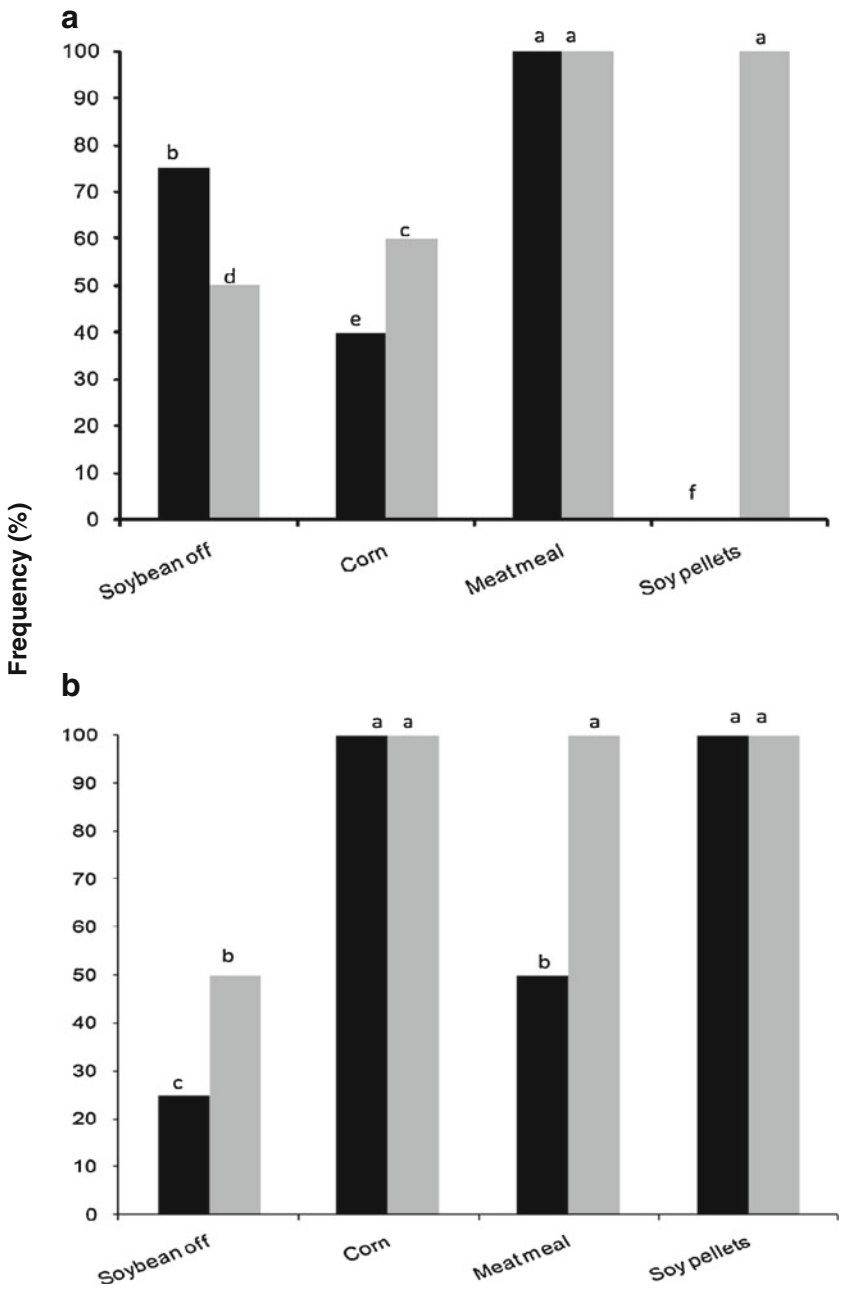

Fig. 2 Frequency of *A. flavus* (**a**) and *F. verticillioides* (**b**) from raw materials in DRBC (■) and DG18 (*black columns*) and (*gray columns*) media. columns with a letter in common are not significantly different according to LSD test ($p<0.05$). n: 40

poultry (GSP and GFP), the variable a_w diminished as *Aspergillus* spp. counts increased, and a positive relationship between a_w, total fungal and *Fusarium* spp. counts was observed (Fig. 4b). In contrast, in pelleted finisher poultry (PFP) samples, a negative relationship between a_w and *Aspergillus* spp. counts was observed.

Discussion

This study shows the presence of toxigenic species belonging to *Aspergillus* section *Flavi* and *Fusarium* species in raw materials and finisher poultry feeds. It has been demonstrated that fungal propagules are helpful indicators of the hygienic quality of feeds. The latest regulations for feeds establish a maximum value of 1×10^4 CFU g^{-1} to assure a good hygienic quality of the product (Good Manufacturing Practices 2008). In this study, 19% of raw materials and a quite high percentage of finisher poultry feed samples (79%) exceeded the maximum allowed. These results suggest a high fungal activity which could affect both the organoleptic properties and nutritive quality of the feed. These results are similar to those obtained earlier from the same substrate (Accensi et al. 2004; Dalcero et al. 1998; Magnoli et al. 2005; Rosa et al. 2006; Krnjaja et al. 2008; Shareef 2010; Astoreca et al. 2011). In these reports, some

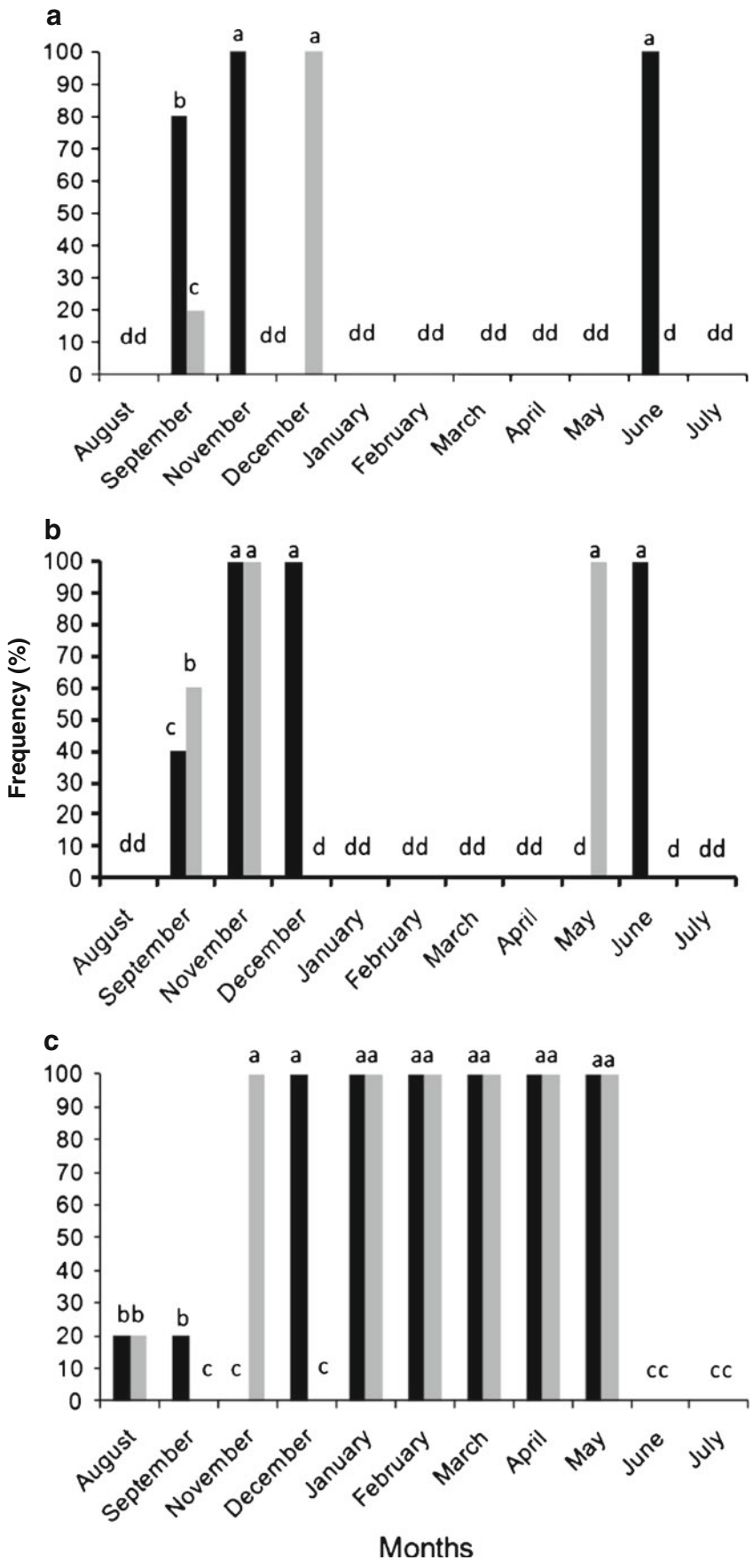

Fig. 3 Frequency of *A. flavus* in ground starter poultry (**a**), ground finisher poultry (**b**) and pelleted finisher poultry (**c**) in DRBC (*black columns*) and DG18 (*gray columns*) media. Columns with a letter in common are not significantly different according to LSD test ($p<0.05$). $n = 108$

Table 4 Aflatoxin B_1 production by *A. flavus* strains isolated from raw materials and poultry feeds

Raw materials and feeds	Producing strains/total strains	Range AFB_1 ($\mu g\ g^{-1}$)	Mean levels ($\mu g\ g^{-1}$)±SD
Soybean off	8/16	<1.0–2.5	1.4±1.0
Corn grains	2/12	3.5–4.2	3.8±0.2
Meat meal	0/3	ND	ND
Soy pellets	3/6	2.5–4.5	3.5±0.7
GSP	14/20	2.7–20.6	13.8±7.8
GFP	6/14	4.1–10.3	7.2±4.3
PFP	13/26	0.9–20.6	6.3±6.7

Detection limits of AFB_1=1 $\mu g\ g^{-1}$

GSP ground starter poultry, *GFP* ground finisher poultry, *PFP* pelleted finisher poultry *SD* standard deviation, *ND* not detected

counts were higher than 10^5 CFU g^{-1}. However, Magnoli et al. (2002), Oliveira et al. (2006), Fraga et al. (2007), and Lincy et al. (2008) found moderate values ranging from 10^3 to 10^4 CFU g^{-1} in all samples. No significant differences in fungal counts were observed between the general DRBC medium which reflects the total fungal count and DG18 which showed high counts of moderately xerophilic fungi such as *Aspergillus* spp. Similar data were observed by Fraga et al. (2007) from samples of maize and poultry feeds.

Poultry feeds undergo a pelleting process during their fabrication (usually 115°C, 60 min). The fungal counts and frequency of *Fusarium* and *Aspergillus* species suggest a fungal resistance to the manufacturing practices and/or the potential contamination after feed elaboration, packing, or storage of finisher product. In this study, the total fungal counts detected in pelleted finisher poultry were significantly lower than those observed in other feeds not subject to the pelleting process (ground starter and finisher poultry). This finding agrees with previous studies (Chelkowski 1991; Dalcero et al. 2002) which reported that fungal counts decreased significantly when the pelleting process was carried out. In contrast with the present results, Fraga et al. (2007) reported that any Brazilian poultry feed samples collected after this process showed fungal contamination, and the counts were below 100 CFU g^{-1}. In the present work, a high frequency of *A. flavus* has been observed in pelleted finisher poultry. In agreement with these results, in previous work, this species has also been isolated in commercial pet feeds after this process (Fernández-Juri et al. 2009; Campos et al. 2009). In addition, *F. verticillioides* was the most frequent species isolated, followed by *A. flavus* during this sampling period, both in corn and finisher feeds, suggesting that this species also tolerates the pelleting process. In contrast, *A. flavus* was reported as the prevalent species followed by *F. verticilliodes* (Rosa et al. 2006; Labuda and Tančinová 2006; Oliveira et al. 2006; Saleemi et al. 2010). *F. verticillioides* and *F. proliferatum* are commonly isolated from maize throughout the world, and are also recognized by their capacity to produce FBs (Fandohan et al. 2003; Glenn 2007). *Fusarium* is one of the major fungal genera associated with maize in Argentina. In previous years, *F. verticillioides*, *F. proliferatum*, and FB_1 were mainly detected in corn grain (Ramirez et al. 1996; Solovey et al. 1999; González et al. 1999; Torres et al. 2001; Pacin et al. 2009). With respect to other species isolated in the present work, *F. subglutinans* has not been recognized as a producer of high levels of FBs, but in recent years, its capacity to produce other mycotoxins such as moniliformin and beauvericin has been demonstrated (Jestoi 2011). The presence of *F. subglutinans* in poultry feeds leads to the possibility of finding samples contaminated with these toxins.

In the present study, 47% of the *A. flavus* strains were AFB_1 producers. The incidence of aflatoxigenic strains found in the present study is similar to that reported in previous work in Argentina and Brazil (Magnoli et al. 1999; Fraga et al. 2007), and the levels produced are relatively lower than those reported by other authors in poultry feeds (Saleemi et al. 2010; Astoreca et al. 2011). The presence of aflatoxigenic strains suggests the potential risk of AFs production in raw materials or feeds if storage conditions are inadequate.

The prevalent species isolated in this study, *A. flavus* and *F. verticillioides*, produce mycotoxins of greater concern for poultry health. In general, relatively low water content present in stored products is most often related with *Aspergillus* spp. growth (Krnjaja et al. 2008), while *F. verticillioides* and *F. proliferatum* presence in substrates such as corn grains are related to high water content (Samapundo et al. 2005). In the present study, a positive relationship between *F. verticillioides* and FB_1 has not been detected. However, positive relationships between high a_w level and *Fusarium* spp. counts and low a_w levels and *Aspergillus* counts have been found in some samples.

Regarding the mycotoxin contamination, FB_1 was present in all poultry feed samples, while AFB_1, gliotoxin, DAS, HT-2 toxin, and T-2 toxin were not detected. In previous sampling in Argentina, the presence of AFB_1, ZEA, DON, OTA, and FBs were reported in this substrate, and FB_1 levels were lower than those detected in the present work (Dalcero et al. 1997, 1998, 2002; Magnoli et al. 2002). In any sample, the FBs levels exceeded the maximum tolerable limit (20 mg FB_1+FB_2 kg^{-1}) established for avian feeds by the European Commission (UE 2007). The presence of FB_1 in finished feeds may be explained by considering that FBs in processed corn products for poultry consumption vary depending on cultivar growth conditions, and then are also influenced by environmental factors such as temperature, humidity, and rainfall during pre-harvest and harvest

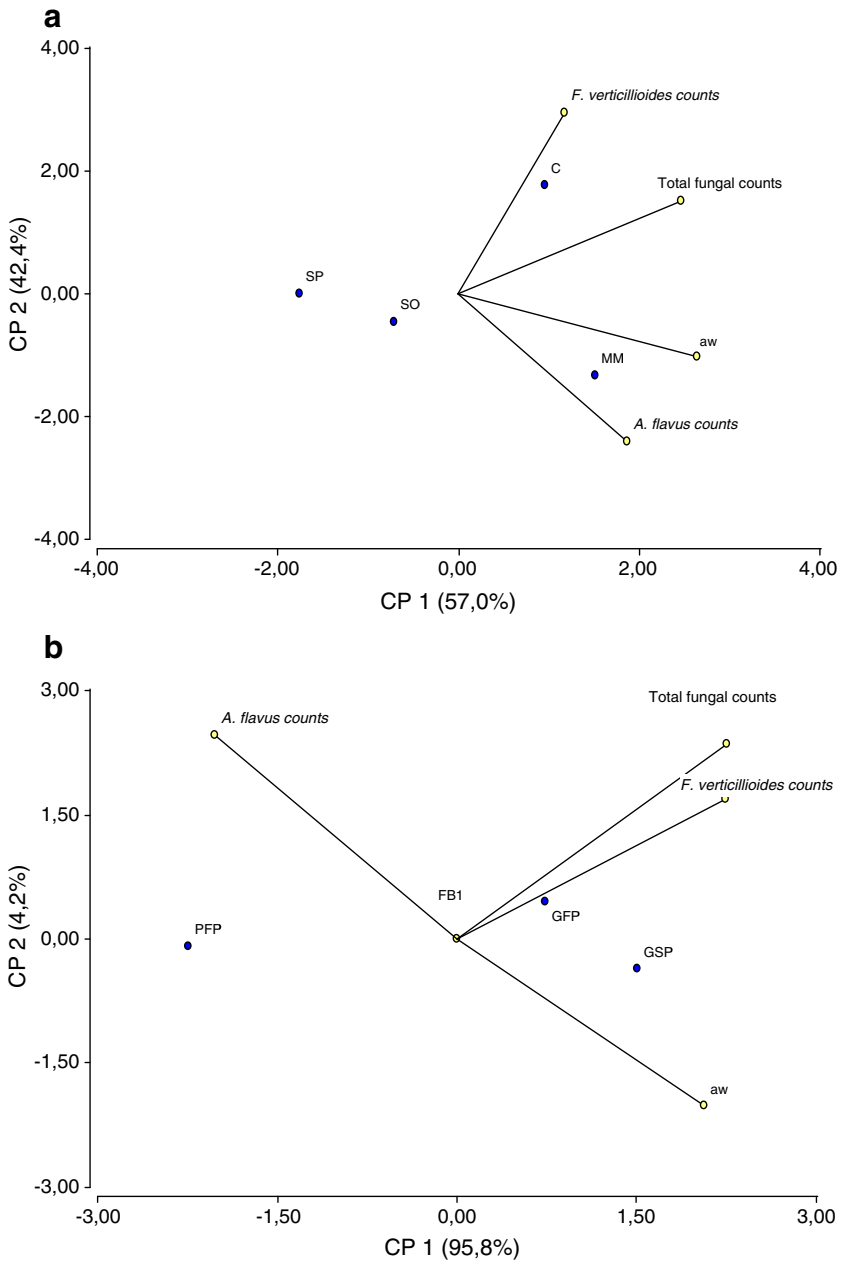

Fig. 4 Biplot graph with principal component analysis (PCA) for the variables in this study (a_w, total fungal, *A. flavus* and *F. verticillioides* counts, and FB_1 levels) in relation to feed type. *C* corn grains, *MM* meat meal, *SO* soybean off, *SP* soy pellets, *GFP* ground finisher poultry feed, *PFP* pelleted finisher poultry feed, *GSP* ground starter poultry feed, (**a**) raw materials, (**b**) poultry feeds

periods. Then, while in storage, these mycotoxins can be produced when the air humidity is appropriate (Bacon and Nelson 1994).

Recently, Astoreca et al. (2011) reported the co-occurrence of AFB_1 and cyclopiazonic acid in 16% of poultry feed samples. A few surveys have reported the natural occurrence of trichothecenes such as T-2 toxin, HT-2 toxin, and DAS in poultry feeds. Labuda et al. (2005) reported the presence of ZEA and trichothecenes in Slovakia. The most frequent mycotoxin detected was T-2, which was found in 90% of the samples in relatively low concentrations (1–130 μg/kg), followed by ZEA that was found in 88%. Lower frequencies were observed with HT-2 and DON (76 and 56%, respectively) with contamination levels ranging from 2 to 173 μg/kg and 64 to 1,230 μg/kg, respectively. A combination of four simultaneously co-occurring mycotoxins, i.e. T-2, HT-2, ZEA, and DON, was found in 44% of the samples. In another study from India, T-2 toxin and trichothecenes producing *Fusarium* were detected only in just 1 sample out of 40, by PCR assays from poultry feed (Lincy et al. 2008). A small number of poultry feed samples from Croatia contained T-2 toxin and DAS. Positive samples were correlated with evident clinical symptoms of toxicosis in poultry (Sokolovic and Simpraga 2006). In contrast, in the present sampling period, trichothecenes and *Fusarium* producer species were not detected, although *F. graminearum* and DON have been

previously detected in Argentinean poultry feeds (Dalcero et al. 1997, 1998).

Previous surveys have shown that mycotoxin contamination depends on the location and the particular year (Dalcero et al. 1997, 1998, 2002; Magnoli et al. 2002). Marked differences in terms of FBs contamination were observed for the same maize variety between two consecutive growing seasons in Argentina (Hennigen et al. 2000). Differences in environmental conditions from one season to another might be responsible for mycotoxin contamination of poultry feed. According to the Argentina Meteorological Service reports, the period from August 2008 to July 2009 were extremely dry and warm (Servicio Meteorológico Nacional 2007). Therefore, the lack of trichothecenes in the sampling period could be explained because their production is favored at low temperatures and high air humidity.

Although each mycotoxin is produced under specific environmental conditions, during the storage the available water and the temperature play a key role in controlling mycotoxin production. In general, due to their composition, feeds are, at high moisture (>12%) and temperature, an excellent substrate for the growth of fungi.

Taking into account that mycotoxicoses affect health and production efficiency and cause economic losses, this survey shows that *Aspergillus* and *Fusarium* species represent a potential toxicological risk and should be regarded as a potential source of mycotoxins. Because there is a wide range of different mycotoxins with different chemical structures that can sporadically occur in agricultural-based feeds, a regular monitoring of toxigenic mycobiota and the application of methodologies for detecting multiple mycotoxins is becoming an essential pre-requisite for the development of strategies to control or prevent mycotoxin exposure in the ploutry industry. The scarcity of available mycotoxicological studies from Argentinean poultry feed using a multitoxin analysis technique enhances the contribution of the findings of this report.

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Conflict of Interest None.

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