Survey of toxigenic fungi and natural co-occurrence of mycotoxins in poultry feeds from Entre Ríos, Argentina

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Abstract:	A total of 120 pelleted poultry feed samples from Entre Ríos Province, Argentina were evaluated. The aim was to investigate: i) the presence of significant toxigenic fungi, as well as to determine the ability to produce aflatoxins (AFs) by Aspergillus section Flavi strains isolated, and ii) to evaluate the natural co-occurrence of AFs, fumonisin (FBs), gliotoxin, diacetoxyscirpenol (DAS), HT-2 and T-2 toxin by HPLC-MS/MS. Total fungal counts are below of the established value (1 x 104 CFU g-1). Aspergillus flavus and A. parasiticus were the only aflatoxigenic species isolated. Co-occurrence of fumonisin B1 (FB1), HT-2 and T-2 toxin were detected in 100% of the feeds in mean levels ranged from 4501.7 to 5813.4; 6.7 to 21.6 and 19.6 to 30.3 µg kg-1, respectively. A high number of starter samples were co-contaminated with aflatoxin B1 (AFB1), FB1, HT-2 and T-2 toxins. Gliotoxin and DAS were not found in this survey.

Survey of toxigenic fungi and natural co-occurrence of mycotoxins in poultry feeds from Entre Ríos, Argentina

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Abstract

A total of 120 pelleted poultry feed samples from Entre Ríos Province, Argentina were evaluated. The aim was to investigate: i) the presence of significant toxigenic fungi, as well as to determine the ability to produce aflatoxins (AFs) by *Aspergillus* section *Flavi* strains isolated, and ii) to evaluate the natural co-occurrence of AFs, fumonisin (FBs), gliotoxin, diacetoxyscirpenol (DAS), HT-2 and T-2 toxin by HPLC-MS/MS. Total fungal counts are below of the established value (1 x 10^4 CFU g^{-1}). *Aspergillus flavus* and *A. parasiticus* were the only aflatoxigenic species isolated. Co- occurrence of fumonisin B_1 (FB_1), HT-2 and T-2 toxin were detected in 100% of the feeds in mean levels ranged from 4501.7 to 5813.4; 6.7 to 21.6 and 19.6 to 30.3 µg kg^{-1}, respectively. A high number of starter samples were co-contaminated with aflatoxin B_1 (AFB_1), FB_1, HT-2 and T-2 toxins. Gliotoxin and DAS were not found in this survey.

Key words: *Aspergillus flavus*, *Aspergillus parasiticus,* aflatoxins, fumonisins, T-2 toxin, HT-2 toxin, starter and finisher poultry feeds.

Introduction

Mycotoxins are structurally diverse group of toxic substances produced mostly by filamentous fungi that grown on several types of grains (cereals, oilseed or their by-products) used as raw materials in animal feeds elaboration. The most common mycotoxin producing fungi in feeds belonging to *Aspergillus*, *Fusarium* and *Penicillium* genera (Kumar et al. 2008; Bryden 2009). Generally, mixed feeds by self-composition, especially under favorable conditions, such as appropriate moisture percentage (> 12%) and moderate temperature (25 to 28ºC) represent an excellent substrate for fungal growth (Zain 2011; Bryden 2012).

Development of toxigenic fungi and consequent mycotoxins contamination on poultry feed, constitute a serious problem in South American countries, where poultry production and processing are expanding rapidly (MAGPyA, 2012). Farmers are often tempted to incorporate moldy grain into animal diets to reduce feed costs. This practice represent the major problem by livestock industry does carry negative effects on the growth and health poultry; and the negative impact on nutritive and organoleptic properties of the final feeds. The economic impact are produced by decreased productivity, weight gain, feed efficiency, meat and egg production, among others (Hussein and Brasel 2001; CAST 2003; Bryden 2012).

Aflatoxins (AFs), fumonisisns (FBs) and trichothecenes (TCT) are common contaminants of feedstuffs and their adverse effects on poultry health and productivity have been studied extensively. Aflatoxins are the principal classes of mycotoxins mainly produced in cereals and oilseeds by *A. flavus* and *A. parasiticus;* which have been extensively reported as the most potent hepatocarcinogenic substance known. The liver is the primary target organ, with liver damage in cases where poultry have been fed with aflatoxin B_1 (AFB_1). Fumonisins are cancer-promoting metabolites produced by *Fusarium proliferatum* and *F. verticillioides*; and commonly are present in co-occurrence with AFs in poultry feeds. Fumonisin B_1 (FB_1) is the most toxic metabolite and

their effects have been clearly reported in some animal species as rats, equines and pigs. In poultry relatively high levels are required to produce negative effects. However, FBs in combination with other mycotoxins as diacetoxyscirpenol (DAS), AFs and ochratoxin A (OTA) appear to exert a greater negative impact on the health and productivity of livestock in comparison to their individual effects (Iheshiulor et al. 2011). The TCT comprise a vast group of metabolites since *Fusarium* genus the main producers in cereals and derives. These mycotoxins contain the same basic structure (an epoxide at the C12,13 positions) which is responsible for their toxicological activity. In poultry the toxic action results in extensive necrosis of the oral mucosa and skin in contact with the toxin, acute effect on the digestive tract and decreased bone marrow and immune function. The TCT causing most concern in animals are T-2 toxin (which is the most acute toxicity), HT-2 toxin, nivalenol (NIV) and deoxynivalenol (DON), the most frequently occurring TCT (Sundstøl and Pettersson, 2004).

The greatest significant mycotoxin groups that have been reported in poultry feeds from tropical and subtropical countries are AFs and FBs (Shetty and Bhat 1997; Siame et al. 1998; Ali et al. 1998; Scudamore et al. 1998; Scudamore and Patel 2009; Dalcero et al. 1997, 1998, 2002; Magnoli et al. 2002, 2005; Accensi et al. 2004; Oliveira et al. 2006; Rosa et al. 2006; Osho et al. 2007; Sharef 2010). Despite the several risk assessments for TCT in humans and animals, limited works have been published worldwide on the occurrence of these mycotoxins in poultry feeds. In Argentina, there are several data about the occurrence of TCT and *Fusarium* producers species from cereals grains; however limited information is available from animals´ feeds (Dalcero et al. 1997, 1998; Solovey et al. 1999; Labuda et al. 2005; Sokolovic and Simpraga 2006; Lincy et al. 2008; González et al. 2008; Roige et al. 2009; Scudamore and Patel 2009; Gónzalez Pereyra et al. 2008; Monge et al. 2012; Reynoso et al. 2011; Barros et al. 2011, 2012; Sampietro et al. 2012).On the other hand, recently studies by Monge et al. (2012) from Córdoba province,

Argentina reported that *A. flavus* and *F. verticillioides* isolated from poultry feeds were the main toxigenics fungi. Respecting mycotoxin occurrence, one hundred percent of poultry feed samples were contaminated with FB_1, while AFB_1, gliotoxin, DAS, HT-2 toxin and T-2 toxin were not detected.

The HPLC-MS/MS multi-analyte techniques are used to monitoring the occurrence of mycotoxins in several agricultural commodities. In addition, this determination is essential for evaluate the risk of feed consumption and/or confirming the diagnosis of a possible mycotoxicosis. Mycotoxins occur sporadically both seasonally and geographically throughout the world, with significant regional differences especially between tropical and temperate areas. Hence, the aim of this study was to investigate: i) the presence of significant toxigenic fungi in starter and finisher poultry feeds from Entre Ríos Province, as well as to determine the ability to produce aflatoxins by *Aspergillus* section *Flavi* strains isolated, and ii) to evaluate the natural co-occurrence of aflatoxins, fumonisin, gliotoxin, diacetoxyscirpenol, HT-2 toxin and T-2 toxin by HPLC-MS/MS.

Materials and Methods

Samples

A total of 120 samples of different types of starter and finisher pelleted feeds: 20 starter 1 (S1), 20 starter 2 (S2), 20 starter 3 (S3), 20 finisher (F), 10 finisher off (FO), 10 finisher split (FS), 10 finisher parallel half (FPH) and 10 finisher half (FH), were analyzed. Samples were collected from a feed-processing plant located in Entre Ríos Province, Argentina in December 2010. This province is located between 30º 9' and 34º 2'of south latitude and between 57º 48' and 60º 47' of west latitude. The sampling area is inserted into the transition area of temperate and subtropical climates. This region is characterized by abundant rainfall throughout the year. Sampling was

performed manually from the silos in transects at three levels (from upper, middle and low). At each site, two sub-samples (2 kg each) of the same products were collected. In the laboratory, the samples were homogenized and quartered to obtain 1 kg primary sample. These representative samples were finely ground and were analyzed immediately to determine water activity (a_w) and fungal contamination; and then stored at 4°C for mycotoxin analyses. Water activity determinations of the samples were done with AQUALAB CX2 (Decagon Devices, Pullman, WA, USA) using the methodology previously described by Monge et al. (2012).

Mycobiota isolation and identification

Quantitative enumeration of fungal propagules was done on solid media using the surface spread method by blending 10 g portion of each sample with 90 ml of 0.1% peptone water solution for 30 min. Serial dilutions of 10^{-1} to 10^{-3} concentrations were made and 0.1 ml aliquots were inoculated in triplicates on two culture media: Dichloran Rose Bengal Chloranphenicol Agar (DRBC) and Dichloran 18% Glycerol Agar (DG18) (Pitt and Hocking 2009). The plates were incubated in darkness at 25°C during 7 days. On the last day of incubation, only plates containing 10 to 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* was transferred on Malt Extract Agar (MEA). Taxonomic identification of *Aspergillus* section *Flavi* species was achieved through macroscopic and microscopic studies following the schemes proposed by Pitt and Hocking (2009) and Klich (2002). Cultures were grown on Czapek Yeast Extract Agar (CYA), at 25 and 37°C; Malt Extract Agar (MEA), 25% Glycerol Nitrate Agar (G25N), and Czapek Yeast Extract with 20% Sucrose Agar (CY20S) at 25 °C. All plates were incubated for 7 days. The total fungal counts and percentage of samples contaminated with *Aspergillus* section *Flavi* were determined.

Aflatoxin production by Aspergillus section Flavi strains

Thirty-four *A. flavus* and *A. parasiticus* strains were evaluated for their ability to produce AFs following the methodology described by Geisen (1996). 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 4,000 rpm during 10 min. The chloroform phase was transferred to a clean microtube, evaporated to dryness and stored at 4ºC until AFs analysis. The extract was 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 was used. Plates were then examined under long wave UV light (365 nm) and AFs concentration was determined throughout visual comparison with standard solutions. The detection limit of the analytical method was 0.5 µg g^{-1}.

Samples processing and extraction of mycotoxins from poultry feeds

Mycotoxins extraction of the samples and detection were performed according to Sulyok et al. (2007) with some modifications described previously by Monge et al. (2012).

Assay of spiking and recovery of mycotoxins from poultry feeds

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: different 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, mycotoxin extraction was performed. Matrix blank extraction was also included for comparison.

Statistical analyses

Data were analyzed with an 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, percentage of samples contaminated by *Aspergillus* section *Flavi* species and presence of mycotoxins in poultry feeds ($p< 0.05$). Principal component analysis (PCA) of the data was carried done in order to determine the relationships among water activities, percentage of samples contaminated by *Aspergillus* section *Flavi* and presence of mycotoxin in different poultry feeds The analysis was conducted using software InfoStat, 2008 version; group InfoStat, National University of Córdoba, Argentina.

Results

Mycological assays and aflatoxigenic capacity of Aspergillus section Flavi strains

Table 1 shows a_w determination of different poultry feed samples. Water activity throughout the sampling period ranged from 0.579 to 0.662 in starter feeds and from 0.529 to 0.677 in finisher feeds. No statistical significant differences were observed among different poultry feed types ($p< 0.05$).

Fungal total counts in all samples were below 10^2 CFU g^{-1} (data not shown). Although there were no significant differences between the both medium used when fungal total counts from starter and finisher feeds were compared. In general, DRBC medium reflected the total

mycobiota present whereas DG18 showed prevalence of xerophilic fungi ($p< 0.05$) (Data no shown).

Regarding toxigenic genera only *Aspergillus* spp. were isolated from all feeds. Figure 1 shows the distribution of *Aspergillus* section *Flavi* in different feeds analyzed. *Aspergillus flavus* and *A. parasiticus* were the only aflatoxigenic species isolated from poultry feed; and the distribution varied according to the poultry feeds analyzed. *Aspergillus flavus* was isolated from two starter feeds (S1 and S2), in percentages from 45 to 55 and 53 to 48% in DRBC and DG18, respectively. From these strains 14% were AFB_1 producers in mean levels of 0.8 µg/g. *A. parasiticus* strains were isolated in 100% of the F and FPH samples; and a high percentage of these strains (83%) was AFB_1 and AFG_1 producers with similar produced mean levels of both AFs (1.5 µg g^{-1}).

Mycotoxins determination

Mean recoveries for AFB_1, FB_1, T2 and HT2 toxin at three levels of spiking were 56, 68, 89 and 68%, respectively. The limit of detection (LOD) for the method was 1.6 and 2.42 µg kg^{-1} for AFB_1 and FB_1; and 13 for T2 and HT2 toxin based on a signal-to-noise ratio 3:1 and the limit of quantification (LOQ) was established as three times the LOD. The LOQ for AFB_1 and FB_1 were 5.3 and 7.28 µg/kg; and 40 µg kg^{-1} for T2 and HT2 toxin.

Co- occurrence of FB_1, HT-2 and T-2 toxin were detected in 100% of the starter and finisher feeds in mean levels ranged from 4501.7 to 5813.4; 6.7 to 21.6 and 19.6 to 30.3 µg kg^{-1}, respectively. A high number of starter samples (77%) were co-contaminated with four mycotoxins, AFB_1, FB_1, HT-2 and T-2 toxins; and the levels of AFB_1 detected ranged from 3.7 to 8.7 µg kg^{-1}. The highest levels of AFB_1 and FB_1 were detected in S2 and FS feeds, respectively; while S3 and F samples containing the highest levels of HT-2 and T-2 toxin ($p < 0.05$). Gliotoxin and DAS were not detected in any poultry feed samples (Table 2).

Multivariate statistical analysis

Figure 2 shows the bi-plot for principal component analysis (PCA) in order to determine the influence of type poultry feed on AFB_1, FB_1, T-2 and HT-2 toxin levels. As can be observed S2, S3, FP and FH are closely related to AFB_1 and FB_1. Regarding to S3 and F, closely relation is then observed between T-2 and HT-2 toxin levels. Regarding to FO, FH and FPH a positive relation was observed respect to HT-2 toxin levels.

Discussion

Mycological assays show the presence of aflatoxigenic species belonging to *Aspergillus* section *Flavi* in starter and finisher poultry feeds. Fungal propagules detected are below of the established value (1×10^4 CFU g^{-1}) in the latest laws for determining the hygienic quality in feed intended for animal consumption (GMP, 2008). These total fungal counts were lower than those obtained earlier by other researchers in this substrate (Dalcero et al. 1998; Accensi et al. 2004; Magnoli et al. 2005; Rosa et al. 2006; Krnjaja et al. 2008; Shareef 2010; Astoreca et al. 2011). In other studies (Magnoli et al. 2002; Oliveira et al. 2006; Lincy et al. 2008) found moderates values ranged from 10^3 to 10^4 CFU g^{-1} in poultry feeds. Our results are in agreement with Fraga et al. (2007) who informed that in Brazilian pelleted poultry feed samples the total fungal counts were below the detection limit of technique (100 CFU g^{-1}). In a previous study from Córdoba province Monge et al. (2012) observed that the 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 samples either ground or pelleted.

In the present work despite the low fungal total counts a high frequency of samples were contaminated with *A. flavus* and *A. parasiticus* potential aflatoxigenic strains of greater concern

for the poultry health. These fact suggesting that these *Aspergillus* species tolerate the pelleting process applied during their elaboration (usually 115ºC, 60 min). In agreement with these results in previous works these species has also been isolated with different prevalence in commercial pelleted feeds (Dalcero et al. 1997, 1998; Magnoli et al. 2005; Fraga et al. 2007; Campos et al. 2009; Fernández Juri et al. 2009; Astoreca et al. 2011; Monge et al. 2012). In the present study, 26% of the *A. flavus*- *A. parasiticus* strains were AFs producers. The incidence of aflatoxigenic strains found in the present study is similar at reported from Argentina and Brazil by Magnoli et al. (1999) and Fraga et al. (2007), respectively. While the percentages of aflatoxigenic strains informed by Saleemi et al. (2010), Astoreca et al. (2011) and Monge et al. (2012) were higher than those the reported in the present study. Respecting, AFs contamination is important to note that the levels of AFB_1 detected in poultry samples did not exceed the regulated value (AFB_1: 20 $\mu g\ kg^{-1}$) established by the European Commission for feeds (EC, 2003). However, the presence of aflatoxigenic species suggests that the potential risk of AFs production if environmental stored conditions of raw materials and poultry feeds are adequate for the production of these mycotoxins.

This work showed that the co-ocurrence of three mycotoxins (FB_1, T-2 and HT-2 toxin) in all poultry feed samples and co-occurrence of four mycotoxins (AFB_1, FB_1, T-2 and HT-2 toxins) in 77% of the samples. These results are partially in agreement with recently sampling from other Argentinean region (Córdoba, Province), where FB_1 was the only mycotoxin detected in all poultry feed samples, in levels lower than those detected in the present work (Monge et al. 2012). In another sampling from this region, Magnoli et al. (2002) showed that FBs had the highest incidence, and were found in 97% of the samples in levels lower than those found in the present work; followed by AFB_1 (46%), zearalenone (18%) and deoxynivalenol (6%).

Fusarium verticillioides and *F. proliferatum* are the two main species with the capacity to produce FBs associated to feedstuffs (Glenn 2007). Weather in Entre Ríos Province is included in the transition area from subtropical to temperate climates, with abundant rainfall all year round. Maize grown in temperate regions is an appropriate substrate for *Fusarium* species growth and FBs production. In this sampling although FBs producing strains were not isolated, FB_1 was detected in a high percentage of the samples and the FB_1 levels not exceeded the maximum tolerable limit (20 mg FB_1+FB_2 kg^{-1}) established for avian feeds by the European Commission (EC, 2007). No isolation of FBs producing strains may take place due to raw material dilution involved in elaboration process, or the susceptibility of these strains especially at pelleted process. The simultaneous presence of *F. verticillioides* and FBs have been previously detected in Argentinean poultry feed (Magnoli et al. 2002, Monge et al. 2012).

Regarding the TCT contamination, T-2 toxin and HT-2 toxin were found in high frequency of samples, while any sample contained DAS. These results partially agree with the obtained previously from Córdoba region, due to these mycotoxins were not found in any samples (Monge et al. 2012). The regulations of T-2 toxin and HT-2 toxin maximum levels are under discussion (EC, 2006) and considering the future maximum limits proposed about 100-150 μg kg^{-1} for the sum of T-2 and HT-2 (Trebstein et al. 2008; Edwards et al. 2010), the levels detected in the present work are below of this values.

There are few reports from other countries regarding the natural co-occurrence of TCT as T-2 toxin, HT-2 toxin and DAS from poultry feeds. All of the incidence data show wide variation in mycotoxins co-occurrence, especially in the type of TCT and levels detected. From Slovakia, Labuda et al. (2005) reported the simultaneous presence of ZEA and TCT. The most frequent TCT detected was T-2 toxin followed by HT-2 toxin and DON; the percentages of contaminated samples were lower and the levels higher than those observed in the present work. From Croatia

and India, T-2 toxin and DAS have been reported in variable percentages from 3 to 69%, in levels higher than detected in Argentinean poultry feeds (10 to 410 and 180 to 480 μg kg^{-1} from T-2 toxin and DAS, respectively (Sokolovic and Simpraga 2006; Lincy et al. 2008).

In the present sampling period, the simultaneous presence of trichothecenes and *Fusarium* producer species were not detected in Argentinean poultry feeds. These results are in agreement with found recently by Monge et al. (2012), but in disagreement with previous sampling years from this region (Dalcero et al. 1997, 1998).

The results obtained confirm the observed previously, the occurrence of fungal and mycotoxins shows important variations in poultry feeds from one year to another (Dalcero et al. 1997; 1998; 2002; Magnoli et al. 2002; Monge et al. 2012). This marked difference found in terms of mycotoxin presence between different geographic regions for the same substrate, could be due to the fact that environmental conditions differ from one growing grains season to another. In addition, the mycological quality of raw materials used in feed elaboration would be conditioning the final feeds.

The presence of multiple mycotoxins in feeds can also exert additive, synergistic or antagonistic effects exacerbating the problems associated with each particular mycotoxin; and conduced to mycotoxicosis in animals depending on the conditions of birds, the age and levels of exposure. It should be noted that besides the gravity of the introduction of mycotoxins or metabolites in the human food chain. The levels of mycotoxins detected in our survey were generally below the one that induces disease with typical clinical symptoms in poultry. Nevertheless a significant percentage of samples contained more than one mycotoxin at low dietary levels can conduce at subtle changes as decreased feed intake and gain rate as well as an increased risk to infectious diseases mycotoxins. In other hand in poultry farms this situation produce a large economic losses that affect the productive or performance of birds.

The data obtained from this work highlights the need of toxigenic fungi and mycotoxins continual vigilance in commercial poultry feeds in the processing plant; known the mycotoxicological quality of feeds allows planning further prevention measures as adsorbents addition during production.

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Figure caption

Figure 1. Isolation frequency (%) of *A. flavus* and *A. parasiticus* in starter (S1: starter 1, S2: starter 2) and finisher poultry samples (F: finisher, FPH: finisher parallel half) in DRBC and DG18 media. (n: 120).

Figure 2. Biplot graph with principal component analysis (PCA) for the variables in this study (fumonisin B_1, aflatoxin B_1, T-2 toxin, HT-2 toxin) in relation to feed type. S1: starter 1, S2: starter 2, S3: starter 3, F: finisher, FO: finisher off, FS: finisher split, FPH: finisher parallel half and FH: finisher half. (n: 120).

Table 1. Water activity of started and finisher poultry feed.

Feeds	Water activity (a_W)	
	Range	Mean ± SD
Starter		
S 1	0.579 - 0.662	0.608 ± 0.033
S 2	0.599 - 0.630	0.633 ± 0.017
S 3	0.611 - 0.619	1.232 ± 0.003
Finisher		
F	0.529 – 0.664	0.603 ± 0.052
FO	0.611 – 0.619	0.615 ± 0.004
FP	0.529 – 0.625	0.591 ± 0.054
FPH	0.582 – 0.629	0.611 ± 0.020
FH	0.582 – 0.677	0.628 ± 0.030

S1: starter 1, S2: starter 2, S3: starter 3, F: finisher, FO: finisher off, FS: finisher split, FPH: finisher parallel half, FH: finisher half. SD: standard deviation. (n: 120).

Table 2. Incidence of mycotoxins in starter and finisher poultry feed samples.

Feeds	AFB_1		FB_1		HT-2 toxin		T-2 toxin	
	Contaminated samples (%)	Mean levels (µg Kg^{-1}) ± SD	Contaminated samples (%)	Mean levels (µg Kg^{-1}) ± SD	Contaminated samples (%)	Mean levels (µg Kg^{-1}) ± SD	Contaminated samples (%)	Mean levels (µg Kg^{-1}) ± SD
S1	100	6.7 ± 2.6 [b]	100	4987.3 ± 969.0 [b]	100	7.8 ± 2.1 [d]	100	21.1 ± 5.1 [d]
S2	100	8.7 ± 3.4 [a]	100	4961.5 ±576.2 [b]	100	6.7 ± 2.2 [d]	100	24.0 ± 3.6 [c]
S3	30	1.3 ± 0.5 [c]	100	5345.6 ± 577.7 [b]	100	17.6 ± 11.7 [a]	100	30.3 ± 18.6 [a]
F	ND	ND	100	4574.1 ±1271.6 [c]	100	21.6 ± 10.6 [a]	100	28.9 ± 12.8 [b]
FO	ND	ND	100	4578.0 ± 752.8 [c]	100	9.3 ± 1.7 [c]	100	20.5 ± 3.1 [e]
FS	ND	ND	100	5813.4 ± 726.1 [a]	100	8.7 ± 2.7 [c]	100	21.9 ± 2.8 [d]
FH	ND	ND	100	4501.7 ±1292.8 [c]	100	13.1 ± 3.3 [b]	100	22.1 ± 4.1 [d]
FPH	ND	ND	100	4506.8 ± 452.4 [c]	100	14.8 ± 3.9 [b]	100	19.6 ± 2.8 [e]

S1: starter 1, S2: starter 2, S3: starter 3, F: Finisher, FO: Finisher off, FS: finisher split, FH: finisher half, FPH: finisher parallel half. SD: standard deviation.

ND: not detected.

[a, e] Mean in a row with a letter in common is not significantly different according to LSD test ($p < 0.05$). (n: 120).

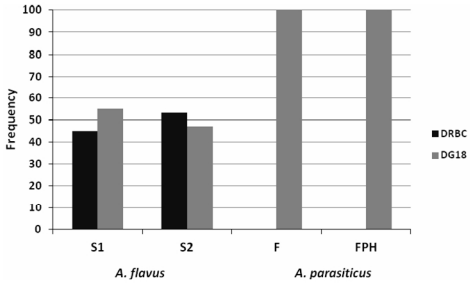

Figure 1.

Isolation frequency (%) of A. flavus and A. parasiticus in starter (S1: starter 1, S2: starter 2) and finisher poultry samples (F: finisher, FPH: finisher parallel half) in DRBC and DG18 media. (n: 120).
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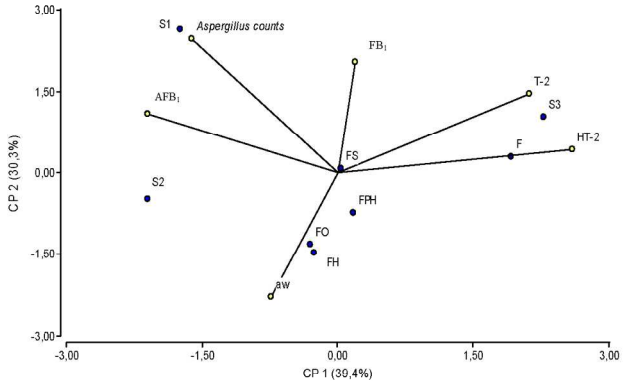

Figure 2.

Biplot graph with principal component analysis (PCA) for the variables in this study (fumonisin B1, aflatoxin B1, T-2 toxin, HT-2 toxin) in relation to feed type. S1: starter 1, S2: starter 2, S3: starter 3, F: finisher, FO: finisher off, FS: finisher split, FPH: finisher parallel half and FH: finisher half. (n: 120).
209x296mm (200 x 200 DPI)

Additional Table. Validation results in poultry feed samples.

Mycotoxin	y= ax + b*	r^2	Cal. Range (µg kg^{-1})	Validation levels (µg kg^{-1})	Recovery (%)	RSD (%)	LOQ (µg kg^{-1})	LOD (µg kg^{-1})
AFB_1	y= 1x 10^6 x + 295.51	0.99	32 - 480	200 400	56	11.26	5.3	1.6
FB_1	y= 1x10^6 x + 295.51	0.99	32 - 480	20000 40000	68	7.60	7.28	2.42
Gliotoxin	y= 6x10^6 x 5434.7	0.98	160 - 2400	1040 2080	82	3.32	133	44
DAS	y= 2x10^7 x 31052	0.99	320 - 4816	2000 4000	54	8.70	40	13
T2 toxin	y= 5x10^6 x 13291	0.98	320 - 4816	2000 4000	89	2.63	40	13
HT-2 toxin	y= 1x10^6 x 295.51	0.99	32 - 480	2000 4000	68	7.60	40	13

* *x* is the injected concentration in ng and y is the quotient of analyte peak area to peak area of internal standard.
LOQ: limit of quantification. LOD: limit of detection. RSD: relative standard deviation. n: 120.