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Mycoflora and mycotoxins in finished fish feed and feed ingredients from smallholder farms in East Africa

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ABSTRACT

A total of 52 samples of finished fish feeds and ingredients were collected from smallholder farmers in Kenya, Tanzania, Rwanda and Uganda, and analyzed. Culture and molecular techniques were used to identify fungal isolates from the feedstock, and mycotoxin profiles were determined using liquid chromatography–tandem mass spectrometry. The most prevalent fungal species recovered in the samples was *Asperigillus flavus* (54.5%). Other fungal species recovered from the samples were *Aspergillus tamarii* (9.1%), *Mucor velutinosus* (9%), *Phoma* sp. (6.1%), *Aspergillus niger* (6%), *Eurotium rubrum* (3%) and *Penicillium chrysogenum* (3%). Fourteen mycotoxins were identified: aflatoxins B_1, B_2, G_1 and G_2, fumonisin B_1 and B_3, deoxynivalenol (DON) and acetyldeoxynivalenol (sum of 3-ADONand 15-ADON), ochratoxin A, roquefortine C, alternariol, T-2 toxin, and nivalenol. DON (92.9%), aflatoxins (64.3%) and fumonisins (57.1%) were the most prevalent within locally manufactured feeds, while no contamination was found in imported feed. Samples from Kenya were the most contaminated with aflatoxin (maximum 806.9 $\mu g \cdot kg^{-1}$). The high levels of aflatoxin and trichothecene type A and B contamination found in this study point to potential risks to fish performance and to the health of consumers of the fish and derived products.

Introduction

Fish feed is the major cost item in the aquaculture industry and constitutes 40–50% of the total production costs in intensive culture systems (Enyidi et al., 2017). Feed cost may be reduced by incorporating vegetable oil, increasing levels of plant ingredients, and reduction in the level of costly fishmeal (Enyidi et al., 2017). However, plant-based ingredients have been associated with contaminants produced by fungi during the initial stages of crop production (Embaby et al., 2015). During processing, feed can be contaminated with fungal spores, particularly when grains are ground and the feed pelleted (Embaby et al., 2015). Feed storage practices and processing methods, environmental temperatures >27 °C, humidity levels >62%, and moisture levels in the feed >14% are some of the factors that can increase fungal growth in feed, and this may result in mycotoxin production (Mahfouz and Sherif, 2015). Exposing fish to mycotoxigenic fungi would subsequently reduce their growth rate, damage the liver, reduce immune responsiveness, increase mortality, and lead to a steady and gradual decline in quality of reared fish stock, posing serious challenges to aquaculture development (Fallah et al., 2014).

Mycotoxins are toxic secondary fungal metabolites produced by mycotoxigenic fungi, mainly of the genera *Aspergillus, Fusarium* and *Penicillium* and have been identified as a worldwide food and feed safety issue. Fungi can readily colonize any plant substrate and produce a multitude of mycotoxins with different toxicological

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effects (Sofie et al., 2010). As a result, most agricultural systems find it practically impossible to control the proliferation of fungi and subsequent mycotoxin contamination, aggravating food and feed safety concerns (Bryden, 2012).

The most commonly farmed fish species in East Africa are Nile tilapia (*Oreochromis niloticus*) and the African catfish (*Clarias gariepinus*) (Charo-Karisa et al., 2013). Most farmers use on-farm or locally-made commercial fish feed produced using locally available ingredients, rarely using imported feed. East African countries lie entirely in the tropics, which are highly favorable to the proliferation of mycotoxin-producing fungi. Controlling mycotoxin accumulation throughout the fish feed value chain requires proper handling of the ingredients and prepared feeds (Bryden, 2012), which must be controlled through standardization. However, although fish feed quality standards exist in the East African countries, standards for manufacture, distribution, storage and handling of ingredients are either non-existent or not strictly regulated by law.

Although a number of studies on mycotoxin contamination in fish feeds have been undertaken in many parts of the world, only a few such studies have been reported in Sub-Saharan Africa (Bryden, 2012; Njobeh et al., 2012). Furthermore, in eastern Africa, no studies have been undertaken on the identification of mycoflora and the co-occurrence of multiple mycotoxins in fish feed. In this article, we provide a pioneer snapshot of the co-occurrence of aflatoxins, fumonisin and ochratoxin A, as well as other mycotoxins, in fish feed and ingredients from East Africa.

Materials and methods

Sample collection

A total of 52 samples were collected from Kisumu, Kenya (n = 16), Ukerewe, Tanzania (n = 13), Kigembe, Rwanda (n = 10), and Jinja, Uganda (n = 13). These regions are major sites of fish farming in these countries. Farmers practicing Nile tilapia and African catfish aquaculture and using floating-pellet fish feed were identified with the help of regional fisheries officers. We categorized farmers into three groups: farmers who manufacture their own feed at the farm level (n = 14), those who use feed from local fish feed millers (n = 14), and those who use imported feed (from Israel and India) (n = 12). In each of the regions, four farmers were selected and samples were categorized either as fish feed ingredients, on-farm feed, local commercial feed, or imported fish feed (Table 1). Each bag of 20 kg was linearly divided into three equal parts by imaginary divisions in its length (upper layer, central layer, and lower layer), from which samples (1 kg each) were collected.

Feed ingredients were collected from farmers who formulate their own feed, as follows: sunflower seed cake (n = 2), rice bran (n = 2), cotton seed cake (n = 3), maize bran (n = 3) and soybean (n = 2). The number of samples of ingredients that were collected depended on availability at the time of collection. Samples collected were packed in sealed polyethylene bags and stored at 4 °C before transportation for investigation at the Laboratory of Food Analysis, Ghent University, Belgium. Samples of pellets, cottonseed cake, and sunflower seed cake were finely ground using a Romer Mill (Romer series II® MILL) and thoroughly mixed before mycotoxin analysis.

Fungal isolation and identification

Fungi were isolated using the dilution plate technique. One gram of each sample was mixed with 9.0 mL of sterile distilled water on a horizontal shaker (New Brunswick Co. Inc., Edison, NJ, USA) at 220 rpm for 20 min at 25 °C to produce a homogenate. Ten-fold appropriate serial dilutions were prepared and aliquots consisting of 1.0 mL of each dilution (in triplicate) were spread over Dichloran Glycerol Agar (DG18) plates, which were then incubated for 7 days at 30 °C (Kana et al., 2013a). A pure culture of each colony type on each plate was obtained by sub-culturing each of the different colonies onto Potato Dextrose Agar plates, which were incubated at room temperature for 5 days. Pure fungal isolates were identified from their macroscopic and microscopic characteristics according to Samson et al. (2010). Fungal isolates initially cultured on PDA were subsequently sub-cultured on Malt Extract Agar (MEA) for isolation of pure cultures for purification and DNA extraction, following Kana et al. (2013a,b).

Molecular characterization of fungi

Pure fungal cultures identified based on morphology were further confirmed using DNA sequencing. Fungal genera were identified using Internal Transcribed Spacer gene (ITS) primers, as described by White et al. (1990). Primer pair ITS1 (5′-TCCGTAGGT GAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) was used to amplify a fragment of ~500 bp of the ITS region. The PCR protocol used was described by Kana et al. (2013a,b).

PCR amplicons were purified using a GeneJetPCR purification kit (Thermo Scientific, Catalog No.K0702). Bidirectional sequencing of DNA samples was carried out using an ABI3730 DNA Analyzer and a Big Dye terminator v3.1 kit. Base calling for each sequence run was done using Sequence Analysis v5.2 software at BecA-ILRI Lab., Nairobi, Kenya. Consensus sequences from the forward and reverse strands were generated using CLC Bio DNA workbench.

The consensus sequence was used to assess the percentage of identity and similarities using the Basic Local Alignment Search Tool (www.ncbi.nlm.nih.gov/blast). DNA sequences used in the present study were deposited in GenBank under accession numbers KY203942–KY203944, KY203946, KY203947–KY203950, KY203954, KY203958–KY203960, KY234265–KY234271, KY234274–234279, and KY234281. Phylogenetic analysis of the ITS sequences was carried out using MEGA 5.0 software and the neighbor-joining method (*Helgoeca nana* was used as an outgroup). All the DNA sequences were aligned using the program Clustal X v1.8.

Multiple mycotoxin analysis using liquid chromatography–tandem mass spectrometry

Chemicals and standards

Mycotoxin-reference standards 3-acetyldeoxynivalenol (3-ADON), 15-acetyldeoxynivalenol (15-ADON), aflatoxin B_1 (AFB_1), aflatoxin B_2 (AFB_2), aflatoxin G_1 (AFG_1), aflatoxin G_2 (AFG_2), fusarenon-X (FX),

Table 1
Sample collection from four East African countries.

Feed Category	Kenya	Tanzania	Uganda	Rwanda	Total
Feed Ingredients	3	6	3	0	12
On-farm made fish feed	9	5	0	0	14
Local commercial fish feed	2	2	10	0	14
Commercial imported feed	2	0	0	10	12
Total	16	13	13	10	52

nivalenol (NIV), deoxynivalenol (DON), HT-2 toxin (HT-2), alternariol (AOH), alternariol methyl ether (AME), altenuene (ALT), ochratoxin A (OTA), zearalenone (ZEN), fumonisin B_1 (FB_1), fumonisin B_2 (FB_2), sterigmatocystin (STERIG), and deepoxy-deoxynivalenol (DOM) were obtained from Sigma-Aldrich (Bornem, Belgium). Diacetoxyscirpenol (DAS) and T-2 toxin were obtained from RomerLabs (Tulln, Austria), while fumonisin B_3 (FB_3) and roquefortine C (ROQC) were purchased from Promec Unit (Tygerberg, South Africa) and Enzo Life Science (Lorrach, Germany) respectively. The mycotoxin stock and working solutions were prepared following Sofie et al. (2010). DOM was used as an internal standard for DON, 3-ADON, and 15-ADON, while ZEN was used as internal standard for all other compounds. The acetylated derivatives of DON were quantified as a sum (∑3-ADON+15-ADON). The purpose of the internal standards was to correct for losses during extraction-clean-up and thus facilitate quantification.

Sample pretreatment

The sample preparation protocol has been reported previously (Sofie et al., 2010). A 5 g portion of each fish feed was weighed into a 50-mL extraction tube. In addition, six extraction tubes containing maize flour were weighed out. Five of the extraction tubes were used for generation of the calibration curve, with the remaining extraction tube used as a blank. Maize was selected to generate the calibration curve due to similarity to the matrix under analysis. Internal standards ZEN (50 μL of a 10 μg mL^{-1} solution) and DOM (25 μL of a 50 μg mL^{-1}working solution) were added to each of the samples and left in the dark for 15 min. The calibration curve was generated with the following points; 0.5 Xμg·kg^{-1}, 0.75 Xμg·kg^{-1}, 1 Xμg·kg^{-1}, 1.5 Xμg·$kg^{-1}$and 2 Xμg·kg^{-1}, where X represents the following concentrations of mycotoxin: DAS (5 μg·kg^{-1}), ROQ-C (10 μg·kg^{-1}); AFB_1, B_2, G_1 and G_2 (20 μg·kg^{-1}); 15-ADON (25 μg·kg^{-1}); ALT, OTA, 3-ADON, STERIG (50 μg·kg^{-1}); ZEN, NEO, AOH, T-2 and HT-2 (100 μg·kg^{-1}); NIV, F-X, AME (200 μg·kg^{-1}); FB_3 (250 μg·kg^{-1}); and DON, FB_1 and FB_2 (400 μg·kg^{-1}).

Extraction and solid phase extraction clean-up

Extraction and clean-up procedures were performed as described by (Sofie et al., 2010). Briefly, to each of the samples, 20 mL of the extraction solvent acetonitrile/water/acetic acid (79/20/1, v/v/v) was added and subsequently vortexed for 1 min. All samples were then wrapped in aluminum foil, extracted for 1 h in an overhead shaker, and centrifuged for 10 min at 3000×g. SPE C_{18} columns were mounted on a vacuum manifold and equilibrated twice with 5 mL extraction solvent. The supernatant was then transferred into the column and the eluent was collected into a 25-mL volumetric flask at two drops per min. The extraction procedure was repeated as described above with 5 mL of extraction solvent.

Defatting and MultiSep 226-column clean-up

The contents of the volumetric flasks were transferred to 50-mL extraction tubes with the addition of 10 mL of *n*-hexane. The mixture was agitated on an overhead shaker for 10 min and centrifuged for 15 min at 4000g. The hexane layer was removed using a Pasteur pipette. The defatted extract was split into two portions: portion 1 (10 mL) and portion 2 (15 mL). More details on the defatting and MultiSep 226-column clean-up method can be found in Ediage et al. (2011) and Sofie et al. (2010).

Liquid chromatography–mass spectrometry (LC–MS/MS) analysis

Multiple mycotoxins were analyzed using LC–MS. The LC–MS/MS equipment (Quattro Premier XE, Micromass, Waters) conditions were: Symmetry C_{18} (150 × 2.1 mm, 5 μm) column, Symmetry C_{18} guard column(Waters, Zellik, Belgium) with an injection volume 20 μL, flow rate 0.3 mL/min, and run time 30 min. Mobile phase A consisted of water/methanol/acetic acid (94/5/1, v/v/v) containing 5 mM ammonium acetate, while mobile phase B was composed of water/methanol/acetic acid (2/97/1, v/v/v) containing 5 mM ammonium acetate. Samples were ionized in positive electrospray mode (ESI+) and analyzed in the Multiple Reaction Monitoring mode. More details on the LC–MS/MS method were described by Sofie et al. (2010).

Twelve blank feed samples were spiked with a known concentration of mycotoxin mixture at four different concentration levels (μg·kg^{-1}), as follows: 0.5, 0.75, 1, and 1.5 times the cutoff level, and analyzed (Sofie et al., 2010). Validation of the method was performed according to European Commission Decision 2002/657/EC (Commission, 2002). The typical parameters for validation of methods were accuracy, specificity, linearity and detection limits, which were determined following Sofie et al. (2010).

Results and discussion

Mycoflora in fish feed and feed ingredients

Among ten fungal species recovered, *A. flavus* (found in 54.5% of samples) was the most prevalent (Fig. 1a). The highest isolation frequency of *A. flavus* was recorded in feed ingredients (58.3%), on-farm produced feed (50%), and in locally-produced commercial feed (28.6%). Imported feed did not have any *A. flavus* contamination (Fig. 1b). These results show that ingredients and locally-produced feed are at high risk of aflatoxin contamination and handling of the items along the value chain will determine their safety. This result agrees with Barbosa et al. (2013) and Fallah et al. (2014), who obtained 35 and 48.1% frequencies of *A. flavus*, respectively, in locally produced fish feed from Brazil and Iran. In the present study, *A. tamarii* and *A. niger*, which are also aflatoxigenic fungi, were recovered at 9.1 and 6% respectively.

Other fungi recovered from the samples were *M. velutinosus* (9%), *Phoma* sp. (6.1%), *Hyphopichia burtonii* (6%), *E. rubrum* (3%) and *P. chrysogenum* (3%). Barbosa et al. (2013) and Greco et al. (2015) isolated *Penicillium*, *Eurotium* and *Mucor* sp. from finished fish feed and rainbow trout feed, respectively, at higher frequencies than in the present study. The occurrence of *Penicillium, Mucor* and *Eurotium* in feedstuffs is reported to cause a decrease in the storage life of the product (Piotrowska et al., 2013). Moreover, while *M. velutinosus* is not mycotoxigenic, *E. rubrum* produces afla-

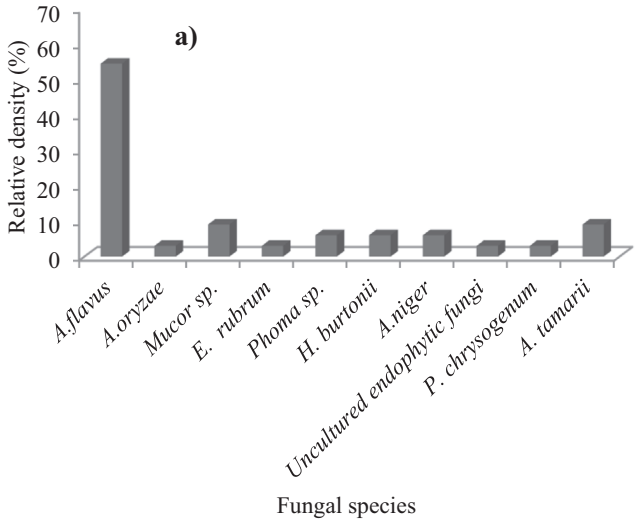

Fig. 1a. Relative density (%) of fungal species in finished fish feeds and ingredients.

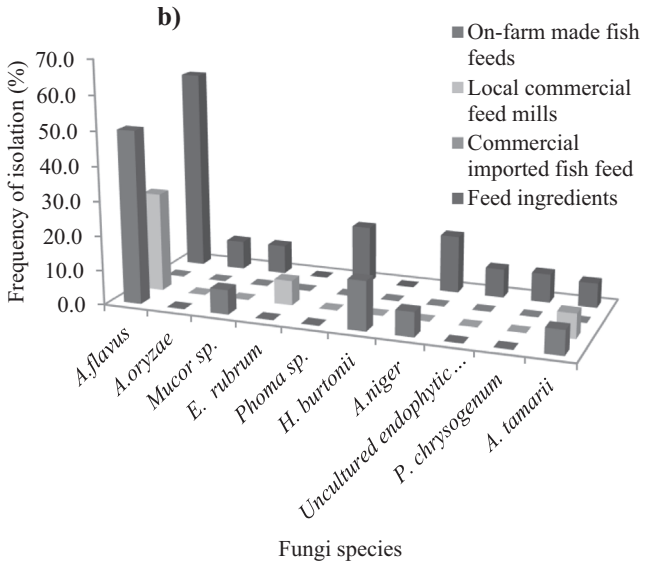

Fig. 1b. Frequency (%) of fungal species isolated from feed ingredients, local commercial feed millers, on-farm produced feed and commercial imported feed.

toxin and *P. chrysogenum* produces ROQ-C (Greco et al., 2015; Nielsen, 2003). Studies on the effects of ROQ-C on animals and fish are limited (Nielsen, 2003).

In the present study, the phylogenetic relationships of fungal species isolated from the feeds, using ITS gene sequences resolved into nine main clades, which were clustered based on fungal species (Fig. 2). We found no concordance between geographical sampling sites or feed type between and across all fungal isolates. This suggests that fungi existing in the agricultural growing regions of the sampled countries emanate from one major evolutionary lineage. Our results contrast with the finding of Donner et al. (2009) who reported that there are geographic distribution patterns among *Aspergillus* isolates from Nigeria, Benin and Uganda.

Mycotoxin occurrence in fish feed and feed ingredients

In the present study, fourteen different mycotoxins were detected in finished fish feed and feed ingredients. In finished fish feed, 12 mycotoxins were detected: DON, NIV, AFB_2, AFB_1, FB_1, FB_3, $\sum$3ADON+15ADON, AFG_1, AFG_2, AOH, and DAS, while 13 mycotoxins were detected in feed ingredients: 3-ADON, 15-ADON, DON, AFG_2, AFG_1, AFB_2, AFB_1, DAS, AOH, FB_1, FB_3, OTA and ROQ-C (Tables 2 and 3). Of the fourteen mycotoxins identified in the samples, the most prevalent were DON, AF and FB (Table 2). Our results show a wide range of mycotoxins compared to Njobeh et al. (2012), who found only AF, FB, OTA, DON and ZEA in compound feeds from South Africa. Cereals and seed cakes, which are widely used as major ingredients for feed formulation, are frequently subject to mycotoxin contamination (Bryden, 2012) due to factors such as high protein and energy content, poor handling and climatic changes (Enyidi et al., 2017; Van der Fels-Klerx et al., 2016). In

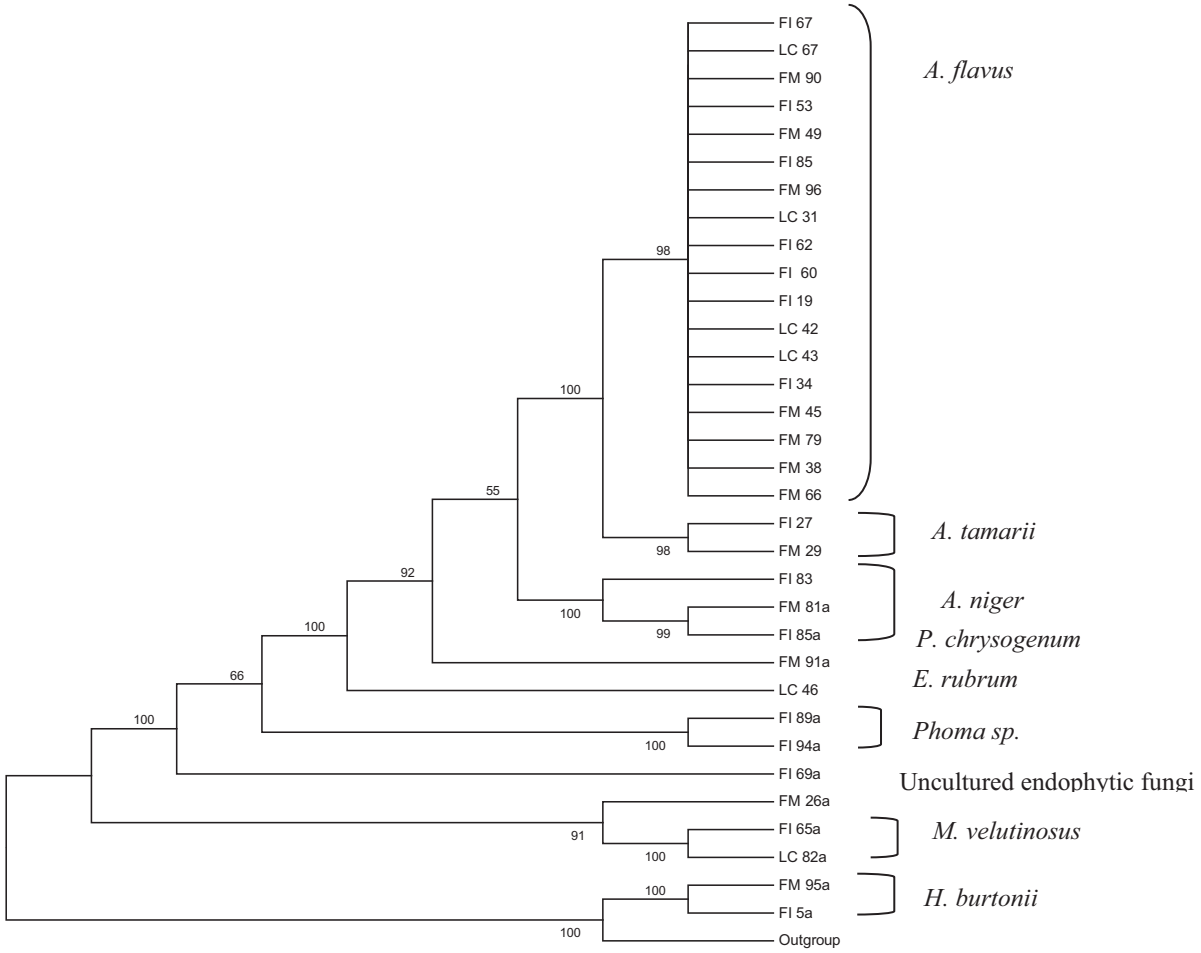

Fig. 2. Phylogenetic tree of fungal isolates from fish feed based on ITS-region sequence homology. The tree was constructed using MEGA5 software and then neighbor joining method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (2000 replicates) are shown next to the branches.

the present study, samples were collected in four East African regions that experience high temperature and relative humidity due to their tropical location. During the time of sample collection, the average temperature and relative humidity were 32 °C and 78% respectively; together with inappropriate handling and storage practices, these factors increase the risk of mycotoxin production.

Our data show that samples from Kenya were the most contaminated with AFB_1, with mean concentration of 90.05 ± 32.62 $\mu g \cdot kg^{-1}$, followed by Tanzania (55.52 ± 31.73 $\mu g \cdot kg^{-1}$), Uganda (9.05 ± 2.60 $\mu g \cdot kg^{-1}$) and Rwanda (<2 $\mu g \cdot kg^{-1}$), (Table 4). During human aflatoxicosis outbreaks in 2005 and 2006 in Kenya, maize samples collected were heavily contaminated with aflatoxin with maximum levels of 48,000 and 24,000 $\mu g \cdot kg^{-1}$ respectively (Daniel et al., 2011). Drought and famines followed by unseasonable rains during harvest and improper storage of homegrown maize in moist conditions were the reasons behind the high incidence of aflatoxicosis (Daniel et al., 2011). The few published studies on the occurrence of mycotoxins in feedstuffs from Africa have reported that aflatoxin was found in broiler chicken feed and compounded feed, with mean concentrations of 11.1 ± 2.2 and 14.7 ± 22.8 $\mu g \cdot kg^{-1}$ respectively (Kana et al., 2013b; Njobeh et al., 2012). The present study provides insight into the contamination level of aflatoxin in fish feed, and the use of aflatoxin-contaminated feed may result in economic losses due to a decrease in productivity and higher mortality rates, as documented by Barbosa et al. (2013) and Rodrigues et al. (2011). Samples from Uganda and Rwanda were less contaminated by aflatoxin and other mycotoxins than those from Kenya and Tanzania because

Table 2
Mycotoxin levels ($\mu g \cdot kg^{-1}$) in analyzed fish feed and fish feed ingredients.

Feed Category	Mycotoxin contamination[c]									
	AFs[d]	FBs[e]	DON	NIV	OTA	∑15-ADON +3-ADON	DAS	AOH	T2	ROQ-C
On-farm made fish feed										
Samples/+vesamples[a]	14/9 (64.3%)*	14/8 (57.1%)	14/13 (92.90%)	14/2(14.30%)	–	14/2 (14.30%)	14/2 (14.30%)	14/1 (7.1)%)	<LOD	–
Mean[b]	71.0 ± 31.5	1136.5 ± 717.9	245.8 ± 190.1	388.8 ± 202.5	–	74.1 ± 20.4	0.9 ± 0.3	91.3 ± 25.3	<LOD	–
Minimum	2.4	33.2	69.1	45	–	22.5	0.7	<2	<LOD	–
Maximum	126	2834.6	755.4	732.5	–	63.2	1.1	91.3	<LOD	–
Feed ingredients										
Samples/+ve samples[a]	12/5 (50%)	12/4 (33.3%)	12/4(33.3%)	12/0	12/3 (25%)	12/4(33.3%)	–	12/1(8.3%)	12/1 (8.3%)	12/1 (8.3%)
Mean[b]	469.9 ± 130.9	1594.9 ± 820.2	633.4 ± 342.8	–	16.3 ± 8.3	94.2 ± 29.7	–	3.41 ± 0.34	2.8 ± 0.20	0.14 ± 0.1
Minimum	<2	62.8	165.5	–	6.5	17	–	<2	<2	0
Maximum	806	3970.1	984.3	–	24.4	98.3	–	44.4	36.5	1.8
Local commercial fish feed										
Samples/+ve samples[a]	14/5(35.7%)	–	–	–	<LOD	–	–	–	<LOD	–
Mean[b]	11.6 ± 0.7	–	–	–	<LOD	–	–	–	<LOD	–
Minimum	<2	–	–	–	<LOD	–	–	–	<LOD	–
Maximum	28	–	–	–	<LOD	–	–	–	<LOD	–
Commercial imported fish feed										
Samples/+ve samples[a]	12/0	–	–	–	–	–	12/1 (8.3%)	–	–	–
Mean[b]	1.4 ± 0.9	–	–	–	–	–	2.2 ± 0.7	–	–	–
Minimum	<2	–	–	–	–	–	<2	–	–	–
Maximum	2.6	–	–	–	–	–	2.7	–	–	–

– = not detected. Values in parentheses indicate the % incidence.
[a] Positive samples(+ve) were samples with mycotoxin above the limit of detection (LOD); <LOD, below limit of detection.
[b] Mean levels of mycotoxin content in positive samples above the limit of quantification (LOQ) expressed as mean ± SD (standard deviation).
[c] NEO,FX and STERIG were analyzed but not detected in any of the samples.
[d] AFs = AFB_1,AFB_2, AFG_1 and AFG_2.
[e] FBs = FB_1, FB_2 and FB_3.

Table 3
Concentrations of mycotoxins ($\mu g \cdot kg^{-1}$) in feed ingredients collected from smallholder fish farmers in East Africa.

Sample ID	Mycotoxin contamination*												
	DON	3-ADON	15-ADON	AFG_2	AFG_1	AFB_2	AFB_1	DAS	AOH	FB_1	FB_3	OTA	ROQ-C
Soya beans	–	–	<LOQ	–	–	<LOQ	–	–	–	–	–	–	–
Soya beans	165.5	–	–	–	–	–	<LOQ	–	–	1402.3	118.1	–	–
Cotton seed cake	<LOQ	31.7	–	–	–	–	38.2	–	–	–	–	6.5	–
Cotton seed cake	<LOQ	–	–	3.8	21	28.4	377.9	<LOQ	–	–	–	24.42	–
Cotton seed cake	–	–	–	9	29.1	24.3	173.6	1.6	–	–	–	17.92	–
Sunflower oil cake	–	–	–	65.6	265.6	74.4	806.9	–	–	–	–	–	–
Sunflower oil cake	–	–	–	–	–	–	128	–	–	–	–	–	–
Maize bran	984.3	–	98.3	–	–	1.6	–	–	–	62.8	–	–	–
Maize bran	734.1	–	41.4	–	–	–	135	–	<LOQ	74.5	–	–	1.8
Maize bran	649.5	–	–	–	–	–	–	–	–	3970.1	316.9	–	–
Rice bran	–	17	–	–	–	–	–	2.7	<LOQ	–	–	–	–
Rice bran	–	–	–	–	–	–	<LOQ	–	–	–	–	–	–

* FB_2, NIV, T-2 and Sterigmatocystin were analyzed but not detected in any of the samples; <LOQ, below limit of quantification; – = not detected.

Table 4
Mycotoxin levels (μg·kg^{-1}) in analyzed fish feed and fish ingredients from four countries.

Country	Mycotoxin contamination										
	AFB_1	AFB2	AFG_1	AFG_2	FB_1	FB_3	DON	∑15-ADON+3-ADON	OTA	DAS	NIV
Kenya											
Mean[a]	90.05 ± 32.62	9.88 ± 3.03	22.07 ± 9.94	5.69 ± 2.42	494.98 ± 200.97	34.49 ± 13.89	201.40 ± 53.26	8.97 ± 4.65	1.28 ± 1.12	<LOQ	3.21 ± 0.32
Range	<2–806.9	<2–74.40	<2–265.60	<2–65.60	<2–2077	<2–137.20	0–755.40	<2–63.20	<2–17.92		0–45.00
Rwanda											
Mean[a]	1.51 ± 35.24	–	–	–	–	–	–	–	–	0.32 ± 0.13	–
Range	<2–4.80									0–2.70	
Tanzania											
Mean	55.52 ± 31.73										
	5.04 ± 2.82	6.36 ± 3.61									
	0.32 ± 0.31										
	578.50 ± 217.07	26.41 ± 15.0	242.37 ± 57.53	15.69 ± 5.03	2.57 ± 1.22	–	61.04 ± 29.27				
Range	<2–377.90	<2–28.40	<2–38.50	<2–3.80	<2–3970	<2–3970.10	0–984.30	0–98.30	<2–24.42		0–732.5
Uganda											
Mean[a]	9.05 ± 2.60	–	–	–	–	–	<LOQ	–	0.46 ± 0.10	–	–
Range	<2–28.00								<2–6.51		

[a] Mean = mean level of mycotoxin content in positive samples above the LOQ, expressed as mean ± SE (standard error); - = not detected: <LOQ, below limit of quantification.

most samples collected from these countries were from farmers who use feed from local commercial feed millers and imported feed. On the other hand, samples from Tanzania and Kenya were collected from farmers who produced their own feed. The use of antifungal agents by millers to minimize fungal growth may be a reason for low mycotoxin contamination of samples from Rwanda and Uganda (Dalié et al., 2010).

Aflatoxin content in feed ranged from <2 to 806 μg·kg^{-1} (Table 2). This toxin group was detected in on-farm produced feed (64.3%), feed ingredients (50%) and local commercial feed mills (35.7%), but not in imported feed. AFB_1, which is the most toxic aflatoxin chemotype, had concentrations that ranged from <2 to 806 μg·kg^{-1}, while AFB_2 ranged from <2 to 74.4 μg·kg^{-1}. These concentrations are higher than those recorded in previous studies of fish feed. Fallah et al. (2014) and Rodrigues et al. (2011) reported fish feed from Iran and Brazil as having aflatoxin concentrations that ranged from 0.46 to 68.5 μg·kg^{-1} and 1.83 to 67.35 μg·kg^{-1}, respectively. Aflatoxicosis in fish has been reported even at the low concentration of 20 μg·kg^{-1} of aflatoxin B_1 in feed (Mahfouz and Sherif, 2015).

The most prevalent trichothecene found was DON, with concentration range 69.1 to 984.3 μg·kg^{-1} (Table 1). The incidence of DON contamination was observed mostly in feed produced on the farm (92.9%), followed by that in feed ingredients (33.3%). However, all samples tested were below the regulatory limits of 5000 μg·kg^{-1} FDA (2010). Our findings are contrary to the previous study on compounded feed from South Africa, which was reported to contain very high concentrations of DON (max. 11,022 μg·kg^{-1}) (Griessler, n.d.). Wide occurrence of DON, even at low levels, may be of concern, since it can cause growth retardation and immunotoxic effects in fish (Tola et al., 2015). Regular monitoring of the presence of DON in fish feeds and their ingredients is recommended.

Fumonisin was the third most dominant mycotoxin after aflatoxin and DON, with concentrations ranging from 33.2 to 3970.1 μg·kg^{-1} in the samples tested in the present study (Table 2). The concentrations of fumonisin reported in this study exceed those in Barbosa et al. (2013) and Njobeh et al. (2012), who found 104–2371 μg·kg^{-1} and 0.3–4.94 μg·kg^{-1}fumonisin in fish feeds and compound animal feeds in Brazil and South Africa, respectively. In our study, all samples containing fumonisin were below the acceptable limits of 5000 μg·kg^{-1} recommended by WHO (2012). However, exposing fish even to low fumonisin concentrations may cause adverse effects (Barbosa et al., 2013).

Maize bran and cottonseed cake feed ingredients were contaminated with a diverse range of mycotoxins, including AFs, DON, ∑3-ADON+15-ADON, OTA, FBs, ROQ-C (Table 3). This high diversity of mycotoxins can be attributed to the diversity of fungal strains that readily colonize maize and cottonseed cake samples. DAS was recovered at very low concentrations from samples from Rwanda. This might be because the DAS producer *Fusarium* sp. is confined to temperate regions (Parikka et al., 2012) and the Rwandan fish feeds were imported from Israel. Among the feed ingredients collected, rice bran had low levels of mycotoxin contamination. OTA contamination only occurred in cottonseed cake samples, with a concentration range 6.50–24.42 μg·kg^{-1} (Table 3). Cotton seed oil cake or full-fat cotton seed from South Africa, which are sole feeds for ruminants, were reported to contain high levels of OTA (Njobeh et al., 2012). Sunflower seed cake was the only ingredient contaminated with mycotoxins belonging to a single mycotoxin group (AFB_1, AFB_2, AFG_1, and AFG_2), and it contained the highest observed AFB_1 concentration, 806.9 μg·kg^{-1} (Table 3). Our results are inconsistent with those of Mmongoyo et al. (2017), who found sunflower seed cake from Tanzania was contaminated with aflatoxin with a maximum concentration of 662.7 μg·kg^{-1}.

Other important mycotoxins such as T-2 and AOH were found in this study. AOH was found in 7.1% and 8.3% of on-farm produced

feed and feed ingredients, with maximum concentrations of 44.4 and 91.3 $\mu g \cdot kg^{-1}$respectively (Tables 1 and 2). Meanwhile, T-2 was detected in feed ingredients only, with concentration 36.5 $\mu g \cdot kg^{-1}$. Although Sofie et al. (2010) reported the presence of T-2 and AOH in animal feed, this was at lower concentrations than in the present study. However, compared to other *Alternaria* group, AOH is less toxic to animals (EFSA, 2014). NIV was detected only in on-farm produced feed, at a concentration of 733 $\mu g \cdot kg^{-1}$. Minor pathological changes were observed in chicken fed a 1000 $\mu g \cdot kg^{-1}$NIV-contaminated diet (Eriksen and Pettersson, 2004). Hence, preventive measures should be taken to minimize the NIV levels in fish feeds as the toxicity of NIV in fish is not yet documented (Eriksen and Pettersson, 2004).

Co-occurrence of mycotoxins in fish feed

Mycotoxins often occur concurrently in feed (Barbosa et al., 2013). In the current study, we used the LC–MS/MS technique, which determines co-occurrence and concentration of several mycotoxins within a given sample in a single run (Ediage et al., 2011; Malachová et al., 2014). Our results show that aflatoxin co-occurred with fumonisin in 13 of 24 feed samples. There was a diverse co-occurrence of mycotoxins in both mixed feed and feed ingredients. These findings concur with Njobeh et al. (2012), who reported co-occurrence of aflatoxin and fumonisin in poultry and cattle feed from South Africa. Also in our study, a significant number of samples (12 of 24 feed samples) showed the co-occurrence of DON and FB_1. Some interactions of DON and FB_1, exhibiting synergistic and additive effects on growth depression, have been reported in broiler chicks and pigs by Murugesan et al. (2015) and Pierron et al. (2016) respectively, and such effects might also be expected in fish.

Conclusions

The present study indicates high levels of contamination of finished fish feed and feed ingredients with different fungal species, but especially the highly-mycotoxigenic fungus *A. flavus*. Of the 14 mycotoxins found in this study AF, FB and DON were dominant. The co-occurrence of AFB_1, FB_1, DON, and OTA presents a health risk because of their synergistic and/or additive effects. Mycotoxins such as AF and OTA can be carried over to human food of animal origin; human exposure to these mycotoxin types may cause health threats. This study showed that feed ingredients are important vehicles for contaminating finished fish feed as they may be heavily contaminated by AF, FB and DON. Feed manufacturers should monitor feeds routinely, and appropriate mycotoxin absorbents should be selected to reduce mycotoxin contamination in feed ingredients and finished feed.

Conflicts of interest

No conflicts of interest declared by the authors.

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