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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

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