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Mycotoxigenic Fungi and Mycotoxins Contamination in Fish Feed from selected Retailers and Fish Farms in Southwest States, Nigeria

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Abstracts: Mycotoxins are prevalent in fish feeds and feed ingredients resulting in colonization of fungal species. Oral feeding by fish cultured with mycotoxin colonized feed/feed ingredients could result in loss of fish. Therefore, this work was intended to assess the mycotoxigenic fungi and mycotoxins contamination in fish feed from retailers and fish farms in Ibadan (Oyo State,), Akure (Ondo State), Osogbo (Osun State), Ijebu Ode (Ogun State) and Ikorodu (Lagos State), Southwest States, Nigeria using standard methods. A total of 17 samples made up of 5 composite fish feed samples were collected. The moisture content and the pH ranged between 1.45 – 11.85% and 6.77 – 7.64 respectively. The total fungi count ranged between 2.0 $x10^1$ – 3.0 x 10^3 cfu/g. All the samples that had fungal growth were also contaminated with Aflatoxin and Ochratoxin A, 86% contaminated with Zeralenone (ZON) and 43% were contaminated with Deoxynivalenol (DON). The total Aflatoxin, Ochratoxin A, ZON and Deoxynivalenol (DON) in the samples ranged between 0.38 – 41.1 ppb, 0.55 – 40.76 ppb, 30.20 – 140.20 ppb and 27.50 – 230.20 ppb respectively. The levels of mycotoxins contamination were within the maximum permissible limits. The *Aspergillus flavus, A. niger, Penicillium chrysogenum* and *Fusarium chlamydosporium* produced aflatoxin, Ochratoxin A, ZON and DON which ranged from 10.20 – 15.70 ppb, 0.05 – 0.2 ppb, 30.20 – 35.20 ppb and 22.0 – 37.50 ppb respectively. The presence of mycotoxin-producing fungi and mycotoxin levels in the fish feed samples is of public health concern and proper attention is needed for the control of quality of fish feed for fish consumption which in turn affect humans.

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Fish feeds are substances or products, including additives, intended to be used for oral feeding in fish culture systems producing fish for human and animal consumptions. Fish feeds are composed of proteins, fat, ash, crude fibre, nitrogen-free extract and water. Most of these components maybe the sources of fungi attack (Klitch, 2003).

The term "mycotoxin" is derived from the Greek word, "mykes" meaning fungus (mould) and the Latin word "toxicum" meaning poison (Mazumder *et al.*, 2002; Darwish, 2019); as such, mycotoxins refer to poisons produced by fungi. Specifically, mycotoxins are fungal secondary metabolites, which are toxic to vertebrates and other animal species in low concentrations (Bennett and Klich, 2003). They are produced mainly by fungi belonging to *Fusarium*, *Aspergillus*, *Claviceps* and *Penicillium* genera (Reverberi *et al.*, 2010). The largest group is *Aspergillus* species, then *Fusarium* sp., and, finally, one species in the genus *Penicillium*. They are thought to be produced to assist the fungi to cope with oxidative stress and also as a defense mechanism

against other organisms sharing the same trophic niche (Reverberi *et al.,* 2010). Over 300 mycotoxins have been identified (Alshannaq and Yu, 2017), and the most common mycotoxins are: aflatoxins (AF); ochratoxin A (OTA); citrinin; patulin; deoxynivalenol (DON), T-2 toxin (T2) and HT-2 toxin (HT2); fumonisins (FUM) and zearalenone (ZON) (Freire and Sant'Ana, 2018). Mycotoxins are prevalent in a majority of feeds and feed ingredients (Murugesan *et al.,* 2015; Gruber-Dorninger *et al*., 2018). Co-occurrence of different mycotoxin may occur due to colonization of feed/feed ingredients by more than one fungal species or colonization by one fungal species that produces more than one type of mycotoxin (Muthomi *et al*., 2008). Exposure of animals and humans to multiple mycotoxins may lead to additive, synergistic or antagonistic toxic effects (Smith *et al*., 2016). Studies on the combined effects of multiple mycotoxins suggest possible greater toxic effects of the mixtures compared to individual mycotoxins (Gruber-Dorninger *et al.,* 2019). There are reported loss of fish and other aquatic animals through diseases and other routes of which the feed maybe responsible for such. Thus, the need to access some selected fish feeds for fungal growth which in turn may cause mycotoxin, determine the concentration of the various mycotoxins produced which can lead to fish death. Therefore, the objective of this work is to assess the mycotoxigenic fungi and mycotoxins contamination in fish feed from retailers and fish farms in Ibadan (Oyo State,), Akure (Ondo State), Osogbo (Osun State), Ijebu Ode (Ogun State) and Ikorodu (Lagos State), Southwest States, Nigeria.

MATERIALS AND METHODS

Sample Collection: Fish feeds were purchased from farmers and retail stands in five different states of the southwestern Nigeria, all totaling seventeen (17), lumped together to make 5 composite samples each representing the sample states, labelled and transported to the Microbiology Laboratory of the Fish Technology and Products Development, NIOMR, Lagos for analysis.

Determination of pH and Moisture content: The pH values were obtained using the digital pH meter while moisture content was determined using the oven method of Love, 1975.

Isolation and Characterization of Fungi Associated with the Samples: Isolation of fungi from the fish feed samples were made on sterile Potato Dextrose Agar (PDA) using the dilution method (Fawole and Oso, 2004). Fungal isolates were identified based on their morphological and cultural characteristics as recommended by Domsch and Gams (1980) and Singh *et al.* (1991).

Determination of Aflatoxigenic activity of the isolated Fungi: Determination of aflatoxigenic activity of fungi was carried out using the method of Davis *et al*., (1987). Pure fungal isolates were cultured on Neutral Red Desiccated Coconut Agar (NRDCA). The plates were incubated for 3days at 28^{o}C. Production of an orange-yellow pigmentation by the mycelium prior to the production of blue fluorescence on the reverse side of the colony under ultraviolet (UV) light (365nm) indicate aflatoxigenic activity.

Analysis of Sample for mycotoxin: A high performance liquid chromatography with fluorescence detection (HPLC-FD) was used for the extraction and detection of the mycotoxins. The detection limit of the analysis was determined as 0.2 ppb (Vicam, 2007). Fifty grams of fish feed samples were extracted with 100 mL methanol and 25 mL water by using a blender at high speed for 1-2 min. Whatman No. 4 filter paper were used for filtration of the extract. A 10 mL aliquot of the filtrate was diluted with 40 mL ultrapure water, shaken vigorously and then filtered once more by a glass microfiber filter. The final volume of 10 mL filtrate was swiftly passed through column at a rate of 1-2 drop/sec.

Estimation of Ochratoxin A: The samples were extracted using the modified method of Toscani *et al*., 2007. The sample (15 g) was blended in 50 ml of acetonitrile - water (45:05, v/v), using high speed blending and then the extract was filtered through filter paper. 5 ml of the filtrate was mixed with 50 ml of phosphate buffer saline (PBS) and filtered through a glass microfiber. Then 10 ml of the filtrate was passed through immunoaffinity columns. OTA was eluted from the column by passing 1.5 ml of methanol (HPLC grade) and collected in a vial.

RESULTS AND DISCUSSION

Table 1 shows the distribution of the fungal isolates obtained from the fish feed samples. *Aspergillus flavus* and *A. niger* were present in all the samples from the locations (100% occurrence) while *Fusarium chlamydosporium* was present in only one location (20% occurrence). Table 2 shows the type of mycotoxin and their quantities in the fish feed samples. Total Aflatoxin was detected in all the fish feed samples.

Sample AL_1 from Akure had the highest total aflatoxin quantity present (42.10 part per billion, ppb) while the sample UIF_1 from Ibadan has the least. Sample AR_1 from Akure had the highest Ochratoxin A present (40.76 ppb) while the sample UIF_1 from Ibadan had the

least (0.86 ppb) Ochratoxin-A detected. Like total aflatoxin, Ochratoxin A was also detected in all the samples. Zeralenone was not detected in a sample, LHAF, making the sample free of the toxin. The highest quantity of Zeralenone was also reported from sample AR_1 from Akure (140.20 ppb) while the least Zeralenone was recorded in sample UIF_1 from Ibadan (30.20). Deoxynivalenol was not detected in samples from Ibadan (UIF_1) and Osogbo (OHAF) but was reported in other samples. The highest deoxynivalenol was reported in the sample LHAF from Lagos (230.20 ppb) while the least was reported in sample IOC from Ijebu Ode (27.50 ppb).

Table 1: Distribution of Fungal strains in fish feed samples

Fungal Strains	**Locations**				
	Ibadan	Osogbo	Ijebu Ode	Lagos	Akure
Rhizopus stolonifer	+	+	+	-	+
Aspergillus flavus	+	+	+	+	+
Aspergillus niger	+	+	+	+	+
Fusarium oxysporum	+	-	-	-	+
Aspergillus versicolor	-	-	+	-	-
Penicillium citrinum	-	+	-	+	-
Fusarium sacchari	-	-	+	+	-
Cladosporium sphaerospherium	+	+	-	-	-
Fusarium chlamydosporium	-	-	+	-	-
Fusarium compactum	+	+	-	-	+
Alternaria sp.	-	+	-	-	+
Penicillium chrysogenum	+	+	+	-	-
Aspergillus aculeatus	+	+	-	-	-
Aspergillus tamarii	+	-	+	-	+

Keys: + = present; - = absent

Table 3 below shows the concentrations of mycotoxins in the fungal isolates. Isolates IOE_1-A, IOE-C and AL_1-C were identified to be *Aspergillus niger*. Highest Aflatoxin B_1 (AfB_1), AfB_2, AfG_1, and AfG_2 production were found in isolates IOE-C and AL_1-C to be 5.70 ppb, 5.78 ppb, 5.43 ppb and 5.30 ppb respectively. Isolate AL_1-C produced the highest Ochratoxin A (OTA), 26.30 ppb. The highest Zeralenone (ZON) was produced by isolate AL_1-C (26.30 ppb) and the highest deoxynivalenol (DON) was produced by isolate IOE_1-A which was 27.50 ppb. Isolate IOE_1-A had the least AfB_1, AfB_2, AfG_1, AfG_2, OTA and ZON production. This was detected to be 0.10ppb, 0.05ppb, 0.03ppb, 0.20ppb, 0.75ppb and 30.20ppb respectively. The least DON production was by isolate IOE-C which was 22.03 ppb. DON was not detected in the isolate AL_1-C. Isolates IOE* and IOE_1–B were identified to be *Aspergillus flavus*. The highest AfB_1, AfB_2, AfG_1, AfG_2 and DON quantified to be 15.70ppb, 15.75ppb, 15.43ppb, 15.22ppb and 240.60ppb respectively were from the isolate IOE* while the highest OTA and ZON production was by the isolate IOE_1–B quantified to be 22.36 and 230.20 respectively. The least AfB_1, AfB_2, AfG_1, AfG_2 and DON production from the *A. flavus* was by isolate IOE_1–B quantified to be 10.20ppb, 10.35ppb, 10.26ppb, 10.20ppb, and 20.20ppb while the least OTA and ZON production was recorded from the isolate IOE* quantified to be 20.76 and 40.20 respectively. For *Alternaria* sp., isolate UIF_1-A had higher AfB_1, AfB_2, AfG_1, AfG_2, OTA, DON and ZON production quantified to be 10.70ppb, 10.75ppb, 10.43ppb, 10.22ppb, 210.76ppb, 140.20ppb and 240.26ppb respectively while the least toxins were recorded from the isolate IBC-A quantified to be 0.50ppb, 0.65ppb, 0.24ppb, 0.25ppb, 0.86ppb and 30.20ppb. IBC-A did not produce deoxynivalenol. *Fusarium oxysporum.* Isolates IOC-B and AL_1-D were identified to be *F. oxysporum.* Highest AfB_1, AfB_2, AfG_1, AfG_2, OTA and DON production was by the isolate AL_1-D quantified to be 3.10ppb, 3.05ppb, 3.03ppb, 3.20ppb, 4.75ppb and 37.50ppb respectively while the highest ZON production was by the isolate IOC-B which was quantified to be 35.20ppb.

Table 2: Concentrations of Mycotoxins in fish feed samples

Cities	Mycotoxins concentrations (ppb)			
	TA	OTA	ZON	DON
Akure	42.10	40.76	140.20	220.26
Ibadan	1.60	0.86	30.20	-
Ijebu Ode	22.59	7.05	60.40	27.50
Ikorodu	41.10	22.36	-	230.20
Osogbo	3.42	1.25	67.10	-

TA= Total Aflatoxin; OTA= Ochratoxin A; ZON= Zeralenone; DON= Deoxynivalenol

The least AfB_1, AfB_2, AfG_1, AfG_2 and OTA production was by the isolate IOC-B quantified to be 0.52ppb, 0.75ppb, 0.25ppb, 0.22ppb, 0.70ppb respectively while the least ZON production was by the isolate AL_1-D quantified to be 32.20ppb. Isolate IOC-B did not produce DON. Of all the isolates whose mycotoxins were quantified, IOE* had the highest AfB_1, AfB_2, AfG_1, AfG_2 and DON production quantified to be 15.70ppb, 15.75ppb, 15.43ppb, 15.22ppb and 240.60ppb respectively, UIF_1-A produced the highest OTA quantified to be 210.76ppb

while IOE_1-B produced the highest ZON quantified to be 230.20ppb. The isolate with least AfB_1, AfB_2, AfG_1, AfG_2 and ZON was IOE_1-A which was quantified to be 0.10ppb, 0.05ppb, 0.03ppb, 0.20ppb and 30.20ppb respectively, IOC-B produced the least OTA quantified to be 0.70ppb while the least DON quantified to be 20.20ppb was produced by IOE_1-B.

Table 3: Mycotoxin profiles of the fungal isolates from the fish feed samples

Fungi	Isolate Code	Mycotoxins Detected (ppb)						
		AfB_1	AfG_1	AfB_2	AfG_2	OTA	ZON	DON
A. niger	IOE_1-A	0.10	0.05	0.03	0.20	0.75	30.20	27.50
	IOE-C	5.70	5.78	5.43	5.30	6.30	35.20	22.03
	AL1-C	5.70	5.78	5.43	5.30	26.30	32.20	-
A. flavus	IOE*	15.70	15.75	15.43	15.22	20.76	40.20	240.60
	IOE1-B	10.20	10.35	10.26	10.20	22.36	230.20	20.20
Alternaria sp.	UIF1-A	10.70	10.75	10.43	10.22	210.76	140.20	240.26
	IBC-A	0.50	0.65	0.24	0.25	0.86	30.20	-
Fusarium oxysporum	IOC-B	0.52	0.75	0.25	0.22	0.70	35.20	-
	AL1-D	3.10	3.05	3.03	3.20	4.75	32.20	37.50
Cladosporium sphaerospermum	OHAF	10.70	10.75	10.43	10.22	200.76	140.20	220.26
A. aculeatus	IOC-A	4.48	4.72	4.26	4.22	4.55	34.90	-
Penicillium citrinum	LHAF	0.48	0.72	0.26	0.22	30.55	34.40	-

The fungi distribution in the fish feed were also reported by Adebayo-Tayo, 2008 and Kana *et al.*, 2013 in their different work. The results obtained for the detection of aflatoxigenic moulds in the fish feed samples was in line with the work of Esther *et al.* (2017) who reported the presence of aflatoxigenic fungi in fish feeds and feed ingredients. Sekar *et al.* (2008) also reported the presence of aflatoxigenic fungi in dried fruits and grains. Kana *et al.* (2013) also detected aflatoxigenic fungi in food (grains and maize) and poultry feeds. Aflatoxigenic capacity of fungi isolated from the fish feed samples were detected using Neutral Red Desiccated Coconut Agar (NRDCA) medium. All *Aspergillus flavus* strains screened showed the ability to produce aflatoxins, with the production of blue pigmentation in the medium when viewed under long wavelength of 620nm inside a dark chamber. This was corroborated by the work of Al-Jaal *et al*., (2019) on food and feed. The mycotoxins detected, Aflatoxin, Ochratoxin A, Zeralenone and Deoxynivalenol, in these samples are similar to those reported by Njobeh *et al.* (2012), who found the same AF, FB, OTA, DON and ZEA in compound feeds from South Africa. The total aflatoxin quantified ranged between 1.68 – 42.10 ppb which falls below the Nigerian government (NAFDAC) limits of 50ppb but higher than that of Food and Drug Administration (FDA) set at 20ppb, (van Egmond *et al.*, 1989). This was in agreement with the work of Adebayo-Tayo, 2008 and Kana *et al.*, 2013, who reported the concentration to have ranged from 1.65-3.56 ppb. Ochratoxin A quantified ranged between 0.55 – 40.76 ppb which falls within the FDA standard of between 5 – 100 ppb as contained in mycotoxin handbook of September, 2015. This was in agreement with the work of Bryden, 2012 and Esther *et al.*, 2017 who reported Ochratoxin A in local and imported fish feeds in South Africa within the range of 0.25 – 43.20 ppb. Zeralenone, ZON, was also detected and quantified to range between 30.20 – 140.20 ppb and thid falls within the limit of FDA standard of 100 – 1000 ppb. This means with regards to ZON, the feed is still safe for fish consumption. This was corroborated by Van Egmond and Jonker, 2004 who reported the presence of ZON in feed samples at a concentration below the permissible limits. Deoxynivalenol, DON, was also detected and quantified to range between 27.50 – 230.20 ppb. This was also lower than the FDA standard set at 5000 ppb for dairy products and animal feedstuffs. This finding was contrary to the previous study on compounded feed from South Africa, which was reported to contain very high concentrations of DON (max. 352ppb) (Griessler, 2013). 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. All the isolates were also assayed for; Aflatoxin, Zeralenone, Deoxynivalenol and Ochratoxin A production. The highest producers in the order above were isolate coded *A. flavus* which had 62.10 ppb, *Cladosporium sphaerospermum* with 140.22 ppb, *A. flavus* with 230.22 ppb and *Alternaria* sp. with 210.76 ppb respectively. The co-occurrence of AFB_1, DON and OTA presents a serious health risk because of their synergistic and additive effects (Smith *et. al.*, 2016). Mycotoxins such as AFB and OTA can be carried over to human food of animal origin such as fish in this case; human exposure to these mycotoxin types may cause health threats which include but not

limited to sterility, brain and liver damage, death (Bennett and Klitch, 2003).

Conclusion: Most fish feeds sold and used by farmers in some major cities within the South western states of Nigeria are contaminated with mycotoxigenic fungi which is of public health concern. Therefore, it is likely that the fishes fed with these feeds are likely to be affected which may in turn have a carry-over effects on humans who consume these fishes. Although, these toxins are in very small concentrations, continuous consumption may pose risks as high as death to the consumers. To avoid these risks, proper processing of the feeds should be of high priority. If all these are adequately put in place, the incidence of mycoses in fishes and humans will be greatly reduced.

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