AFLATOXINS ASSOCIATED WITH STORAGE FUNGI IN FISH FEED

***Samuel, T. O. and Odunigba, O.**
Department of Botany, University of Lagos, Akoka, Lagos State
*Corresponding author: tosamuel@unilag.edu.ng; temmitade@yahoo.com +2348036410449
(Received: 24th June, 2015; Accepted: 27th July, 2015)

ABSTRACT

Cereals and legumes are a very important part of feed used in culturing fishes. Feed, when not properly stored, enhances the growth of storage fungi which is a source of mycotoxins, secondary metabolites produced by storage fungi. This study investigates storage fungi and aflatoxin in fish feed stored under three different storage conditions. Storage fungi were isolated and identified using direct isolation technique; detection and identification of aflatoxins using the High Performance Liquid Chromatography and Proximate analysis of the stored feed were also carried out. Two fungi species (*Mucor species* and *Fusarium oxysporium*) were isolated from the stored feed. It was observed that it takes five weeks after sporulation of storage fungi for production of mycotoxins to take place. Four types of Aflatoxins (G1, G2, B1 & B2) were identified in the stored feed. The results on the proximate analysis on the stored feed prior to and after storage periods revealed decrease in the carbohydrate, protein, ash and the crude fiber content of the stored feed and increase in the moisture and crude fat content of the feed. The results from this study revealed that good storage condition is very essential and there is need for further work to assess the implication of aflatoxin on the health of fish and human (final consumer).

Keywords: Storage fungi, aflatoxins, fish feed, proximate analysis.

INTRODUCTION

Fish farming is a vast business around the Southwest area of Nigeria. A lot of people own fish ponds in order to meet with the needs of their immediate families and cater for the community. Aqua feed given to fishes in order to help them continue their normal metabolic activities comes in two categories; the imported and the locally-made. Examples of some imported fish feeds include Coppens and Gold coin aqua feed. The locally-made feed comprises ingredients like wheat and maize which are mixed and ground after which it is pelleted and stored. The imported aqua feed is more costly than the locally-made one. As a result, many fish farmers resort only to locally-made aqua feed and usually produce and store the feed in large quantities in order to cut-down cost of feeding the fishes and make more profit. Cereals as energy source and legumes as protein feedstuffs are the main part of feed (up to 90%) for all kinds and categories of fish. Cereal feedstuffs are vulnerable to fungal contamination either prior to harvesting of the cereal or due to poor storage conditions. In such condition the storage fungi multiply and their metabolites cause changes in the feed quality. Mycotoxins are secondary metabolites of fungi which are formed during enzymatic reactions. The chemical characteristics and biological activities of mycotoxins are very wide and able to cause different pathological and patho-histological changes in fish which can in-turn cause serious effects in humans, the final consumers, at certain dosages (Amadi and Adeniyi, 2009). Recently, some chemical compounds like mycotoxin binders (Hydrated sodium calcium aluminosilicales) and mould inhibitors (Calcium propionate) have been used to eliminate the presence of storage fungi. However, studies have shown that most of these compounds appear to bind to only a small group of toxins while showing very little or no binding to others. The use of these additives is therefore not recommended to farmers since a fungus can produce a variety of mycotoxins (Avantaggiato *et al.* 2005; Bintvihok and Kositcharoenkul, 2006).

This study aims at isolating and identifying mycotoxin-producing fungi present (if any) in stored aqua feeds, identify the mycotoxins they produce, determine the nutritional value of the feed sample prior and after storage and to ascertain the kind of storage condition(s) that enhances the growth of the mycotoxin-producing fungi.

MATERIALS AND METHODS

Sample Source

Freshly-compounded feed sample with known constituents, (Table 1) ready for storage was collected from the Aqua-culture unit of the Department of Marine Sciences, Faculty of Science, University of Lagos Akoka, Lagos. The collected feed was divided into three equal weighs of ten kilograms and stored under three different storage conditions. The first portion was stored in a tightly-covered storage bowl, this was labelled A; the second portion was stored in a covered storage bowl, not tightly covered in order to enhance ventilation and this was labelled B. The last portion was stored in an open storage container (no cover) and this was labelled C. These three storage containers containing sample feed were stored in a cool dry place of the research laboratory of the Department of Botany, University of Lagos for six weeks.

Table 1: Fish Feed Constituents

S/N	CONSTITUENT	QTY (KG)	RATE (%)
1	Wheat Offal	10	39
2	Maize	16	65
3	G. N. C	20	95
4	F. F. S. M	10	145
5	P. K. C	12	35
6	Fish Meal	12	450
7	Fish	2	1000
8	Indomie	18	171

*PKC – Palm Kernel cake meal *GNC – Groundnut cake meal
*FFSM – Full fat soya bean meal

Medium Preparation

Twenty grammmes of commercially produced Potato dextrose agar (PDA) was dissolved in five hundred millilitres (500ml) of distilled water and allowed to homogenize in a water bath. The medium was later sterilized by autoclaving in an autoclave at 121^0 C for 15 minutes. Chloramphenicol was added to the medium (the antibiotic is to inhibit the growth of any bacterial species that could contaminate the isolate), before pouring the melted medium into sterile Petri plates (15 cm diameter Petri plates at a depth of 4.0 mm), and cool at room temperature to solidify.

Isolation and Identification of Storage Fungi

A little portion of the stored sample feed was collected from each of the storage containers at an interval of seven days for six consecutive weeks. Using the direct inoculation method by Watanabe (2002), each of these collected feed was inoculated on the prepared PDA medium plate and incubated at 26^0 C. Developed colonies of fungi were sub-cultured to obtain pure cultures. The fungi cultures were examined both macroscopically and microscopically for colony, mycelia and spore characteristics. The characteristics were compared with those in a standard Mycology textbook (Vashishta and Sinha, 2005).

Aflatoxins (Mycotoxin) Detection and Identification

Five grammes of the stored aqua feed were collected from each of the storage containers on weekly basis for six consecutive weeks. Ten millilitres (10ml) of acetonitrile was added to each of the collected feed in a "sample bottle" and mixed with a vortex mixer, then placed in a sonicator and centrifuged at 4000 revolutions per minute for 10 minutes. With the aid of a Millipore filter (0.45μm) and a syringe, the supernatant was extracted into another set of "sample bottle" and kept. The identification was carried out by

employing the use of the High Performance Liquid Chromatography (HPLC) method. The electronic Chromatographic machine used is the Agilent Machine which was connected to a computer system. It has an injection port in which the sample was injected and the reading was shown on the computer screen. The computer displayed the reading on the screen indicating the time of run and the area where mycotoxins were detected.

Proximate Composition of the Stored Feed

Proximate compositions of the feed samples prior to and after storage period were carried out according to the method of A.O.A.C. (1990) and Akubor *et al.* (2000). This includes determination of ash content, crude fibre, crude protein, crude fat, nitrogen free extract and moisture content.

Statistical Analysis

Descriptive statistics for all parameters were calculated in triplicate for freshly-prepared feed sample and six weeks old stored feed sample in the three storage containers. All these were reported as means ± standard deviation**.**

RESULTS

In the first week of storage, no fungus was isolated from the three storage bowls. At the end of the second week of storage; two fungi species were isolated from the sample container labelled B. In the third week of storage, the sample feed in the storage bowl labelled C, showed presence of the same fungi species isolated from the storage container B. The sample feed in the storage container A showed no fungi growth throughout the storage period. These two fungi are *Mucor species* and *Fusarium oxysporium*. By the end of the third week of storage, it was noted that there was change in the coloration of the stored sample feed in the storage container labelled B and C.

The concentration of the various Aflatoxins present in the storage sample feed tested was given using the formula: $y = mx + c$, where Y is the mean peak area, x is the concentration of the Aflatoxin and m is the slope and c is the intercept. Therefore, $x = y - c/m$.

Four types of Aflatoxins (G1, G2, B1 & B2) were present in the sample feed of the container labelled B and C. Of the four aflatoxins present, the G2 had the highest concentration in the sample feed stored in an open container (labelled C) with the concentration of 1.237 *ug/ml*. Aflatoxins B2 had the least concentration in the sample feed stored in storage container labelled C with the concentration of 0.072 *ug/ml* (Table 2). In the sample feed stored in the container labelled B, aflatoxins G1 had the highest concentration of 1.182 *ug/ml* while aflatoxins G2 had the least concentration of 0.012 *ug/ml* (Table 2).

Table 2: Aflatoxins Concentration in the Stored Fish Feed

Parameters	**Container label B (ug/ml)**				**Container label C (ug/ml)**			
	Aflatoxin B_1	**Aflatoxin B_2**	**Aflatoxin G_1**	**Aflatoxin G_2**	**Aflatoxin B_1**	**Aflatoxin B_2**	**Aflatoxin G_1**	**Aflatoxin G_2**
y	1.774	5.505	25.571	3.212	7.171	7.171	25.743	27.745
m	31.21	38.63	10.82	20.03	31.21	38.63	10.82	20.03
c	12.46	4.368	12.77	2.97	12.46	4.368	12.77	2.97
X =y-c/m	0.342	0.029	1.183	0.012	0.170	0.072	1.199	1.237

*Where **y** = mean peak area, **m** = slope, **c** = intercept and **x** = aflatoxin concentration.

From the proximate analysis, it was showed that at the end of the storage period, there was decrease in the carbohydrate, protein, ash and the crude fiber content of the feed sample stored. The carbohydrate content percentage decreased from 64.18 to 58.13 and 56.16 in storage container labelled B and C respectively. The protein content percentage also decreased from 17.04 to 15.74 and 13.21 in storage container labelled B and C respectively. Reduction was also noted in the percentage of the crude fiber content of the stored sample feed. It reduced from 2.53 to 1.64 and 1.38 in the storage container labelled B and C respectively. The ash content percentage also decreased from7.1 to 4.22 and 4.21 in storage container labelled B and C respectively (Table 3).

Table 3: Proximate Composition of Stored Fish Feed Prior to and After Storage Period

Proximate component	Freshly prepared feed (%)	Stored feed in container labelled B (%)	Stored feed in container labelled C (%)
Nitrogen free extract	64.17	58.13	56.16
Crude protein	17.04	15.74	13.70
Crude ash	7.10	4.22	4.21
Crude fibres	2.53	0.68	1.38
Crude fat content	2.96	11.78	12.81
Moisture content	6.20	9.45	11.74

In contrast to the reduction noted in the carbohydrate, protein, ash and the crude fiber contents of the stored sample feed, there was increase in the moisture and crude fat content of the feed. The moisture content percentage increased from 6.2 to 9.45 and 11.74 in the storage container B and C respectively. The same trend was observed in the crude fat content of the stored sample feed. Prior to storage, the crude fat was 2.96 percent. This was increased to 8.78 and 12.81 percent in the sample stored in the container labelled B and C (Table 3).

DISCUSSION

The results from this study revealed that good storage condition is very essential in eliminating the presence of storage fungi in stored aquaculture feed. This observation was noted as the sample feed stored in a tightly covered storage bowl labelled A showed no fungus invasion throughout the storage period. Agboola (1992) reported in his work that fungi are generally aerobic organisms, therefore storage atmosphere deficient in oxygen would lead to reduction in fungi invasion and aflatoxin (mycotoxin) production. Absence of storage fungi from the storage container labelled A showed that isolated fungi in this study are not prior to the harvesting of the cereals used in the production of the feed sample as these were only observed in the other two storage containers. It was also deduced from this study that it takes about five weeks after sporulation of storage fungi for production of mycotoxins to take place. European Commission according to Commission Directive (2003) set the limit level of aflatoxin in all animal feed to 0.02mg/kg and 1.237 *ug/ml* (0.0012 mg/kg) of aflatoxin G2 was the highest concentration obtained in this study. Koirala *et al.* (2005) in their report noted that continuous consumption of small doses of aflatoxins can lead to health issues of great concerns.

According to Al-Abdalall and Al-Jaraifani (2013), the change in coloration observed at the third week of storage was due to the reaction between nitrogenous compounds and reducing sugars. However, Osman *et al.* (1988) had reported changes in the coloration of stored grains to be as a result of oxidation of fats and fatty acids.

The result from the proximate analysis on the decrease in carbohydrate, protein, ash and fibre content agreed with the reports of Embaby *et al.* (2006) and Kakde and Chavan (2011). These authors reported that the presence of these storage fungi (*Mucor* and *Fusarium*) is responsible

for the reduction. It was reported that these storage fungi utilized these biochemical composition as substrate for their growth. Unlike the carbohydrate, protein, ash and fibre content, moisture and crude fat content was found to increase in the result; this corresponds with the reports of Christensen (1967); Oladele and Osipitan (2011). Oladele and Osipitan (2011) in their work reported that the increase in the moisture content was due to the presence of the storage fungi. It was reported that as the storage fungi grow in the stored sample feed, they convert into water the part of the stored sample feed they consume. Christensen (1967) reported that increase in storage fungi population will increase production of carbon dioxide and free acids due to the peculiar structure of the seed coat which gave fungi easy access to the interior of moist stored sample feed.

There is need for further work/research into the implication of aflatoxin on the health of fish and human (final consumer).

ACKNOWLEDGMENTS

The authors thank the Department of Marine Sciences, Faculty of Science, University of Lagos, for granting the permission to access fish feed from their aquaculture unit.

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