



Molecular and phylogenetic characterization of fungi and their mycotoxins in locally processed dried beef meats from Ekiti State, Nigeria

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DECLARATION

I, Toluwase Adeseye Dada, assert that the thesis entitled “**Molecular and Phylogenetic Characterization of Fungi and their Mycotoxins in Locally Processed Dried Beef Meats from Ekiti State, Nigeria**”, hereby submitted for the degree of Doctor of Philosophy in Agriculture, Animal Health has not been formerly submitted by me for a degree at this or any other university. I also declare that this work was designed and executed by myself and that all materials used herein have been duly acknowledged.

Name: Toluwase Adeseye Dada

Signature: 

Date: 14/09/2019

DEDICATION

This thesis is dedicated to God Almighty and my beloved wife and daughter (Oluwakemi Dada and Toluwanimi Dada) for their sacrifices, encouragement, patience, understanding and support given to me all through this journey. I couldn't make this happen without you. The thesis is also dedicated to my parents Chief Benjamin Oladimeji Dada, Mrs. Julianah Feyisayo Dada and my siblings for their financial support.

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GENERAL ABSTRACT

This study report on the status of mycological safety of sun-dried meat and its nutritional content as well as their correlation with mycotoxin production from toxigenic fungi. The dried meat is both economically and nutritionally essential to the consumers and sellers of the beef dried meat across southern part of Nigeria. However, safety of processed meat has remained a major concern largely due to its processing method, mode of packaging, transportation, storage and mode of selling in open air. To assess the fungi biodiversity of the dried meat consumed in Ekiti State, dried meat samples were collected from selected locations in the 10 local governments out of 16 local governments (North, South, East, West and Central) were sampled. The mycological analysis was done to establish the occurrences of fungi and the mycotoxins level in the samples. Fungi isolation was done through serial dilution of the dried meat samples, and subsequent purification of the isolates was done using a different media. The isolates from the dried beef samples were morphologically and molecularly identified, with the results obtained show a different pattern of fungi contamination. The mycobiota of the dried meat revealed that *Aspergillus* was the most prevalent fungi isolated with (43%), then *Rhizopus* (42%), *Penicillium* (3%), *Cladosporium* (3%), *Fusarium* (2%) and remaining genera were (7%).

Aspergillus genera had the highest frequency of 57.6%, in all the fungal isolates contaminating the dry meat. *Aspergillus niger* and *A. flavus* having 13.2 and 11.8% of the *Aspergillus*, respectively, with *A. ustus* and *A. fumigatus* recording 10.3% each. While *A. aeneus*, *A. alabamensis*, *A. awamori*, *A. chevalieri*, *A. melleus*, *A. ruber*, *A. tubingensis* and *A. unguis* had the least densities (1.5%) respectively when considering the entire number of *Aspergillus* isolates. The contamination by *A. flavus*, *A. niger* and *A. parasiticus* was found in almost all the locations where dried meat was sampled. The *Aspergillus* isolates were examined for their aflatoxin potential using 5 aflatoxin biosynthetic regulatory primers of (*aflR*, *aflJ*, *aflD*, *aflM*, and *omt-A*). For the majority of the *aspergillus* isolates detected, the essential genes for aflatoxin biosynthetic regulatory genes were observed in them (*omt-A*, *aflD* and *aflM*). Although, the genes were expressed in 30.9% of the *Aspergillus* species, their occurrence should be a source of worry because they also have the ability to produce aflatoxins in conducive environments.

Nonetheless, the phylogenetic relationship based on Maximum Likelihood tree showed that the isolated and identified *Aspergillus*, *Fusarium*, *Penicillium* and other identified fungi species were homologous to the fungi species deposited in GenBank which were isolated from other different

sources. This result for *Aspergillus* species was in six main clades, with nearly all of them having high homology percentage. This shows that they possessed nearly the same nucleotide signature with their relative counterpart from the GenBank especially *Aspergillus unguis* and *A. Aeneus* shared 98% and 99% with the reference of the same species from GenBank. The two *Fusarium* identified from this study clustered with 68% similarity index with *F. Equseti* reference strain from the GenBank. This similarity index is quite low because it was below the 70% expected borderline for the degree of relatedness. However, the *Penicillium* species had between 97% - 99% similarity index with the same species from the GenBank. This high homology percentage indicates that they possessed nearly the same nucleotide signature with their relative counterpart from the GenBank. The *Epicoccum sorghinum* isolated from this study exhibited low similarity index of 67% and less than 50% with *Epicoccum sorghinum* and *Phoma* sp. respectively. This similarity index is quite low because it was below the 70% expected borderline for the degree of relatedness.

However, the mycotoxin analysis obtained from 108 dried meat samples revealed that 92.7% of the beef meat was contaminated with OTA, although not up to the maximum acceptable level of (1 µg/kg). Sixty-six samples contained AF with an average mean estimate (0.44 µg/kg and 10.80 µg/kg) for Elisa and LC-MS respectively. While the result of the total aflatoxins detected in this study by LC-MS showed that AFB₁ have an average mean estimate of 105.4 µg/kg, AFB₂ has an estimated value of 6.92 µg/kg. AFG₁ average mean value was 40.49 µg/kg, while AFG₂ average mean value was 2.60 µg/kg. In this study, the average mean value recorded for aflatoxin total was 10 µg/kg, a value that was far higher than the permissible level of 4 µg/kg in food by the European Union. The concentrations of α -Zea and cyclopiazonic acid were relatively higher with α -Zea having an average mean value of 113.82 µg/kg, while cyclopiazonic acid estimated average mean value was 51.99 µg/kg.

The proximate content and mineral analysis of the meat as determined showed that the protein and fat content ranged from 76 -78%, 7.48-11.79% respectively. The moisture content has an average mean of 7.56- 11.57. The mineral content varied with the average mean value of Sodium, potassium, calcium, iron, zinc, magnesium, phosphorous, copper, selenium, manganese and chromium in order of 76.35, 208.49, 14.64, 4.70, 1.76, 22.55, 111.09, 1.57, 17.32, 1.47 and 0.09% respectively. This study showed that dried meat contains an average value of 77.1% of

protein as against the average 20% protein value found in raw beef meat, the minerals content were also high which indicates that its consumption can support good human nutrition.

This study also established the relationship between the nutritional composition, aflatoxin level and the aflatoxigenic fungi in the dried meat. The relationship between the nutritional composition and aflatoxin concentration showed a positive correlation with protein, ash, carbohydrate, potassium, magnesium and manganese ($r = 0.66, r = 0.13, r = 0.15, r = 0.19, r = 0.23, r = 0.50$; $p < .05$) while it correlated negatively with fungi contamination, moisture, fat, sodium, calcium, iron, zinc, phosphorus, copper, chromium and the pH value ($r = -0.28, r = -0.13, r = -0.34, r = -0.27, r = -0.15, r = -0.52, r = -0.29, r = -0.48, r = -0.68, r = -0.31, r = -0.14$; $p < .05$) respectively. However, it was observed that the AFB₁, AFB₂, AFG₁ and AFG₂, CPA, OTA including Ster correlated negatively ($r = -0.15, r = -0.35, r = -0.39, r = -0.46, r = -0.16, r = -0.3, r = -0.09$; $p < 0.05$), while α Zea, TCS and OTB had a positive relationship ($r = 0.28, r = 0.44, r = 0.57$) respectively with fungi contamination level. The correlation result showed that even though aflatoxigenic fungi were present in the dried meat, the molecular result of the fungi species tested for aflatoxin production potentials showed that many of them have the essential aflatoxin genes (*omt-A*, *aflD* and *aflM*) that are necessary to trigger *aflR* in aflatoxin production but were not expressed. Therefore the Aflatoxin and other mycotoxin detected might not have been produced as a result of the presence of the detected toxigenic fungi. This is because our findings revealed that most of the aflatoxigenic fungi were not found in the dried meat in some locations and the samples from these locations were contaminated with aflatoxin.

This present study is the first of survey on biodiversity of fungi contaminating dried beef meat that is sold in Ekiti State. The study established that different toxigenic fungi were contaminating the dried meat and that the contamination trend was not consistent as dried meat from the different locations had different types of toxigenic fungi contaminating them. This led to the conclusion that although toxigenic fungi were found on the dried meat, they may not be responsible for the mycotoxin detected in the dried meat. These mycotoxins might be due to carry-over effect from feed into the bovine. In this study, the occurrence of various mycotoxins were demonstrated and also established the possibility of concurrent exposure of dried meat consumers to multiple mycotoxins.

CHAPTER ONE

GENERAL INTRODUCTION

Meat is defined as flesh including skeletal muscle and any attached connective tissue and/or fat (Lawrie & Ledward, 2006b). It is a good source of many minerals (iron, zinc and selenium) and vitamins (vitamin D, riboflavin, B₁₂) and contains all the essential amino acids (Millward & Garnett, 2010). Fresh meat is highly perishable due to its biological structure, its storage and freshness could be affected by several factors ranging from temperature, packaging, endogenous enzymes, moisture, light, and microorganisms among several others (Zhou *et al.*, 2010). The presence of bacteria in meat may influence spoilage and render it toxic or unhealthy for human consumption.

Traditional methods of processing and preserving dried meat are still very much popular among the rural populace in developing countries, this is due largely to the non-existence or availability of functional modern storage facilities needed to preserve them (Egbunike & Okubanjo, 1999). Consequently, meats are process into dry form for preservation, which include but not limited to Banda (boiled and sundried meat), Balangu (smoked chunks of meat) and Kilishi (sliced and sundried meat) which are the most prominent products. The major difference between them is in their size reduction, which is necessary to enhance quick and uniform cooking, drying, packaging and distribution. They also vary as to whether they are hot smoked with or without prior boiling and with seasoned coatings or not (Egbunike & Okubanjo, 1999). They can be stored for six to twelve months.

These traditional methods expose the dried meat to fungal and other food pathogen contamination, right from the processing time, drying and up to the storage level (Fonkem *et al.*, 2010). When traditionally processed, the usual practice for its storage is to store the dried meat products in wooden baskets of various sizes covered with clean cement paper bags, clothes, polyethylene bags jute bags, trays; cardboard boxes while others are heaped on polyethylene carelessly on the floor in unventilated stores. Other factors responsible for the proliferation of fungi on the dried meat include; quality of raw materials, the physical and biochemical factors, processing practices and the hygienic quality of the processing environment (Mižáková *et al.*, 2002; Samson *et al.*, 2004).

Majority of the meat products start developing filamentous fungi prior to selling, and in certain cases, they become visibly mouldy (high level of discolouration) when displayed in the market (Ajiboye *et al.*, 2011). This practice of preserving meat in dried form is common among the rural people of Nigeria. Aged people that are in their late 50s as a way of avoiding the consumption of meat that are high in fat content consume dried meat. It is also use for light refreshment for the entertainment of visitors, or eaten as a delicacy with or without drinks (alcoholic or non-alcoholic beverages).

The presence and growth of fungi on the dried meats reduces the quality and question the safety of the meat, which triggers a substantial monetary damage (Samson *et al.*, 2004). In a study by Fakolade and Omojola (2008), they observed that the microbial load on dried meat during storage has a direct financial implication on the economy of the sellers. They are snub by the supposed buyers due to the discolouration and reduced quality that is visible on the meat product. There have been several reports on different bacterial contaminating surfaces of various meat. Many of them showed the potential of producing secondary metabolites called mycotoxins, and they are capable of remaining in the product long after the fungi has died (Ajiboye *et al.*, 2011; Berwal & Dinchev, 1991; Fagbohun *et al.*, 2016; Oladejo & Adebayo-Tayo, 2011).

Mycotoxin is the toxic secondary metabolites that are naturally produced by filamentous fungi, it is not desirable in food for humans and feed for animals consumption (da Rocha *et al.*, 2014). The secondary metabolites that are toxic to bacteria are called antibiotics while the ones that are toxic to plants are called phytotoxins (Bennett & Klich, 2003). Mycotoxin is a combination of the Greek word for fungus '*mykes*' and the Latin word '*toxicum*' meaning poison (Köppen *et al.*, 2010). Mycotoxins are known contaminant of several food crops and food produces all through the food chain (Wagacha & Muthomi, 2008). Development of mycotoxins usually occurs in the course of production, harvesting and storage of food crops (Bhat & Vasanthi, 2003; Krska *et al.*, 2008). Also, infestation by insects and humid conditions such as high temperatures and dampness can promote fungal increase and the production of mycotoxin in food crops (Bhat & Vasanthi, 2003; D'Mello *et al.*, 1997). Erroneous practices during picking, keeping, poor handling, in addition to transportation as well as marketing also promotes fungal and mycotoxin manifestation (Sforza *et al.*, 2006).

The most commonly encountered genera of mycotoxigenic fungi include those of *Aspergillus*, *Penicillium* and *Fusarium* genera. Other important food and plants pathogenic fungi include *Alternaria*, *Trichothecium* and *Trichoderma* etc (Oladejo & Adebayo-Tayo, 2011; Smith & Moss, 1985). Aflatoxins are carcinogenic in nature and have immunosuppressive properties, which can induce infections in people. They are linked to promoting liver disease in hepatitis B (VHB) patients who are acutely ill (Groopman *et al.*, 2008). They are also believed to be involved in the hepatocellular tumour in Asia as well as Africa (Scholl & Groopman, 2008). Ochratoxins A, is one of the most common and deadly toxins of *Aspergillus* as well as *Penicillium* species that commonly contaminate coffee, cereals, bread including other food produces (Speijers & Van Egmond, 1993). Fumonisin are other sets of mycotoxins occurring worldwide. They are usually produced by *Fusarium moniliforme* and related species when they grow in grains. The fumonisins B₁ and B₂ are of toxicological significance while the others (A₁, A₂, B₃ and B₄) occur in very low concentrations and are less toxic (Peraica *et al.*, 1999).

1.1 Problem Statement

Traditional methods of preserving meats are still very much popular among the rural populace in Nigeria and most developing countries where most of the modern preservative equipment are lacking, affordable, available or accessible due to the epileptic power supply (electricity). They usually process the raw meat by cutting them into smaller pieces, boiled, sun-dried or smoked dried in some cases. Storage of dried meats usually employs wooden baskets covered with clean cement paper bags, clothes, polyethylene bags, Jute bags, trays or cardboard boxes, while some are heap on polyethylene carelessly on the floor in unventilated stores. These traditional methods of preparation and storage expose the dried meat to fungal contamination as well as the drying and storage environment (Fonkem, *et al.*, 2010).

For example, the majority of the filamentous fungi are frequent microbes obtained in the surroundings (Gołofit-Szymczak & Górny, 2010). In general, dried meats which are usually displayed openly in the markets are sometimes found to be visibly mouldy and occasionally with fungal and microbial contamination. Most of these fungi are known toxin producers considered to be detrimental to both human and animal health on exposure to the toxins (Muri *et al.*, 2009; Wagacha & Muthomi, 2008).

This represents a significant challenge in meat industry because consumers are not likely to sufficiently eradicate microbial pathogens or eliminate toxins produced in the dried meats, since melting point for most mycotoxin is around 250 °C and above. In spite of this, the increasing safety consciousness of food internationally and the paucity of data on the fungi biodiversity, mycotoxin contamination on dried meat sold in open markets in Ekiti State. This study aim was to determine the fungi biodiversity and their mycotoxins, on locally processed dried meats sold in Ekiti State, Nigeria

1.2 Main Objective

The main objective of the study was to determine the fungi biodiversity, their mycotoxin production and the possible health risk associated with dried meats sold in Ekiti, Nigeria.

1.3 Specific Objectives

The primary purposes of this study are to:

1. Determine fungi biodiversity in the dried meats
2. Characterize fungi isolates for their potential to produce mycotoxins
3. Determine the mycotoxin contamination level in the dried meats.
4. Determine the influence of chemical and mineral content of the dried beef meat on their fungi and mycotoxin contamination

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CHAPTER TWO

LITERATURE REVIEW

2.1 Meat

The beef meat is essential to human nutrition as well as economy, health and beliefs internationally (Paredi *et al.*, 2013). Meat production includes raising various domestic animal species. The choice of these species depends on many factors such as religious and traditional beliefs, suitability, and readiness among others. Meat definition include the entire mammal fleshy tissue that is appropriate for consumption. Commonly meat refers merely to beef including any associated tissue and fat (Lawrie & Ledward, 2006a). It is an excellent source of several minerals zinc, iron, selenium, riboflavin, vitamin B₁₂ vitamin D. It is a rich source of the entire major amino acids (Millward & Garnett, 2010). Thus, it is a valuable source of some key nutrients.

2.1.1 Global meat production and consumption

Since 1960, global meat production has further increased as a result of population growth and an increase in incomes in developing countries (Meade & Rosen, 2013). Internationally, production of meat is projected at 16% increase by 2025, believing that developing countries will provide the bulk of the meat increase (OECD-FAO, 2016). Meat and meat products are an important source of protein in human diets, and their consumption depends on socio-economic factors, ethics or religious beliefs, and tradition. Globally, pork is consumed the most (15.8 kg/capita/year), followed by poultry (13.6 kg/capita/year), beef (9.6 kg/capita/year) and finally sheep and goat meat (1.9 kg/capita/year) (FAOSTAT, 2014).

The consumption varies among and within countries. For instance, in Muslim countries pork consumption is extremely low or absent, whereas it can exceed 50 kg/capita/year in countries such as Austria, Poland, Germany and Lithuania (FAOSTAT, 2014). It is estimated that the world yearly meat consumption per person is projected to attain 35.3 kg by 2025 (OECD-FAO, 2016). Meat consumption makes a bit of a typical stable food meant for adults residing in both the developing and developed countries (Cosgrove *et al.*, 2005). It is acknowledged that many years of humans evolution has changed meat consumption in large quantities (Mann, 2000).

The consumption of meat has been associated with the standard of living, food, animal production, purchaser costs including macro-economic insecurity to GDP. The demand for meat is linked to higher earnings, food eating style in urban cities and the rise in proteins of animal origin in food intake (Tester & Langridge, 2010). While great increase exists in world meat production, the mode of intake differs. In the US, meat consumption per person is 124 kg per year, which is quite different when compared to the world average meat consumption of 38 kg per person. The world least meat consumptions countries are in Africa and South Asia with an estimated meat consumption of between 3-5 kg per individual every year (Speedy, 2003).

2.1.2 Future meat consumption

Against a backdrop of a generally favourable global economic situation, and growing world population, the future for meat consumption overall is likely to be positive. Indeed world meat consumption growth is expected to be second only to vegetable oil in terms of growth rates of the major agricultural commodities (1.7% per annum for meat vs. 2% for vegetable oil by 2021 (OECD FAO, 2013)). On a geographic basis, most growth is expected from Asia, Latin America and the Middle East, and from developing countries in line. According to Rosegrant, Paiser, Meijer, and Witcover (2001) diet upgrades, made possible by income growth, are expected to double the quantity of meat demanded by consumers in developing countries by the year 2020 (using 1997 as the base year). In contrast, a contraction in consumption is expected in some developed countries as a result of lower rates of income growth and declining populations (European-Commission, 2012). Furthermore, meat consumption per capita appears saturated in these countries (OECD-FAO, 2013) and aging populations, changing demographics as well as increased health and dietary awareness is likely to result in a pattern of slowing consumption growth

2.1.3 Risk associated with meat consumption

In recent times, several epidemiological findings have linked red processed meat intake to an increase in cardiovascular disease risk (CVD) including malign cells (Fraser, 1999; Micha *et al.*, 2010; Zheng & Lee, 2009). However, the “Beef in an Optimal Lean Diet” study (Roussell *et al.*, 2012) showed that diets including 3–5 ounces (85–142 g) of lean beef per day lessened cardiovascular disease (CVD) risk (Roussell *et al.*, 2014). Other investigations have also indicated that lean red meat is unlikely to increase the risk of CVD or colon cancer (McAfee *et*

al., 2010) or of weight gain (Melanson *et al.*, 2003). Furthermore, red meat consumption can improve nutritional status (Asp *et al.*, 2012) and reduce the risk of noncommunicable diseases worldwide (McNeill and Van Elswyk, 2012). Up-to-date nutrient data for beef are essential to enable researchers to accurately evaluate beef's role in health and to inform consumers about making healthy selections.

2.2 Fungi

Several species of fungi of the genus *Aspergillus*, *Fusarium*, *Penicillium*, *Alternaria* and *Claviceps* are producers of mycotoxins (Ruyck *et al.*, 2015). *Aspergillus* species are common food contaminants in subtropical and tropical countries, and usually requires higher temperatures for optimal growth. *Aspergillus ochraceus*, *Aspergillus versicolor*, *Penicillium nordicum*, *Penicillium verrucosum*, *Penicillium brevicompactum*, *Penicillium chrysogenum* and *Penicillium polonicum* produces toxic mycotoxins (Iacumin *et al.*, 2011; Sonjak *et al.*, 2011; Ferrara *et al.*, 2015). In certain environmental and substrate conditions, they grow in meat and meat products (Perrone *et al.*, 2015) as well as posing as a potential risk to the consumers (Asefa *et al.*, 2009; Iacumin *et al.*, 2011; Plavsic *et al.*, 2015). In a study conducted in Egypt, the most frequent fungal species found in prepared meat samples from two manufacturers were *Aspergillus niger*, *Aspergillus flavus* and *Penicillium chrysogenum* (Ismail and Zaky, 1999).

Penicillium nordicum is a contaminant that may be present in foods rich in proteins (Dall'Asta *et al.*, 2010; Sonjak *et al.*, 2011; Ferrara *et al.*, 2015), can grow well at low temperatures (15 °C) and increased salt content (> 5% NaCl) and is often isolated from refrigerated protein foods such as dry-cured ham, salami and salted fish (Sonjak *et al.*, 2011).

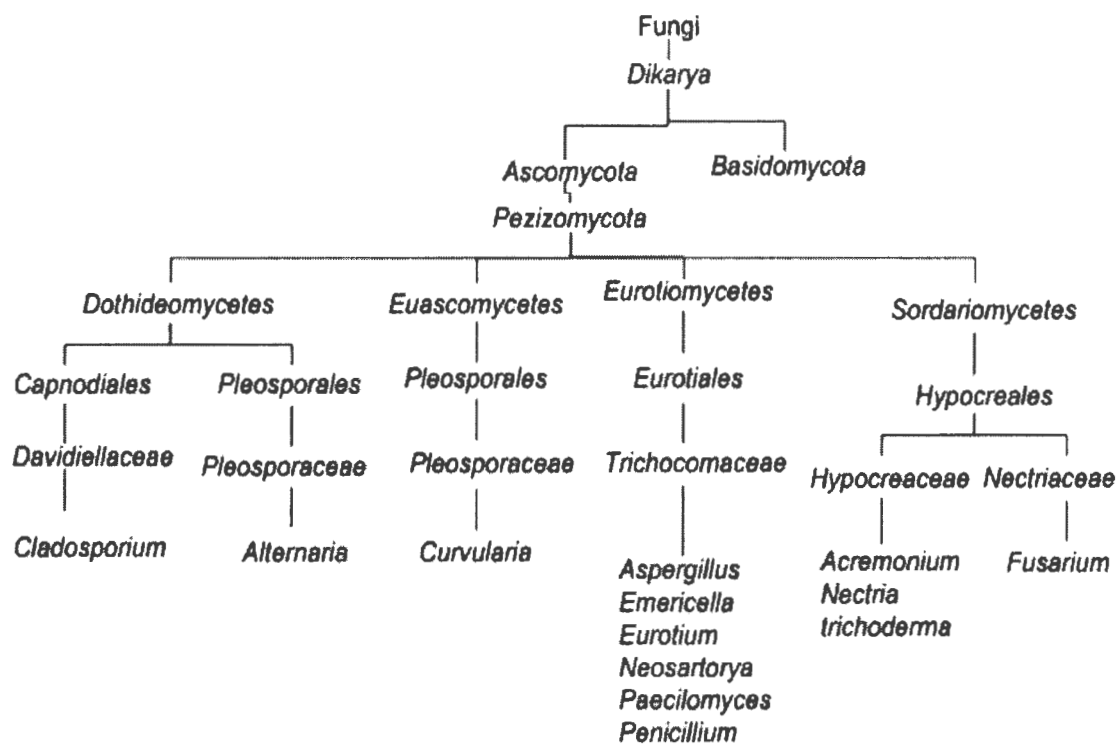
Fungi are prolific in nature and discreet due to their small shapes and their obscure way of life on materials they inhabit (JI *et al.*, 2000). Due to their enormous existence in the ecosystem, their beneficial and detrimental uses has been broadly study in relation to human and animal (Egbuta *et al.*, 2017; Klich, 2009; Pihet *et al.*, 2009; Seidl, 2008; Siegel *et al.*, 1990; Yu & Keller, 2005). A variety of filamentous fungi that belong to diverse groups have been linked to various diseases in human bodies like eyes, ears, nasal cavity, skin etc (Wilson, 2005).

2.2.1 Toxigenic fungi

2.2.2 Definition and Concepts

Toxicogenic fungi have the capability to synthesise a range of normal products as main and ancillary metabolites, particularly those *Aspergillus* and *Penicillium* species. Their beneficial value both in the medical and food productions as a result of the secondary metabolites they produce are well documented (Couto & Sanromán, 2006; Laich *et al.*, 2002). There are reports which have linked these filamentlike fungi with infections and diseases (Fisher *et al.*, 2012; Nucci & Marr, 2005). Almost all the filamentlike fungi have been implicated in causing both external infections (skin and nail) and internal infections predominantly in vulnerable individuals (Georgiadou & Kontoyiannis, 2012; Shoham & Levitz, 2005).

The secretion of mycotoxin through some reaction and certain situations that favours them, for instance, dampness and heat (D'Mello *et al.*, 1997; Kuiper-Goodman, 1995) could cause risk in person as well as in the animal. Infections like oesophagus and liver cancer are linked to mycotoxins in humans, besides illnesses, suppression of the immune system that can lead to death in animals (Dutton & Evans, 1996; Grollman & Jelaković, 2007; Grollman *et al.*, 2007). As shown in figure 2.1, a large known numbers of filamentlike fungi have a tendency to come from the sub- Phylum "*Pezizomycotina*" (Reeb *et al.*, 2004).



Source : (Ainsworth, 2008; Egbuta *et al.*, 2016; Ji *et al.*, 2000; Moretti, 2009)

Figure 2. 1 Distribution of filamentous fungi in phylum Ascomycota

2.2.3 The *Aspergillus* species

Aspergillus is the most prolific and commonly dispersed filament like fungi in the world, even though they are common in the warmer areas, their occurrence is pronounced in the temperate zones than in warmer regions (Egbuta *et al.*, 2016; Klich, 2002). *Aspergillus* species are universal, diverse and generally isolated from dust, plant rubbles and enclosed surroundings (Klich, 2002). They can grow on stored foods and feed with low moisture content with a dark, black, grey, green, white or milky colour characteristic of. More than 185 species of the *Aspergillus* genera have been isolated, *A. fumigatus*, *A. flavus* including *A. niger* are commonly isolated species among them (Klich, 2002). Other less commonly isolated *Aspergillus* species includes *A. ustus*, *A. clavatus* and *A. versicolor* etc (Klich, 2002).

2.2.4 *Fusarium* species

Fusarium is a big diverse group of fungi that is of major concern due to its various species that are significant plant pathogens (Austwick, 1984). *Fusarium* species have peculiar secondary metabolites that causes fungal infection in animals and in humans. These group of fungi was first reported in 1809, and they are commonly distributed in plants and soil (Leslie & Summerell, 2006). They have been connected to different diseases and infections in humans and animals aside from their pathogenicity (Nucci & Anaissie, 2007). Among the several known *Fusarium* groups, the commonly isolated ones are *Fusarium proliferatum*, *F. verticillioides*, *F. sporotrichioides*, *F. graminearum* including *F. chlamydosporum*.

2.2.5 The *Penicillium* species

The economically essential fungal genera are the *Penicillium* group; they are useful in food product development such as cheese and salami among others. They are also useful in the production of antibiotic drug, like penicillin in the pharmaceutical trade. Even though they are not a major threat in plant infection, they are likely to be accountable in some diseases affecting plants (Varga *et al.*, 2004). They are allied with *Aspergillus* group, even though they are less able to survive high temperatures and they are more noticeable in cooler areas (Dix & Webster, 1995; Egbuta *et al.*, 2016).

2.2.6 *Cladosporium* species

The genus *Cladosporium* was described by H.F. Link in 1816 with *C. herbarium* as type species (Ogórek *et al.*, 2012). *Cladosporium* groups are widespread in nature throughout the world, and their spores occur in the sand, atmosphere including inside water (Domsch *et al.*, 1980). They are generally recovered from houses as well as communal areas and from food products (Ogórek & Płaskowska, 2011; Ogórek *et al.*, 2011). *Cladosporium* spp. needs cool, damp weather conditions for vibrant growth, sporulation, spore release, germination and disease development. The genus of *Cladosporium* are effective during low temperatures including high moisture (Kwon *et al.*, 2001). The most frequently isolated species of *Cladosporium* on fresh fruits is *Cladosporium fulvum*, it is majorly responsible for tomato plant diseases.

2.2.7 *Alternaria*, *Acremonium* and *Curvularia* species

There exist nearly 100 species of *Alternaria* genus of dematiaceous hyphomycetes, occurring in different environments worldwide (Ellis, 1971; Joly, 1964; Pryor & Gilbertson, 2000). Few species derive their nutrient from non-living or decaying organic matter that are usually found in soil, dying or dead plant tissue. Also, they are involved in causing a variety of economically important infections on a variety of crops globally (Rotem, 1994; Strandberg, 1992). *Alternaria* species belong to the family of *Pleosporaceae*, they occur ubiquitously with green, black or grey thick colonies. *Alternaria. alternate*, *A. solani*, *A. longipes* among others are frequently isolated.

The species of *Acremonium* genus are generally considered a highly polyphyletic form containing distantly related fungi (An *et al.*, 1992). This genus is described by their smooth and big hyaline hyphae and different genus that are mostly produced by unfussy phialides and one-celled conidia (Ellis *et al.*, 2007). They are usually isolated from soil and dead plant materials, with *A. stratum* frequently isolated on foodstuff.

The genus *Curvularia* consists of species known globally to be pathogenic to both plant and human. Additionally, they have been isolated from the soil, fresh water and air (da Cunha *et al.*, 2013; Manamgoda *et al.*, 2012; Sivanesan, 1987). They possess the capability to tolerate high temperatures (40°C), a condition that favours their prevalence in humid regions (Pitt & Hocking, 2009). The species that are generally isolated include: *C. protuberate*, *C. penniseti*, *C. trifolii* and *C. tuberculata* among others (Pitt & Hocking, 2009).

2.2.8 *Emericella* and *Eurotium* species

The *Emericella* genus comprises of species that are of considerable importance due to their well-clarified genetics (MacCabe *et al.*, 2001), this is due to the penicillin production of some species. (Dulaney, 1947; Gill-Carey, 1949). Some particular types of *Emericella* produces toxin called sterigmatocystin, especially, *E. nidulans* species that has been constantly implicated in aflatoxin production (Frisvad & Samson, 2004). They are usually found together with their comparable *Aspergillus* species in the course of continuous growth (Verweij *et al.*, 2008). They propagate quickly and are generally found in humid and damp areas of the ecosphere, they comprise of more than thirty (30) species, for instance, *E. nidulans*, *E. olivicola*, *E. discophora* and *E. stellmaris* (Matsuzawa *et al.*, 2010).

Eurotium species are additional anamorph of *Aspergillus* species frequently found in humid and moist areas of the world (Kirk *et al.*, 2008). *Eurotium* is characterize with chains and is rough walled growth of the ellipsoidal spores that grow fast. Their clusters are predictably yellow, with the capability of growing fine at little water activity. Widely reported among the *Eurotium* species comprise *Eurotium herbariorum*, *E. rubrum*, *E. amstelodami* and *E. repens* (Butinar *et al.*, 2005).

2.2.9 The *Paecilomyces* species

Paecilomyces is similar to the *Eurotium*, and *Aspergillus* and usually mistaken for the *Penicillium* genus due to their near morphological similarity (Kirk *et al.*, 2008). Emerging types of *Paecilomyces* are considered thermophilic organisms as a result of their ability to propagate well in high temperatures (Inglis & Tigano, 2006). *Paecilomyces* species that are commonly isolated includes *Paecilomyces variotii*, *P. lilacinus* and *P. fulvus* (Assmann *et al.*, 2013; Egbuta *et al.*, 2014; Inglis & Tigano, 2006; Pastor & Guarro, 2006).

2.3 Commercial value of filamentous fungi

Currently, importance of fungi in the agricultural and industrial regions worldwide serve as raw materials in pharmaceutical, cosmetic, chemical and food. Table 2.1 shows the beneficial and detrimental economic effects of filament like fungi.

Table 2. 1 Distribution of filamentous fungi in phylum Ascomycota

Fungi	Agriculture	Industry	Medical	References
<i>A. flavus</i>	Bioremediation			(Kurniati <i>et al.</i> , 2014)
<i>A. niger</i>		Production of citric acid in food, cosmetics and adhesives; source of enzymes and production of gluconic acid		(Schuster <i>et al.</i> , 2002)
<i>A. oryzae</i>		Production of Kojic acid used in the cosmetics and food industries		(Ogawa <i>et al.</i> , 1995)
<i>A. terreus</i>		Production of itaconic acid, a synthetic polymer	Source of antibiotics (Lovostatin)	(van der Straat <i>et al.</i> , 2014)
<i>F. venenatum</i>		Industrially produced as food		(Katona & Kaminski, 2002)
<i>F. oxysporum</i>	Biocontrol agent			(Kaur <i>et al.</i> , 2010)
<i>P. adametzioides</i>	Biocontrol agent			(Ahmed <i>et al.</i> , 2015)
<i>P. aethiopicum</i>			Production of antibiotic (griseofulvin)	(Frisvad <i>et al.</i> , 2004)
<i>P. brevicompactum</i>		Confectionary production		(Barthomeuf <i>et al.</i> , 1991)
<i>P. camamberti</i>		Used in cheese production		(Michelson, 2010)
<i>P. chrysogenum</i>			Production of antibiotic (penicillin)	(Laich <i>et al.</i> , 2002)

<i>P. citrinum</i>			Production of antibiotic (mevastatin)	(Faseleh Jahromi <i>et al.</i> , 2012)
<i>P. expansum</i>			Production of antibiotic (patulum)	(Frisvad <i>et al.</i> , 2004).
<i>P. funiculosum</i>	used in animal feed processing			(Sahasrabudhe & Sankpal, 2001)
<i>P. glaucum</i>			Production of immunosuppressant drug	(Frisvad <i>et al.</i> , 2004).
<i>P. griseofulvum</i>			Production of antibiotics (griseofulvin, patulin and penicillin)	(Laich <i>et al.</i> , 2002)
<i>P. janezewski</i>			Production of antibiotics (griseofulvin)	(Frisvad <i>et al.</i> , 2004)
<i>P. nalgiovense</i>			Production of antibiotic penicillin	(Laich <i>et al.</i> , 2002)
<i>P. patulum</i>			Production of antibiotics (griseofulvin and patulin)	(Frisvad <i>et al.</i> , 2004)
<i>P. purpurogenum</i>		Confectionary production		(Barthomeuf <i>et al.</i> , 1991)
<i>P. roqueforti</i>		Cheese production, flavour and fragrances		(Nelson, 1970)

Source:(Egbuta *et al.*, 2016)

2.3.1 The use of fungi in agriculture

Fungi are used as an agent in bioremediation; they are useful in removing chemical contaminants from the soil and thus decreasing soil toxicity. The class of *A. flavus* including *Paecilomyces farinosus* proved useful in the reduction of Benzo. Some of the bioremediation activity of fungi has stimulated further research on the potential of bioremediation properties in naturally occurring soil fungi. For instance, Kurniati *et al.* (2014), investigated the reduction of mercury in soil using filamentous fungi. Their prospect in biofuel production has also been studied and reported to be feasible (Zheng *et al.*, 2012)

Many of the fungi have been implicated in food spoilage, plant infections and in agriculture, they usually contaminate crops at various steps of production (Dutton & Evans, 1996). Food crops and food product fungal contaminations lead to a decrease in the nutritional content, food quality and subsequent economic loss (Perrone *et al.*, 2007; Zain, 2011).

2.3.2 The use of fungi in manufacturing industry

According to Jerusik (2010), fungi have been successfully used to produce a superior paper that is appropriate in printing as well as writing. Their mycelium makes up almost 10% of the paper manufacturing industry. Mohammad *et al.* (2012), described their importance in biodegrading organic materials in the industries. This indicates their effectiveness as a potential agent in removing generated compost in the production of palm.

2.3.3 The use of fungi in food industry

The notable area in the food industry that fungi have been successfully used is food fermentation and this has made it important in food manufacturing industries (Kirk *et al.*, 2008). For instance, the production of citric acid through fermentation of *Aspergillus niger*, which has become the leading sources of citric acid production. Likewise, the flavours, vitamins, enzymes proteins, lipid and other compounds of interest by the filament like fungi in food manufacturing has been studied (Sahasrabudhe & Sankpal, 2001).

2.3.4 The use of fungi in pharmaceutical/medical industry

The secondary metabolites by filament like fungi are of great importance to the pharmaceutical and medical industry, in that it could have beneficial or detrimental properties on both persons and wildlife. Their produced toxins have been proven to have various hindering effects in chemical reactions pathways (Goswami *et al.*, 2012). For instance, statins and Lovastatin which

A. terreus secondary metabolites, Statins prevent the enzyme that is responsible for cholesterol reduction in blood levels of individuals with high cholesterol levels (Manzoni & Rollini, 2002). While production of cyclosporine-A (an immunosuppressant) is linked to *Fusarium oxysporum*, with their secondary metabolite reported to be effective in cancer management.

Their additional benefit is their ability in preventing bacterial progression (He *et al.*, 2002). These properties were reported effective in prevention of *Candida albicans* and *Escherichia coli* growth (Svahn *et al.*, 2015). Biofilm produced by Filamentous fungi is used to speed up (catalyze) chemical reactions in the production of the human drug due to their proven effectiveness for a longer time (Amadio *et al.*, 2013). This has positively affect drug development and contributes to their toxicity assessment.

2.4 Mycotoxins produced by Filamentous fungi

As earlier stated (paragraph 2.2.2), mycotoxins are the products of toxigenic fungi. In general, secondary metabolites are toxigenic to both human and animal. These secondary metabolites produced by filament like fungi comprises of aflatoxins, fumonisins, ochratoxin, deoxynivalenol, trichothecenes, gliotoxins, zearalenone, etc., with more than 300 known synthesized mycotoxins worldwide (Hussein & Brasel, 2001). However, more than one fungi species or genera can potentially produce a particular type of mycotoxin. For example, *A. ochraceus*, *A. niger* including *P. viridicatum* are the producers of ochratoxin A mycotoxin. While on the other hand, one fungal species or genera can potentially produce other types of mycotoxins. Such as *F. culmorum* and *F. verticillioides* are known producers of nivalenol, deoxynivalenol, fumonisin B₁ and some other mycotoxins under certain favourable conditions. Table 2.2 show details of the fungi genera and their produced mycotoxins.

Table 2. 2 Some of the major mycotoxins produced by toxigenic fungi

Fungal Genera	Mycotoxins Produced
<i>Aspergillus</i> species	
<i>A. carneus</i>	Citrinin
<i>A. clavatus</i>	Cytochlasin E, Patulin, Tryptoquivalene
<i>A. flavus</i>	Aflatoxins, Sterigmatocystin
<i>A. fumigatus</i>	Fumagilin, Gliotoxin, Verruculogen, viriditoxin
<i>A. nidulans</i>	Sterigmatocystin
<i>A. niger</i>	Malformin, Oxalic acid, Ochratoxin A
<i>A. ochraceus</i>	Ochratoxin A, Penicillic acid, Destruxin
<i>A. terreus</i>	Citrinin, Citreoviridin
<i>A. ustus</i>	Austdiol, Austamide, Austocystin
<i>A. versicolor</i>	Cyclopiazonic acid, Sterigmatocystin
<i>A. parasiticus</i>	Aflatoxins
<i>Fusarium</i> species	
<i>F. avenaceum</i>	Enniatins, Fructagenin +1, HT-2 toxin, Ipomeanine, Lateritin +1, Lycomerasmin +1, Moniliformin, Monoacetoxyscirpenol, Neosolaniol, Nivalenol, Sambucynin
<i>F. culmorum</i>	Deoxynivalenol, Fructagenin +1, HT-2 toxin, Ipomeanine, Lateritin +1, Lycomerasmin +1, Moniliformin, Neosolaniol
<i>F. equiseti</i>	Moniliformin, Nivelenol, Monoacetoxyscirpenol, Acetoxyscirpenediol, Acetyldeoxynivalenol, Acetylneosolaniol, Acetyl T-2 toxin, Avenacein +1, Beauvericin +2, Butenolide, Calonectrin, Deacetylcalonectrin, T-1 toxin, zearalenol, T-1 toxin, T-2 toxin
<i>F. nivale</i>	Deoxynivalenol diacetate, HT-2 toxin, Ipomeanine, Lateritin +1, Lycomerasmin +1, Moniliformin, Monoacetoxyscirpenol, Sambucynin
<i>F. oxysporum</i>	Moniliformin, Monoacetoxyscirpenol, Neosolaniol, Nivalenol, Acetoxyscirpenediol, Acetyldeoxynivalenol, Acetylneosolaniol, Acetyl T-2 toxin, Avenacein +1, Beauvericin +2, Butenolide, Calonectrin, Deacetylcalonectrin, zearalenone
<i>F. roseum</i>	Fructagenin +1, Moniliformin, Monoacetoxyscirpenol, Neosolaniol, NT-1 toxin, N-2 toxin

<i>F. solani</i>	Enniatins, T-1 toxin, T-2 toxin, Sambucynin, Scirpentriol
<i>F. verticillioides</i>	Fumonisin, Monoacetoxyscirpenol, Neosolaniol, Ipomeanine, Avenacein +1, Beauvericin +2, Fusaric acid, Fusarin
<i>F. graminearum</i>	Zearalenone, Yavanicin +1
<i>Penicillium</i> species	
<i>P. viridicatum</i>	Ochratoxin A, Rubrosulphin, Viopurpurin, Viomellein
<i>P. citrinin</i>	Citrinin
<i>P. verrucosum</i>	Citrinin
<i>P. hirsutum</i>	Citrinin
<i>P. citreoviride</i>	Citreoviridin
<i>P. islandicum</i>	Islanditoxin
<i>P. expansum</i>	Patulin
<i>P. roqueforti</i>	Patulin
<i>P. griseofulvum</i>	Patulin
<i>P. claviforme</i>	Patulin
<i>P. crustosum</i>	Penitrem, Viomellein
<i>P. rubrum</i>	Rubratoxin
<i>P. brunneum</i>	Rugulosin
<i>P. kloeckeri</i>	Rugulosin
<i>P. rugulosum</i>	Sterigmatocystin, Rugulosin
<i>P. aurantiogriseum</i>	Viomellein

Source: (Egbuta *et al.*, 2017)

There are various types of mycotoxins from the *Aspergillus* groups, among which are aflatoxins as well as sterigmatocystin that is usually produced by *A. flavus*, while oxalic acid, malformin, ochratoxin A and fumonisin B₂ are generally produced by *A. niger*. The gliotoxin and viriditoxin mycotoxins are produced by *A. fumigatus* etc (Bennett & Klich, 2003). Also, mycotoxins such as ochratoxin A, gliotoxin, rubrosulphin, rugulosin, citrinin patulin and islanditoxin are produced by *Penicillium* species (Sweeney & Dobson, 1998).

The report by Richard (2007) on fungi mycotoxins production has been linked with diseases. Mycotoxin causes genetic effects when it is prolonged through consumption and could result in detrimental health effects (Steyn, 1995). Among the five major important mycotoxins of public health concern is Aflatoxins, which are Hepatotoxic and immune-suppressive predominantly affect the liver (Humans *et al.*, 2002). The most potent aflatoxin group that is categorized to be carcinogenic to human is AFB₁ (IARC, 1994). Another major mycotoxin classified to be a human carcinogen is Ochratoxin A, with kidney as its main target. Ochratoxin A an immuno-suppressive, teratogenic, nephrotoxic and carcinogenic mycotoxin to human and animals (Stoev, 1998). Other public health important mycotoxins that are immune-suppressive, mutagenic, carcinogenic, teratogenic include deoxynivalenol, fumonisins and zearalenone (Hussein & Brasel, 2001; Richard, 2007)(Table 2.3).

Table 2. 3 Frequent mycotoxins and their health impacts including the focus organs

Metabolites	Health impacts	Focus organs	References
<i>Aflatoxins</i>	Hepatotoxic and immune-suppressive	Liver	(Steyn, 1995)
<i>Ochratoxin A</i>	Carcinogenic, genotoxic, Immuno-suppressive, nephrotoxic and causing upper urinary tract disease	Kidney, liver	(Pfohl-Leszkowicz <i>et al.</i> , 2002)
<i>Fumonisin</i>	Carcinogenic, hepatotoxic, nephrotoxic, immunosuppressive	Gastro-intestinal tract (GIT), liver, kidney	(Chu & Li, 1994; Soriano <i>et al.</i> , 2005).
<i>Deoxynivalenol</i>	Nausea, vomiting, diarrhea, reproductive effects and toxicoses	Reproductive organs, GIT	(Kuiper-Goodman, 1995; Richard, 2007).
<i>T-2 toxin</i>	Hepatotoxic, genotoxic and immune-suppressive	GIT, Immune system	(Hymery <i>et al.</i> , 2009; Li <i>et al.</i> , 2006)
<i>Zearalenone</i>	Carcinogenic, hormonal imbalance and reproductive effects	Reproductive organs	(D'Mello <i>et al.</i> , 1997; Miller, 1994)
<i>Nivalenol</i>	Anorexic, immunotoxic, haematotoxic and genotoxic	GIT, immune system	(Bony <i>et al.</i> , 2007; Kubosaki <i>et al.</i> , 2008)
<i>Sterigmatocystin</i>	Genotoxic, cytotoxic, immunotoxic and carcinogenic	Liver, immune system, kidney	(Huang <i>et al.</i> , 2014; Terao <i>et al.</i> , 1978)
<i>Cyclopiazonic acid</i>	Immunotoxic and hepatotoxic	Muscle, hepatic tissue and spleen	(Antony <i>et al.</i> , 2003; Burdock & Flamm, 2000)
<i>Moniliformin</i>	Cardiotoxic, muscular disorders, immunotoxic	Heart, kidney, and muscles	(Peltonen <i>et al.</i> , 2010; Zhang <i>et al.</i> , 2010)
<i>Enniatins</i>	Immunotoxic, cytotoxic	Immune system	(Juan-García <i>et al.</i> , 2013; Prosperini <i>et al.</i> , 2014)
<i>Gliotoxin</i>	Immunotoxic, nephrotoxic, hepatotoxic and genotoxic	Kidney, liver, immune system	(DeWitte-Orr & Bols, 2005; Mueller <i>et al.</i> , 2013; Niide <i>et al.</i> , 2006)
<i>Citreoviridin</i>	Teratogenic and immunotoxic	Not specific	(Hou <i>et al.</i> , 2014; Morrissey & Vesonder, 1986)
<i>Citrinin</i>	Nephrotoxic	Kidney	(Bennett & Klich, 2003)

Source: (Egbuta *et al.*, 2017)

Table 2. 4 Infections induced by fungi species and thier targeted organs

<i>Aspergillus candidus</i>	Respiratory tract, brain, ear and nails	Respiratory disease, otomycosis, onychomycosis, brain granuloma	(Ahmadi <i>et al.</i> , 2012; Ribeiro <i>et al.</i> , 2005).
<i>Aspergillus flavus</i>	Nails, respiratory tract, bone and eye	Sinusitis, keratitis, aspergillosis, osteomyelitis	(Hedayati <i>et al.</i> , 2007; Zhang <i>et al.</i> , 2005)
<i>Aspergillus fumigatus</i>	Respiratory tract	Pulmonary infections	(Latge, 2003).
<i>Aspergillus niger</i>	Ears, throat and respiratory tract	Otomycosis, pulmonary aspergillosis	(Georgiadou & Kontoyiannis, 2012).
<i>Aspergillus versicolor</i>	Nose, eyes, throat, nails,	Invasive aspergillosis, onychomycosis	(Benndorf <i>et al.</i> , 2008; Charles <i>et al.</i> , 2011)
<i>Fusarium oxysporum</i>	Eyes and Nails	Keratitis, onychomycosis	(Jain <i>et al.</i> , 2011).
<i>Fusarium solani</i>	Eyes, respiratory tract, nails, skin and bone	Keratitis, sinusitis, endophtalmitis, onychomycosis, cutaneous infections, mycetoma and arthritis	(Jain <i>et al.</i> , 2011).
<i>Fusarium verticillioides</i>	Eyes, skin, internal organs such as lungs, etc.	Keratomycosis, skin lesions, proliferation of internal organs	(Cocchi <i>et al.</i> , 2011; Georgiadou <i>et al.</i> , 2014; Mochizuki <i>et al.</i> , 2012).
<i>Penicillium citrinum</i>	Eyes and respiratory tract	Keratitis, asthma, pneumonia	(Mok <i>et al.</i> , 1997; Walsh <i>et al.</i> , 2004).
<i>Penicillium marneffeii</i>	Blood, skin and respiratory tract	Fungemia, skin lesions, anaemia	(Walsh <i>et al.</i> , 2004)

Source:(Egbuta *et al.*, 2017)

2.5 Infections caused by filamentous fungi

Filamentous fungal species are widespread in the environment and have been reported in association with some human and animal infections and diseases (Howard, 2002). Several fungal infections are associated with *Aspergillus*, *Fusarium* and *Penicillium* (Table 2.3). These fungal genera induce infections in a specific manner attacking specific organs and parts of the body (Vonberg & Gastmeier, 2006).

2.5.1 *Aspergillus* species

More than 20 species of *Aspergillus* are causative agents of opportunistic infections in man. *Aspergillus* species are well-known to play a role in three different clinical settings in man: opportunistic infections, allergic states and toxicoses, apart from the production of mycotoxins (Howard, 2002) mostly through inhalation of spores (Vonberg & Gastmeier, 2006). *Aspergillus* is responsible for a range of infections such as allergic bronchopulmonary aspergillosis (ABPA), aspergilloma, chronic necrotizing aspergillosis (CNA) and invasive pulmonary aspergillosis (IPA) generally referred to as aspergillosis. It usually affects people with existing health conditions, especially those with injured lungs and suppressed immunity (Khan & Karuppaiyil, 2012) with usage of drugs and medications reported to have also contributed to reducing immunity in healthy individuals. Aspergillosis symptoms include difficulty in breathing, increased thirst, diarrhoea and fever (Patron, 2006). Among all the species of genus *Aspergillus*, *A. niger*, *A. flavus*, *A. fumigatus*, *A. versicolor* and *A. nidulans* are reported to have the ability to induce disease and infection (Galimberti *et al.*, 2012). Despite the fact that *A. ochraceus* has the ability to produce mycotoxins, there is no report of this species inducing any form of infection in humans or animals.

2.5.1.1 *Aspergillus fumigatus*

The most frequently occurring aerial pathogen with life-threatening properties is *Aspergillus fumigatus* (Chaudhuri *et al.*, 2011). It is usually isolated from blood and tissues of persons showing symptoms associated with *A. fumigatus* infection (Galimberti *et al.*, 2012). *Aspergillus fumigatus* is responsible for a range of pulmonary infections. Its metabolites includes polypeptide allergens liable for asthma and rhinitis (Latge, 2003). They also produce mycotoxins such as gliotoxins that have negative health effects on humans and animals as well as β -1, 3 glucans that are known modulators of the immune system (Fischer *et al.*, 2000). *Aspergillus fumigatus* is a source of amplified immune-suppression and perhaps mortality in immuno-

compromised individuals, with the characteristic of immune-suppression credited to the production of the toxic metabolite gliotoxin during hyphal growth under certain favourable conditions (Kupfahl *et al.*, 2008). In a study by Wartenberg *et al.* (2011), it was revealed that *A. fumigatus* has the ability to produce proteins and other allergens that can induce infections and diseases.

2.5.1.2 *Aspergillus flavus*

Aspergillus flavus is a frequently isolated *Aspergillus* species from soil, contaminated food commodities and fungal pathogen associated with a range of fungal infections. A common causative agent in invasive aspergillosis and superficial infections, *A. flavus* has been implicated with clinical syndromes such as prolonged granulomatous sinusitis, keratitis, cutaneous aspergillosis, wound infections and osteomyelitis (Hedayati *et al.*, 2007; Zhang *et al.*, 2005). It has also been reported to cause adverse health illnesses in immuno-compromised individuals which could at times be deadly (Vonberg & Gastmeier, 2006) and can be lethal in healthy individuals exposed to the spores.

2.5.1.3 *Aspergillus versicolor*

Aspergillus versicolor species are usually found in humid indoor surroundings and food products (Engelhart *et al.*, 2002). They produce hepatotoxic and carcinogenic mycotoxin sterigmatocystin (Nielsen, 2003). This adaptable pathogen is known to contain over 20 allergens and it irritates the nose, eyes and throat (Benndorf *et al.*, 2008). It has been reported to be a causative agent of invasive aspergillosis with the fungi being the main cause of onychomycosis, a fungal infection of the nails (Charles *et al.*, 2011; Torres-Rodriguez *et al.*, 1998).

2.5.1.4 *Aspergillus candidus*

Aspergillus candidus, a common contaminant of grain dust and common producer of potent cytotoxic materials, p-terphenyl metabolites and terpenins (Krysińska-Traczyk & Dutkiewicz, 2000) it has been implicated in lung diseases in humans. It is a potential respiratory threat for individuals who are exposed to them. Different types of infections have been ascribed to this *Aspergillus* species which consist of invasive aspergillosis, otomycosis, brain granuloma, onychomycosis, allergic alveolitis and mycotoxicosis (Ahmadi *et al.*, 2012; Ribeiro *et al.*, 2005).

2.5.1.5 *Aspergillus niger*

Aspergillus niger is a common contaminant of food, soil and indoor environment with the ability to grow on a wide range of substances. Although its spores are widespread, the fungus has been reported to be unlikely cause of human diseases compared to other *Aspergillus* species (Person *et al.*, 2010). *Aspergillus niger* usually attacks tissues that are already susceptible due to bacterial infections, physical injury or accumulation of cerumen in the external auditory canal. Like other *Aspergillus* species, *A. niger* is a known causative agent of otomycosis, a superficial fungal infection in the ear, throat or nose that can be sub-acute or chronic. As is the case with most filamentous fungal species, immune compromised individuals are also susceptible to *A. niger* infections. In most cases, they cause invasive pulmonary aspergillosis characterized by chronic productive cough and coughing up blood (Georgiadou & Kontoyiannis, 2012; Person *et al.*, 2010).

2.6.2 *Fusarium* species

Fusarium species contain more than fifty species commonly found in the soil and in association with plants such as cereals and grains, is a causative agent of superficial and systemic infections. *Fusarium* can infect humans and animals through inhalation of air-borne conidia and wounds in the skin (Georgiadou *et al.*, 2014). Additionally it causes a series of opportunistic infections, while some *Fusarium* species produce mycotoxins that affect human and animal health through food chain. *Fusarium* infections are normally referred to as fusariosis which is mostly dependent on the immune status of the host and mode of entry (Nucci & Anaissie, 2007).

The common *Fusarium* infections among healthy hosts are keratitis and onychomycosis with additional less common illnesses such as sinusitis, pneumonia, thrombophlebitis and fungemia (Nucci & Anaissie, 2007). While those at great risk of fusariosis are individuals with protracted and intense neutropenia as well as T-cell immunodeficiency among the immune-compromised individuals (Consigny *et al.*, 2003). Although it is not all the *Fusarium* that possess the ability to induce disease or infection, there are a few causing infections such as *F. solani*, *F. verticillioides*, complex, *F. oxysporum* and *F. proliferatum*, with *F. solani* being the most frequent (Nucci & Anaissie, 2007).

2.6.2.1 *Fusarium solani*

The often and most virulent of all *Fusarium* species reported in relation to fusariosis, is *F. solani*, it is ubiquitous in nature (Zhang *et al.*, 2006). *Fusarium solani*, a plant pathogen, has been reported to induce a range of diseases and infections in both strong immune and weak immune compromised individuals (Jain *et al.*, 2011). Although a variety of *Fusarium* species are causative agents of mycotic keratitis, *F. solani* is the most frequently reported unlike other species (Howard, 2003; Zhang *et al.*, 2006). Symptoms of *F. solani* infections include ulcers, fevers, skin lesions and organ membrane disruption.

2.6.2.2 *Fusarium verticillioides*

Normally contaminating maize, rice, other cereals and grains, *F. verticillioides* is one of the major producers of fumonisin mycotoxin known to have adverse negative health effects including oesophageal cancer (Chu & Li, 1994). Infection by *F. verticillioides* has been reported to occur in immune-compromised individuals who have undergone major organ re-transplant due to rejections by their bodies (Consigny *et al.*, 2003). Another known infection induced by *F. verticillioides* is *Fusarium* keratomycosis, a fungal infection of the cornea considered by red and painful eyes in addition to signs of ulcers (Mochizuki *et al.*, 2012). Warning signs associated with *F. verticillioides* infections include necrotic lesions on the skin, typically on the legs, fever and endothelial proliferation of internal organs (Cocchi *et al.*, 2011; Georgiadou *et al.*, 2014).

2.6.2.3 *Fusarium oxysporum*

Fusarium oxysporum, which consist of all the species, ranges and forms within a group called section *Elegans*, is an abundant *Fusarium* species with the ability to live in different environments such as deserts, tropical and temperate forests as well as grasslands (Gordon & Martyn, 1997). Many of its strains have been classified as non-pathogenic, human pathogenic and plant pathogenic (Howard, 2002). *F. oxysporum* of human pathogenic strains are known to cause infections in both humans and animals whether immune-competent or immune-compromised (Jain *et al.*, 2011). Infections such as keratitis, onychomycosis are some of the infections associated with *F. oxysporum* as well as localized necrotic diseases (Jain *et al.*, 2011).

2.6.3 *Penicillium* species

Penicillium species are abundant and broadly distributed in the environment. In spite of their abundance and diversity in the environment, they are not usually associated with human and

animal infections (Mok *et al.*, 1997). More or less of the few pathogenic species affecting plants, *P. citrinum*, *P. chrysogenum*, *P. digitatum*, *P. expansum* and *P. marneffei* are normally associated with humans and animals with manner of infection commonly through inhalation and occasionally ingestion (Walsh *et al.*, 2004). Ailments resulting from *Penicillium* infection are called Penicilliosis. Species of this genus have been revealed in relationship with infections such as keratitis, endophthalmitis, otomycosis, pneumonia, endocarditis and urinary tract infections (Deshpande & Koppikar, 1999).

2.6.3.1 *Penicillium citrinum*

Known as one of the frequently occurring *Penicillium* species, this fungus is important in plant, human and animal. It produces ochratoxin and citrinin mycotoxins, which has been implicated in relationship with some fungal infections and other diseases such as keratitis, asthma and pneumonia which in some cases, may be deadly (Mok *et al.*, 1997; Walsh *et al.*, 2004).

2.6.3.2 *Penicillium marneffei*

Penicillium marneffei is the third most common opportunistic pathogen that attacks persons suffering from acquired immune deficiency syndrome (AIDS) especially in regions where it is prevalent (Mok *et al.*, 1997). They affects individuals with hematological malignancies as well as persons getting immune-suppressive therapy when exposed to it. In parts of Southeast Asia, this fungus has continued to cause sickness and death in human immunodeficiency virus (HIV) positive patients who are either ignorant of their status or unable to take anti- retroviral therapy (Walsh *et al.*, 2004). People exposed to *P. marneffei* infections display clinical signs such as weight loss, skin injuries, fungemia, pulmonary lesions, anaemia, cough and low-grade fever (Walsh *et al.*, 2004)

2.6.3.3 Other less common pathogenic *Penicillium* species

Penicillium expansum, *P. digitatum* and *P. chrysogenum* are some other less common pathogenic fungi belonging to the genus *Penicillium*. Although, not frequently mentioned in relation to Penicilliosis, these fungal species cause infections in human that might be lethal. *Penicillium chrysogenum* and *P. expansum* have been implicated in necrotizing esophagitis, endophthalmitis, keratitis and asthma (Walsh *et al.*, 2004). *Penicillium digitatum*, which is a post-harvest plant pathogen, was reported in relation to deadly pneumonia (Oshikata *et al.*, 2013).

2.7 Mycotoxins

2.7.1 Definition and Concepts

As previously stated, that mycotoxins are secondary fungal metabolites of small molecular weight, capable of causing disease and death in both humans and other animals (D'mello & Macdonald, 2003; Moss, 1992) known as mycotoxicosis (Piva & Galvano, 1999). The term mycotoxin is a derivative Greek word 'Mykes' which means 'fungus' and the Latin expression 'Toxicum' denoting poison (Chu, 1991; Njobeh, 2009). Mycotoxins can be considered as naturally occurring toxins that cause health interrelated problems through the natural path of ingestion, inhalation or absorbed through the skin (Pitt & Hocking, 1997). Mycotoxins are derived from living organisms and are produced through non-anthropogenic fungal actions and are endogenously created in foods as a result of secondary metabolism (Scimeca, 1997), which may also be lethal to plants (phytotoxins) or other micro-organisms (antibiotics) as shown by Bhatnagar *et al.* (2002).

According to Berthiller *et al.* (2007), about 300-400 mycotoxins are known to exist in nature, while only 30 are known to be relevant to agriculture (Binder *et al.*, 2007; IARC, 1993) owing to their incident and the relative vital role they play in human and animal health. There are other evolving mycotoxins whose characteristics, structures as well as general characteristics has not been fully explained, at times, such mycotoxins may incite more shocking toxic effects than the previously known mycotoxin effects. Nevertheless, this review will center chiefly on *aflatoxins*, *deoxynivalenol*, *ochratoxin*, *fumonisin* and *zearalenone* that are more of public health concerned (D'Mello *et al.*, 1997; D'mello *et al.*, 1999; Newman, 2000; Njobeh, 2009; Raju & Devegowda, 2000), and expected to infect several foods from Nigeria.

An important additional consideration is the potential of mycotoxins in causing severe harmful health effects such as carcinogenic or immunosuppressive effects. Many of the important mycotoxins such as *T-2 toxins*, *moniliformin*, *nivalenol*, *citrinin*, *patulin*, *Kojic acid*, *sterigmatocystin*, *tenuazonic acid*, *cyclopiazonic acid* and will be concisely discussed in this

review. In addition, mycotoxin production by air-borne fungi is poorly defined, apart from under laboratory environments or in situations, where the management of mycotoxin contaminated foodstuffs is involved. Conversely, their existence in enclosed environments is presently getting various consideration. The presence of mycotoxin is detrimental and so plans to control their pollution levels both in the environment and in foods are of main concern.

2.7.2 Origin, Nature, Chemistry and Distribution of Mycotoxins

Mycotoxins or secondary metabolites are produced by a number of moulds, together with members of the *Aspergillus*, *Penicillium*, *Fusarium* (Yiannikouris & Jouany, 2002), *Alternaria* (D'Mello et al., 1997) and *Claviceps* spp (Chu, 1991) in grains along with fodders (Yiannikouris & Jouany, 2002). Specific mycotoxins that are produce within each family of fungal species, individually or in combinations depends on their toxigenic potentials. Not like principal metabolism, ancillary metabolism (toxin production) qualitatively is influenced by fungal species and quantitatively on the fungal strains (Le Bars & Le Bars, 1998). A straight link subsists between the occurrence of mycotoxins in foodstuffs and forages and the existence of connected moulds in that specific food or their components.

Mycotoxins are different in their compound structures, in addition to biological activities such as carcinogenicity, hepatotoxicity, nephrotoxic, neurotoxic, oestrogenic, dermatoxic or immunosuppressive activities (Njobeh, 2009). They exhibit close association in their structural diversity in terms of some reactions such as condensation, oxidation, reduction, alkylation and halogenation (chlorination) with multiple branching of pathways (Bakutis *et al.*, 2005). These compounds classification are in different ways based on their chemical composition, bio-origin or the ill effects they cause in animal species. There are generally some difficulties when considering the fact that a certain type of mycotoxin can be produce by several species of fungi other than from one genus. In addition, multiple symptoms or many damages can be from more than one mycotoxin in more organs, depending on the animal species.

According to Bhatnagar *et al.* (2002) and Steyn (1995), mycotoxins are classified into polyketides (aflatoxins, ochratoxin, zearalenone and fumonisins), terpenes (trichothecenes), tetramic acids (cyclopiazonic acid and tenuazonic acid) and nitrogen-containing metabolites (ergotamine) for convenience and based on their bio-origin. They have various kinds of pharmacological effects in mammals, including human being. The carry-over of mycotoxins to

edible animal products made from animals fed with contaminated feeds is well known (McEvoy, 2002). The pathways involved in the metabolism of mycotoxins, specifically the terpenes and steroids-derived mycotoxins have been widely studied by several authors (Zamir, 1989).

2.7.3 Occurrence of mycotoxins in food and feed worldwide

Occurrence of mycotoxins worldwide in animal feeds and human food commodities predominantly in cereal grains have been reviewed by Jelinek *et al.* (1989) and Binder *et al.* (2007), while Doko *et al.* (1996) and Makun *et al.* (2011a) have also carried out similar studies in South Africa and Nigeria respectively. The Occurrence of mycotoxin contamination in forages and foodstuffs differs from region to region (Clark, 2003). This also depends on the prevalent climatic situations such as temperature and relative humidity (Paterson & Lima, 2010; Sanchis & Magan, 2004), chemical composition (fat, moisture, protein) of the substrate, diversity of consumed foods in addition to the local guidelines of the food quality control policies (Shah *et al.*, 2010). According to Binder *et al.* (2007), their result revealed the occurrence of deoxynivalenol, zearalenone and T2 toxin as the foremost contaminants in the European samples, while aflatoxins, fumonisins, deoxynivalenol and zearalenone were mainly found in the samples from Asia and Pacific regions. Also, CAST (2003) reported that fumonisins B (FB) is common in maize, aflatoxin (AF) in peanuts in the tropics, ochratoxin A (OTA) in barley in the Scandinavian countries, while deoxynivalenol (DON) is predominant in wheat in America, Canada and Europe. In South Africa, the occurrence of FB in maize is a common phenomenon (Marasas, 1995; Sydenham *et al.*, 1990). In Cameroon, according to the reports of Ngoko *et al.* (2001), they found FB to be also common in maize. A survey by Reddy *et al.* (2009) revealed the presence of several species of *Aspergillus* and aflatoxin B₁ in rice samples comprising of paddy and milled rice. Similarly, the occurrence of *Aspergillus* and aflatoxins in rice grains from other countries like Canada, China, Nigeria and Pakistan have been reported (Anthony *et al.*, 2009; Bansal *et al.*, 2011; Iqbal *et al.*, 2012; Lai *et al.*, 2015). Furthermore, Herzallah (2009) reported the presence of aflatoxin M₁ and M₂ in milk samples and aflatoxins B₁, B₂, G₁ and G₂ in meat samples collected from the local markets in Jordan.

2.7.4 Mycotoxins in medicinal herbs

Studies in India revealed the natural occurrence of aflatoxin B₁ and Citrinin in seeds of some medicinal plants under storage (Roy & Kumari, 1991). The occurrence of toxigenic fungi producing *aflatoxins*, *ochratoxin A* and *fumonisin* on medicinal herbs was also reported from Argentina by Rizzo *et al.* (2004). A research from South Africa showed the presence of *fumonisin* B₁ in dietary and medicinal wild plants (Sewram *et al.*, 2006). In Korea, a survey was conducted on spices and processed spice products for aflatoxin contamination, where aflatoxin B₁ was detected in 13.6% of the spices (Cho *et al.*, 2008). Also, While multi-contamination of mycotoxins with *T2 toxin*, *zearalenone*, *aflatoxins*, *ochratoxin A*, *deoxynivalenol*, *citrinin* and *fumonisin* were detected in medicinal herbs surveyed in Spain (Aiko & Mehta, 2015).

2.7.5 Mycotoxins in meat

Mycotoxins in meat products may originate from residue in animal feed, direct growth of toxigenic moulds, commonly on the outer layer of meat products (Bailly & Guerre, 2009) or from the addition of flavoring ingredients, such as spices (Fazekas *et al.*, 2005). However, the intrinsic properties of fresh meat including relatively high water activity, slightly acidic pH and the availability of carbohydrate (glycogen) and proteins make it a good substrate for microbial growth and consequently it is a highly perishable commodity. Therefore, the shelf life of meat products is limited by enzymatic and microbiological spoilage. To extend the shelf life of meat and meat products, traditional preservation methods using sun drying and salting techniques have been used.

Many developing countries are still preparing traditional dried products for human consumption (Campbell-Platt, 1987). Drying techniques mostly rely on extending the keeping properties of the meat by reducing the water activity i.e. the ratio of the equilibrium vapor pressure of the product to the equilibrium vapor pressure of pure water at the same temperature (Scott, 1953, 1957). In many developing countries, including Nigeria it is a routine practice to dry the meat strips hanging over rope in uncovered areas. Mothershaw *et al.* (2003) indicated there is a risk of high levels of fungal contamination during the extensive handling of meat during butchery and preparation of strips for drying. Occurrence of mycotoxins in meat and meat products have been reviewed by Biswas *et al.* (2010) and Bailly and Guerre (2009). *Ochratoxin* produces by *P. nordicum* has been reported in cheese and fermented meats by Cabañes *et al.* (2002) and Sonjak *et al.* (2011).

Mycotoxins occur in meat primarily because of an unintended transfer from naturally contaminated feed. In case of meat products, the occurrence is often influenced by the recipe used with their production, specifically by the origin of meat and the use of edible tissues, blood and spices that can be contaminated by mycotoxins (Gareis & Scheuer, 2000), in addition to their production and storage conditions (Asefa *et al.*, 2011; Rodríguez *et al.*, 2012). According to Ostry *et al.* (2013) favorable conditions for the production of mycotoxins in meat and meat products are: the presence of oxygen, a temperature between 4°C to 40°C, a pH value between 2.5 and 8, minimum water activity of 0.80 and maximum salt concentration of 14%.

2.7.6 Mycotoxin contamination in animal feeds and transfer to foods of animal origin

The exposure of human to mycotoxins can occur through the food supply chain, in which mycotoxins can enter the food/feed processing industry through direct or indirect contamination (Frisvad and Samson, 1991; Rocha *et al.*, 2014). In line with possible natural contamination of the grains used in the food and livestock industry and consequently in the production and processing of foodstuffs for human and animal consumption, there is a high probability of mycotoxins entering the food chain. The products of animal origin such as meat and meat products, may contribute to the human intake of mycotoxins through indirect transfer of production animals, which consume naturally contaminated grains (carry-over effects), as well as through direct contamination of fungi in mixtures of contaminated spices used in meat production (Dall'Asta *et al.*, 2010; Perši *et al.*, 2014; Pleadin *et al.*, 2013, 2015; Turner *et al.*, 2015). This has been demonstrated with the presence of OTA in hams of different origins (Comi and Iacumin, 2013), which can occur in meat and meat products as a result of direct contamination with molds or indirectly through animals fed with contaminated feedstuffs (Bertuzzi *et al.*, 2013; Rodríguez *et al.*, 2014; Lippolis *et al.*, 2016).

The report on salami showed that OTA transferred by accident through the ingestion of contaminated feeds fed to pigs that are later consumed by humans, or through infection with the growth of moulds in the outer layers of carcasses (Lusky *et al.*, 1995; Iacumin *et al.*, 2011). Besides, mycotoxins are capable to resist breakdown or catabolism in the digestive systems of animals, allowing these compounds to remain in the meat (Iqbal *et al.*, 2014). The Indirect contamination of food and feed occurs when an ingredient such as corn, wheat or barley contamination has earlier occurred in the field by toxigenic fungi (*Fusarium* sp.). This

contamination occurs through insects, parasites and other vectors that can carry fungal spores present in the environment, as well as environmental factors such as storms and humidity that damage food predisposing it to contamination.

The occurrence of mycotoxins can also happen in post-harvest during processing and storage of grains or rations when there are no adequate storage temperature and humidity, facilitating the growth of *Aspergillus* sp. and *Penicillium* sp. In feedstuffs fed to animals, thus causing direct contamination. These animals, when given mycotoxin-contaminated feed, accumulate these toxins in their meat, which will later serve as food, thus carrying mycotoxins to the final consumer (Rocha *et al.*, 2014; Piacentini *et al.*, 2015). Therefore, the ingestion of mycotoxins by humans occurs through ingestion of contaminated vegetable products, as well as through consumption of animal origin products, such as meat (Marin *et al.*, 2013).

2.8 Mechanism of toxicity

2.8.1. Aflatoxins

Aflatoxins (AFs) have received considerable attention among the several mycotoxins, due to their effect on human health. AFs are principally produced through a polyketide pathway by several species and unidentified strains of *Aspergillus* Section *Flavi*, which comprises *Aspergillus flavus*, *Aspergillus parasiticus*, *Aspergillus parvisclerotegenus*, and *Aspergillus minisclerotigenes* (Pleadin *et al.*, 2014), Strain SBG (Cotty & Cardwell, 1999) and less regularly *Aspergillus nomius* (Kurtzman *et al.*, 1987). Normally, *Aspergillus flavus* produces only B type, while the other *Aspergillus* species produce both B- and G type AFs (Creppy, 2002; Zinedine & Mañes, 2009). M-type AFs are not typically found on crops, but are metabolites occurring in milk of mammals whose feeds are contaminated with AF-B₁ and AF-B₂ (Iqbal *et al.*, 2012)(Iqbal *et al.*, 2015). About 0.3-6.2% of AF-B₁ is transformed into hydroxylated metabolite AF-M₁, dependent on a number of factors such as the genotype of the animals, milking practices, periodic fluctuations, and ecological conditions (Unusan, 2006). Remarkably, (Wogan & Paglialunga, 1974) stated that AF-M₁ has only 10% of the carcinogenicity of AF-B₁ in vivo.

2.8.2. Ochratoxin

Ochratoxin (OTs) are mainly produced by the genera of *Aspergillus ochraceus* and *Penicillium verrucosum*. Cereals and cocoa are the major crops that are mostly affected by fungi that

produce ochratoxin (Copetti *et al.*, 2010; Gilmour & Lindblom, 2008), coffee (Paulino De Moraes & Luchese, 2003; Romani *et al.*, 2000) and grapes (García-Cela *et al.*, 2012). Among Ochratoxin group, type A is the most deadly compound, nonetheless, type B and C also occur. Ochratoxin primarily affects the kidney of all animal species and at high concentration can affect the liver, where both serious and protracted exposures cause wounds to form on the organs (García-Cela *et al.*, 2012). Other kidney ailment often linked with tumors, called Balkan Endemic Nephropathy, is considered to be caused by OT- A contact (Pfohl-Leszkowicz *et al.*, 2002). Similarly, OT-A has been reported to cause oxidative harm to DNA, leading to mutagenesis and possible cancer (Zepnik *et al.*, 2001). Therefore, OT-A has been termed or regarded as a potential human carcinogen by IARC (Group 2B), quoting suggestive sign of carcinogenesis in animal models, but inadequate evidence from human studies (IARC, 1994).

2.8.3 Fumonisin

Fusarium verticillioides and *Fusarium proliferatum* (FMs) are the primary producers of *Fumonisin* mycotoxins. However, *Aspergillus niger* has also been reported of capable of its production (Richard, 2007). Maize is the foremost food crop affected by FMs, even though incidence has been reported in sorghum and rice (CAST, 2003; Vismer *et al.*, 2015). Among the reported types, FM-B₁ is the most extremely toxic and reported to stimulate lump development (Abel & Gelderblom, 1998), equine leukoencephalomalacia (Marasas, 1995), porcine pulmonary edema (Harrison *et al.*, 1990), nephrotoxicity, and liver cancer in rats (Soriano *et al.*, 2005). FMs can also interrupt sphingolipid metabolism, a serious process in cell regulation, by acting as secondary messengers for growth factors, differentiation factors, and cytokines (Sherif *et al.*, 2009). Relationship between exposure to FM-B₁ and esophageal cancer can be found in several epidemiologic studies (Rheeder *et al.*, 2002). Hence, Fusaria-derived toxins such as FM-B₁ and FM-B₂ are registered as supposed carcinogens in Group 2B of the IARC classification (IARC, 1994). In recent times, (Shirima *et al.*, 2014), who studied the relationship between child growth and AFs and FMs exposure in Tanzania, reported that FMs exposure single-handedly or in combination with AFs, can be casual factors for child growth impairment.

2.8.4 Zearalenone

Zearalenone (ZEA) is an estrogenic mycotoxin that falls under the group of polyketides (Arora & Lillehoj, 1992). They occur globally in nature and similar to DON, are produced by several

Fusarium species with *F. graminearum* and *F. culmorum* as major producers (Kuiper-Goodman *et al.*, 1987). As the name implies, ZEA is most frequently found in maize, nevertheless it can also be detected in other grain produce such as wheat, barley, sorghum, millet, and rice (García-Cela *et al.*, 2012). It can also be found in beverages made with contaminated crops (Chen *et al.*, 1992). Zea shares a structural resemblance with the human sex hormone 17- β -estradiol, which allows it to bind to oestrogen receptors in target cells, resulting in association with potency complications in both humans and animals (Sherif *et al.*, 2009). While IARC found inadequate evidence of Zea carcinogenicity in animals, it is categorized in Group 3 (IARC, 1994). Some current studies portray that Zea may be connected with the premature onset of puberty and possibly increased the risk of cervical cancer Breitenbach *et al.* (2002) and (Sherif *et al.*, 2009) reported that the reproductive system is largely affected by exposure to Zea due to effects on configuration and role of individual organs and may possibly lead to hyperestrogenism in children. Isomers of ZEA are the trans (α -zearalenol) (D'MELLO & Macdonald, 2003) and cis (β -zearalenol) forms, which are capable of being produced in grains and may cause oestrogenic effects in mammals (Richardson *et al.*, 1985) when ingested.

2.8.5 Deoxynivalenol

Deoxynivalenol (DON) is another important group of closely related mycotoxins of the trichothecenes (Moss, 1992). They are the most-known terpenoid mycotoxins (Steyn, 1995) of which over 80 diverse members are found to be toxic in both animal and man as indicated by Bhatnagar *et al.* (2002). Among the trichothecenes (Arora & Lillehoj, 1992; Bakutis *et al.*, 2005), DON even though not the most severely toxic is one of the most important in the group, because of its high incidence in cereals (Visconti, 1996). This noteworthy incidence has been evidently revealed in data existing in assessments of Pittet (1998) and D'MELLO and Macdonald (2003). Deoxynivalenol is largely classified as type B trichothecenes according to (D'mello *et al.*, 1999) containing a non-macrocyclic ring (Bhatnagar *et al.*, 2002). Similar to other trichothecenes, the biosynthesis pathway of DON includes the transformation of mevalonate molecules to a more stable intermediate, trichodiene which is then further catalyzed (Bhatnagar *et al.*, 2002) to produce DON (Zamir, 1989).

2.8.6 Citrinin

Citrinin was first discovered in 1931 by Hetherington and Raistrick, who isolated the compound from several strains of *P. citrinum* (Hetherington & Raistrick, 1931) and was further confirmed by Xu *et al.* (2006). Citrinin, a polyketide mycotoxin (Arora & Lillehoj, 1992; Xu *et al.*, 2006), similar to OTA, has a common structural similarity and often co-occur in cereals with OTA. It can be produced by a minimum of 26 fungal species (Dirheimer, 1998), mainly *Penicillium* (D'MELLO & Macdonald, 2003) and *Aspergillus* species (Kurata, 1990) but mostly by 14 species of *Penicillium* and 3 species of *Aspergillus*. Citrinin incidence has been reported in naturally contaminated products such as agricultural produces, foods and feedstuffs as well as biological fluids in mammals (CAST, 2003). Records obtained from analytical studies on Citrinin are limited when compared to other mycotoxins probably because of its instability in foodstuffs (Xu *et al.*, 2006). Nonetheless, it has been reported from naturally contaminated wheat, oats and bran (D'mello & Macdonald, 2003)

2.8.7 Patulin

Patulin is also a polyketide (Steyn, 1998) produced by several *Penicillium* (D'Mello, 2003) and *Aspergillus* species (Pittet, 1998) with *P. expansum* (Leggott & Shephard, 2001; Moss, 2002) as the most significant and predominantly a naturally contaminant of apples and other fruits. It has not been recognized as natural contaminants of grains according to (Scott, 1991). It is significant to food production because its incidence is often linked with storage deterioration in apple and other fruits such as banana, pineapple, grapes and tomatoes (Koca & EKŞİ, 2005) notwithstanding at low incidence rate and low concentration levels. The presence of patulin in fruits and fruit products results in severe damage and cause severe economic loss (D'mello & Macdonald, 2003)

2.8.8 Cyclopiazonic acid (CPA)

Cyclopiazonic acid is another important mycotoxin of the polyketide group (Bhatnagar *et al.*, 2002), produced by many *Penicillium* spp. such as *P. aurantiogriseum*, *P. crustosum*, *P. camemberti* as well as *A. tamaris* and *A. versicolor* (Huang *et al.*, 1994). It is mainly found in maize and peanuts (CAST, 2003). Co-occurrence of CPA with other *Penicillium* as well as *Aspergillus* produce mycotoxins such as penicillic acid may exist.

2.9. Implications of mycotoxin contamination in Foods

2.9.1 Economic and social implications

Monetary losses arising from mycotoxicosis in Africa are daunting (Otsuki *et al.*, 2001; Wu, 2004). Whereas developed countries may suffer economic losses because of the trade concerns of mycotoxin-contaminated foodstuffs, developing countries, have a tendency to incur both the economic losses along with additional expenses related to health problems. The economic effect of AFs can be perceived through direct drop in saleable volumes of a crop, cheap value of contaminated products in local markets, regulatory rejection of products by high-value markets, and damages suffered via afflicted livestock, including disease, morbidity, mortality, and contamination of animal products (Zain, 2011).

The economic concerns also take account of the cost of controlling and research programs envisioned to decrease health threats for humans and livestock. Hussein and Brasel (2001), pointed that most studies on financial losses are centered on individual factors of mycotoxin exposure or contamination, which is mostly due to the fact that economic facsimilia to measure global impact have been difficult to establish

2.9.2 Health implications

The toxic effects caused by mycotoxins on humans are supported by extrapolations of information from animal studies (Dutton *et al.*, 2001). It is often difficult to make an accurate assessment of their effects on humans with the exception of acute cases of poisoning. Animals in general are usually defenseless when in contact with mycotoxins, because their immune system does not identify them as antigens Hundley (2001), thus resulting in poisoning. Consumption of a concentrated mycotoxin in food within a short period of time causes acute toxicity and ultimate death, whereas consumption of low quantities over an extended period results in protracted effects (Sibanda *et al.*, 1997). People are exposed to mycotoxins in three ways: individuals that are involved in handling contaminated feeds; those involved in laboratory analyses of mycotoxins, and in particular, consumers of contaminated food and feedstuffs or by-products from animals with mycotoxicosis.

2.10 Methods of mycotoxins detection in foods and feeds

Several analytical methods have been described for monitoring of toxic contaminants in food and feedstuffs based on different measurement such as the use of chromatography, spectroscopy,

electrochemistry, colorimetric detection, and rapid test strips (Chauhan *et al.*, 2015; Wang *et al.*, 2015). Most analytical measurements for mycotoxins comprise common techniques such as sampling, homogenization, and extraction followed by clean-up to detached by products in the suspension which may obstruct sample concentration, and final separation and detection stages. Quantification is typically accomplished either by chromatographic procedures in a combination with a range of detectors, or by immunochemical methods (Pereira *et al.*, 2014).

2.10.1 High-performance liquid chromatographic (HPLC)

Current analysis of mycotoxins depend greatly on HPLC using various adsorbents subject to the physical and chemical structure of the mycotoxin. Standard and reversed-phase columns were used for separation and purification of toxins depending on their polarity. Small mini columns were used for sample pretreatment and large scale preparative columns can also be used for preparation of mycotoxin standards (Reinhard & Zimmerli, 1999).

2.10.2 Thin Layer Chromatography (TLC)

Among chromatographic techniques, thin layer chromatography (TLC) is usually used for screening since it presents many benefits, nevertheless it is restricted in its capacity to measure toxins accurately unless quantitative densitometry is used (Gilbert & Anklam, 2002). However, Gnonlonfin *et al.* (2010).

2.10.3 Liquid / Gas Chromatography and Mass Spectrometry (LC/GC-MS)

Presently, liquid chromatography (LC) along with gas chromatography attached to mass spectrometry (GC-MS) are the most commonly used in quantitative analysis of mycotoxins (Lindenmeier *et al.*, 2004; Pereira *et al.*, 2014). Equally, modern LC-MS/MS multitoxin method has been developed to investigate various mycotoxin contaminations, but this method has compromised method accuracy due to matrix effects (Sulyok *et al.*, 2015; Warth *et al.*, 2012). Generally, the above-mentioned methods need extensive clean-up of samples, sophisticated equipment, trained professionals, and relatively long analysis time in addition to high costs and use of dangerous reagents (Singh *et al.*, 2007; Wang *et al.*, 2015).

2.10.4 Enzyme-Linked Immunosorbent Assay (ELISA)

Concerning immunoassay-based methods, enzyme-linked immunosorbent assay has been widely applied for the determination of mycotoxins. Lee *et al.* (2004) described that ELISA is a suitable tool for quick and precise analysis which allows high sampling rate, yet is also cost effective and

needs only small sample volumes for analysis (Sherry, 1997). Conversely with all ELISA techniques, a positive result needs to be confirmed by HPLC (FAO/WHO, 2001).

2.10.5 Near Infrared Spectroscopy (NIRS)

Another relevant application for screening of fungal contamination and presence of toxins in food and feed is Near Infrared Spectroscopy (Berardo *et al.*, 2005; Peiris *et al.*, 2009). As Pearson *et al.* (2001) reported that scattering and absorbance features are influenced by presence of *Aspergillus flavus* in grain kernels since fungal development causes the endosperm to become powdery which changes the absorbance spectra depending on different level of AFs. Berardo *et al.* (2005) showed that it was possible to quantify fungal infection, and metabolites such as mycotoxins, produced in maize grain by *Fusarium verticillioides* using NIRS. On the other hand, NIRS only produces an average spectrum which lacks in spatial information from the sample with respect to distribution of the chemical composition.

2.10.6 Hyperspectral Imaging (HSI)

Alternatively, Hyperspectral Imaging is a promising method that can be used to investigate both the distribution and composition of mycotoxins in contaminated food samples, especially grains. This method can provide both restricted information and a complete spectrum for certain regions of the electromagnetic spectrum in each pixel (Manley *et al.*, 2009). Recently, (Wang *et al.*, 2015) established the potential of HSI for quantitative identification and distinction of AFs in inoculated maize kernels.

2.10.7 Fluorescence spectrometry (FS)

Fluorescence is one of the properties of several mycotoxins is an important characteristic for their detection. A novel biosensor based on surface Plasmon-enhanced fluorescence spectroscopy has been developed for detecting aflatoxin M₁ in milk with a detection as low as 0.6 µg/mL (Wang *et al.* 2009).

2.10.8 Radioimmunoassay (RIA)

The other immunological methods for mycotoxin detection include radioimmunoassay. Sun and Chu (1977) developed a solid-phase RIA for detecting aflatoxin B₁ in corn and wheat. Commercial kit for the application of RIA has been validated for determining *Ochratoxin A* in both human foods and livestock feeds with a detectable limit of 1 µg/kg (Fukal, 1990). RIA was

also used for detecting nivalenol in barley and the result was reported to be consistent with gas chromatographic analysis (Teshima *et al.*, 1990)

2.10.9 Lateral Flow Immunoassay (LFIA)

Lateral flow or dipstick immunoassay was developed using the principle of ELISA, and it has been successfully used to detect *zearalenone* and *deoxynivalenol* simultaneously in wheat samples (Kolosova *et al.*, 2007). Fumonisin has been detected in maize samples using a quantitative lateral flow immunoassay (Anfossi *et al.*, 2010). Other studies include aflatoxin B₁ detection in pig feed (Delmulle *et al.*, 2005), T2 toxin in wheat and oat (Molinelli *et al.*, 2008). This method is very simple and rapid where the result is ready within 10 min and offers a convenient on-site screening tool.

2.11 Detoxification of mycotoxins

Elimination or detoxification of mycotoxins using physical, chemical or biological methods has been widely studied. Effective degradation of mycotoxins is very challenging since most mycotoxins are heat-stable and form toxic degradation products. Although there are various methods of mycotoxins detoxification, only a few have practical use.

2.11.1 Physical decontamination of mycotoxins

Many food-processing operations (boiling, microwave heating, extrusion, irradiation, roasting, cooking, frying, baking) comprise heat treatment. Since mycotoxins vary in their stability under heat treatments, the degree of destruction of any specific mycotoxin must be determined primarily under the thermal conditions that prevail during the particular process to which it is to be subjected to (Volcani Center, 2004). Also, mycotoxin degradation is affected by some added parameters, such as the moisture content of the product, the level of the toxin, the type of food matrix, and the presence of additives (Scott, 1984). Aflatoxins are relatively resistant to thermal inactivation and are destroyed only at temperatures of around 250 °C. Therefore, processes which include heating should reach that temperature range. Under any given type of heat processing, mycotoxin destruction depends, basically, on the temperature and the time of exposure to it. For instance, roasting of green coffee beans at 200 °C for 12 min caused a reduction of 79 % in aflatoxin content, whereas after 15 min, 94 % of the toxin had disappeared (Levi, 1980). Decrease in aflatoxin in the course of heat processing also depends on the moisture content of the product, at high moisture content, degradation was found more efficient (Mann & Rehm, 1976).

Under dry conditions, *Citrinin* was decomposed at 170 °C, whereas under moist condition it was detoxified at 140 °C (Kitabatake *et al.*, 1991). Heating *ochratoxin A* in sodium hydroxide (NaOH) bring about the detoxification of the toxin (Trivedi *et al.*, 1992). Roasting *fumonisin* B₁ contaminated corn meal at 218 °C for 15 min resulted in almost complete degradation of the toxin (Castelo *et al.*, 1998). The presence of ammonia during extrusion of *aflatoxin* B₁ increased the amount of degradation (Castells *et al.*, 2005). *Aflatoxins* are photosensitive in nature; hence, various radiations such as sunlight, UV light and gamma rays have been employed for degradation studies. Sunlight was efficiently used for degrading *aflatoxin* B₁ in olive oil, groundnut oil (Mahjoub & Bullerman, 1988). *Aflatoxin* B₁ was found to be more susceptible to irradiation when present in liquid medium than in solid media. The cytotoxicity and mutagenicity of *aflatoxin* B₁ has been shown to reduce after treatment with UV in aqueous medium (Liu *et al.*, 2011). On the other hand, it was reported that irradiated fungal inoculum may produce increased levels of mycotoxins especially *aflatoxins* Applegate and Chipley (1974) and *ochratoxin* (Paster *et al.*, 1985).

2.11.2 Chemical decontamination of mycotoxins

Degradation of *aflatoxin* B₁ with chemicals has been efficient, however formation of degradation products has been observed. Acids convert *aflatoxin* B₁ into several products such as *aflatoxins* B₂, B_{2a}, D₁, instead of complete degradation (Schuda, 1980). Lee *et al.* (2015) reported the conversion of *aflatoxin* B₁ into *aflatoxin* B₂ and *aflatoxin* G₁ into *aflatoxin* G₂ by lactic acid. Citric acid causes the hydration of *aflatoxin* B₁ at the 8, 9-olefinic bond of the terminal furan ring to form *aflatoxin* B_{2a} (Ciegler & Peterson, 1968). Usage of hydrochloric acid at high temperatures to degrade *aflatoxin* B₁ totally destroyed the toxin devoid of the formation of toxic deposits (Williams & Dutton, 1988). Other acids like salicylic, sulphamic, sulfosalicylic, anthracitic, benzoic, boric, oxalic and propionic acids were efficiently used for degrading *aflatoxin* B₁ by more than 90% in sorghum (Hasan, 1996). Boiling contaminated corn with *aflatoxin* B₁ in NaOH decreased the level of *aflatoxin* B₁ by 93%, with 18% reversion level after treatment with acid (Camou-Arriola & Price, 1989). Nixtamalization (alkaline cooking of grains) was reported to be an effective technique in degrading *fumonisin* (Aiko & Mehta, 2015). Sodium bisulphate and hydrogen peroxide were also used in degrading *aflatoxin* B₁ efficiently (ALTUĞ *et al.*, 1990). Among the many chemicals used for detoxification of mycotoxins, ammonia is the most efficient and it has been accepted for use by the corn production industry.

Ammonia degrades aflatoxin B₁ into aflatoxin D₁ which has reduced toxicity and mutagenic potential (Lee & Cucullu, 1978). Ozone has also been used to degrade aflatoxin B₁ by more than 90% in animal feed (Jr & King, 2002). Maeba *et al.* (1988) showed that ozone-treated aflatoxins were not toxic and mutagenic. Aflatoxin B₂ and G₂, fumonisin, ochratoxin, patulin and zearalenone were also efficiently degraded by ozone.

2.11.3 Biological decontamination of mycotoxins

2.11.3.1 Microorganism

Some strains of lactic acid bacteria have been reported to remove *aflatoxins* B₁ and M₁ by binding non-covalently (Lahtinen & *et al.*, 2004). Heat-treated and acid-treated *Lactobacillus rhomus* GG and *L. rhomus* were able to remove zearalenone, indicating that binding and not metabolism is the mechanism by which the toxins are removed (El-Nezami *et al.*, 2002). Line *et al.* (1994) studied the mechanism and suggested that the degradation of *aflatoxin* B₁ by *F. aurantiacum* is probably a mineralization phenomenon. A number of fungal species, especially *Phoma* *sp.* were reported to prevent the synthesis of *aflatoxin* B₁ and to also degrade the toxin (Shantha, 1999).

Degradation of mycotoxins occurs during fermentation of various foods such as milk Wu *et al.* (2009), dough fermentation in making bread [El-Banna and Scott (1983)] or during beer brewing (Chu *et al.*, 1975). Some toxigenic fungi (*Aspergillus parasiticus*, *A. flavus*) have been reported to degrade their own toxins (Doyle & Marth, 1978; Hamid & Smith, 1987). Degradation of aflatoxin B₁ depends on the type of substrate and the fungal strains used.

2.11.3.2 Enzymes

Several fungal enzymes have been reported to degrade aflatoxin B₁. *Armellaria tabescens* produced a multi-enzyme system which detoxified *aflatoxin* B₁ by opening the difuran ring (Liu *et al.* 1998). The enzyme peroxidase from *A. flavus* and *A. parasiticus* has been shown to degrade aflatoxins B₁ and G₁ (Doyle & Marth, 1979; Singh, 1997). A horse radish peroxidase enzyme from the plant *Raphanus sativa* has also been reported to degrade *aflatoxin* B₁ (Das & Mishra, 2000).

2.11.3.3 Plant extracts

The use of substance obtained from a plant as anti-fungal and anti-mycotoxin agent is considered to be both safe to humans and environmental friendly. Several extracts from plants such as piperine from black and long peppers Singh *et al.* (1994), lutein and xanthophyll from Aztec marigold de Mejia *et al.* (1997) and carotenoids from fruits and vegetables Rauscher *et al.* (1998) were reported to possess properties that subdue the toxicity and mutagenicity of aflatoxin B₁. The important oils of some plants have been recognized to contain strong antimicrobial property (Bansod & Rai, 2008; Lv *et al.*, 2010). The powder and essential oil of *Cymbopogon citratus* have been successfully used for preventing aflatoxin B₁ contamination and maintaining the quality of melon seed under storage (Bankole & Joda, 2004). The essential oils of *Cinnamomum jensenianum* Tian *et al.* (2012), *Ocimum sanctum* Kumar *et al.* (2010) and *Zataria multiflora* Gandomi *et al.* (2009) were used effectively against toxigenic fungi and aflatoxin B₁ and their harmless use as natural preservative of food. Among the number of plants, *Syzygium aromaticum* (clove) has been extensively studied for its anti-microbial property (Aiko & Mehta, 2015).

The clove oil and its main component, eugenol, has been reported to inhibit *Aspergillus* growth and aflatoxin B₁ production by various investigators (Bullerman *et al.*, 1977; Jayashree & Subramanyam, 1999). Total content of the clove has also been shown to hinder the growth of *A. flavus* and *P. citrinum* and their toxins in culture media and rice grains (Aiko & Mehta, 2015). Removal of toxigenic fungi and mycotoxins by botanicals are usually preferred over chemical treatments.

2.12 Legislation and regulations of mycotoxins in foods and feeds

Legislation and regulations are constantly changing, since countries progressively know that addressing mycotoxin contamination in foods and feeds will not only lessen healthcare costs, but also provide benefits with respect to global trade as well as access to high-value markets. Regulations in different countries generally depend on the final use, with the harshest limits defined for human consumption and trade products and the lowest for industrial uses. Indeed, “safe” limits of aflatoxins for human consumption range 4-20 mg/kg. The EU has set the strictest criteria, such that any goods for direct human consumption can only be sold with concentrations of AF-B₁ and total AFs not greater than 2 mg/kg and 4 mg/kg, separately (EC, 2007). Equally, US regulations have indicated the maximum tolerable limit for total AFs at 20 mg/kg (Van Egmond & Jonker, 2016; Wu, 2006). In India, a tolerance limit of 30 mg/kg for AFs in all foods

has been defined. Kenya approved a maximum permissible level of 10 mg/kg of AF-B₁ in groundnuts and several grain foods. Brazil has fixed the limit of total AFs in nuts at 30 mg/kg (Freitas-Silva & Venâncio, 2011). On the other hand the EC also defined the limit for OT-A at 3 mg/kg for all goods making from cereals, while level of 1000 mg/kg for FMs and 200 mg/kg for ZN are set for maize planned for direct human consumption (EC, 2007)

Likewise, the international regulations for the maximum limit of AF-M₁ in milk and dairy products range 0.05-1.0 mg/kg. The EC limits AF-M₁ levels at 0.05 mg/kg for milk and milk products. In this regulation, the EC states that “even if AF-M₁ is viewed as a less risky genotoxic carcinogenic substance than AF-B₁, it is essential to stop its presence in milk, and therefore in dairy products, intended for human consumption and for children in particular”. The EC has also set a limit for AF-B₁ at 5 mg/kg in lactating dairy feeds. According to the USFDA, AF-M₁ in milk should not exceed 0.5 mg/kg (Muhammad Wasim, 2015). Similarly, in Australia and Switzerland, maximum levels of 0.05 mg/ kg for milk, 0.25 mg/kg for cheese, and 0.02 mg/kg for butter are set. In Nigeria, 1.0 mg/kg is fixed as the regulatory limited for milk Iqbal *et al.* (2015) even as South Africa at the moment allows up to 0.05 mg/kg of AF-M₁ in milk and milk products (Mulunda & Mike, 2014). In lieu of animal-based products traded across the EU, the minimum level of OT-A contaminations is established to be 0.052 mg/kg, which is applied mainly to pork-based products (JØrgensen, 2005). Based on the USFDA standards, allowable limits of AFs in maize feed for raising and final processes of cattle and pigs range 100-300 mg/kg, limits for other feedstuffs are established to be 20 mg/kg

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CHAPTER THREE

CHARACTERIZATION OF ISOLATED FUNGI FROM DRIED BEEF

ABSTRACT

The aim of the present study was to examine the fungi diversity in the dried meat, characterize the isolated fungi of the beef meat sold in Ekiti State, and determine the aflatoxin producing ability of the aspergillus *fungi* in the samples. Samples of processed dried beef was collected from different markets in Ekiti State, Nigeria and screened for filamentous fungi contamination using conventional and molecular methods. Samples were cultured aseptically on potato dextrose agar (PDA) and Malt Extract Agar (MEA) prior to fungi isolation. Conventional methods comprising of macroscopic and microscopic evaluation of isolated fungi species were used in the identification of fungi species. Molecular identification involved DNA extraction, Polymerase chain Reaction (PCR) using ITS-1/ITS-4 primer pair and nucleotide sequencing. The results obtained indicated a range of filamentous fungi genus including *Aspergillus*, *Penicillium*, *Fusarium*, *Alternaria*, *Cladosporium* and *Rhizopus* contaminating the dried meat with *Aspergillus* (43%), *Rhizopus* (42%), *Penicillium* (3%), *Fusarium* (2%) and other fungi (7%) species dominating most of the samples. High incidences were recorded for *Aspergillus flavus*, *Aspergillus niger* and *Aspergillus fumigatus* in most of the samples screened. *Aspergillus flavus* and *A.oryzae* isolated in this study which formed (30.9%) of all the *Aspergillus* species isolated expressed the aflatoxin gene. The other three essential genes (*omt-A*, *aflD* and *aflM*) found in the aflatoxin biosynthetic pathway were established in the remaining *Aspergillus* isolates. The occurrences of these filamentous fungal species pose a reason for concern as most of these fungal species are known producers of toxic substances. The Maximum likelihood phylogenetic analysis showed a high level of similarity index which indicates a good relationship between

isolated *Aspergillus*, *Penicillium*, *Fusarium* and other fungi species and the closely related strains from the GenBank that were isolated from different sources. The implication of the finding in this study suggest a high risk for human health because of the possibility of indirect exposure through consumption of contaminated dried meat and other animal products.

Keywords: *Aspergillus*, *Fusarium*, *Penicillium*, filamentous fungi, dried meat, phylogeny

3.1 Introduction

Fungi are a subdivision of the subkingdom Thallophyta. They have a well - defined nucleus but lack the chlorophyll which is a characteristic of most other plants (Mayor and Harold, 1966). They also lack vascular tissue but made up of an assimilative body which may be amoeboid or unicellular in some species, but typically are made up of multicellular branching filaments called hyphae reproducing asexually by the means of spores (Talbot, 1971). They are heterotrophs feeding on substrates of plants and animals. Many fungi are pathogens of plants grown for food, while a smaller number are agents of diseases in animals, including man. They attack and destroy raw and manufactured products resulting in great economic loss. On the other hand, they are useful to man: food yeast (a source of proteins, vitamins, and amino acids), the yeast used for baking and a minor source of food including mushrooms (Talbot, 1971). Fungi do not only cause direct losses but can threaten the health of both man and animals by producing poisons, so - called mycotoxins, which contaminate food and feed.

Fungi *Aspergillus*, *Penicillium*, *Fusarium*, *Alternaria*, *Cladosporium* and among others are common micro-organisms in the environment found in the soil, air and contaminated food (Egbuta *et al.*, 2014; Gołofit-Szymczak & Górny, 2010). These microorganisms spread easily through wind, bugs, dust and rain with the potential to survive within the environment as well as growing on any substrate that favours their growth conditions (Klein & Paschke, 2004). Fungi naturally produce a vast variety of metabolites, referred to as mycotoxins which have been investigated to have adverse health effects on both humans and animals when exposed to them (Muri *et al.*, 2009; Wagacha & Muthomi, 2008). The production of mycotoxins is exasperated by certain environmental conditions that can also influence the extent of contamination which may be distinct with geographic location, agricultural methods and the susceptibility of food products to the penetration of fungi during storage and processing periods (Jonathan & Esho, 2010).

Aflatoxins are the top toxic and carcinogenic compounds among the recognized mycotoxins and, as a result, have been widely studied. These toxins are produced by a number of diverse *Aspergillus* species (Klich *et al.*, 2000). On the other hand, in agricultural produces, they are predominantly produced by *Aspergillus flavus* and *Aspergillus parasiticus* (Yu *et al.*, 2002). There are four major naturally occurring aflatoxin B₁, B₂, G₁ and G₂, with *A. flavus*, *A. pseudotamarii*, and *A. ochraceoroseus* producing only the B aflatoxins, and *A. nomius*, *Aspergillus bombycis*, *A. parasiticus* produces both B and G toxins (Lopez *et al.*, 2002; Yu *et al.*, 2002).

Most developing countries, including Nigeria struggle to meet up with the production and rearing of animal's meat consumption demand, in addition to the preservative technology that is grossly insufficient, hence it results in high level of meat loss or wastage (Eke *et al.*, 2012). Therefore, there is a need to process the available meat into a more stable preservation form. Many states in the Northern part of Nigeria are involved in production of processed beef as a major source of livelihood, they are usually processed using a range of traditional techniques involving salting, drying, cooking, smoking and marinating or a combination of these operations (Collignan *et al.*, 2001). These processes are often long, poorly controlled and stimulate fungi contamination during processing, transportation, storage and displayed methods at the market. This is particularly notable in the product between the time at the processing site of the exporting States and its arrival at the distribution centre of the consuming States (Adeyeye, 2016). For these reasons, there is considerable opportunity for fungi and mycotoxin contamination to take place and this may lead to economic losses.

The exact figures for world economic losses resulting from filamentous fungi contamination may never be available. Apart from the obvious losses of food, there are losses caused by lower productivity; losses of valuable foreign exchange earnings; costs incurred by inspection, sampling and analysis before and after shipments; losses attributable to compensation paid in case of claims etc. When combined, these costs may be very large or shocking (Coulibaly, 1989). The current work aimed to identify fungi associated with dried beef meats sold in Ekiti State, Nigeria and to compare the isolated fungi using maximum likelihood phylogenetic tree based on partial ITS rDNA gene sequence to show the phylogenetic relationships between identified fungi and the most closely related strains from the GenBank.

3.2 Materials and Methods

All chemicals and reagents used in this study were at least of analytical grade, unless otherwise specified.

3.2.1 Study area and sampling locations

The present study was carried out during 2017 in ten major local government area of Ekiti State of Nigeria and these areas are as follow: Ado Ekiti and Igede town representing central area, Ikole and Omuo town represents the eastern area, Aramoko and Ijero represents western area, Oye and Otun represents northern area, Ilawe and Emure represent the southern area.



Figure 3. 1 Sampling locations of dried beef meats in Ekiti State

3.2.1 Sampling and sample preparation

One hundred and eight (108) samples of dry beef each weighing about 200g to 300g were obtained in triplicates from different markets in Ekiti State. The sampling design was as a result of the availability of dry meat at the different markets. Representative samples were collected in plastic bags and sealed for transportation to the Food, Toxicology Research laboratory, North-West University, Mafikeng Campus, South Africa following all necessary custom duty protocols. All samples collected were meant for human consumption. On arrival at the laboratory, meat samples were ground using a laboratory mill (IKA M20, Merck, Darmstadt, Germany) and stored at -20°C for further analysis.

3.2.2 Mycological screening of samples

To determine fungal population in samples, the method described by Njobeh *et al.* (2009) with slight modifications was used, potato dextrose agar (PDA) and Malt Extract Agar (MEA) were used. Potato dextrose agar and Malt Extract Agar were prepared by dissolving PDA (39 g) and MEA (39 g) in 1 litre of distilled water respectively. Both agar were autoclaved at 121 °C and 15 psi for 15 mins, cooled in a 50 °C water bath and eight (8) millilitre (ml) each of 1% chloramphenicol/ 1% streptomycin antibiotic solution added to agar before pouring into sterile petri dishes.

3.2.3 Fungal colony counts

One (1) gram of each sample in triplicate was suspended in 9 ml of sterile Ringer's solution and vortexed for 2 min and serially diluted to make six dilutions (10^{-6}) per sample. Ringers solution was prepared by dissolving two ringers tablets in 1 litre of distilled water and autoclaved at 121 °C and 15psi for 15mins. A 0.1 ml portion of the suspension was spread plated on PDA in

triplicates and incubated at 28 °C for 48 h and subsequently, the colonies in each plate were counted and the average mean value recorded as the fungal load for the 3 replicate in each sample and the Colony Forming Unit (CFU/g) calculated for each sample

$$\text{Cfu/g} = \frac{\text{Number of colonies} \times \text{reciprocal of the dilution factor}}{\text{Plating volume (0.1 ml)}}$$

3.2.4 Fungal Isolation from Sample Cultures

One gram of each sample was suspended in 9 ml of sterile Ringer's solution and vortexed for 2 min. A 0.1 ml aliquot of the suspension was spread plated in triplicates on Potato Dextrose Agar (PDA) and Malt Extract Agar (MEA) and incubated in the dark at 25 °C for 7 days. The individual fungal colony was carefully transferred into sterile solid MEA plates for final purification at 25 °C for 5 days before the fungal DNA was extracted. Identification of the isolated fungal was done using a molecular method by PCR analysis and subsequently sequenced. The isolation occurrence of each species was calculated using the method described by Adetuji *et al.* (2018)

$$\text{Frequency (\%)} = \frac{\text{Number of samples contaminated with a specie or genus}}{\text{Total number of samples}} \times 100$$

$$\text{Relative Density (\%)} = \frac{\text{Number of isolates of species or genus}}{\text{Total number of isolates}} \times 100$$

3.2.5 Molecular characterization of fungal isolates

3.2.5.1 DNA Extraction

Deoxyribonucleotide (DNA) extraction was undertaken using a Fungal/Bacterial DNA extraction kit (Zymo Research Corporation, Southern California, USA). Fungal spores from 4-5-days old cultures were taken into the Bashing Bead™ lysis tubes and 750 µl of DNA lysis solution was added to the tubes. This was followed by lysing of the fungal cells using a disruptor genie bead beater fitted with a 2 ml tube holder assembly (Scientific Industries Inc., USA) and processed at maximum speed for 15 mins and centrifuged at 10,000 rpm for 1 minute. The supernatant was

transferred to a zymo-spin IV spin filter in a collection tube and centrifuged at 10,000 rpm for 1 minute. The filtrate was added to 1200 µl fungal DNA binding buffer and centrifuged in a Zymo-spin IIc column placed in a collection tube at 10,000 × g for 1 minute. This was followed by washing of DNA in the column using a pre-wash buffer 200 µl and wash buffer 500 µl. Finally, after washing, 100 µl of the DNA elution buffer was added to the column and centrifuged at 10,000× g for 30 mins to elute the DNA. While the eluted DNA was stored at -70 °C for further analysis.

3.2.5.2 Agarose Gel DNA Electrophoresis

Deoxyribonucleotide acid (DNA) gel electrophoresis was carried out by preparation of 1% agarose gel 2 g of agarose (Fermentas Life Science, Lithuania) in 98 ml 1x TAE buffer (Fermentas Life Science, Lithuania) which was boiled in a microwave and cooled to approximately 60 °C. This was followed by the addition of 1 ml of ethidium bromide (Sigma-Aldrich, ST Louis, MO, USA) to the agarose solution. The agarose solution was poured into a casting chamber (Bio-Rad Laboratories, California, USA) and allowed to set. Casting chamber containing the solid agarose was further inserted into the electrophoresis tank (Bio-Rad Laboratories, California, USA) filled with 1× TAE buffer. Fungal DNA 5 µl was loaded into the wells. The chamber was closed and run at 400 V and 80 mA for 50 minutes and DNA viewed using the ChemDoc gel imaging system (Bio-Rad Laboratories, California, USA).

3.2.5.3 PCR amplification of extracted genomic DNA

The amplification of the Internal Transcribed Spacer Region (ITS rDNA) of the fungal isolates from the dry meat was done using Polymerase Chain Reaction (PCR) with the universal ITS 1 (TCCGTAGGTGAACCTGCGG) and ITS 4 region primers (TCCTCCGCTTATTGATATGC). These primers were obtained from Inqaba biotechnical Industrial (Pty) Ltd (Pretoria, South Africa). The PCR amplicons were analyzed through electrophoresis on 1% (w/v) agarose gel to check the anticipated size of the amplicons (670 bp) and view by using Chemi Doc Image Analyzer (Sambrook *et al.*, 2001). The final PCR solution consists of 12 µl of master mix, 1 µl each for forward and reverse primer, 3 µl of DNA and constituted to a final volume of 25 µl with nuclease-free water. Polymerase chain reaction was performed using an Eppendorf 96-well

Thermocycler (Eppendorf, USA) and cycling conditions set as Pre-dwelling at 94 °C for 1 min, 35 cycles denaturation at 94 °C for 1 min, annealing at 54 °C for 1 secs, extension at 72 °C for 1 min 30 seconds, post-dwelling at 72 °C for 9 minutes and held at 4 °C until samples were retrieved.

3.2.5.4 Sequencing of PCR Products

The purified PCR products were sequenced at Inqaba Biotechnical Industrial (Pty) Ltd, Pretoria, South Africa using PRISM™ Ready Reaction Dye Terminator Cycle Sequencing Kit with the dideoxy chain cessation method and electrophoresed with a model ABI PRISM® 3500XL DNA Sequencer (Applied Biosystems, Foster City, CA, USA) by means of following the manufacturer's instructions.

3.2.5.5 Sequence Analysis

The Finch TV software version 1.4.0 was employed for the analysis of Chromatograms, (sense and antisense) ensuing from sequencing reaction for superior sequence assertion. The subsequent chromatographs were edited using Bio Edit Sequence Alignment Editor according to Hall (2004) after which, the resultant consensus ITS rDNA sequences gotten were imported into the NCBI database (<http://www.ncbi.nlm.nih.gov>) for blasting using the Basic Local Alignment Search Tool (BLAST) for homology in identification of the likely organisms (Altschul & Koonin, 1998). The sequences were subsequently deposited in the GenBank for allocation of accession number.

3.2.6 Detection of aflatoxin producing genes of *Aspergilla*

The DNA of assumed *Aspergilla* were observed for the possibility of five important aflatoxin producing genes (*aflR*, *aflJ*, *aflM*, *aflD* and *omt-A*) that are usually present in the aflatoxin biosynthesis pathway by using previously used and reported set of primers (Rashid *et al.*, 2008). The selected genes, primer sequences and their product sizes used were those described by Rashid *et al.* (2008) as shown in (Table 4.2). The primers were provided by Inqaba Biotechnical Industrial (Pty) Ltd, Pretoria, South Africa and the PCR were done using PCR Thermal Cycler (Applied Biosystems).

3.2.6.1 Optimization of polymerase chain reaction

Polymerase Chain Reaction conditions were optimized separately for the target genes. A reaction volume of 25 µl, containing: 8.5 µl nuclease-free water, 12.5 µl PCR Master Mix, 1 µl of oligonucleotide forward and reverse primers (10 µM) and 2 µL template DNA mixed in the PCR

tubes (Montso & Ateba, 2014) were used. The thermal cycle conditions are also shown in Table 1 with varying annealing temperatures ranging from 58 to 75 °C for the five genes respectively. The PCR amplified products were checked on 1% gel by electrophoresis and visualized under Gel documentation system for electrophoretic bands at the various base pair regions for each gene.

3.2.7 Phylogenetic analysis.

Evolutionary study was carried out using Molecular Evolutionary Genetics Analysis (MEGA) 5 (Tamura *et al.*, 2011), while the Neighbor-Joining method was used for the evolutionary history according to Saitou and Nei (1987). The evolutionary distances were calculated using the parameter method of Kimura (1980), using the transversional substitutions per site units of the number.

3.2.8 Statistical analyses

Statistical Analyses were performed using Microsoft Excel 2013 for Window 2010. Descriptive statistics was employed to present the data collected from individual species isolated from each dried meat sampled was considered as one isolate.



Figure 3. 2 Different displaying methods of dried meats across markets sampled in Ekiti State

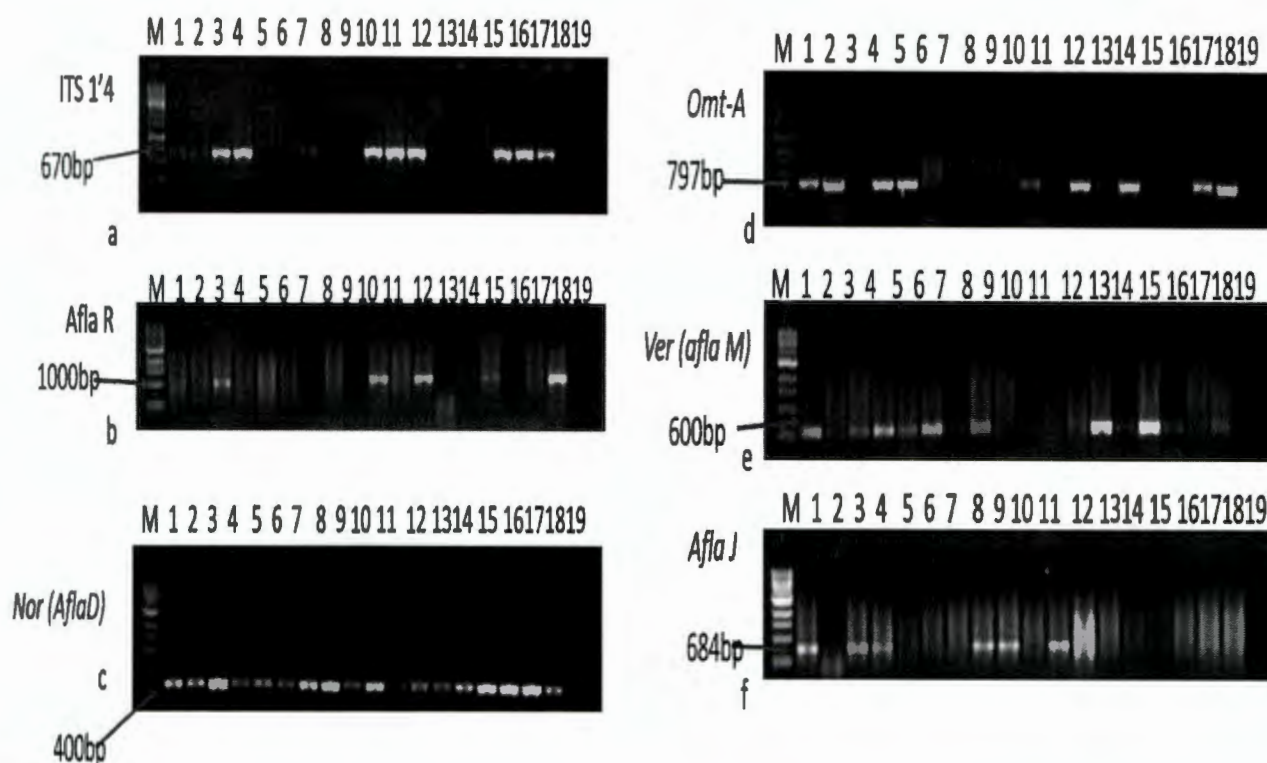


Figure 3. 3 Gel Electrophoretic gel for PCR products showing fungi gene at different bp regions.

M: 1 kb DNA ladder; Lane 1, 3,4,7,10,11,12,16,17,18 and lane 19 as the negative control. b Gel electrophoretic pattern for PCR products expressing *aflR* Gene at 1000 bp region. M: 1 kb DNA ladder; Lane 1, 3, 10, 12, 15, 18: positive isolates, lane 19: negative control, lane 2, 4-9, 11, 13, 14, 16, 17: negative isolates. c Gel electrophoretic arrangement for PCR products expressing *aflD* Gene (Nor-1) at 400 bp region. M: 1 kb DNA ladder; Lane 19: negative control, Lane 1–18: amplification of *nor* gene in positive isolates. d Gel electrophoretic pattern for PCR products expressing *omt-A* gene at 797 bp. M: 1 kb DNA ladder; Lane 1,2,4,5,10,12,14,17,18: positive isolates, Lane 19: negative control, Lane 3, 6–9, 11,13,–15,16: amplification of *omt-A* gene in positive isolates. e Gel electrophoretic pattern for PCR products expressing *aflM* gene (Ver) at 600 bp. M: 1 kb DNA ladder; Lane 4, 5, 6, 8,13, 15: amplification not at the anticipated band size, Lane 19: negative control, Lane 1,3,16,18: positive isolates. f Gel electrophoretic pattern for PCR products expressing *aflJ* gene at 684 bp region. M: 1 kb DNA ladder; Lane 1, 3, 4, 8, 9, 11, 17: positive isolates, Lane 19: negative control. Lane 3, 18, 24: negative isolates

Table 3. 1 Primer name, Target gene Sequence 5'–3'and their product size

Primer pair	Gene	Primer sequence 5'– 3'	PCR product size bp
Nor F	<i>Nor (aflD)</i>	ACCGCTACGCCGGCACTCTCGGCAC	400
Nor R		GTTGGCCGCCAGCTTCGACACTCCG	
Ver F	<i>Ver (aflM)</i>	GCCGCAGGCCGCGGAGAAAGTGGT	600
Ver R		GGGGATATACTCCCGCGACACAGCC	
Omt F	<i>Omt-A</i>	GTGGACGGACCTAGTCCGACATCAC	797
Omt R		GTCGGCGCCACGCACTGGGTTGGGG	
AflR F	<i>AflR</i>	TATCTCCCCCGGGCATCTCCCGG	1000
AflR R		CCGTCAGACAGCCACTGGACACGG	
AflJ F	<i>AflJ</i>	TGAATCCGTACCCTTTGAGG	684
AflJ R		GGAATGGGATGGAGATGAGA	

Reference: (Abd-Elghany & Sallam, 2015; Fakruddin *et al.*, 2015; Gallo *et al.*, 2012; Rashid *et al.*, 2008)

3.3 Results and Discussion

3.3.1 The fungal counts and distribution of fungal isolates in dry meat

A total of 118 fungal isolates were isolated through the ITS section amplification and sequencing of the PCR amplicons of the isolates (Table 3.2). One hundred percent (100%) of the dry meat analyzed were contaminated with fungi of different kinds with fungal counts ranging from 1.0 to $9.5 \log_{10}$ cfu/g with a mean of $3.1 \log_{10}$ cfu/g (Table 3.2). The dried meat sample from Ikole has the highest colony forming unit range of $2.0 \times 10^2 - 1.6 \times 10^4$ with an average mean value of 2.6×10^3 though with 10 different types of fungi species contaminating them. The least colony forming unit range was recorded in Ilawe meat samples with a range value of $1.0 \times 10^1 - 4.5 \times 10^2$ with an average mean value of 1.3×10^2 and a total number of 11 fungi species contaminating them. The low fungal counts of the dried meat might be due to low moisture content which reduced the growth of fungi Ferreira *et al.* (2006) in the dry meat. A similar report of fungal count ($1.8 \times 10^3 - 4.6 \times 10^3$ cfu/g) was reported for sun-dried meat samples (Kundi) obtained from major markets in Ibadan, Oyo State, in Nigeria (Adeyeye, 2016) and also from groundnuts and roasted cashew nuts meant for human consumption from selected locations in Lagos, Nigeria (Adetunji *et al.*, 2018). Likewise, higher fungal counts of $1.8-163 \times 10^2$ cfu/g and $1.0-52.0 \times 10^5$ cfu/g has been previously reported for cashew in Nigeria (Adebajo & Diyaolu, 2003; Adeniyi & Adediji, 2015).

The isolated fungi were classified into fifteen genera (Figure 3.4). *Alternaria*, *Arthrimum*, *Aspergillus*, *Byssochlamys*, *Chaetomium*, *Cladosporium*, *Cochliobolus*, *Epicoccum*, *Fusarium*, *Paecilomyces*, *Penicillium*, *Periconia*, *Phoma*, *Rhizopus* and *Talaromyces* *Aspergillus*, *Penicillium* and *Fusarium*. The *Aspergillus* had the highest frequency of isolation (43%), spp. followed by the *Rhizopus* spp. (42%), *Penicillium* (3%), *Cladosporium* (3%) and *Fusarium* (2%) while the percentage summary of the remaining genera was (7%). The isolation of these fungal genera from dry meat samples aligned with the results of some scholars who have isolated similar fungi from smoked-dried frog, 'gari Ijebu' Banda meats and some human food commodities in Nigeria. (Adebayo-Tayo *et al.*, 2015; Jonathan *et al.*, 2013; Makun *et al.*, 2010; Oladejo & Adebayo-Tayo, 2011). However, the relative density showed that *Aspergillus* genera had the highest relative density (57.6%) of fungal isolates contaminating the dry meat with *A. niger* having the highest percentage of (13.2%), followed by *A. flavus* with (11.8%) *A. aff. Ustus* and *A. fumigatus* are the third highest percentage with (10.3 %) each, while *A. aeneus*, *A. alabamensis*, *A. awamori*, *A. chevalieri* *A. melleus* *A.*

ruber *A. tubingensis* and *A. unguis* had the least densities (1.5%) respectively considering the entire number of *Aspergillus* isolates (Table 3.2).

The meat samples from Ikole were contaminated mainly by fungal types belonging to the genera *Aspergillus* (60%), *Rhizopus* (26.7%), *Penicillium* (10%) and *Talaromyces* having (3.3%). Ilawe samples, *Aspergillus* (56.3%) was the predominant fungi of contamination, followed by *Penicillium*, *Periconia*, *Rhizopus* recording (12.5%) each, while the least was *Chaetomium* with (6.3%). The Aramoko samples had seven genera ranging from *Aspergillus* (56.1%), *Rhizopus* (24.4%), *Penicillium* (9.8%), while *Alternaria*, *Arthrinium*, *Cochliobolus*, and *Phoma* had (2.4%) respectively. In Ijero, *Aspergillus* contamination was (59.4%), *Rhizopus* (25%), *Fusarium* and *Arthrinium* had (6.3%) each, while *Phoma* was (3.1%). The most contaminating fungi in Oye is *Rhizopus* (92.6%), followed by *Alternaria* (3.7%), *Aspergillus* (2.8%), and *Cladosporium* with (0.9%).

The mycological analysis result from Otun meat samples revealed the predominant fungal genera as *Aspergillus* (61.3%), *Cladosporium* and *Rhizopus* having (12.9%) each, *Penicillium* (6.5%) while *Arthrinium* and *Phoma* recorded (3.2%) respectively. Also, Ado had *Aspergillus* (67.2%) as the major fungi contaminant, while *Rhizopus* (19%), *Fusarium* (5.2%) while *Alternaria*, *Arthrinium*, *Cladosporium*, *Penicillium* and *Phoma* recorded (1.7%) respectively. In Omuo, (47.5%) of the meat samples were contaminated with *Aspergillus* species, *Rhizopus* (25%), *Cladosporium* (10%), *Fusarium* and *Chaetomium* recorded (5%) contamination each while *Byssochlamys*, *Epicoccum* and *Talaromyces* have (2.5%) contamination respectively. Igede samples had *Aspergillus*, (58.3%), *Rhizopus* (25%) with *Alternaria* and *Cladosporium* having (8.3%) respectively. Finally, in Ise/Emure, *Aspergillus* was (50%), *Rhizopus* (25%), *Cladosporium*, *Paecilomyces* and *Penicillium* have (8.3%) respectively.

The biodiversity of fungi species according to the different locations sampled revealed that the dried meats had 36 different fungi species belonging to 15 genera contaminating them. The dried meat from Ado Ekiti has the highest fungi contamination with (19 species), followed by Omuo and Aramoko (16 species), Ijero (13 species), Ilawe and Otun (11 species), Ikole (10 species), Ise/Emure (9 species) while Igede and Oye recorded the lowest fungi species contamination (7 and 6 species) respectively as shown in (Figure 3.3). However, the dried meat fungi species contaminating them differ from each location. For example, *Aspergillus flavus* was not found in (Oye and Ise/Emure), *Aspergillus oryzae* was not found

in (Aramoko, Oye, Otun, Omuo and Igede), *Alternaria tenuissima* was found in (Aramoko, Oye, Ado and Igede), *Aspergillus aeneus*, *Cochliobolus sp* (Aramoko), *Aspergillus alabamensis* and *Fusarium equiseti* was found in Ijero, *Aspergillus awamori*, *Aspergillus chevalieri* and *Aspergillus tubingensis* (Ado). Furthermore, *Aspergillus unguis* was found only in (Oye), while *Aspergillus bombycis* (Ilawe, Aramoko and Omuo), *Aspergillus intermedius* (Ijero, Ado and Omuo), while *Fusarium incarnatum*, *Aspergillus melleus*, *Byssochlamys spectabilis* and *Epicoccum sorghinum* (Omuo). Also, *Aspergillus rube* (Igede), *Chaetomium erectum* (Ilawe and Omuo), *Cladosporium cladosporioides* (Oye, Otun, Ado, Omuo, Igede and Ise/Emure), while *Paecilomyces formosus* (Ise/Emure), *Penicillium citrinum*, (Ikole, Ilawe and Aramoko), *Penicillium crustosum* (Otun, Ado and Ise/Emure), *Penicillium mallochii* (Otun), *Periconia macrospinosa* (Ilawe), *Phoma herbarum* (Aramoko, Ijero, Oye and Ado), while *Rhizopus* was found in all the locations, as well as *Aspergillus niger* except in Oye.

This result shows that fungi species isolated from the different locations are peculiar to the sampling environment and have been previously reported from other food samples which implies that their occurrence in dried meat samples could be due to cross contamination as many of the dried meats are seen displayed close to other agricultural commodities. Furthermore, the fungi biodiversity could also be due to storage, selling environment as well as possible interactions with the buyers. Customers are known to usually touch the dried meats when buying them, this is done in order to select the ones that are deemed fit or of good quality. Another likely means of cross-contamination could be from the seller who frequently touch the dried meat either when he or she is helping the buyer to select some good meat, displaying for sale, packing after the end of daily sales, or when given change to the buyers. In addition, vehicular and human movements can also contribute to the meat contamination as they move from one end to the other, this movement sometimes raised dust which eventually perched or contaminate the dried meat as they are not covered or protected from environment where they are sold (Figure 3.1)

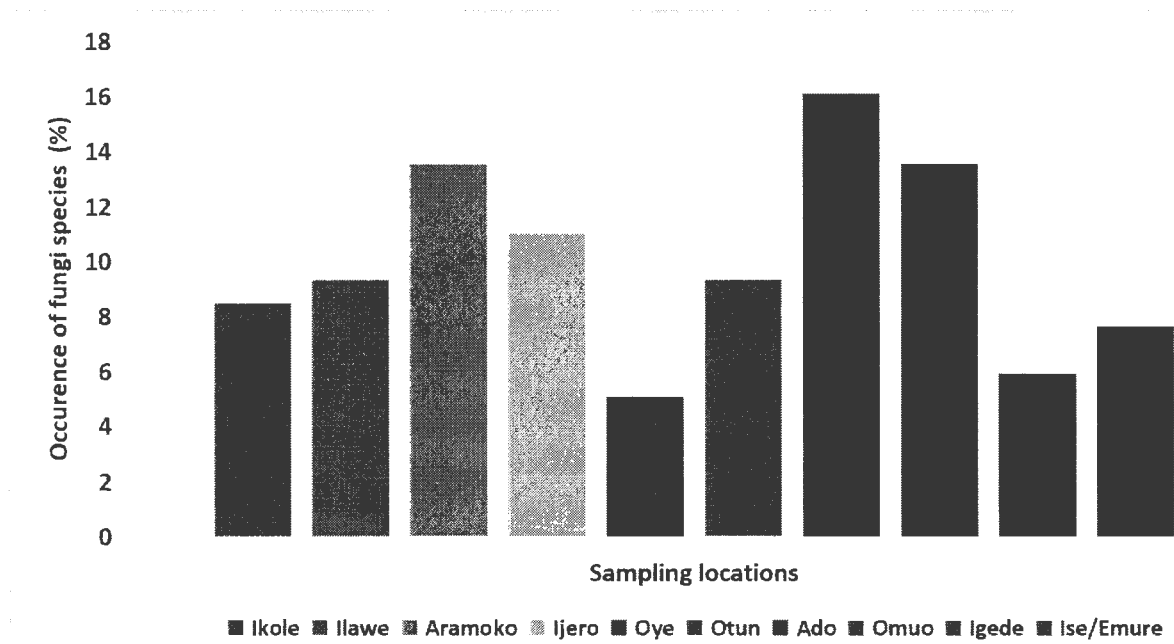


Figure 3. 4 The occurrence of fungi species in dried meat base on the different locations

Table 3. 2 Colony forming unit (Cfu/g) of fungal species recovered per location from dried beef meat.

Location	CFU g ⁻¹ range	Mean	No of fungi isolates
Ikole	$2.0 \times 10^2 - 1.6 \times 10^4$	2.6×10^3	10
Ilawe	$1.0 \times 10^1 - 4.5 \times 10^2$	1.3×10^2	11
Aramoko	$1.0 \times 10^2 - 6.9 \times 10^3$	1.5×10^3	16
Ijero	$2.0 \times 10^2 - 3.2 \times 10^2$	1.5×10^2	13
Oye	$5.0 \times 10^1 - 4.1 \times 10^2$	2.9×10^2	6
Otun	$1.0 \times 10^1 - 1.9 \times 10^2$	5.4×10^1	11
Ado	$2.0 \times 10^1 - 2.1 \times 10^3$	3.2×10^2	19
Omuo	$3.0 \times 10^1 - 6.4 \times 10^2$	1.6×10^2	16
Igede	$9.0 \times 10^1 - 3.2 \times 10^2$	1.0×10^2	7
Ise/Emure	$1.0 \times 10^1 - 2.5 \times 10^2$	9.5×10^1	9

Table 3. 3 Incidence of occurrence (%) of fungal species recovered from dry meat in all the different locations sampled

	Ikole	Ilawe	Aramoko	Ijero	Oye	Otun	Ado	Omuo	Igede	Ise/Enure
<i>Alternaria tenuissima</i>	-	-	8 1(12)	-	67 4(6)	-	5 1(20)	-	17 1(6)	-
<i>Arthrimum phaeospermum</i>	-	-	8 1(12)	20 2(10)	-	7 1(15)	5 1(20)	-	-	-
<i>Aspergillus aeneus</i>	-	-	8 1(12)	-	-	-	-	-	-	-
<i>Aspergillus aff. Ustus</i>	-	17 1(6)	25 3(12)	20 2(10)	17 1(6)	13 2(15)	5 1(20)	14 2(14)	-	-
<i>Aspergillus alabamensis</i>	-	-	-	10 1(10)	-	-	-	-	-	-
<i>Aspergillus amstelodami</i>	7 1(15)	-	-	-	-	-	5 1(20)	-	-	-
<i>Aspergillus awamori</i>	-	-	-	-	-	-	5 1(20)	-	-	-
<i>Aspergillus bombycis</i>	-	17 1(6)	8 1(12)	-	-	-	-	7 1(14)	-	-
<i>Aspergillus chevalieri</i>	-	-	-	-	-	-	5 1(20)	-	-	-
<i>Aspergillus cristatus</i>	-	17 1(6)	17 2(12)	10 1(10)	-	-	-	7 1(14)	17 1(6)	25 1(4)
<i>Aspergillus flavus</i>	27 4(15)	33 2(6)	50 6(12)	50 5(10)	-	40 6(15)	50 10(20)	50 7(14)	50 3(6)	-
<i>Aspergillus fumigatus</i>	13 2(15)	17 1(6)	25 3(12)	-	-	20 3(15)	5 1(20)	7 1(14)	-	25 1(4)
<i>Aspergillus intermedius</i>	-	-	-	10 1(10)	-	-	5 1(20)	7 1(14)	-	-
<i>Aspergillus melleus</i>	-	-	-	-	-	-	-	7 1(14)	-	-
<i>Aspergillus niger</i>	40 6(15)	33 2(6)	42 5(12)	60 6(10)	-	47 7(15)	60 12(20)	29 4(14)	33 2(6)	25 1(4)
<i>Aspergillus ochraceus</i>	13 2(15)	-	8 1(12)	-	17 1(6)	7 1(15)	20 4(20)	7 1(14)	-	-
<i>Aspergillus oryzae</i>	13 2(15)	17 1(6)	-	10 1(10)	-	-	15 3(20)	-	-	50 2(4)
<i>Aspergillus ruber</i>	-	-	-	-	-	-	-	-	17 1(6)	-
<i>Aspergillus tamaraii</i>	7 1(15)	-	8 1(12)	20 2(10)	-	-	15 3(20)	-	-	25 1(4)
<i>Aspergillus tubingensis</i>	-	-	-	-	-	-	5 1(20)	-	-	-
<i>Aspergillus unguis</i>	-	-	-	-	17 1(6)	-	-	-	-	-
<i>Byssochlamys spectabilis</i>	-	-	-	-	-	-	-	7 1(14)	-	-
<i>Chaetomium erectum</i>	-	17 1(6)	-	-	-	-	-	14 2(14)	-	-
<i>Cladosporium cladosporioides</i>	-	-	-	-	17 1(6)	27 4(15)	5 1(20)	29 4(14)	17 1(6)	25 1(4)
<i>Cochliobolus sp</i>	-	-	8 1(12)	-	-	-	-	-	-	-
<i>Epicoccum sorghinum</i>	-	-	-	-	-	-	-	7 1(14)	-	-
<i>Fusarium equiseti</i>	-	-	-	10 1(10)	-	-	-	-	-	-
<i>Fusarium incarnatum</i>	-	-	-	10 1(10)	-	-	15 3(20)	14 2(14)	-	-
<i>Paecilomyces formosus</i>	-	-	-	-	-	-	-	-	-	25 1(4)
<i>Penicillium citrinum</i>	20 3(15)	33 2(6)	17 2(12)	-	-	-	-	-	-	-
<i>Penicillium crustosum</i>	-	-	-	-	-	7 1(15)	5 1(20)	-	-	25 1(4)
<i>Penicillium mallochii</i>	-	-	-	-	-	7 1(15)	-	-	-	-
<i>Periconia macrospinoso</i>	-	33 2(6)	-	-	-	-	-	-	-	-
<i>Phoma herbarum</i>	-	-	8 1(12)	10 1(10)	-	7 1(15)	5 1(20)	-	-	-
<i>Ryzopus</i>	53 8(15)	33 2(6)	83 10(12)	80 8(10)	100 (6)	27 4(15)	55 11(20)	71 10(14)	50 3(6)	75 3(4)
<i>Talaromyces funiculosus</i>	7 1(15)	-	17 2(12)	-	-	-	-	7 1(14)	-	-

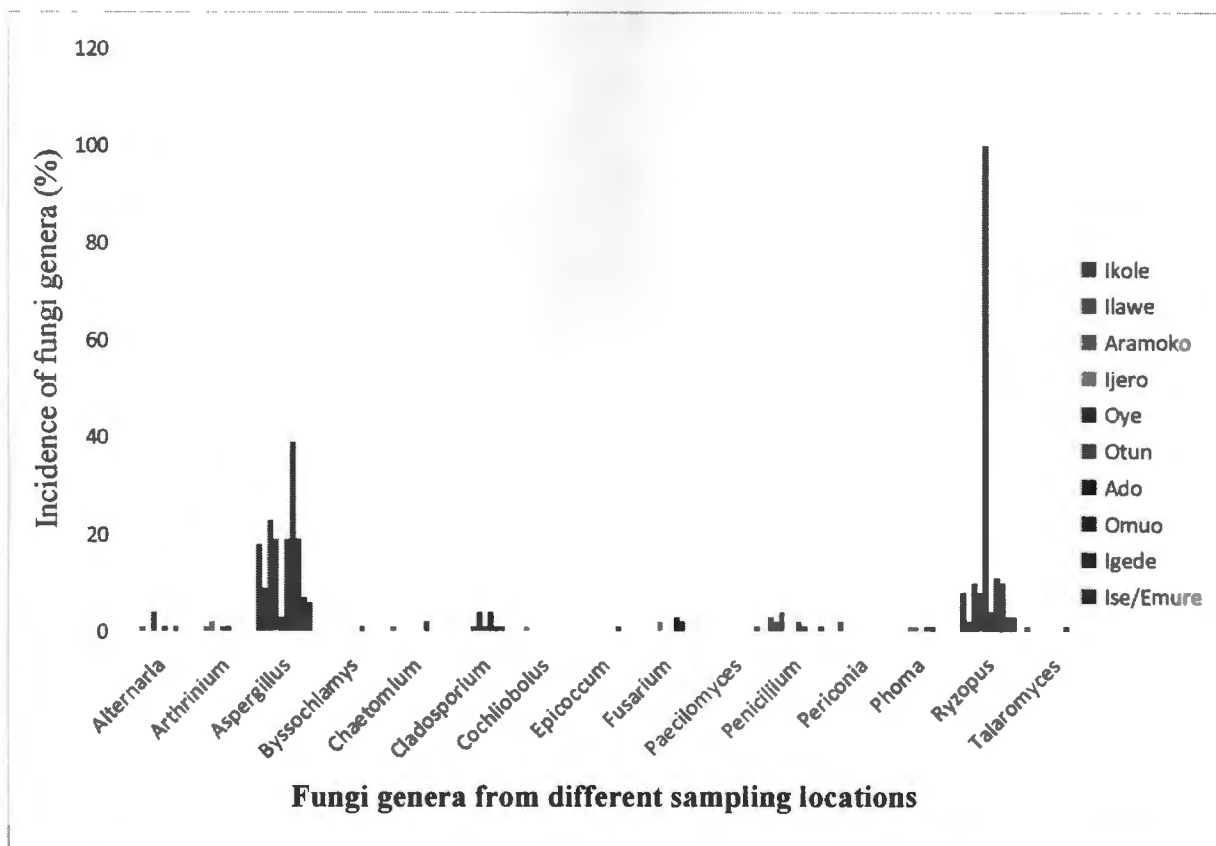


Figure 3. 5 Incidence (%) of different fungi genera contaminating dry meat according to the locations

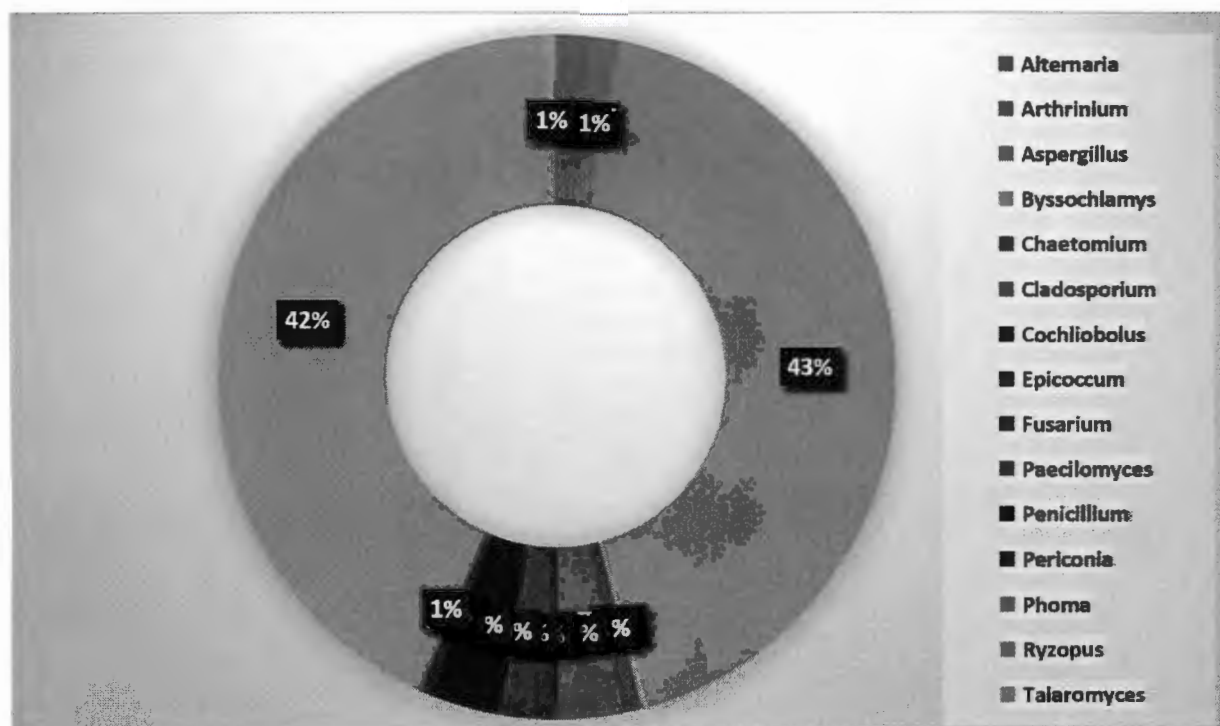


Figure 3. 6 Overall occurrence of all the filamentous fungi genera contaminating dry meat across the markets (%)

The overall fungal contamination result (Figure 3.5) showed that all the 10 sampled locations have *Aspergillus* and *Rhizopus* fungal genera. These were found to contaminate the dry meat across the locations board while *Penicillium* genera were found in 6 out of the 10 locations investigated. The remaining fungal genera were found randomly in dry meat samples from other locations with at least four different types which occurred in Ikole, Oye and Igede respectively while the highest fungal genera contamination with 8 fungal were recorded in Ado and Omuo, followed by Aramoko that had 7 fungal genera contamination.

The dominance of *Aspergillus* and *Rhizopus* species in dry meat samples analyzed corresponds with the high incidence of *Aspergillus* and *Rhizopus* species in regions with high temperatures and humidity (Castellari *et al.*, 2010) with regard to the climatic conditions of Ekiti State (Nigeria). This findings agrees with the known knowledge of *Aspergillus* species to be generally isolated from soil, air and enclosed surroundings and agricultural products (Klich, 2002; Oyetunji, 2006). *Penicillium* and *Cladosporium* species were also seen to contaminate bulk of the sampled meats. *Penicillium* has been reported to infect maize and maize-based produces in West Africa at diverse yield periods (Borgemeister *et al.*, 1998). Additional species of a lesser amount of prevalence found to occur comprised *A. aeneus*, *A. alabamensis*, *A. amstelodami*, *A. awamori*, *A. chevalieri*, *A. melleus*, *A. ruber*, *A. tubingensis*, *A. unguis*, *Byssoschlamys spectabilis*, *Chaetomium erectum*, *Cochliobolus sp.*, *Epicoccum sorghinum*, *Fusarium equiseti*, *Fusarium incarnatum*, *Paecilomyces formosus*, *Penicillium funiculosum*, *Penicillium mallochii*, *Periconia macrospinosa*, *Phoma herbarum* and *Talaromyces funiculosus*.

The higher incidence of *Aspergillus*, *Rhizopus* and *Penicillium* species in dry meat samples collected across the state could be due to the fact that *Aspergillus*, *Rhizopus* and *Penicillium* species are known to be storage fungi that usually contaminate food products under storage (Bankole & Adebajo, 2003; Makun *et al.*, 2011b; Pitt & Hocking, 1997). The occurrence of these fungal species in dry meat is a pointer to possible mycotoxin contamination because majority of them are known producers of main mycotoxins (Sweeney & Dobson, 1998). For instances, *A. flavus* and *A. niger* are known producers of aflatoxins in agricultural produce (Mulunda *et al.*, 2013; Reiter *et al.*, 2010).

Table 3. 4 Toxigenic potential of *Aspergillus* species occurring on dried meat

S/ No	Name of isolates	Nor <i>aflD</i>	Ver <i>aflM</i>	Omt <i>aflP</i>	afl R	aflJ	Aflatoxin Production	Accession number	NCBI % Similarity
1	<i>Aspergillus oryzae</i>	+	-	-	+	+	P	MH345927	99
2	<i>Aspergillus flavus</i>	+	+	+	+	+	P	MH345936	99
3	<i>Aspergillus fumigatus</i>	+	+	-	-	-	NP	MH345871	99
4	<i>Aspergillus tamarii</i>	+	+	+	-	-	NP	MH345873	99
5	<i>Aspergillus oryzae</i>	+	+	+	+	+	P	MH345958	100
6	<i>Aspergillus flavus</i>	+	+	+	+	+	P	MH345870	99
7	<i>Aspergillus awamori</i>	+	-	+	-	-	NP	MH345886	100
8	<i>Aspergillus flavus</i>	+	+	+	+	+	P	MH345846	100
9	<i>Aspergillus flavus</i>	+	+	+	+	+	P	MH345934	100
10	<i>Aspergillus oryzae</i>	+	+	+	+	+	P	MH345933	100
11	<i>Aspergillus unguis</i>	+	-	+	-	-	NP	MH345916	100
12	<i>Aspergillus flavus</i>	+	+	+	+	+	P	MH345922	100
13	<i>Aspergillus oryzae</i>	+	+	+	+	+	P	MH345883	100
14	<i>Aspergillus niger</i>	+	-	-	-	-	NP	MH345930	99
15	<i>Aspergillus niger</i>	+	-	-	-	-	NP	MH345949	99
16	<i>Aspergillus ochraceus</i>	+	-	-	-	-	NP	MH345845	99
17	<i>Aspergillus niger</i>	+	-	-	-	-	NP	MH345918	100
18	<i>Aspergillus candidus</i>	+	+	-	-	-	NP	MH345957	98
19	<i>Aspergillus tamarii</i>	+	+	+	-	-	NP	MH345894	99
20	<i>Aspergillus alabamensis</i>	+	+	+	-	-	NP	MH345915	99
21	<i>Aspergillus flavus</i>	+	+	+	+	+	P	MH345895	99
22	<i>Aspergillus tamarii</i>	+	+	-	-	-	NP	MH345903	100
23	<i>Aspergillus flavus</i>	+	+	-	-	-	NP	MH345924	89
24	<i>Aspergillus niger</i>	+	-	-	-	-	NP	MH345920	99
25	<i>Aspergillus oryzae</i>	+	+	+	+	+	P	MH345954	99
26	<i>Aspergillus niger</i>	+	-	-	-	-	NP	MH345921	99
27	<i>Aspergillus tamarii</i>	+	-	+	-	-	NP	MH345898	99
28	<i>Aspergillus aff. ustus</i>	+	+	-	-	-	NP	MH345862	99
29	<i>Aspergillus cristatus</i>	+	+	+	-	-	NP	MH345941	99
30	<i>Aspergillus niger</i>	+	-	-	-	-	NP	MH345955	97
31	<i>Aspergillus oryzae</i>	+	+	+	-	-	NP	MH345935	99
32	<i>Aspergillus oryzae</i>	+	+	+	+	+	P	MH345900	99
33	<i>Aspergillus flavus</i>	+	+	+	+	+	P	MH345879	99
34	<i>Aspergillus cristatus</i>	+	-	-	-	-	NP	MH345904	99
35	<i>Aspergillus flavus</i>	+	+	+	+	+	P	MH345882	99
36	<i>Aspergillus fumigatus</i>	+	-	+	-	-	NP	MH345948	99
37	<i>Aspergillus oryzae</i>	+	+	-	+	+	P	MH345869	100
38	<i>Aspergillus tamarii</i>	+	+	+	-	-	NP	MH345841	99
39	<i>Aspergillus niger</i>	+	-	-	-	-	NP	MH345917	99
40	<i>Aspergillus flavus</i>	+	+	+	-	-	NP	MH345897	100
41	<i>Aspergillus flavus</i>	+	+	+	+	+	P	MH345959	99

42	<i>Aspergillus niger</i>	+	-	-	-	-	NP	MH345876	99
43	<i>Aspergillus amstelodami</i>	+	+	+	-	-	NP	MH345912	99
44	<i>Aspergillus fumigatus</i>	+	-	-	-	-	NP	MH345848	100
45	<i>Aspergillus niger</i>	+	-	-	-	-	NP	MH345950	100
46	<i>Aspergillus tamarii</i>	+	+	-	-	-	NP	MH345899	99
47	<i>Aspergillus bombycis</i>	+	+	+	-	-	NP	MH345849	99
48	<i>Aspergillus tamarii</i>	+	-	-	-	-	NP	MH345940	100
49	<i>Aspergillus niger</i>	+	-	-	-	-	NP	MH345872	99
50	<i>Aspergillus niger</i>	+	-	+	-	-	NP	MH345878	100
51	<i>Aspergillus flavus</i>	+	+	-	-	-	NP	MH345891	100
52	<i>Aspergillus flavus</i>	+	+	+	+	+	P	MH345842	99
53	<i>Aspergillus tamarii</i>	+	+	-	-	-	NP	MH345906	99
54	<i>Aspergillus tamarii</i>	+	+	-	-	-	NP	MH345874	100
55	<i>Aspergillus oryzae</i>	+	+	+	+	+	P	MH345926	100
56	<i>Aspergillus flavus</i>	+	+	+	+	+	P	MH345932	99
57	<i>Aspergillus fumigatus</i>	+	+	-	-	-	P	MH345864	100
58	<i>Aspergillus fumigatus</i>	+	+	+	-	-	NP	MH345856	100
59	<i>Aspergillus oryzae</i>	+	+	+	-	+	P	MH345880	99
60	<i>Aspergillus flavus</i>	+	+	+	+	+	P	MH345881	100
61	<i>Aspergillus alabamensis</i>	+	+	+	-	-	NP	MH345914	99
62	<i>Aspergillus flavus</i>	+	+	+	+	+	P	MH345952	100
63	<i>Aspergillus flavus</i>	+	+	-	+	+	P	MH345913	99
64	<i>Aspergillus tamarii</i>	+	+	+	-	-	NP	MH345889	100
65	<i>Aspergillus fumigatus</i>	+	+	-	-	-	NP	MH345865	99
66	<i>Aspergillus oryzae</i>	+	+	+	+	+	P	MH345908	99
67	<i>Aspergillus flavus</i>	+	+	+	-	-	NP	MH345892	100
68	<i>Aspergillus intermedius</i>	+	+	-	-	-	NP	MH345890	99
69	<i>Aspergillus fumigatus</i>	+	+	+	-	-	NP	MH345855	100
70	<i>Aspergillus fumigatus</i>	+	-	+	-	-	NP	MH345850	100
71	<i>Aspergillus fumigatus</i>	+	-	-	-	-	NP	MH345857	100
72	<i>Aspergillus flavus</i>	+	+	+	+	+	P	MH345910	100
73	<i>Aspergillus tamarii</i>	+	-	+	-	-	NP	MH345887	99
74	<i>Aspergillus fumigatus</i>	+	-	+	-	-	NP	MH345956	100
75	<i>Aspergillus bombycis</i>	+	+	-	-	-	NP	MH345893	99
76	<i>Aspergillus cristatus</i>	+	+	-	-	-	NP	MH345937	99
77	<i>Aspergillus flavus</i>	+	+	+	+	-	P	MH345946	99
78	<i>Aspergillus niger</i>	+	+	+	-	-	NP	MH345859	96
79	<i>Aspergillus cristatus</i>	+	+	+	-	-	NP	MH345901	99
80	<i>Aspergillus fumigatus</i>	+	-	-	-	-	NP	MH345860	99
81	<i>Aspergillus aeneus</i>	+	-	+	-	-	NP	MH345852	99

NP = No potential to produce aflatoxin, P = Potential to produce aflatoxin

The presence of *aflR* and *aflJ* in aflatoxin biosynthetic regulatory genes with three extra essential genes (*omt-A*, *aflD* and *aflM*) found in the aflatoxin biosynthetic pathway were established in almost all of the isolates, but the genes were simply expressed in 30.9% of the isolates. The occurrence of these genes in the isolates should be a concern as the unexpressed fungal species are likewise capable of producing aflatoxins in favourable environments. The report has it that *aflR*, *omt-A*, *aflD* and *aflM* remain the most vital genes from all the 25 reported genes in the aflatoxin biosynthetic group that control aflatoxin production (Davari *et al.*, 2015). The *aflR* gene controls the initiation of the transcript of pathway genes and aflatoxin biosynthesis (Woloshuk *et al.* (1994) but its occurrence in the isolates may not automatically result in aflatoxin production since aflatoxin pathway is controlled by several mechanisms (Bok & Keller, 2004; Perrin *et al.*, 2007). Also, *aflJ* is likewise an aflatoxin biosynthetic controlling gene identical to the *aflR*, the dual genes are different transcript with separate sponsors. It is essential for the expression of other genes in the aflatoxin group and also involved in the transformation of pathway transitional products to aflatoxins (Georgianna *et al.*, 2008). The occurrence of the aflatoxin controlling (*aflR*) gene was found in the non-producing *A. oryzae* and *A. sojae* types (Kusumoto *et al.*, 1998; Lee *et al.*, 2006), individually *A. flavus* and *A. oryzae* have been reported to be morphologically alike with *A. oryzae* being the ornamental type of *A. flavus* (Payne *et al.*, 2006), the morphological resemblance could be accountable for the occurrence of the aflatoxin biosynthetic genes in the two organisms. Regardless of the presence of aflatoxin biosynthetic genes in all *A. oryzae* and *A. flavus* they might not be producing aflatoxins. Lee *et al.* (2006) hypothesized that while some *A. flavus* strains have the *A. oryzae aflR* gene, they are non-toxigenic. *A. oryzae* also have the aflatoxin biosynthetic group, this might be as a consequence of gene elimination, addition, mutation or structure shift which might have happened in *A. oryzae* in the course of domestication over the years (Tominaga *et al.*, 2006). Equally, *Aspergillus oryzae* is employed in the food industry for the production of fermented foods, the occurrence of the *aflR* gene indicates an imminent threat to food industries as this organism has the propensity of expressing the gene in the future. The *aflR* protein can bind the promoter area of individual aflatoxin synthesis gene and trigger gene expression (Ehrlich *et al.*, 1999). Furthermore, aflatoxin biosynthesis is controlled by many ecological factors, for example, light, Calvo *et al.* (2002), carbon source, temperature, and pH (OBrian *et al.*, 2007).

The Maximum likelihood phylogenetic tree based on partial ITS rDNA gene sequence, showing the phylogenetic relationships between identified *Aspergillus* species and the most closely related *Aspergillus* strains from the GenBank is shown in Fig 3.6. The numbers at the nodes indicate the levels of bootstrap support based on 1000 resampled data sets. Only values greater than 50% are shown. The scale bar indicates 0.5 nucleotide substitution per site. *Aphanophora eugeniae* is set as the out-group. Sequences obtained in this study are denoted with a red circle. This result is in six main clade; comprising the Clade I: *Aspergillus unguis* and *A. aeneus* shared 98% and 99% with the reference of the same species from GenBank, Clade II: *Aspergillus fumigatus* 97% *A. awamori*, *A. niger* (85%), *A. tubingensis* 82%, Clade III: *A. ustus* 68% with *A. ustus* in the GenBank, *A. cristatus* 68% homology with *A. ustus* and 99% with *A. candidus*. *A. candidus* also shared 68% with the reference strain of *A. candidus* from the GenBank. This similarity index is quite low except for *A. ustus* since it is below the 70% anticipated borderline for the degree of similarity according to Wayne *et al.* (1987). *A. ruber* 98% with the *A. amstelodami* has the closest relative. Sample of *A. cristatus*, *A. intermedius*, *A. amstelodami* and *A. chevalieri* shared 81% with the reference strain from the GenBank. Clade IV *A. alabamensis* shared 79% with the reference strain while Clade V *A. oryzae*, *A. flavus*, *A. bombycis* shared 95% with the reference strain. Clade VI *A. tamarii* shared 98% with reference strain. *A. ochraceus* shared 99% with *A. ochraceus* and *A. melleus* reference strain. This high homology percentage shows that they possessed nearly the same nucleotide signature with their relative counterpart from the GenBank (Aremu & Babalola, 2015). These high similarity index expressed by these fungi is beyond 70% borderline of the expected degree of relatedness (Wayne *et al.*, 1987). *A. melleus* from this study shared 60% with *A. ochraceus* and 68% with *A. melleus* reference from the GenBank. This fungus has a different nucleotide signature and its low threshold makes it prone to being wiped out Konstantinidis and Stackebrandt (2013), this could be a novel *Aspergillus* species.

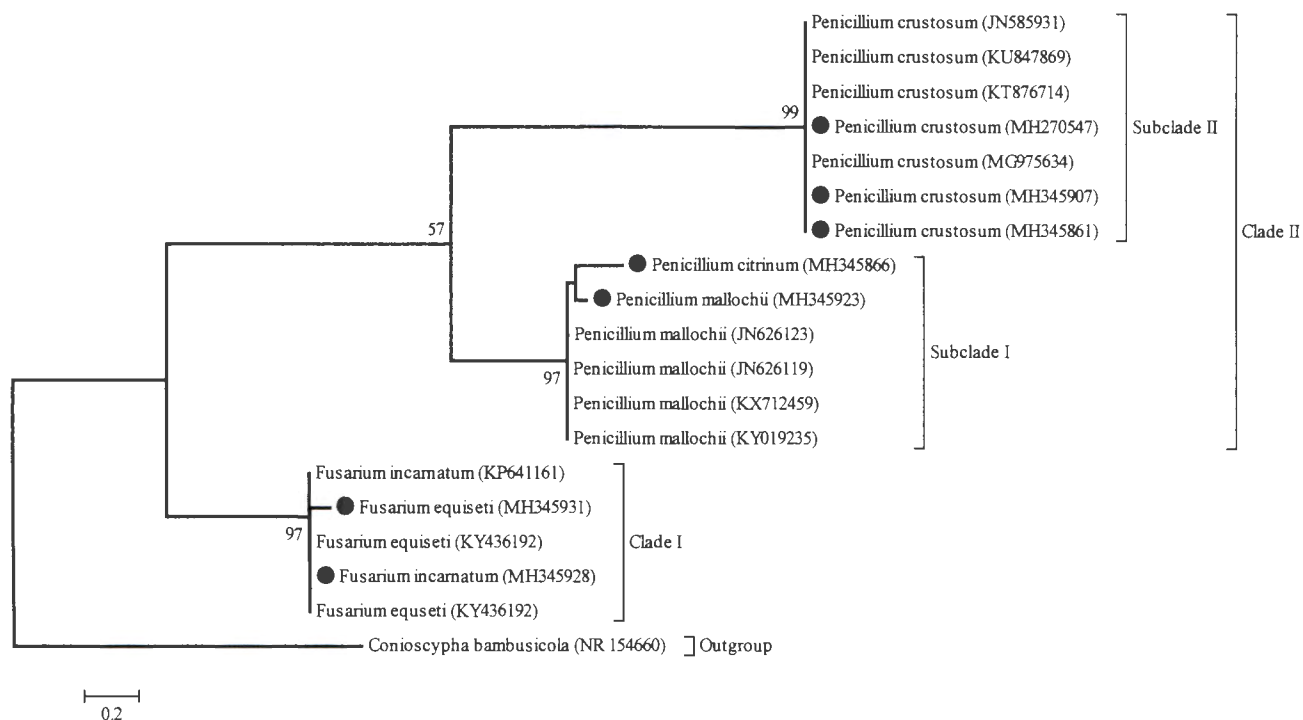


Figure 3. 8 Maximum Likelihood phylogenetic tree for *Penicillium* and *Fusarium* species identified and the most closely related strains from the GenBank.

Maximum likelihood phylogenetic tree based on partial ITS rDNA gene sequence, showing the phylogenetic relationships between identified *Penicillium* and *Fusarium* fungi and the most closely related strains of *Penicillium* and *Fusarium* from the GenBank. The numbers at the nodes indicate the levels of bootstrap support based on 1000 resampled data sets. Only values greater than 50% are shown. The scale bar indicates 0.2 nucleotide substitution per site. *Conioscypha bambusicola* is set as the out-group. Sequences obtained in this study are denoted with a red circle.

This resulted in two main clades; comprising the *Fusarium* (Clade I) and *Penicillium* (Clade II) taxonomic group. In clade I, the two *Fusarium* identified from this study clustered with 68% similarity index with *F. equiseti* reference strain from the GenBank. This similarity index is quite low because it is below the 70% expected borderline for the degree of relatedness according to Wayne *et al.* (1987). Clade II is subdivided into two: subclade I and subclade II. Subclade I comprises the *Penicillium citinum* (identified from this study) clustering with 97% similarity index with the reference strain of *Penicillium malochii* from the GenBank. In addition, *Penicillium malochii* from this study clustered equally with 97% similarity index with the reference strain. Subclade II comprises the *Penicillium crustosum* with 99% similarity index of the same species from the GenBank. This high homology percentage shows that they possessed the nearly the same nucleotide signature with their relative counterpart from the GenBank (Aremu & Babalola, 2015). These high similarity index expressed by these fungi is beyond 70% borderline of the expected degree of relatedness (Wayne *et al.*, 1987). In addition to this, similarities expressed is due to their high similarity value, which result in DNA reassociation values that fall below the 70% threshold values (Stackebrandt *et al.*, 2002). This showed high genetic relatedness that is increasingly reliable because they cannot be wiped out overnight according to (Konstantinidis & Stackebrandt, 2013).

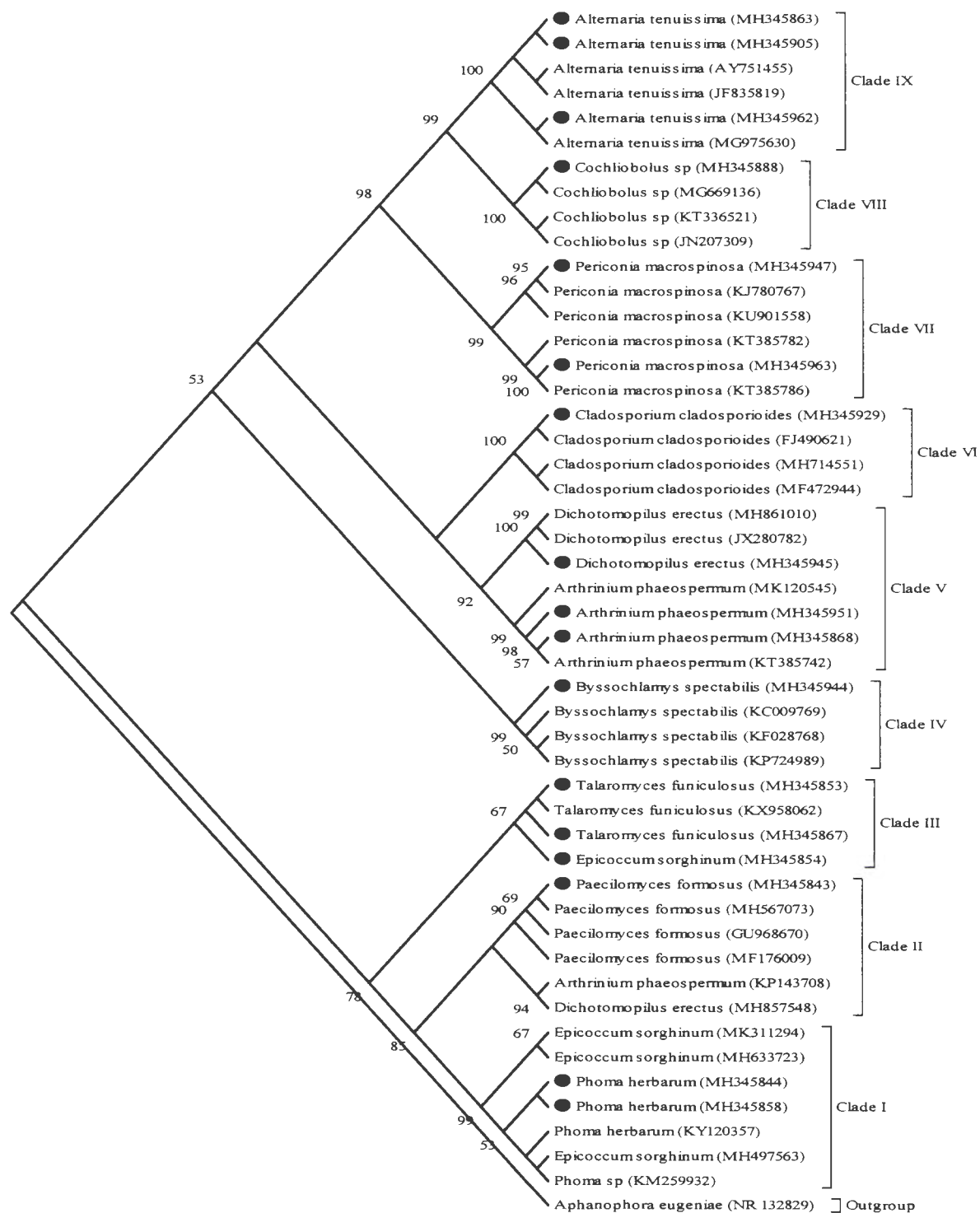


Figure 3. 9 Maximum Likelihood phylogenetic tree for other isolated fungi species and the most closely related strains from the GenBank.

Maximum likelihood phylogenetic tree based on partial ITS gene sequence, showing the phylogenetic relationships between Other identified *Aspergillus* species and the most closely related strains from the GenBank. The numbers at the nodes indicate the levels of bootstrap support based on 1000 resampled data sets. Only values greater than 50% are shown. The scale bar indicates 0.5 nucleotide substitution per site. *Aphanophora eugeniae* is set as the out-group. Sequences obtained in this study are denoted with a red circle

This resulted in nine clades. Clade I comprises *phoma harbarum* isolated from this study exhibited low similarity index of 67% and less than 50% with *Epicoccum sorghinum* and *Phoma* sp. respectively. The *Paecilomyces formosus* in clade II, *Talaromyces funiculosus* in clade III, *Cochiobolus* sp. clade VIII and *Altanaria tenuissima* in clade IX all had similarity index of less than 50%. These similarity index are quite low because they are below the 70% expected borderline for the degree of relatedness according to Wayne *et al.* (1987). The relatedness is just for a while and can be wiped off anytime. They possess a slightly different nucleotide signature from their relative counterpart in the GenBank. Thus a probable novel fungus (Aremu & Babalola, 2015). Conversely, *Periconia macrospinosa* in clade VII and *Dichotomopilus erectus* in clade V, had a very strong similarity index of 95-100% and 100% respectively because they are above the expected 70% borderling for the degree of relatedness.

3.4 Conclusion

In conclusion, the assessment of dried meat sold across different markets in Ekiti States showed, contamination of various species of fungi. The difference in occurrence and types of these fungi are usually associated to the environment of processing, storage and displayed style. The majority of the *Aspergillus* fungal isolated were having the three important basic genes (*omt-A*, *aflD* and *aflM*) which have been previously linked to *aflR* as necessary for the production of aflatoxin. *Aspergillus flavus* and *A. oryzae* constituted (30.9%) of the *aspergillus* genera. *A. flavus* and *A. oryzae* isolated from the dried meat expressed the essential aflatoxin biosynthetic regulatory gene (*AflR*) that is responsible for aflatoxin production that makes them potential mycotoxicogenic fungi. At anytime mycotoxins like aflatoxins, sterigmatocystin and ochratoxin are produced in food, the food product is regarded as unsafe for human consumption. The Maximum likelihood phylogenetic tree based on partial ITS rDNA gene sequence showed that there is a phylogenetic relationship between isolated *Aspergillus*, *Penicillium*, *Fusarium* and other fungi

species and their closely related strains from the GenBank which were isolated from different sources in different countries showed high similarity index (Figure,3.6- 3.8). This study indicates the need for continuous assessment of the mycological status of dried meat in order to create awareness for the need for improvement in food safety, while adequate care should be taken to prevent fungal contamination during processing, handling, storage and selling of these products.

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CHAPTER FOUR

DETERMINATION OF MYCOTOXINS FROM DRIED BEEF MEAT

ABSTRACT

The consumption of food contaminated with mycotoxins results in the development of food-borne mycotoxicosis. This study was carried out using four Elisa kits and also describes an in-house validated method for the determination of 22 mycotoxins in dried meat using a single extraction step followed by liquid chromatography with triple quadrupole mass spectrometry (LC-MS/MS) using dilute and shoot method. The Elisa results showed that 92.7% of dried meat samples were found to be contaminated with OTA although at a level below the maximum permissible levels of 1 µg/kg by EU. There was no significant difference in the average contamination value of OTA from Elisa and the LC-MS as they both have 0.44 and 0.5 µg/kg respectively even though the percentage of contamination varies. The total AFs contamination was found in 66% of the examined dried beef samples, with range values of 6.91 µg/kg and 23.56 µg/kg for Elisa and LC-MS method respectively. While the result of the total aflatoxins detected in this study by LC-MS showed that AFB₁ had a mean value of 105.4 µg/kg, AFB₂ mean value of 6.92 µg/kg, AFG₁ and AFG₂ had an average mean value of 40.49 µg/kg and 2.60 µg/kg, respectively. The total aflatoxins recorded in this study far exceeded the permissible level allowed in the food of up to 10 µg/kg, although in the EU, 4 µg/kg is the threshold. The concentrations of α -Zea were relatively higher with an average mean value of 113.82 µg/kg in all the meat sampled across the various selling locations. Also, high concentrations of cyclopiazonic acid with an average mean value of 51.99 µg/kg were similarly detected in meat intended for human consumption. In some of the samples, up to 10 different mycotoxin co-occurrence were detected, this underlining the abundant range of mycotoxin co-occurrence. Most of the mycotoxins found have not been previously reported together in dried meat, the occurrence of these mycotoxins in the dried meat is an indication of possible exposure of consumers to dangers of mycotoxin consumption which is usually associated with severe health problems.

Keywords: Aflatoxins, ochratoxin A, zearalenone, dried meat, Elisa, LC-MS/MS,

4.1. Introduction

Mycotoxins contamination in food has remained a major concern in food safety as they are toxic secondary metabolites produced by several molds and often contaminate food and feed worldwide (Binder *et al.*, 2007). Owing to the likely natural contamination of grains used by the food and livestock industry, there is a high probability of mycotoxins to enter the food chain (El Khoury & Atoui, 2010; Pleadin *et al.*, 2015). Their incidence is influenced by various factors, such as the food product type, climatic conditions, agricultural practices, storage conditions, and periodic variances (Duarte *et al.*, 2010a; Magan & Aldred, 2007). Mycotoxins have the potential to affect human health by acute and chronic effects such as the induction of hepatocellular carcinoma (HCC) or unexpected death due to acute toxicity in the case of aflatoxins (Lewis *et al.*, 2005).

Prior research has revealed that products of animal sources, for example meat and meat products, can also increase the human mycotoxins' intake coming either from indirect transfer from farm animals exposed to naturally contaminated grains and feed (carry-over effects) or direct contamination with fungi or naturally contaminated seasoning blends used in meat production (Duarte *et al.*, 2010b; Perši *et al.*, 2014). Among mycotoxins of health importance in relation to their toxicity and incidence, aflatoxin (AFs), ochratoxin A (OTA) fumonisins (FBs) and zearalenone (ZEN) are the most frequently encountered contaminants (Iqbal *et al.*, 2014). AFB₁ is the most potent liver carcinogen recognized in mammals, classified by the International Agency for Research on Cancer (IARC) as a Group 1 carcinogen, OTA in group 2B and ZEN in group 3 carcinogens (IARC, 1994).

The occurrence of mycotoxins in food and feed is potentially hazardous to the health of humans in addition to animals because of their carcinogenic, toxic and mutagenic properties (Royer *et al.*, 2004). Among farm animals, pigs are known to be predominantly sensitive to mycotoxins, although ruminants such as sheep and cows are less predisposed as their rumen fluid is capable of enzymatically degrading these mycotoxins into less toxic metabolites (Duarte *et al.*, 2010b). Wagacha and Muthomi (2008) stated that immune modulation effects of some mycotoxins increases health impacts of major illnesses disturbing Africa for instance, malaria, kwashiorkor, and HIV/AIDS. It is apparent that sub-Saharan residents are prone to prolonged dietary mycotoxin exposure since they regularly eat affected crops and due to the fact that crops in

tropical and subtropical areas are more vulnerable to contamination owing to the suitable climatic conditions (Bankole & Adebajo, 2003; Thompson & Henke, 2000).

A number of studies have shown that fungi species belonging to *Penicillium* and *Aspergillus* genera are producers of these mycotoxins, and have been isolated from meat products such as processed sausages or dry-cured hams (Markov *et al.*, 2013; Rodríguez *et al.*, 2012; Tabuc *et al.*, 2004). There are other studies that showed that significant OTA levels are likely to be found in meat products produced from contaminated raw materials (Perši *et al.*, 2014; Pleadin *et al.*, 2015), as well as in smoked meat and meat products (Dall'Asta *et al.*, 2010; Pfohl-Leszkowicz & Manderville, 2007). It is also acknowledged that aflatoxins can be found in meat and as well as in meat products if the animals eat sufficient amounts of AFB₁ occurring in their feed (Herzallah, 2009; Markov *et al.*, 2013; Richard, 2007).

In Nigeria, the consumption of dried meat is increasing especially in the south-west region and there are studies indicating the presence of fungi capable of producing mycotoxins on the dried meat from other states in the south-west region (Adeyeye, 2016; Okoro, 2015). This might be as result of the preservation method that is usually employed to lower the water activity such as dehydration, and the addition of salt in some cases. However, as the meat surfaces dry, there is increased possibility of growth of undesirable fungi, several of which can produce potent mycotoxins, due to the favourable conditions during the drying, storage and selling process. The presence of fungi on regular food items in Nigeria is not in doubt since the prevalence of mycotoxins in sub-Saharan Africa has been reported (Rubert *et al.*, 2013). However, there is dearth of information on multi-mycotoxins on dried meat in Nigeria while a lot of attention is placed on other food products. In lieu of the above facts, the present study is aimed at investigating the level of AFs, OTA, FBs and ZEN using Elisa screening method and LC-MS/MS to assess a wider range of potential mycotoxins that contaminates dried meat samples sold in Ekiti State for human consumption.

4.2 Materials and methods

4.2.1 Reagents, chemicals and Extraction Kits

Milli-Q quality water (Millipore, M.A.S. 67120 Molsheim, France) was used during the whole analysis. Formic acid, acetonitrile and methanol were of MS grade, and purchased from Merck

(Darmstadt, Germany). The Elisa kits used in the present study were purchased from Sigma-Aldrich, Steinem, Germany, while the Enzyme immunoassay Elisa kits (RIDASCREEN® Ochratoxin, Aflatoxin Total, Fumonisin and Zearalenone) were purchased from R-Biopharm AG, An der Neuen Bergstraße 17 64297 Darmstadt, Germany. Distilled water was used and all other chemicals used were of analytical grade.

4.2.2. Standards for LC-MS

The standards of aflatoxin B₁ (AFB₁), aflatoxin B₂ (AFB₂), aflatoxin G₁ (AFG₁), aflatoxin G₂ (AFG₂), ochratoxin A (OTA), ochratoxin B (OTB), sterigmatocystin (STE), a-zearalenol (a-ZEA), β ZEA zearalenone (ZEN), nivalenol (NIV), deoxynivalenol (DON), 3-acetyldeoxynivalenol (3-ADON), 15-acetyldeoxynivalenol (15-ADON), fumonisin B₁ (FB₁), fumonisin B₂ (FB₂), fumonisin B₃ (FB₃), alternariol monomethyl ether (AME), Kojic Acid (KA), Citrinin, Sterigmatocystin (STER), Cyclopiazonic acid (CPA), Tenuazonic copper salt (TCS), T-2 and HT-2 toxins, were supplied from the National Metrology Institute of South Africa (NMISA). The mycotoxins stock solutions were prepared according to (Rubert *et al.*, 2012).

4.3. Sampling

One hundred and eight dried meats samples were purchased from selected ten major open markets from Ten Local Government Areas of Ekiti State, Nigeria. The markets includes Oye, Ilawe, Ise/Emure, Otun, Omuo, Igede, Ikole, Aramoko and Ado. Sampling locations comprised 2 cities each from 5 geographical zones North, South, East, West and Central. Each sample was collected from random points of trader's trays as 12-14 sub samples (50-100 g) and mixed together to form the bulk sample (200 -400 g) and pulverized. Fifty grams representative sample was obtained from each bulk and stored at -20 °C until analyzed.

4.3.1 Elisa extraction procedure for aflatoxin total

Two (2) grams of ground and homogenized samples were blended with 10 ml of 70% methanol in a 15 ml centrifuge tube and were briskly shaken for 10 min on an electric shaker. The samples were centrifuged for 10 min at 4000 g at room temperature. The supernatant from each sample was mixed with 6 µl of hexane and centrifuge for 5 min at 4000 rpm at room temperature. The supernatant (100 µl) from each sample further diluted with 600 µl of distilled water per well in the test. 50 µl of the diluted filtrate was used per well. Fifty (50) µl of the standard solutions and

prepared samples, including those artificially contaminated (spiked), were added into each single wells in duplicate. Thereafter, 50 µl of the enzyme conjugate was added to each well, additional 50 µl of antibody solution was added to each well and gently mixed by shaking the plate by hand and incubated for 30 min at room temperature (20 – 25 °C). After 30 min of incubation, the liquids were emptied out of the wells and tapped with the microwell holder upside down vigorously (3 times in a row) against absorbent paper to ensure complete removal of liquid from the wells. The wells were washed 3 times with 250 µl distilled water and were completely emptied. After the washing step, 50 µl substrate and 50 µl chromogen was added to each of the wells and was gently mixed by shaking the plate manually and incubated for 30 min at room temperature in the dark. Lastly, the reaction was stopped by adding 100 µl of stop solution to each of the wells and gently mixed by shaking the plate manually and the absorbance was measured at 450 nm within 10 min after adding the stop solution, using Full-Automatic Microplate Reader (MB-580, HEALES, Shenzhen, China). The zero standard was made equal to 100% and the absorbance values quoted as percentages. The values calculated for the standard are recorded in a system of coordinates on semi-logarithmic graph paper against the zearalenone concentration. The aflatoxin concentration (ng/kg) corresponded to the absorbance of each sample and read directly from calibration curve.

$$\% \text{ absorbance} = \frac{\text{absorbance standard (or sample)}}{\text{absorbance zero standard}} \times 100$$

In addition, to assess the accuracy of the ELISA measurements, two negative meat samples of a preliminary testing were spiked with 5 and 10 µg/kg total aflatoxin.

4.3.2 Elisa extraction procedure for ochratoxin

Two grams (2 g) of the representative sample was thoroughly mixed into a centrifugal screw cap vial and 5 ml of 1 N HCl and shaken for 5 min. Then 10 ml of dichloromethane was shaken vigorously and centrifuged for 15 min at 3500 rpm. The upper aqueous layer was completely removed up to the sample cake level and discarded. After, the dichloromethane layer was filtered from the cake into a new centrifugal screw cap an equivalent volume of 0.13 M sodium hydrogen carbonate buffer was added to it and shake vigorously for 15 min. Thereafter, it was centrifuged for 15 min at 3500 rpm, afterwards, 100 µl of the upper aqueous phase was diluted with 400 µl of 0.13 M sodium hydrogen carbonate buffer. Fifty (50) µl of the standard solution

and prepared samples, were added into each single well in duplicate. Subsequently, 50 µl of the enzyme conjugate was added to each well, additional 50 µl of antibody solution was added to each well and gently mixed by shaking the plate by hand and incubated for 30 min at room temperature (20 – 25 °C) in the dark. The liquids were emptied out of the wells after 30 min of incubation and tapped with the microwell holder upside down vigorously (3 times in a row) against absorbent paper to ensure complete removal of liquid from the wells. The wells were washed 3 times with 250 µl distilled water and were completely emptied. After the washing step, 100 µl substrate/chromogen was added to each of the wells and mixed gently by shaking the plate manually and incubated for 15 min at room temperature in the dark. Finally, the reaction was stopped by adding 100 µl of stop solution to each of the wells and gently mixed by shaking the plate manually and the absorbance was measured at 450 nm within 10 min after adding the stop solution, using Full-Automatic Microplate Reader (MB-580, HEALES, Shenzhen, China). The zero standard was made equal to 100% and the absorbance values quoted as percentages. The values calculated for the standard are recorded in a system of coordinates on semi-logarithmic graph paper against the zearalenone concentration. The aflatoxin concentration (mg/kg) corresponded to the absorbance of each sample and read directly from the calibration curve.

$$\% \text{ absorbance} = \frac{\text{absorbance standard (or sample)}}{\text{absorbance zero standard}} \times 100$$

4.3.3 Elisa extraction procedure for fumonisin

Five (5) grams of ground and homogenized samples were blended with 25 ml of 70 % methanol in a 50 mL centrifuge tube and shaken vigorously for 3 min on electric auto shaker (SK-O180-S, LED digital Orbital Shaker, Pocklington York, UK). Thereafter, the samples were centrifuged for 10 min at 4500 rpm at room temperature using (Shuke Centrifuge TD-600, Sichuan China). The supernatant (100 µl) from each of the samples were diluted with 1300 µl of distilled water and vortex for 2 min. Furthermore, to determine the accuracy of ELISA measurement, two negative beef samples that had been previously tested were spiked with 5 and 10 µg/kg fumonisin (Andreasson *et al.*, 2015). Fifty (50) µl of the standard solutions and prepared samples, comprising those artificially contaminated (spiked), were added into each separate wells in duplicate. Subsequently, 50 µl of the fumonisin enzyme conjugate was added to each well,

additional 50 µl of anti-fumonisin antibody solution was added to each well and gently mixed by shaking the plate manually and incubated for 30 min at room temperature (20 – 25 °C). After 30 min of incubation, the liquids were poured out of the wells and tapped with the microwell holder upside down vigorously (3 times in a row) against absorbent paper to ensure complete removal of liquid from the wells. The wells were washed 3 times with 250 µl distilled water and were completely emptied. After the washing step, 100 µl substrate/chromogen was added to each of the wells and mixed gently by shaking the plate manually and incubated for 15 min at room temperature in the dark. Finally, the reaction was stopped by adding 100 µl of stop solution to each of the wells and gently mixed by shaking the plate manually and the absorbance was measured at 450 nm within 10 min after adding the stop solution, using Full-Automatic Microplate Reader (MB-580, HEALES, Shenzhen, China). The zero standard was made equal to 100% and the absorbance values quoted as percentages. The values calculated for the standard are recorded in a system of coordinates on semi-logarithmic graph paper against the fumonisin concentration. The fumonisin concentration (mg/kg) corresponded to the absorbance of each sample and read directly from the calibration curve.

$$\% \text{ absorbance} = \frac{\text{absorbance standard (or sample)}}{\text{absorbance zero standard}} \times 100$$

4.3.4 Elisa extraction procedure for zearalenone

Five grams of ground meat sample was weighed into a suitable container, and 25 ml of methanol/water solution (70:30%) was added to it. The solution was shaken vigorously for 3 min using an electric shaker. After shaking the extract was centrifuged (Shuke Centrifuge TD- 600, Germany) at 4500 rpm for 10 min at room temperature (20 – 25°C/68-77°F). The supernatant was diluted with the sample dilution buffer (1:7) and 50 µl of the diluted extract were used per well in the test. Fifty (50) µl of the standard solution and prepared samples, including those artificially contaminated (spiked), were added into each separate wells in duplicate. Subsequently, 50 µl of the enzyme conjugate was added to each well and gently mixed by shaking the plate manually and incubated for 2 h at room temperature (20 – 25 °C). After the incubation, the liquids were poured out of the wells and tapped with the microwell holder upside down vigorously (3 times in a row) against absorbent paper to ensure complete removal of liquid from the wells. The wells were washed 3 times with 250 µl distilled water and were completely

emptied. After the washing step, 50 µl substrate and 50 µl chromogen was added to each of the wells and mixed gently by shaking the plate manually and incubated for 30 min at room temperature in the dark. Lastly, the reaction was stopped by adding 100 µl of stop solution to each of the wells and gently mixed by shaking the plate manually and the absorbance was measured at 450 nm within 10 min after adding the stop solution, using Full-Automatic Microplate Reader (MB-580, HEALES, Shenzhen, China). The zero standard was made equal to 100% and the absorbance values quoted as percentages. The values calculated for the standard are recorded in a system of coordinates on semi-logarithmic graph paper against the zearalenone concentration. The zearalenone concentration (ng/kg) corresponded to the absorbance of each sample and read directly from the calibration curve.

$$\% \text{ absorbance} = \frac{\text{absorbance standard (or sample)}}{\text{absorbance zero standard}} \times 100$$

4.3.5 Extraction procedure for LC-MS

The representative samples of particle size between 0.5 and 1 mm were weighed into a 50 mL polypropylene tube (Sarstedt, Nümbrecht, Germany) and enclosed with extraction solvent made up of acetonitrile/water/acetic acid (79:20:1, v/v/v) in 20 mL solvent/ 5g sample. For spiking and recovery experiments 5 g of samples were used for extraction. Samples were extracted for 90 min at 180 rpm on a Labcon FS16 rotary shaker (Labcon, South Africa), and centrifuged for 15 min at 3500 rpm. The supernatant was carefully transferred into another centrifuge tube and 10 mL of n-hexane was added to breakdown the fat sample (Adekoya *et al.*, 2017). The mixture was vortexed for 2 min and allowed to settle for 20 min at room temperature before the n-hexane phase was removed, and diluted with an equal volume of dilution solvent (acetonitrile/water/ acetic acid 79:20: 1, v/v/v), filtered through a 0.22 µm syringe nylon filter (Membrane Solutions, Tokyo) into 1.5 mL HPLC vial bottles for injection into the LC-MS/MS system (Sulyok *et al.*, 2007).

4.4 Instrumentation for LC-MS

A Shimadzu UHPLC instrument LC-MS/MS 8030 equipment (Shimadzu Corporation, Tokyo, Japan), with an ultrafast scan speed of 15,000 u/sec, and a polarity switching of 15 msec was used for the identification and quantification of the analytes. The chromatograph was a LC-30AD Nexera which was connected to a SIL-30 AC Nexera auto sampler and a CTO-20 AC

Prominence Column Oven. The oven was equipped with a Raptor™ ARC-18 column from Restek (2.7 µm, 2.1 mm × 100 mm) (Restek Corporation, Pennsylvania USA), and maintained at a constant temperature of 40 °C. The mobile phases used consisted of (A) 0.1% formic acid in deionized water, and (B) 0.1% formic acid in acetonitrile: methanol (50:50 v/v), was delivered at a constant flow rate of 0.2 mL/min with a sample injection volume set at 5 µl. The elution gradient program had a total run time of 20 min, and started with 10% B for 0.01 min and increased steadily to 95% B in 10 min, at which point it was kept constant for 3.5 min, and then the initial condition (10% B) was re-established for 1 min, and the column allowed to re-equilibrate for 5 min for the next run.

Following chromatographic separation, analyte were committed to a Shimadzu triple quad mass spectrometry detector model 8030 (Shimadzu Corporation, Kyoto, Japan) for detection and quantitation of analyte. The ionization source was an electron spray ionization (ESI) operated in positive mode at an event time of 0.206 sec. Data was acquired by a multiple reaction monitoring (MRM) method at optimized MS conditions for the analyte (Table 4. 1). The interface nebulizing gas flow rate was 3 L/min, DL temperature was 250 °C, heat block temperature was 400 °C, and drying gas flow rate was 15 L/min.

4.5 Validation of the method for LC-MS

The performance characteristics of the method such as matrix effect, linearity, trueness, intra-day and inter-day precision, limits of detection (LOD) and quantification (LOQ) and selectivity were evaluated.

Matrix effects were estimated using a 12 point matrix-matched calibration curves and a neat standard solution calibration curves within the linear range of 0.9, to 2000 µg/kg. Signal suppression/enhancement (SSE) values, which were calculated by comparing the slopes of matrix-matched calibrations with those of pure standard solution calibrations, were used for the evaluation of the matrix effects (Han *et al.*, 2010).

Calibration curves were generated by plotting the area under peak against the concentration of individual mycotoxin in the meat matrices. The method sensitivity was calculated by determining the limit of detection (LOD) which is given as $LOD = 3.3 \times \frac{Con}{S/N}$ and limit of

quantification (LOQ) given as $LOQ = 10 \times \frac{Conc.}{S/N}$. The LOD and LOQ values of each mycotoxin were determined by mixing the stock standard solutions with the blank matrices.

The recoveries were determined at three different levels using blank samples with nine replicates per concentration level, the spiking was done at low (25 µg/kg), intermediate (50 µg/kg) and high (100 µg/kg) levels of the mycotoxin concentrations. Preparation of samples was carried out in triplicate as described above. Extraction recovery for each analyte was evaluated by matching the mean peak areas of the samples previously spiked before extraction with that of the samples spiked after extraction.

Precision was evaluated for intra-day and inter-day in non-contaminated samples using standard addition. The samples were spiked with three different concentration levels of each standard (25, 50 and 100 µg/kg) and prepared in triplicate replicates for calculating the intra-day precision, while two samples spiked with 50 and 100 µg/kg of each standard were analyzed daily in triplicate for 5 consecutive days for inter-day precision. The calculation for recovery and precision were based on matrix-matched calibrations.

4.6 Statistical data analysis

4.6.1 Statistical Analysis for Elisa Data

The data of examined mycotoxins (µg/kg) were calculated based on calibration curves using mathematical interpolation and multiplication by the sample's dilution factor. Statistical analysis was done using descriptive statistics (IBM SPSS version 25.0 software) and the resultant data presented as mean ± standard deviation (SD) of two separated determinations and given to the analysis of variance (ANOVA) using Duncan's multiple range test at ($p < 0.05$), to find the dissimilarity among means.

4.6.2 Statistical Analysis for LC-MS Data and Coefficients of Correlation

The mycotoxin concentrations (µg/kg) were calculated using calibration curves with mathematical interpolation and multiplication by the sample's dilution factor. While final concentrations were calculated based on the average recovery values acquired for each analyte and the applied method. Statistical analysis was done using IBM SPSS version 25 Software with the statistical significance level set at 95% ($P = 0.05$). Correlation analyses were carried out with

the Excel software 2013, using the zero-order Pearson correlation coefficients (r) to identify possible associations between the fungi isolates, their genera and mycotoxin production in the dried meat samples evaluated. Interpretation of values was performed according to Pearson's coefficient (r) which are: very weak $0.000 \leq r \leq 0.200$; weak $0.201 \leq r \leq 0.400$; moderate $0.401 \leq r \leq 0.600$; strong $0.601 \leq r \leq 0.800$ and very strong $0.801 \leq r \leq 1.000$ (Christmann & Badgett, 2009). Once one variable increases and the other variable has a tendency to increase, the relationship indicates positive r values. If one variable increases and the other decreases, the correlation coefficient will be negative.

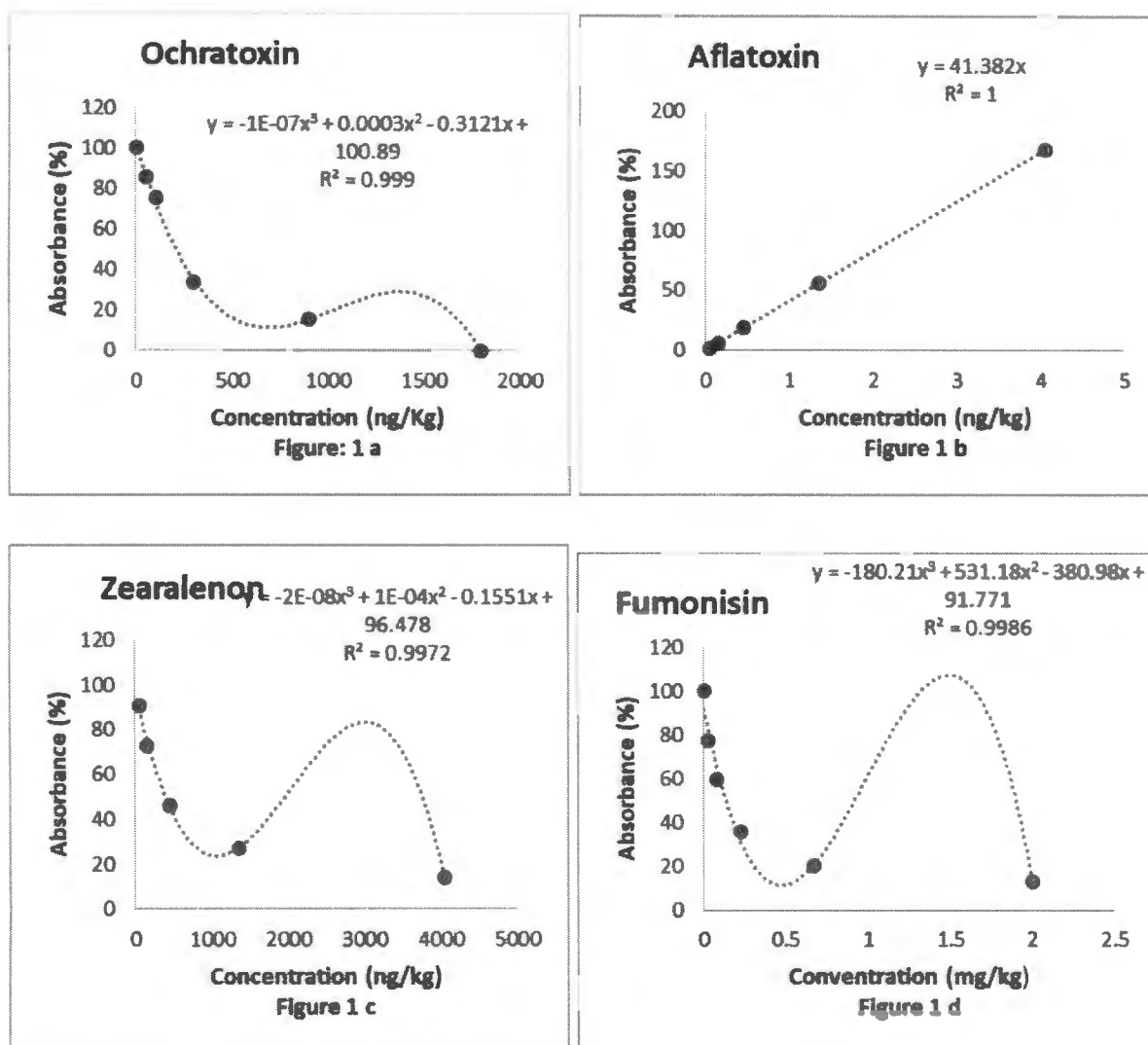


Figure 4. 1 Elisa standard calibration curve for Ochratoxin (1a), Aflatoxin (1b), Zearalenone (1c) and Fumonisin (1d)

Table 4 1 Frequency and concentration of ochratoxin level in dried meat using (Elisa)

Market location	Samples		OTA content (µg/kg)		Samples with Level	
	Analyzed	Positive	Range	Mean ± SD	<1* µg/kg	>1 µg/kg
Ikole	15	15 (100)	0.45 – 0.47	0.46 ± 0.00 ^a	15	0
Ilawe	6	6 (100)	0.44 - 0.45	0.45 ± 0.00 ^a	6	0
Aramoko	12	11 (92)	0 - 0.47	0.42± 0.04 ^{ba}	12	0
Ijero	10	10 (100)	0.44 - 0.46	0.45 ± 0.00 ^a	10	0
Oye	6	6 (100)	0.44 - 0.46	0.45 ± 0.00 ^a	6	0
Otun	15	15 (100)	0.44 - 0.47	0.45 ± 0.00 ^a	15	0
Ado	20	19 (95)	0 – 0.77	0.44 ± 0.03 ^a	20	0
Omuo	14	14 (100)	0.44 - 0.47	0.45 ± 0.00 ^a	14	0
Igede	6	6 (100)	0.44 - 0.47	0.46 ± 0.00 ^a	6	0
Ise/Emure	4	3 (75)	0 - 0.47	0.35 ± 0.12 ^b	4	0
Average mean	108	105 (97)	0 - 0.77	0.44 ± 0.01	10.8	0

The data in parentheses represents the percentage (%) of positive samples Ochratoxin A (OTA), Standard deviation (SD)

Table 4 2 Frequency and concentration of aflatoxin level in dried meat using (Elisa)

Market location	Samples		Aflatoxin content (µg/kg)		Samples with Level	
	Analyzed	Positive	Range	Mean ± SD	<10* µg/kg	>10 µg/kg
Ikole	15	8 (53)	0 - 24.09	6.91 ± 2.42 ^j	10	5
Ilawe	6	4 (67)	0 - 24.83	13.15 ± 4.39 ^c	2	4
Aramoko	12	7 (58)	0 - 24.35	10.35 ± 2.91 ^f	6	6
Ijero	10	6 (60)	0 - 24.83	9.92 ± 3.15 ^g	6	4
Oye	6	6 (100)	21.74- 24.83	23.56 ± 0.58 ^a	0	6
Otun	15	13 (87)	0 - 24.83	14.12 ± 2.86 ^b	7	8
Ado	20	12 (60)	0 - 24.83	7.56 ± 2.03 ⁱ	15	5
Omuo	14	10 (71)	0 - 24.03	11.04 ± 2.82 ^e	8	6
Igede	6	3 (50)	0 - 19.63	8.71 ± 3.93 ^h	3	3
Ise/Emure	4	2 (50)	0 - 24.83	12.41 ± 7.17 ^d	2	2
Average mean	108	71 (66)	0 - 24.83	10.80 ± 0.99	5.9	4.9

The data in parentheses represents the percentage (%) of positive samples

Standard deviation (SD)

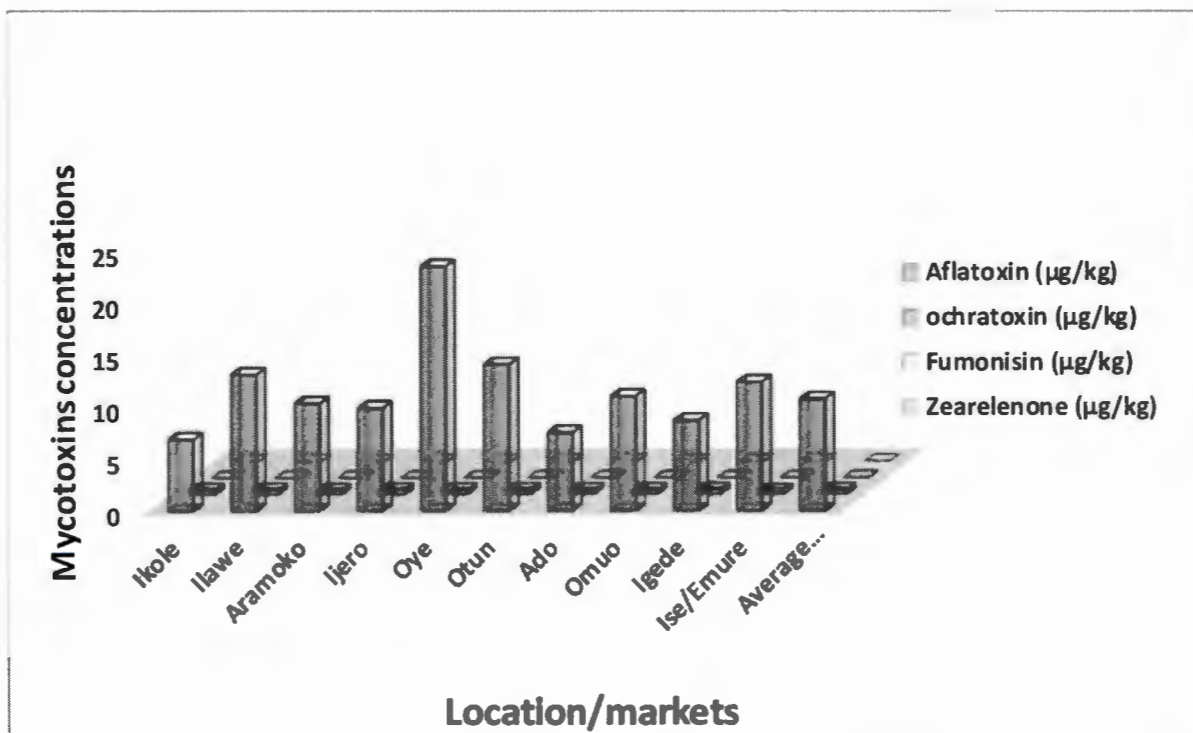


Figure 4. 2 Overall concentration of aflatoxin, ochratoxin, fumonisin and zearelenone in dried meat sold Ekiti State using Elisa

Table 4 3 Multiple reaction monitoring transitions, mass spectrometer conditions and retention times of the determined mycotoxins

S/No	Mycotoxin	Ret. Time (min)	Precursor (mz)	Products (mz)	Q1 Pre Bias (V)	CE	Q3 Pre Bias (V)
1	15ACDON	6.54	355.9	260.9	-12	-16	-30
				136.9	-26	-20	-22
2	3ACDON	6.589	338.9	231	-24	-15	-22
				213	-12	-17	-20
3	HT-2	8.817	425	263	-20	-12	-18
				104.9	-16	-47	-19
4	AFG2	7.507	331	245.1	-12	-32	-24
				313	-12	-24	-20
5	AFG1	7.767	329	243	-12	-28	-23
				311.1	-16	-24	-14
6	AFB1	8.25	313	241	-22	-41	-23
				285.1	-22	-24	-29
7	FB3	8.754	706.3	336.3	-40	-39	-11
				354.4	-20	-35	-24
8	FB2	8.95	706.1	336.3	-20	-38	-22
				318.3	-26	-41	-22
9	B-ZEA	8.949	323.1	277.2	-16	-16	-18
				305.2	-16	-11	-20
10	a-ZEA	9.415	323.1	277.2	-17	-17	-18
				305.2	-24	-9	-20
11	T-2 Toxin	9.674	467.2	245.1	-13	-11	-16
				305.2	-22	-11	-20
12	ZEA	10.063	319.1	185	-12	-27	-30
				187.1	-15	-21	-19
13	AME	10.125	273	128.1	-10	-49	-21
				115.05	-18	-54	-19
14	OTB	9.331	370.1	205	-13	-22	-21
				324.1	-13	-14	-22
15	DON	3.874	297.1	231	-21	-13	-26
				249.1	-14	-12	-25
16	OTA	10.132	403.8	239	-15	-27	-24
				221	-12	-38	-21
17	FB1	7.97	722.2	352.5	-20	-44	-19
				334.4	-26	-42	-11
18	AFB2	8.007	315	259.1	-22	-31	-25
				287	-23	-26	-30
19	TCS	5.02	198.15	125.05	-10	-18	-21
				-	-	-	-

20	Citrinin	5.00	251	205.1	-13	-26	-21
				233	-17	-17	-24
21	STECY	5.01	324.90	310	-22	-24	-30
				281.1	-22	-40	-27
22	CPA	5.01	337.1	196.1	-10	-23	-18
				182.1	-10	-20	-20

Table 4 4 Analytical parameters for LC-MS/MS method validation: matrix effect, linearity, limits of detection (LOD), limits of quantitation (LOQ), recoveries at three spiked concentration levels, intra-day and inter-day precision for the studied mycotoxins.

Mycotoxin	Matrix Effect (%)	Linearity r^2	LOD $\mu\text{g/kg}$	LOQ $\mu\text{g/kg}$	Recovery %			Intra-day(RSD) %			Inter-day (RSD) %		
					Spiked Level			Spiked Level			Spiked Level		
					25	50	100	25	50	100	1	3	5
AFB ₁	73	0.998	2	6.8	114	121	124	10.9	6.5	4.8	4.22	3.71	3.75
AFB ₂	75	0.999	6	17	121	125	127	4.8	2.7	3.15	3.42	4.35	3.59
AFG ₁	77	0.997	6	16	130	129	130	6.68	6.73	3.07	3.63	4.48	3.69
AFG ₂	83	0.994	44	134	114	120	126	8.2	9.03	3.63	3.57	2.12	3.47
STER	47	0.999	8	23	73	71	71	3.71	6.89	13.1	13.2	15.1	14.7
OTA	60	0.997	0.13	0.4	71	70	70	12.4	5.01	3.29	5.18	5.38	6.81
OTB	48	0.999	0.41	1.2	36	36	41	5.43	5.47	7.33	9.51	5.81	6.46
ZEA	73	0.999	123	374	108	113	114	5.47	5.56	4.7	3.12	5.38	3.1
FB ₁	33	0.986	25	75	NR	NR	NR	NR	NR	NR	NR	NR	NR
FB ₂	62	0.997	5	15	NR	NR	NR	NR	NR	NR	NR	NR	NR
FB ₃	68	0.996	17	52	NR	NR	NR	NR	NR	NR	NR	NR	NR
T-2	52	0.992	84	254	59	64	58	4.64	6.31	3.85	6.19	6.09	6.14
HT-2	35	0.999	3	10	81	79	78	16.4	7.62	4.59	4.86	4.62	4.58
15-ACDON	16	0.999	72	217	NR	NR	NR	NR	NR	NR	NR	NR	NR
3-ACDON	39	0.997	25	75	48	52	57	2.33	10.1	6.92	6.94	6.18	6.28
DON	64	0.996	597	1811	NR	NR	NR	NR	NR	NR	NR	NR	NR
AME	66	0.994	55	166	112	114	111	4.41	3.85	2.12	2.34	2.45	2.71
α -ZEA	72	0.998	127	385	117	116	114	3.49	5.54	2.57	3.29	3.55	3.3
β -ZEA	70	0.998	19	57	102	99	99	2.75	4.42	1.81	2.49	3.1	3.37
Citrinin	87	0.998	18	54	101	107	107	2.17	2.6	3.09	4.59	3.33	4.71
CPA	75	0.996	7	20	81	71	71	5.23	4.61	3.15	6.91	6.12	6.11
TCS	43	0.998	8	25	53	26	11	8.39	5.23	5.65	6.38	6.27	6.21

*NR = No recovery for the spiked samples

DON:deoxynivalenol, 3-ADON: 3-acetyldeoxynivalenol, 15-ACDON: 15-acetyldeoxynivalenol, AFG₂: aflatoxin G₂, AFG₁: aflatoxin G₁, AFB₂: aflatoxin B₂, AFB₁: aflatoxin B₁, CIT: citrinin, HT-2: HT-2 toxin, FB₁: fumonisin B₁, T-2: T-2 toxin, α -ZOL: alpha zearalenol, FB₂: fumonisin B₂, ZEN: zearalenone, OTA: ochratoxin A, STE: sterigmatocystin, FB₃: fumonisin B₃: AME: Alternariol Methyl Ether

Table 4 5 Concentration (µg/kg) of mycotoxins in analyzed dried meat samples (n = 108).

Location	Ikole	Ilawe	Aramoko	Ijero	Oye	Otun	Ado	Omuo	Igede	Ise/Emure
HT 2	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
CPA	118.87 ± 52.05 ^{ab}	6.42 ± 4.74 ^{ab}	13.36 ± 5.11 ^{ab}	25.81 ± 10.54 ^{ab}	163.93 ± 94.75 ^a	10.36 ± 8.51 ^{ab}	92.62 ± 44.61 ^{ab}	56.43 ± 38.35 ^{ab}	30.03 ± 14.90 ^{ab}	2.11 ± 1.11 ^b
TCS	35.88 ± 28.74	0.30 ± 0.30	55.83 ± 55.83	0	9.27 ± 4.47	0	18.79 ± 11.19	15.6 ± 14.93	0.16 ± 0.16	0
AFB ₁	282.75 ± 136.66	5.85 ± 5.85	15.6 ± 9.62	66.57 ± 45.71	14.1 ± 8.87	59.81 ± 58.53	137.87 ± 60.05	70.69 ± 51.14	295.41 ± 272.73	3.91 ± 3.91
AFB ₂	15.69 ± 7.32 ^{ab}	0	0.65 ± 0.47 ^b	1.72 ± 0.67 ^b	1.69 ± 1.24 ^b	2.5 ± 2.31 ^b	7.42 ± 2.88 ^b	3.6 ± 2.51 ^b	33.13 ± 31.02 ^a	2.85 ± 2.48 ^b
AFG ₁	88.66 ± 39.09 ^b	0	4.94 ± 1.91 ^b	13.95 ± 9.96 ^b	0	6.82 ± 5.78 ^b	15.45 ± 8.21 ^b	15.56 ± 8.81 ^b	257.35 ± 208.95 ^a	2.24 ± 2.24 ^b
AFG ₂	5.53 ± 2.55 ^b	0	0	0	0.64 ± 0.64 ^b	0.45 ± 0.45 ^b	0	0.38 ± 0.38 ^b	19.08 ± 14.87 ^a	0
ZEA	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
α Zea	137.2 ± 49.00	148.89 ± 124.93	167.34 ± 70.53	47.6 ± 39.14	81.64 ± 51.73	100.83 ± 44.62	103.35 ± 44.46	136.96 ± 52.71	80.33 ± 50.97	134.11 ± 77.59
β Zea	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
CIT	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
T2	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
OTA	0.02 ± 0.02 ^b	0	0.03 ± 0.03 ^b	0.28 ± 0.10 ^b	3.96 ± 3.60 ^a	0	1.12 ± 0.64 ^b	0.02 ± 0.02 ^b	0	0
OTB	0.03 ± 0.02	0	0.01 ± 0.05	0.03 ± 0.03	0.03 ± 0.03	0.01 ± 0.00	0.22 ± 0.19	0.01 ± 0.00	0.01 ± 0.01	0
STER	25.37 ± 13.77	0	2.64 ± 2.64	3.56 ± 1.78	3.06 ± 2.38	1.08 ± 0.59	4.36 ± 3.42	4.98 ± 4.62	5.89 ± 5.89	0
AME	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
3-AC DON	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
15-AC DON	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND

*Letters with different superscript along the same row indicates significant difference,

*Concentration value is the mean of all the samples from the same location.

*values without superscript mean there is no significant difference

Table 4 6 Occurrence (%) of mycotoxins in analyzed samples (n = 108)

Location	HT 2	CPA	TCS	AFB ₁	AFB ₂	AFG ₁	AFG ₂	ZEA	α ZEA	β ZEA	CIT	T2	OTA	OTB	STER	AME	3ACDON	15ACDON
Ikole	0(15)	14(15)	4(15)	8(15)	9(15)	7(15)	4(15)	0(15)	6(15)	0(15)	0(15)	0(15)	1(15)	3(15)	6(15)	0(15)	0(15)	0(15)
Ilawe	0(6)	5(6)	1(6)	1(6)	0(6)	0(6)	0(6)	0(6)	2(6)	0(6)	0(6)	0(6)	0(6)	0(6)	0(6)	0(6)	0(6)	0(6)
Aramoko	0(12)	12(12)	1(12)	6(12)	2(12)	7(12)	0(12)	0(12)	5(12)	0(12)	0(12)	0(12)	1(12)	1(12)	1(12)	0(12)	0(12)	0(12)
Ijero	0(10)	10(10)	0(10)	7(10)	5(10)	4(10)	0(10)	0(10)	2(10)	0(10)	0(10)	0(10)	5(10)	1(10)	4(10)	0(10)	0(10)	0(10)
Oye	0(6)	6(6)	3(6)	4(6)	2(6)	0(6)	1(6)	0(6)	2(6)	0(6)	0(6)	0(6)	3(6)	1(6)	2(6)	0(6)	0(6)	0(6)
Otun	0(15)	13(15)	0(15)	4(15)	2(15)	4(15)	1(15)	0(15)	5(15)	0(15)	0(15)	0(15)	0(15)	1(15)	3(15)	0(15)	0(15)	0(15)
Ado	0(20)	20(20)	3(20)	12(20)	11(20)	6(20)	0(20)	0(20)	5(20)	0(20)	0(20)	0(20)	6(20)	4(20)	5(20)	0(20)	0(20)	0(20)
Omuo	0(14)	13(14)	2(14)	7(14)	5(14)	4(14)	1(14)	0(14)	5(14)	0(14)	0(14)	0(14)	1(14)	2(14)	2(14)	0(14)	0(14)	0(14)
Igede	0(6)	6(6)	1(6)	4(6)	3(6)	3(6)	2(6)	0(6)	2(6)	0(6)	0(6)	0(6)	0(6)	1(6)	1(6)	0(6)	0(6)	0(6)
Ise/Emure	0(4)	3(4)	0(4)	1(4)	2(4)	1(4)	0(4)	0(4)	2(4)	0(4)	0(4)	0(4)	0(4)	0(4)	0(4)	0(4)	0(4)	0(4)
Total %	0%	102	15	54	41	36	9	0%	36	0%	0%	0%	17	14	24	0%	0%	0%
		94%	14%	50%	38%	33%	8%		33%				16%	13%	22%			

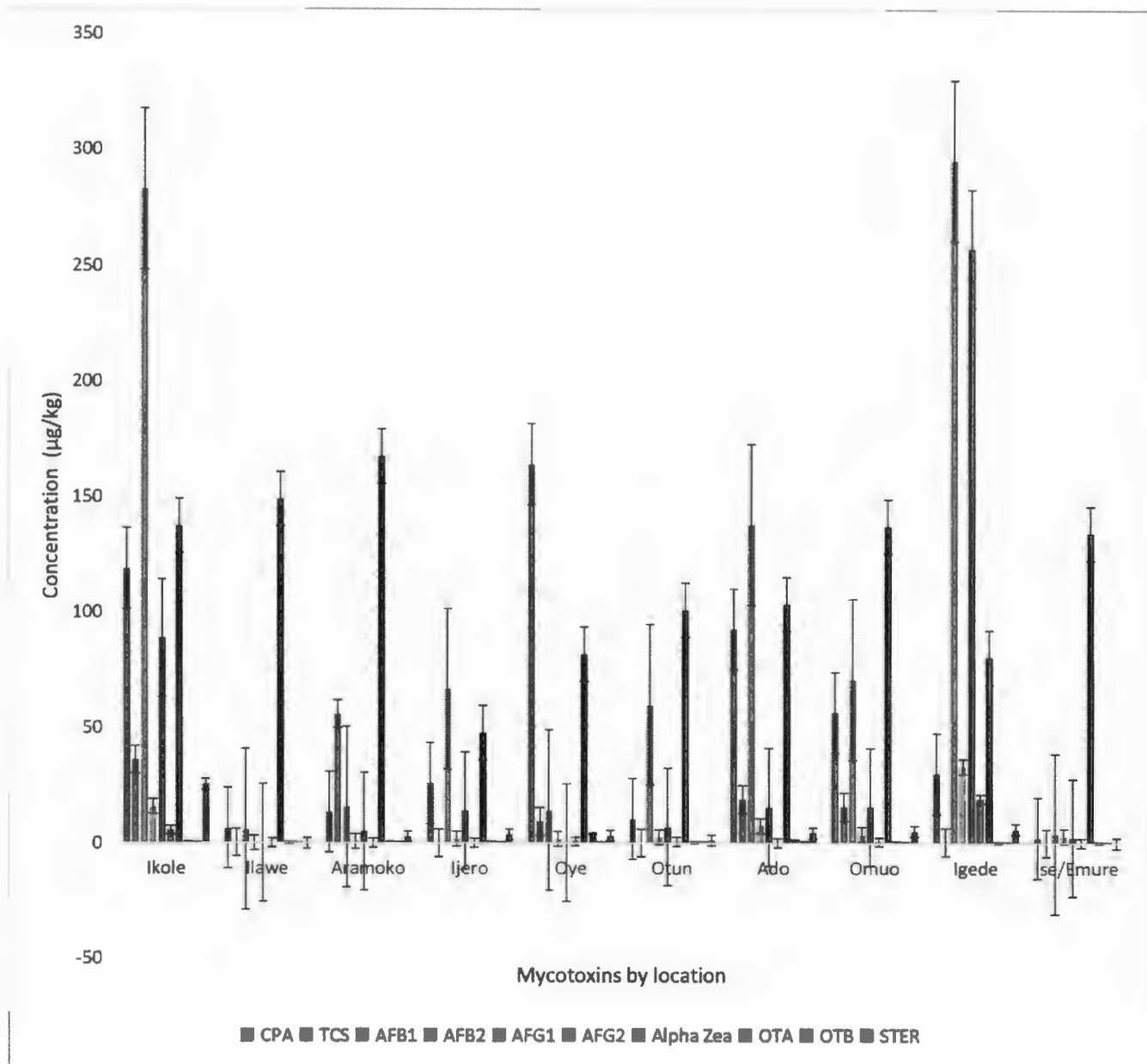


Figure 4. 3 Concentration of mycotoxins in different locations/markets of analyzed samples

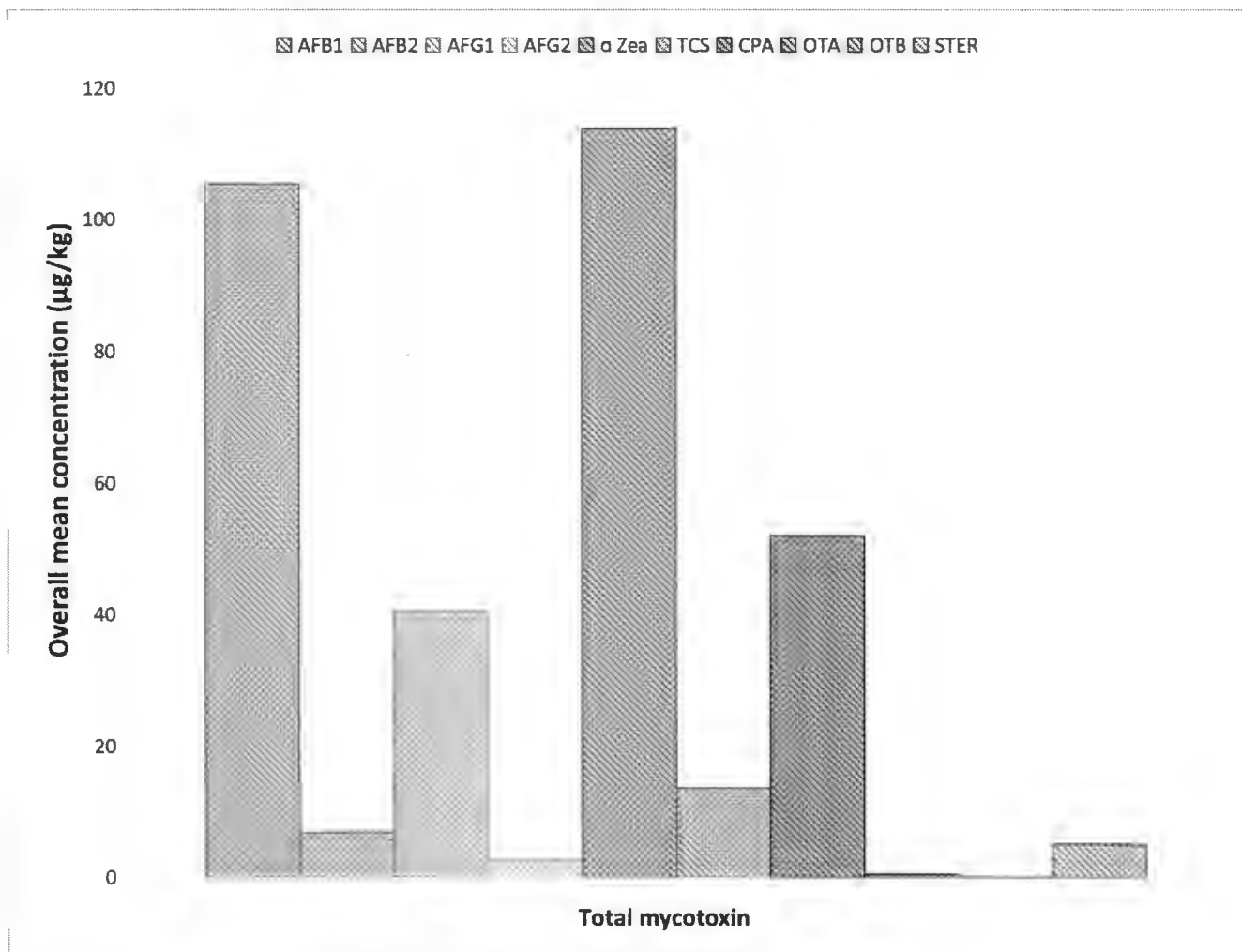


Figure 4. 4 Total mean mycotoxins concentration in analyzed dried meat samples in Ekiti State

Table 4 7 Zero-order correlation coefficients for total fungi isolates, genera and mycotoxin level across the different locations

	1.	2.	3.	4.	5.	6.	7.	8.	9.	10.	11.	12.
1. fungi isolates	---											
2. fungi genera	0.91	---										
3. T. Aflatoxin	-0.28	-0.36	---									
4. AFB ₁	-0.15	-0.29	0.97	---								
5. AFB ₂	-0.35	-0.38	0.98	0.90	---							
6. AFG ₁	-0.39	-0.42	0.95	0.84	0.98	---						
7. AFG ₂	-0.46	-0.46	0.93	0.80	0.97	1.00	---					
8. α Zea	0.28	0.33	-0.24	-0.21	-0.25	-0.26	-0.26	---				
9. TCS	0.44	0.33	0.01	0.10	-0.07	-0.11	-0.14	0.58	---			
10. CPA	-0.16	-0.18	0.15	0.27	0.10	0.00	0.00	-0.22	0.22	---		
11. OTA	-0.31	-0.21	-0.23	-0.23	-0.19	-0.22	-0.17	-0.37	-0.06	0.77	---	
12. OTB	0.57	0.47	0.06	0.19	0.03	-0.10	-0.15	-0.19	0.13	0.38	0.25	---
13. STER	-0.09	-0.29	0.59	0.74	0.45	0.36	0.31	0.10	0.44	0.51	-0.11	0.06

Note. Bold coefficients are significant (*p* < 0.05)

4.7 Results and discussion

4.7.1 Method validation

The results obtained from the validation of the extraction and LC-MS method used for the determination of the mycotoxins in the dried meat samples are shown in (Table 4.1 and 4.2). Recovery values ranged from 114 -130% for all the aflatoxin B₁, B₂, G₁ and G₂. For STE and OTA, the recovery ranged from 70 -73%, while HT-2 had 78-81% recovery values. The matrix effects (ME) evaluation was performed using matrix-matched calibration curves; the suppression of the signal (SS) obtained for all the mycotoxins ranged from 16 to 87%. The obtained limits of detection (LOD) and limits of quantification (LOQ) values for each mycotoxin ranged between 0.13 µg/kg (OTA) – 127 µg/kg (α-ZEA) and 0.4 µg/kg (OTA) – 385 µg/kg (α-ZEA), respectively. For the evaluation of the linearity, calibration curves were constructed for all mycotoxins at twelve different levels concentrations from 0.9 to 2000 µg/kg for all the mycotoxins. The validation results showed strong correlation coefficients ($R^2 > 0.992$). The intra-day accuracy was evaluated by nine determinations at each count level of morning, afternoon and night on the same day, while inter-day precision was determined for three days. The range of relative standard deviations were between 1.81 and 16.4% for the intra-day precision and between 2.12 and 15.1% for the inter-day precision. The recovery results ranged from 70 to 130%, except for OTB, T-2, 3-ACDON and TCS (36-41, 58-64, 48-57, 11-52) respectively. Interestingly, it was discovered that all the standards used for fumonisins (FBs), 15-ACDON and DON to spike blank samples for recovery purpose at the three different concentrations levels could not be recovered. This is because fumonisins are not quantitatively extracted by the extraction solvent used, which was optimized as a compromise between polar, apolar, acidic, and basic components (Warth *et al.*, 2012).

4.7.2 Occurrence of mycotoxins in meats

The analyzed samples in the current study were contaminated with at least four mycotoxins. Most samples (60%) showed co-occurrence of 4 to 10 different mycotoxins. The occurrence of mycotoxins in the samples are shown in Table 4.4. The CPA was the most prevalent mycotoxin which was detected in 94% of the samples with an average mean of 51.99 ppb, followed by AFB₁ with (50%), ranged from 3.91-295.41 with an average mean of 95.25 ppb, AFB₂ has (38%) with a range of 0.65-33.13 and an average mean value of 6.93 ppb, AFG₁ and α-ZEA recording

(33%) with different average mean value of 40.49 and 113.82 ppb, STE (22%) mean value was 5.09 ppb, OTA (16%) has mean value of 0.54 ppb, TCS (14%) has the mean value of 13.58 ppb, OTB (13%) has an average mean value of 0.04 ppb and AFG₂ (8%) recorded an average mean value of 2.61 ppb. However, HT-2, ZEA, β ZEA, CIT, T2, AME, 3-ACDON and 15-ACDON were not detected in any of the samples.

4.7.3 Co-contamination of mycotoxins in meats from the different sampling locations

The occurrence based on mean concentration of each mycotoxin are shown according to the different locations in Figure 4.1. Contamination levels of AFB₁ were higher and were similar in Ikole and Igede market, followed by α -ZEA and then AFG₁ in Igede market. In Ilawe, Aramoko, Otun, Omuo and Ise/Emure, α -ZEA was the dominant mycotoxin. Ilawe and Ise/Emure samples had the lowest mycotoxin co-contamination of 4 and 6 out of the (18) analyzed mycotoxins respectively, followed by Ijero and Otun both with 8 different types of mycotoxins in that order. Ikole and Omuo had 10 different types of mycotoxins that are present in at least one of the samples, this make them the highest locations simultaneous mycotoxin contaminations followed by Aramoko, Oye, Ado and Igede with each recording 9 different mycotoxins separately. This trend of contamination could be as a result of different fungi peculiarity to the different locations where the cattle were fed and reared, as the meat samples are sourced from about three different States from the Northern part of Nigeria. This could also be influenced by storage, air pollution and human contact with the product as well as displaying method. Foods are frequently contaminated with several fungi, and once the temperature and relative humidity at the peak after contamination, there might be likely mycotoxin production (Styriak *et al.*, 1998). It has also been established that relatively low water activity ($a_w < 0.9$) with low pH-values that is less than 6.0 are principally favourable for fungi growth (Hanssen, 1995). The dried meat samples analyzed had low water activity and pH average value of 5.0. This is in congruent with favourable water activity and pH value known to favour fungi and mycotoxin production, hence the likely level of fungi contamination and mycotoxin level discovered in the dried meats.

Previous studies have shown that mycotoxin contamination of both raw and final meat products from pork is very common (Markov *et al.*, 2013; Pietri *et al.*, 2006). Although mycotoxins naturally occurs in meat primarily as a result of an indirect transfer from naturally contaminated feed, Gareis and Scheuer (2000), production, transportation, storage and displaying conditions

can also influence the occurrence of mycotoxins on dried meat sold in open markets where they are not protected from their environment. These findings showed that there are mycotoxins residues in the meat sold in Ekiti markets.

4.7.3.1 Trichothecenes in meat by LC-MS

Some of the most common trichothecenes investigated (DON, 3-ACDON and 15-ACDON) were not detected in this study. DON, 15-ACDON could not be recovered even in the spiked samples. Recovery for 3-ACDON and T-2 was low (48-57% and 58-64 %) respectively, with the exception of HT-2 which recorded recovery rate of 78-81%. The other trichothecenes were detected at low concentrations and low occurrences. Comparism with other studies is very difficult, because a very limited number of publications are available.

4.7.3.2 Ochratoxin A levels in meat by Elisa and LC-MS

The Elisa result showed that out of the total 108 meat samples tested, assessable amounts of ochratoxin A were found in 105 meat samples tested which accounted for 97.2% of all samples. All the 105 samples have low levels of ochratoxin A ranging from 0.00 to 0.77 µg/kg, with an average value of 0.44 µg/kg which is below 1 µg/kg. These values were less than the maximum permissible levels of (1 µg/kg) set by Ministry of Health in Italy since 1999 and in some European Union countries guidelines for the OTA level in pork and pork-derived products, meat products included as provided under the applicable regulations (Dall'Asta *et al.*, 2010).

The LC-MS result showed that OTA was detected in 14% (n=108) of the meat samples at concentrations ranging from 0.02 – 3.96 µg/kg with an average mean of 0.5 µg/kg. Other studies Pleadin *et al.* (2015) reported a natural occurrence in a range of 0.96 -1.29 µg/kg for dry sausage product, and 5.23 µg/kg mean value for all the meat products studied in Egypt (Abd-Elghany & Sallam, 2015). OTA contamination levels observed herein are comparable to the one reported by Sirot *et al.* (2013) where mean value for OTA in French delicatessen meats was 0.25 µg/kg, but lower than the OTA mean value of 5.23 µg/kg reported by Abd-Elghany and Sallam (2015) in beef luncheon and 4.55 µg/kg in beef burger samples respectively.

The comparison between Elisa and LC-MS result showed that 92.7 % of the analyzed meat samples were positive for ochratoxin A using ELISA, while 14 % were positive for LC-MS. The

huge difference could be as a result of ELISA being a screening tests that is capable of showing a high matrix dependence and possible overestimation of levels of contamination due to its speed, ease of operation, sensitivity and high sample throughput (Krska *et al.*, 2008). Although the LC-MS is more accurate in the actual determination of the amount of contamination, the average mean value is of both the LC-MS and Elisa methods were very similar despite the difference in their percentage level of contamination. This study showed that ochratoxin A levels in meat in Ekiti State were lower than the maximum permissible levels of (1 µg/kg) set by Ministry of Health in Italy and in some European Union countries guidelines for the OTA level in pork and pork-derived products.

4.7.3.3 Aflatoxin total levels in meat by Elisa and LC-MS

The Elisa result in this study revealed that the levels of total aflatoxin were in the range of 6.91 – 23.56 µg/kg. Omuo, Ise/Emure, Ilawe, Otun and Oye markets recorded the highest aflatoxin values that are more than the maximal recommended levels for meat products in the values of 11.04 ± 2.82 , 12.41 ± 7.17 , 13.15 ± 4.39 , 14.12 ± 2.86 and 23.56 ± 0.58 respectively. In over 75 countries around the world, total aflatoxins in food are allowed to be up to 10 µg/kg, although in the EU 4 µg/kg is permitted (Herzallah, 2009). The contamination of dried meat samples examined in this study may possibly be the result of inadequate hygienic conditions under which the meat was processed. Consequently, dried meat produced and sold under uncontrolled situations and regularly consumed can denote a likely source of mycotoxins and pose a threat to human health.

While the result of the total aflatoxins detected in this study by LC-MS as shown in Table 4. 3 and Figure 4.2, showed that AFB₁ values ranged between 3.91-295.41µg/kg with a mean value of 105.4 µg/kg, AFB₂ ranged from 0.65-33.13 µg/kg and mean value of 6.92 µg/kg. AFG₁ and AFG₂ ranged from 2.24-257.35 µg/kg, 0.38-19.08 µg/kg with mean value of 40.49 µg/kg and 2.60 µg/kg respectively. The range and mean value recorded in this present study is higher than the range of 1.10-8.32 µg/kg and 0.15-6.36 µg/kg reported by (Herzallah, 2009). AFB₁ is the predominant contaminant among the AFs, contaminating 50% of the meat samples analyzed which is far higher than the 10% reported in chicken meat from Croatia (Iqbal *et al.*, 2014). It is also far above the maximum permissible level (MPLs) adopted by over 75 countries around the

world for AFB₁ and total aflatoxins of 5 and 10 µg/kg (Pleadin *et al.*, 2015) and not more than 2 and 4 µg/kg stipulated by European Union (Herzallah, 2009).

4.7.3.4 Fumonisin and Zearalenone levels in meat by Elisa

Fumonisin and zearalenone were not detected in this study, the findings deviate from the report of Iqbal *et al.* (2014). Their report showed that 52% chicken and 32% egg from central areas of Punjab in Pakistan were contaminated with Zen with the mean value of 2.01 µg/kg and 1.58 µg/kg respectively.

4.7.3.5 Other mycotoxins by LC-MS

Other important mycotoxins detected in the meat samples included, cyclopiazonic acid, sterigmatocystin, α-ZEA interestingly, the concentrations of α-ZEA were relatively higher and ranged from 47.60 µg/kg – 167.34 µg/kg with an average mean value of 113.82 µg/kg in all the meat sampled across the various selling locations. The exact occurrence data and concentration ranges are displayed in Table 4.3 and Figure 4.2. Also, high concentrations of cyclopiazonic acid (from 2.11µg/kg – 163.9 µg/kg) with an average mean value of 51.99 µg/kg were similarly detected in meat intended for human consumption.

4.7.4 The zero-order correlation coefficient of total fungi isolates, their genera and mycotoxin production by LC-MS

The correlation showed that total fungi isolates and their genera tend to have a strong relationship of 0.91 correlation value. However, the relationship tends to be negative with the amount of mycotoxins detected from the dried meat samples. These findings is similar to the findings of Gareis and Scheuer (2000), that mycotoxins occur in meat majorly as a result of an indirect transfer from naturally contaminated feed. This explains the non-relationship between fungi and mycotoxin level. The alpha Zea tends to have a weak positive correlation of 0.28 with the total number of fungi isolated. While OTB and Tenuazonic copper salt inclines to have a moderate positive correlation value of 0.57 and 0.44 respectively with the total number of fungi isolates.

4.8 Potential health risks for dried meat consumers.

Almost all the analyzed meat samples had significant amounts of different mycotoxins, and most people in these areas consume these meat products as a delicacy, as such, average exposure to a multitude of mycotoxins is possible. The continuous exposure of consumers to the dried meat that is contaminated with cyclopiazonic acid (CPA) could result in immunotoxic and hepatotoxic effects that target muscle, hepatic tissue and spleen organ of human (Antony *et al.*, 2003; Burdock & Flamm, 2000). In the case of alpha zearalenone, it is known to be carcinogenic, it causes hormonal imbalance and have effects on reproductive organ (D'Mello *et al.*, 1997; Miller, 1994). The aflatoxin consumption is well-known to be hepatotoxic and suppress the immune system, its target organ is the liver (Steyn, 1995). While the ochratoxin are recognized to be carcinogenic, genotoxic, immune-suppressive, nephrotic and causes upper urinary tract diseases with its target organ being kidney and liver (Pfohl-Leszkowicz *et al.*, 2002). For sterigmatocystin, it is identified as genotoxic, cytotoxic, immunotoxic and carcinogenic which mainly attack the liver, immune system and kidney (Huang *et al.*, 2014; Terao *et al.*, 1978). Tenuazonic acid (copper salt) has been described to exert antiviral, antitumor, antiseptic, cytotoxic, phytotoxic material, and likewise to be highly toxic in living organisms (Asam & Rychlik, 2013).

4.9 Conclusion

In summary, a comprehensive screening for regulated as well as other mycotoxins was conducted in dried meat samples from Nigeria. According to the available literature, this is the first report on the natural contamination of dried meat in Ekiti. Also the efficiency of the LC-MS/MS and extraction solvent employed showed that it was effective for the determination of (CPA, AFB₁, AFB₂, AFG₁, AFG₂, α ZEA, STER, OTA, TCS and OTB) in dried meat. However, it was not effective for the of fumonisin as it was practically impossible to recover the spiked known fumonisin concentration used for recovery in method validation. This might be due to fumonisin polarity and the fact that it is water soluble, or consequence of matrix co-elution that affects its extraction (Griessler & Encarnaç o, 2009). The presence of these mycotoxins in dried meat could be attributed to using meat from animals fed with mycotoxin-contaminated feed or the presence of *Aspergillus* and *oryzae* that showed full potential for the aflatoxin production in the dried meats where they are isolated. This result is in agreement with the observations of

Abdel-Kareem (2010) who found that *A. flavus* isolates have the ability to produce aflatoxin B₁ and B₂ and extracted Aflatoxin B₁, B₂, G₁, G₂ and sterigmatocystin from meat products. The obtained data offers additional material for discuss about the incident of mycotoxins in dried meat and their impact on the food safety. Although further research and expansive exposure studies might be necessary, the findings has provided a new outlook for food safety policy makers for the production, handling, storage and mode of selling dried meats products across the country. It is recommended that further studies be done to know the extent of mycotoxin carry-over into edible tissues like meat when the animals are fed with contaminated feed.

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CHAPTER FIVE

PROXIMATE, MINERAL COMPOSITION AND THEIR CORRELATION WITH TOTAL AFLATOXIN LEVEL AND FUNGAL CONTAMINATION IN DRIED BEEF SOLD IN EKITI STATE.

ABSTRACT

The objective of the present study was to determine chemical composition, mineral content and the possible relationship with the fungal isolates and total aflatoxin of the meat. The crude Protein was determined by the Kjeldahl method using an automatic Kjeldahl system (Automatic Distillation and Titration System Velp Scientifica, UDK 159, Europe) (AOAC, 2002) method. The crude fat was determined according to the described method of AOAC (2002) using (ANKOM Ether Extractor XT10I, USA), crude fiber (ANKOM Fibre Analyzer, A220, USA). While the mineral analysis were determined using inductively coupled plasma mass spectrometry (ICP-MS), Perkin- Elmer, Model NexIONTM 300Q. The chemical analysis showed that the protein content of meat from Igede and Ise Emure had an average value of 78.50 and 78.17%, respectively that is slightly higher than the other values from other locations. The lipid content and ash ranged from 1.97-10.23%, 1.15-3.33% of the fresh weight, correspondingly. The concentrations of a number of elements in the dried meats were not significantly different across the sampled markets, except in their Na and K which were significantly different across the different location at ($p < 0.05$). Chromium was highest in meat from Aramoko market with 0.12 mg/kg in value. The values of Fe, Ca, Zn and Mg showed closed relationship across the locations. Phosphorus value was highest in the Oye market (114.11 mg/kg), whereas Mn and Zn were highest in meat from Igede and Ilawe markets. Nevertheless, the mean chemical and mineral values of the dried meats sampled across the various markets in this study showed that dried meat is very high in protein as against the average protein value found in unprocessed meat, the minerals content is also high which indicates that its consumption can support good human nutrition. Interestingly, the protein, ash, carbohydrate, potassium, magnesium and manganese contents tends to have a strong correlation with total aflatoxin value of the dried meat. While fungi showed weak relationship with the total aflatoxin content of the meat.

Key words: Chemical composition, mineral, nutrient quality, meat, markets, correlations

5.1 Introduction

Meat has previously defines to refere to all animal tissues appropriate as food for human consumption. Commonly meat refers only to meat flesh (skeletal muscle plus any attached connective tissue and/or fat) (Lawrie & Ledward, 2006b). Meat is a good source of many minerals (iron, zinc and selenium) and vitamins (vitamin D, riboflavin, B₁₂) and contains all the essential amino acids (Millward & Garnett, 2010) Meat production involves numerous domestic species, depending on many factors like religious and cultural beliefs, convenience, availability, and so forth (Paredi *et al.*, 2013). The importance of meat is measured in relations to its major chemical constituents such as proteins, fats, carbohydrates, minerals and fatty acids contents Karakok et al. 2010 (Karakök *et al.*, 2010).

Fresh meat is highly perishable due to its biological structure, its storage and freshness could be affected by several factors ranging from temperature, packaging, endogenous enzymes, moisture, light, and microorganisms among several others (Zhou *et al.*, 2010). In this sense, meat processing and other forms of preservations are applied to fresh meat with the aim of increasing its shelf life (Zhou *et al.*, 2010). In several of the developing countries, the infrastructural facilities required to preserve these highly perishable meats in a wholesome state for a longer period of time virtually do not exist or are scarce (Egbunike & Okubanjo, 1999). Consequently, meat is processed to a stable product using the sun and smoke drying to preserve the meat in order to supply the increasing populations with sufficient quantities, good-quality and affordable meat products.

The dried meat is prepared from quality beef by boiling or cooking for 15–30 min, smoking or sun-drying chunk of the beef for about 18–30 h, cooling, stored and packaged in sacks and jute/mat bags. This process reduces its moisture content and makes it less bulky with increase in its protein value to nearly three or four times value of raw protein, while the nutritional quality of dried meat is similar to that of the average cooked meat, with no changes in the vitamin and mineral contents (Egbunike & Okubanjo, 1999; Kinsman, 1982). Dried beef is a stable product with a shelf-life of 6–12 months or even up to two years under ambient temperature (Idufueko, 1984, Okonkwo & Obanu, 1984). Its consumption can be with or without rehydration, and stable under humid condition without refrigeration. Although, it may require occasional sun drying in

some situation to prevent moisture gain, it also gives a unique flavour that makes it appealing to the consumers in great demand.

Metals such as iron, zinc, copper, chromium and manganese are crucial for human metabolism, while some other metals such as lead, cadmium, nickel and mercury are lethal at certain levels (Onianwa *et al.*, 1999). The essential metals play significant roles in biological systems. For instance, iron is an important part for many proteins and enzymes and is a central component of proteins that transport oxygen, regulation of cell growth and differentiation, iron insufficient in the human lead to fatigue, decreased immunity and death (Lemos *et al.*, 2008; Shakerian *et al.*, 2008). Chromium is considered to be an essential trace element for the maintenance of effective glucose, lipid and protein metabolism in mammals (Sacmaci *et al.*, 2012). Dietary that is insufficient in copper have been associated with some various medical ailments such as anaemia, depression of growth, diminished reproductive performance, heart failure and gastrointestinal troubles (Gjerde *et al.*, 1993; Kolachi *et al.*, 2011). Zinc is an important part of over 200 enzymes that are responsible for absorption, metabolism, and reproduction and wound healing. Zinc shortage can lead to cognitive flaws and damages the immune system (Sacmaci *et al.*, 2012). The occurrence of these elements in different environments are largely due to manufacturing processes such as mining, agricultural facilities and mechanization. Air, surface and ground waters, soil, foods and plants are affected by the trace elements that are emitted by these facilities (Behbahani *et al.*, 2014).

The dried meat is mostly produced from cattle particularly from the northern states of Nigeria like Borno, Kano, Kebbi and Kaduna State among others, which were subsequently conveyed to different markets in the south-west zone of the country including Ekiti State. However, scanty information exists on the relationship between nutritional content, fungi contamination as well as the mycotoxin production level in the dried beef meat sold in Ekiti State Nigeria. The chemical characteristics of meat are very essential to both consumers and researchers due to their positive and negative effects (Lebert *et al.*, 2005). Therefore, this study seek to explore the possible relationship between the proximate and mineral composition, fungi and total aflatoxin content of the dried meat.

5.2 Materials and Methods

5.2.1 Sample collection

As previously mentioned in other chapters, One hundred and eight dried meats samples from different markets during 2017 were used. The samples was randomly picked in 10 major markets across 10 local government areas representing North, South, East, West, and Central of Ekiti State, Nigeria. Samples collected from March to May 2017, ground to a fine powder with an electric grinder (0.5 mm) and stored in clean GLAD Zip Seal Bags at -18°C until further analysis. The entire meat sampled were for human consumption.

5.2.2 Chemical composition analysis.

The proximate analyses were carry out according to the procedure of the Association of Official Analytical Chemist (AOAC, 2002). The moisture content of the samples was determined in an oven (Memmert-500, Germany) at 105 °C, to a constant weight (Egan et al., 1990). crude ash was determined by incineration in a muffle furnace (Heidolph-3001k, Germany) at 550 for 10 hrs (Egan et al., 1990) and crude Protein was determined by the Kjeldahl method using an automatic Kjeldahl system (Automatic Distillation and Titration System Velp Scientifica, UDK 159, Europe) (AOAC, 2002) method. Finally, the crude fat was determined according to the described method of AOAC (2002) using (ANKOM Ether Extractor XT10I, USA), crude fiber (ANKOM Fibre Analyzer, A220, USA).

5.2.3 Physico-chemical analysis.

Kent pH meter (model 7020, Kent Ind. Measurement Ltd., Surrey, UK) equipped with a glass electrode was used to measure the pH of the beef. 10 g of meat was weigh and homogenized in 10 ml of distilled water, and these were determined in triplicate in all cases.

5.2.4 Mineral analysis

One gram of ground meat was dried in an oven at 100 °C for 16 h, thereafter; the samples were ashed in a furnace at 600 °C for 6 h. The ash samples were digested in a solution of 8 ml nitric acid (55%) and 2 ml hydrochloric acid (33%). The digested samples were heated on a preheated general purpose laboratory Hotplate (Model 502, South Africa) at 80 °C for 35 min. After cooling, the solution was rinsed into 100 ml volumetric flask and made up to 100 ml with

deionized water. The solution was allowed to rest for 12 h and later filter into 15 ml centrifuge tube in duplicate using (Whatman no.1). The digested samples were analyzed for all elements by inductively coupled plasma mass spectrometry (ICP-MS), Perkin- Elmer, Model NexION™ 300Q in Faculty of Agriculture, Science and Technology, North-West University, Mafikeng, South Africa. Metal concentrations were computed from the mean of two replicates and reported as mg/l.

5.3 Statistical analysis.

The data obtained were analyzed using descriptive statistics (IBM SPSS version 25.0 software) and the resulting data presented as mean $\pm$ standard deviation (SD) of Three separated determinations and given to the analysis of variance (ANOVA) using Duncan's multiple range test at ($p < 0.05$), to find the difference between means.

The Correlation coefficient (r), for the relationship between the aflatoxins, fungi, proximate and mineral content of the dried meat as shown in the preceding chapters, was determined using Microsoft excel application. The interpretation of values was performed according to Pearson's coefficient (r) which are: very weak $0.000 \leq r \leq 0.200$; weak $0.201 \leq r \leq 0.400$; moderate $0.401 \leq r \leq 0.600$; strong $0.601 \leq r \leq 0.800$ and very strong $0.801 \leq r \leq 1.000$ (Christmann & Badgett, 2009). Once one variable increases and the other variable has a tendency to increase, the relationship indicates positive r -value. If one variable increases and the other decreases, the correlation coefficient will be negative.

5.4 Results and Discussion

5.4.1 Chemical composition of the dried meat

The chemical composition of the dried meat samples is in Table 5.1. The fat content ranged from 7.48 – 11.79%, with an average mean of 9.38%. This value is higher than the value that many countries considered as low-fat meat for intramuscular fat (IMF) of <5% (Scollan *et al.*, 2006). The crude protein content ranged from 76.42 – 78.50% with an average mean of 77.13%, crude ash ranged from 0.85 – 0.89 with an average mean of 0.87%. The result of moisture content ranged (from 7.56 – 11.57%). The protein content reported in this study is comparable with the findings of Faleye and Fagbohun (2012)., In their study on the proximate composition of “Tinko” dried meat reported 2.05 ± 0.01 to 3.07 ± 0.40 range for ash content, crude protein

69.98±0.15 to 74.56±0.80 and carbohydrate 4.08±0.05 to 4.48±0.15. The moisture content of the dried meat in this study was the same the range of 7.62 – 9.91% reported by Oladejo and Adebayo-Tayo (2011), in their work on the proximate composition of Tinko sold in Oyo State, Nigeria. According to the Chatfield (1926), meat naturally contains moisture being made up of approximately 75% water, 20% protein and 5% fat, carbohydrates and minerals. The high protein content and the reduced moisture content observed in the dried meat samples could be ascribed to an increase in the dry matter content per unit weight following the drying process (Silva *et al.*, 2008).

Table 5. 1 Chemical composition (%) of dried beef meat sold in Ekiti State

Major markets	Moisture %	Protein %	Fat %	Ash %	Carbohydrate %	pH
Ikole	10.99±1.47 ^{ba}	77.65± 1.52 ^a	7.48±0.50 ^b	0.85±0.01 ^{ba}	3.03±0.74 ^b	5.90±0.01
Ilawe	7.56±0.54 ^b	76.61± 1.73 ^a	11.79±1.48 ^a	0.89±0.00 ^a	3.15±0.80 ^b	5.91±0.01
Aramoko	8.94±1.01 ^{ba}	77.17± 1.99 ^a	9.90±1.45 ^{ba}	0.87±0.01 ^{ba}	3.12±0.83 ^b	5.91±0.01
Ijero	8.82±1.19 ^{ba}	76.52± 1.12 ^a	10.21±0.77 ^{ba}	0.88±0.01 ^{ba}	3.57±0.54 ^b	5.90±0.01
Oye	10.39±1.97 ^{ba}	76.44± 4.14 ^a	9.64±1.15 ^{ba}	0.87±0.02 ^{ba}	3.66±1.74 ^b	5.90±0.01
Otun	9.05±0.25 ^{ba}	76.70± 0.83 ^a	10.25±0.84 ^{ba}	0.87±0.00 ^{ba}	3.13±0.42 ^b	5.90±0.00
Ado	8.43±0.64 ^{ba}	77.21± 2.38 ^a	9.46±0.68 ^{ba}	0.88±0.00 ^{ba}	4.04±1.01 ^a	5.91±0.01
Omuo	11.57±0.74 ^a	76.42± 1.49 ^a	8.06±0.99 ^{ba}	0.85±0.00 ^b	3.10±0.62 ^b	5.90±0.01
Igede	7.56±0.37 ^b	78.50± 2.70 ^a	9.25±0.76 ^{ba}	0.89±0.00 ^{ba}	3.80±1.20 ^b	5.90±0.01
Ise/Emure	9.29±0.70 ^{ba}	78.17± 3.30 ^a	7.80±0.66 ^b	0.86±0.01 ^{ba}	3.88±1.45 ^b	5.90±0.02
Average mean	9.26	77.13	9.38	0.87	3.36	5.90±0.00

Mean proximate ± SD; mean values with different superscript in the same column are significantly different (p < 0.05).

5.4.2 Mineral results of meat samples

The minerals have a physiological interaction and do not play autonomous roles in the human body. For example, calcium and phosphorus are in a fixed relationship in the development of bones and teeth (Engmann *et al.*, 2013; Giovannucci *et al.*, 1998), likewise, iron and zinc were shown to decrease the sickness of diarrhea and pneumonia (Walker *et al.*, 2005). The results obtained from the mineral analysis as shown in Table 5.2.

The sodium (Na) level in the dried beef meat, differs significantly from one location to the other. It ranged from 55.07 - 98.96 mg/kg with an average mean of 76.35 mg/g, this values are higher than the 51mg/g value reported for Australian red meat (Williams *et al.*, 2007), but comparable to sodium of 63 mg/g in raw lean of red meats (Wood *et al.*, 1999). Potassium (K) in all samples ranged from 138.35 mg/kg - 266.49 mg/kg. Potassium is essential for the normal functioning of the nervous system and muscle, the sugar digestion, acid-base stability and oxygen metabolism in the brain. Sodium normalizes the electrolyte and acid-alkali equilibriums, the conductive ability of the nerves, muscle contractions and the production of adrenaline and amino acids. About 3 g sodium per day is required for human and most people consume between 2-4 g per day on the average (Tolonen, 1990). There was a significant difference ($P>0.05$) in Na and K value among the dried meat samples, even though their values were generally high. The relatively high to moderate percentage of Na and K may be attributed to the animal feeding habit, species or breed, but the observed difference in value could be owing to absorption capability and conversion potentials of essential nutrients from their diets or their local environment (Adewoye & Omotosho, 1997).

Calcium serves as the main structural element in the body with ninety-nine percent of body calcium found in the bones and teeth and only one per cent in the soft tissues and body fluids (Engmann *et al.*, 2013). Consequently, meat and offal are mostly low in their calcium content (between 9 - 11 mg/100 g) even though it's readily absorbed by the body (Chan *et al.*, 1995). The calcium level ranged from 14. 60 - 14.75 mg/kg, and its similar across the 10 sampled markets for all the meats. Iron (Fe) level was the same in all the samples with 4.66 - 4.73 mg/kg. Meat is a major source of Fe for adults and children. Its absence in the diet causes anaemia (Demirezen & Uruç, 2006). The average intake of Fe is pegged at 18 mg per day by RDA

(Tolonen, 1990). The results attained in this study revealed that the zinc (Zn) contents of samples were between 1.41 – 2.14 mg/kg. As shown in Table 2, the lowest Zn value was in Oye with (1.41 mg/ kg). Zinc is an important element in the human diet. The low Zn levels can cause problems; however, too much Zn is dangerous to human health (Demirezen & Aksoy, 2006). Views about Zn levels are generally divergent. The adult RDA for Zn is 15 mg (Tolonen, 1990). The results gotten from this study revealed that the magnesium (Mg) and phosphorous (P) contents of samples were 22.42 -22.64 mg/kg and 109.89 -114.11 mg/kg, respectively. The Mg value shows no significant difference in all the meats, while P value had the highest concentrations in Oye (114.11 mg/kg). Magnesium is an important mineral for cell physiological processes and it occupies a key role in entire reactions with phosphate. The cells also need Mg for cell division and enzyme production. The RDA for Mg and P is 2000 and 300-450 mg per day, respectively (Tolonen, 1990).

The average copper (Cu) concentration in the examined samples was 1.27- 1.87 mg/kg, with average concentrations of Cu of (1.57 mg/kg). Copper is important for human well-being, but excess intakes can cause health problems such as liver and kidney damage (Javed & Usmani, 2013) The RDA for Cu is 2 mg. Selenium (Se) levels ranged from 15.52 mg/kg – 18.64 mg/kg which corresponded to each other in the study and nearly similar values were found in all meats. Selenium is an antioxidant, which inhibits the damage of the cells owing to rancidification and delays the pathological aging process. It prevents chromosome damage, mutations and cancer, an intake of 70 µg for healthy men and 50 µg for healthy women is adequate to keep the kinetics in balance (Lobo *et al.*, 2010). The levels of manganese (Mn) in all samples ranged from 0.51 to 2.96 mg/kg, although a small amount of daily intake of Mn is required for growth and good health in humans., If deficient, Mn can cause nervous system problems (Demirezen & Uruç, 2006). The RDA for Mn is 3.8 mg, and studies have shown that actual daily intake is about 5-6 mg (Tolonen, 1990). The concentration of chromium (Cr) in meat samples was between 0.08 – 0.12 mg/kg. Even though Cr is an important element in assisting the body in utilizing sugar, protein and fat, all the same, it is cancer-causing for organisms (Valko *et al.*, 2006). High level of Cr may cause adverse health effects (Smith & Steinmaus, 2009). Requirements for Cr is not yet determined. Based on American estimates, a daily intake of 50-200 µg is a nontoxic and satisfactory dosage (Tolonen, 1990).

Table 5. 2 Mineral compositions of dried meat given as mg/kg dry weight

Mineral	Na	K	Ca	Fe	Zn	Mg	P	Cu	Se	Mn	Cr
Ikole	58.74±0.01 ⁱ	138.35±0.47 ^{ef}	14.62±0.06 ^a	4.69±0.02 ^a	1.66±0.07 ^{ab}	22.64±0.11 ^a	110.31±0.90 ^c	1.41±0.19 ^a	18.03±0.53 ^{ab}	1.01±0.16 ^{bc}	0.08±0.00 ^a
Ilawe	62.00±0.22 ^h	230.69±0.41 ^c	14.67±0.06 ^a	4.73±0.00 ^a	2.14±0.22 ^a	22.61±0.18 ^a	111.92±0.74 ^{abc}	1.70±0.16 ^a	18.57±0.21 ^a	2.09±0.60 ^{ab}	0.09±0.00 ^a
Aramoko	76.95±0.21 ^e	146.96±0.29 ^e	14.75±0.11 ^a	4.73±0.02 ^a	1.64±0.18 ^{ab}	22.51±0.13 ^a	110.69±0.78 ^{bc}	1.87±0.20 ^a	17.43±0.70 ^{ab}	1.21±0.16 ^{bc}	0.12±0.13 ^a
Ijero	79.72±0.01 ^d	206.15±0.53 ^d	14.62±0.07 ^a	4.68±0.03 ^a	1.86±0.21 ^{ab}	22.55±0.14 ^a	110.28±0.91 ^c	1.77±0.20 ^a	15.52±0.79 ^b	1.19±0.27 ^{bc}	0.08±0.00 ^a
Oye	55.07±0.28 ^j	119.83±0.14 ^b	14.65±0.11 ^a	4.73±0.01 ^a	1.41±0.14 ^b	22.42±0.18 ^a	114.11±1.64 ^a	1.40±0.26 ^a	18.64±0.74 ^a	1.20±0.53 ^{bc}	0.08±0.00 ^a
Otun	98.96±0.36 ^a	199.39±0.55 ^d	14.62±0.06 ^a	4.70±0.02 ^a	1.60±0.08 ^{ab}	22.51±0.12 ^a	111.17±0.62 ^{abc}	1.54±0.08 ^a	17.04±0.70 ^{ab}	0.51±0.13 ^c	0.08±0.00 ^a
Ado	83.13±0.11 ^c	261.18±7.58 ^{ab}	14.62±0.05 ^a	4.71±0.00 ^a	1.97±0.15 ^{ab}	22.63±0.10 ^a	111.63±0.79 ^{abc}	1.43±0.12 ^a	17.04±0.60 ^{ab}	2.00±0.42 ^{ab}	0.08±0.00 ^a
Omuo	71.59±0.00 ^f	266.49±8.51 ^a	14.60±0.06 ^a	4.66±0.02 ^a	1.81±0.11 ^{ab}	22.46±0.12 ^a	109.89±0.64 ^c	1.69±0.11 ^a	16.89±0.73 ^{ab}	1.82±0.00 ^b	0.09±0.00 ^a
Igede	68.85±0.03 ^b	265.54±13.66 ^a	14.67±0.11 ^a	4.68±0.06 ^a	1.59±0.13 ^{ab}	22.52±0.20 ^a	110.28±0.99 ^c	1.27±0.25 ^a	17.84±0.53 ^{ab}	2.96±0.00 ^a	0.08±0.00 ^a
Ise/Emure	94.85±0.03 ^b	243.68±16.01 ^{bc}	14.69±0.11 ^a	4.73±0.00 ^a	1.94±0.35 ^{ab}	22.56±0.26 ^a	113.74±0.94 ^{ab}	1.59±0.18 ^a	17.97±0.73 ^{ab}	1.56±0.53 ^{bc}	0.08±0.00 ^a
T mean	76.35±1.29	208.49±5.43	14.64±0.02 ^a	4.70±0.00 ^a	1.76±0.05 ^{ab}	22.55±0.04 ^a	111.09±0.29	1.57±0.55 ^a	17.32±0.23	1.47±0.11	0.09±0.00 ^a

Mean proximate ± SD; mean values with different superscript in the same column are significantly different (p < 0.05). Na = Sodium, K = Potassium, Ca = calcium, Fe = Iron, Zn = Zinc, Mg = Magnesium, P = Phosphorus, Cu = Copper, Se = Selenium, Mn = Manganese, Cr = Chromium

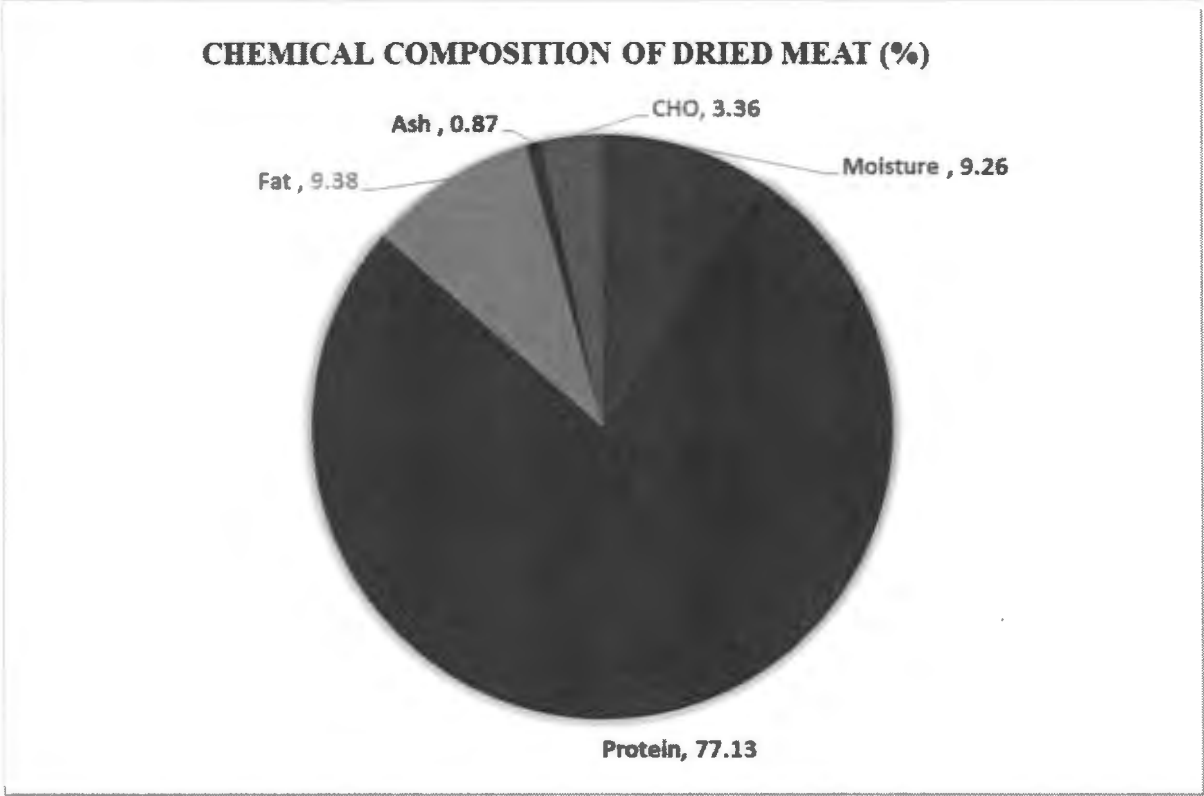


Figure 5. 1 Overall chemical composition of dried meat sold in Ekiti State

Table 5. 3 Zero-order correlation coefficients for all dried meat variables in the study

	1.	2.	3.	4.	5.	6.	7.	8.	9.	10.	11.	12.	13.	14.	15.	16.	17.	18.
1. Total Aflatoxin	---																	
2. T. fungi	-0.28	---																
3. Moisture	-0.13	0.04	---															
4. Protein	0.66	-0.31	-0.29	---														
5. Fat	-0.34	0.05	-0.67	-0.47	---													
6. Ash	0.13	-0.09	-0.92	0.05	0.79	---												
7. CHO	0.15	-0.09	-0.37	0.41	-0.10	0.39	---											
8. Na	-0.27	0.30	-0.22	0.14	-0.03	-0.03	0.22	---										
9. K	0.19	0.30	-0.36	0.25	-0.02	0.27	0.38	0.39	---									
10. Ca	-0.15	-0.11	-0.40	0.36	0.19	0.24	0.02	0.03	-0.23	---								
11. Fe	-0.52	-0.17	-0.32	0.03	0.35	0.22	0.17	0.02	-0.38	0.66	---							
12. Zn	-0.29	0.45	-0.36	-0.05	0.23	0.23	0.11	0.21	0.60	-0.05	0.10	---						
13. Mg	0.23	0.27	-0.34	0.33	0.00	0.15	0.06	0.07	0.19	-0.08	0.09	0.63	---					
14. P	-0.48	-0.43	-0.04	0.00	0.06	0.04	0.46	0.03	-0.22	0.25	0.77	-0.02	-0.18	---				
15. Cu	-0.68	0.50	0.02	-0.48	0.31	-0.09	-0.45	0.23	-0.07	0.33	0.13	0.38	-0.07	-0.19	---			
16. Se	0.07	-0.56	-0.02	0.27	-0.02	0.01	-0.03	-0.49	-0.27	0.37	0.58	-0.14	0.00	0.56	-0.40	---		
17. Mn	0.50	-0.03	-0.47	0.46	0.04	0.50	0.45	-0.27	0.67	0.13	-0.16	0.31	0.12	-0.11	-0.32	0.21	---	
18. Cr	-0.31	0.42	-0.04	-0.13	0.21	-0.04	-0.45	-0.05	-0.26	0.71	0.29	-0.02	-0.17	-0.23	0.68	0.02	-0.07	
19. pH	-0.14	0.72	-0.22	0.04	0.12	0.15	0.18	0.18	-0.04	0.40	0.33	0.10	0.21	-0.09	0.23	-0.15	0.04	0.58

Note. Bold coefficients are significant ($p < 0.05$). T. fungi = Total number of fungi isolate

5.4.3 Correlation between the meat nutrient, aflatoxin level in dried meat

In this study, the number of fungi isolates, moisture, fat, sodium, calcium, iron, zinc, phosphorous, copper, selenium, chromium and the pH, shows a negative relationship ($r=-0.28$, $r=-0.13$, $r=-0.34$, $r=-0.27$, $r=-0.15$, $r=-0.52$, $r=-0.29$, $r=-0.29$, $r=-0.48$, $r=-0.68$, $r=-0.31$, $r=-0.14$; $p<0.05$) value with total aflatoxin level in the dried meat. However, the result also indicated that total aflatoxin has a strong positive correlation ($r=0.66$, $p<0.05$) with the protein content. It also has a moderate correlation ($r=0.50$, $p<0.05$) with Manganese and a weak correlation ($r=0.13$, $r=0.15$, $r=0.19$ and $r=0.23$, $p<0.05$) with ash, carbohydrate, potassium and magnesium respectively. There is no available information to back this finding, as this might be the first time of reporting correlation analysis that involved proximate, mineral, pH of dried meat as substrate for fungi and aflatoxin synthesis, and the level of the toxin.

Equally, in terms of fungal nutritional need and the mineral content relationship, the coefficient of correlation revealed that moisture and fat has a very weak positive relationship ($r=0.04$, $r=0.05$; $p<0.05$) while magnesium, sodium, potassium exhibited a weak positive correlation ($r=0.27$, $r=0.30$, $r=0.30$; $p<0.05$). Furthermore, zinc, copper and chromium showed moderate positive correlation ($r=0.45$, $r=0.50$, $r=0.42$; $p<0.05$). However, the pH had a strong positive correlation ($r=0.72$) with fungal contamination. There was no previous report on this that we can also use to buttress our findings. This study established that the mineral and chemical composition of the dried meat to some extent played a positive role in the aflatoxin production as well as the fungi contamination isolated from the dried meat samples.

5.5 Conclusion

This study has shown that dried meat sold in major markets in Ekiti State in Nigeria, has high protein content and commensurable amount of essential minerals. It has also revealed that the meat had slight presence of heavy metals. The detected quantities were, however generally below the maximum permissible levels, set by the World Health Organization for Pb (0.3 ppm), Cd (0.2 ppm), Hg (0.2ppm) and Cr (0.5ppm) and hence pose no risk to consumers (Amos-Tautua *et al.*, 2013). These findings also concludes that dried meat sold in major markets in Ekiti State in Nigeria, need appropriate monitoring as processing, storage, handling and marketing practices could have serious effect on the safety of the meat product.

This dried meat, if properly processed and preserved can be used to tackle the protein malnutrition in developing countries and in areas where conflicts, drought and climatic change has hinder and prevent animal production. Additionally, the correlation coefficients of the possible relationship between some of the chemical content and the mineral composition of the dried meat revealed a strong potential of some of the nutrient and mineral to influence the level of fungi contamination as well as the total aflatoxin discovered in the analyzed meat samples sold in Ekiti State.

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CHAPTER SIX

GENERAL DISCUSSION, CONCLUSION AND RECOMMENDATION

6.1 General discussion

The fungal contamination of dried beef meat could be due to animal's feeding on contaminated feeds, improper sanitation during slaughtering, handling, storage, transportation and mode of selling the dried meat in an open market without protection from the environment. These contaminations reduces the quality of the product and make it unfit for human consumption resulting in significant economic losses or public health hazard to the consumer. One hundred per cent (100%) of the dry meat analyzed were contaminated with fungi of different kinds with a total fungal count of 1.0 to 9.5 log₁₀ cfu/g and an average mean of 3.1 log₁₀ cfu/ g (Table 3.1). In total, 118 fungal species were isolated through the ITS section amplification and sequencing of the PCR amplicons of the isolates (Table 3.2).

Results obtained for biodiversity of fungi showed a range of filamentous fungi genus including *Aspergillus*, *Penicillium*, *Fusarium*, *Alternaria*, *Cladosporium* and *Rhizopus* contaminating the dried meat with *Aspergillus* (43%), *Rhizopus* (42%), *Penicillium* (3%), *Fusarium* (2%) and other fungi (7%) species dominating most of the samples. High incidences were recorded for *Aspergillus flavus*, *Aspergillus niger* and *Aspergillus fumigatus* in most of the samples screened. The occurrences of these filamentous fungal species pose a reason for concern, as most of these fungal species produces toxic substance

For the aflatoxin production ability of *Aspergillus* fungi isolated in this study, the presence of essential aflatoxin biosynthetic regulatory genes, especially *aflD* and *aflM*, have been linked to *aflR* in the aflatoxin biosynthetic pathway, were established in almost all of the *Aspergillus* isolates, even though 39 % of the isolates expressed the *AflR* gene among them. Most of the isolated *Aspergillus flavus* shown potential to produce mycotoxins under suitable condition and this can lead to public health risk for the consumers. The high occurrence of toxigenic fungi might be due to the widespread distribution of these fungi in the environment and

cross-contamination from other agricultural products that were displayed close to the dried meat in the markets (Fig 3.1).

This research likewise established a relationship between the isolated and identified fungi from the dried meat with other fungi species reported from different sources in other countries from GenBank. The *Aspergillus* species showed good and strong nucleotide signature with their relative counterpart from the GenBank. This demonstrates that these fungal species have high similarity index with their counterpart from GenBank as they have above 70% expected borderline for the degree of relatedness.

Mycotoxin analysis and data obtained from 108 dried meat samples revealed that 92.7% of the samples contained OTA although at a level below the maximum permissible levels of 1 µg/kg. Sixty-six samples contained AF, with mean values of 0.44 µg/kg and 10.80 µg/kg respectively for Elisa and LC-MS. Aflatoxin total result showed that AFB₁ with the mean value of 105.4 µg/kg, AFB₂ (6.92 µg/kg), AFG₁ and AFG₂ have an average mean value of 40.49 µg/kg and 2.60 µg/kg respectively. The established levels of AFs were far above the permissible level in food (10 µg/kg), although in the EU, not more than 4 µg/kg threshold is allowed. The concentrations of α-Zea and cyclopiazonic acid were relatively higher with an average mean value of 113.82 µg/kg and 51.99 µg/kg respectively.

This study as shown in mean average chemical composition (Fig.2 and Table 2), shows that the dried meat sold in Ekiti State is rich in protein and mineral content. This dried meat, if properly processed, preserved, packaged and protected during the storage and selling environments could be use to tackle the protein malnutrition in developing countries, especially in areas where conflicts, drought, and climatic change has hindered and prevented animal production. The mineral content of the dried meat is in abundance, which can help millions of people to meet their essential mineral requirement through adequate consumption. It also showed that the meat is generally low in the heavy metals that when compared to the maximum permissible levels set by the World Health Organization for Pb (0.3 ppm), Cd (0.2 ppm), Hg (0.2ppm) and Cr (0.5ppm) and hence pose no risk to consumers.

The correlation study between fungi isolates, moisture, fat, sodium, calcium, iron, zinc, phosphorous, copper, selenium, chromium and the pH with aflatoxin production showed a negative relationship ($r=-0.28$, $r=-0.13$, $r=-0.34$, $r=-0.27$, $r=-0.15$, $r=-0.52$, $r=-0.29$, $r=-0.29$, $r=-0.48$, $r=-0.68$, $r=-0.31$, $r=-0.14$; $p<0.05$) in the dried meat samples. However, the result

also indicated that total aflatoxin has a strong positive correlation ($r=0.66$, $p<0.05$) with the protein content, and moderate correlation ($r=0.50$, $p<0.05$) with Manganese. Meanwhile, it showed a weak correlation ($r=0.13$, $r=0.15$, $r=0.19$ and $r=0.23$, $p<0.05$) with ash, carbohydrate, potassium and magnesium respectively. Likewise, fungal level and the mineral content revealed that moisture and fat has a very weak positive relationship ($r=0.04$, $r=0.05$; $p<0.05$) while magnesium, sodium, potassium exhibited a weak positive correlation ($r=0.27$, $r=0.30$, $r=0.30$; $p<0.05$) also zinc, copper and chromium showed moderate positive correlation ($r=0.45$, $r=0.50$, $r=0.42$; $p<0.05$). However, the pH had a strong positive correlation ($r=0.72$) with fungal contamination. There was no previous report on this that we can also use to buttress our findings. This study established that the mineral and chemical composition of the dried meat to some extent played a positive role in the aflatoxin production as well as the fungi contamination isolated from the dried meat samples.

6.2 Conclusion

This study has shown that dried meat sold in major markets in Ekiti State in Nigeria, although rich in protein with commensurable amount of essential minerals are contaminated with mycotoxigenic fungi and mycotoxins that are of public health concern. Similarly, it concluded that dried meat sold in major markets in Ekiti State- Nigeria, needs appropriate monitoring as production, handling, storage and marketing practices could have a severe effect on the safety of the dried meat product as well as the health of their consumers. These findings however put forward that the information obtained from this study should be embrace for mycotoxin regulations, while instituting investigation programmes on a constant basis to protect public health.

6.3 Recommendations

Further study should be design to elucidate the factors responsible for the fungal and mycotoxin profiles of the product. I.e. evaluate the weather conditions and feeds of animals used for the dried meats.

Effort towards periodic examination of fungi and mycotoxin contamination of dried meat might be necessary to keep track of their safety and create awareness among consumers, producers and the sellers.

The risk assessment of mycotoxin from dried meat consumption should be determine, through dietary intake levels of the toxins from the dried meat, toxicity of the levels of toxins founds using cytotoxicity evaluation or animal studies and/or cultured cell.