Molecular Characterization of Aflatoxigenic Moulds and Effects of Processing on Aflatoxin Contamination of Some Wheat Mills from Northern Nigeria

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A THESIS SUBMITTED TO THE SCHOOL OF POST GRADUATE STUDIES, AHMADU BELLO UNIVERSITY, ZARIA IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE AWARD OF DOCTOR OF PHILOSOPHY DEGREE IN MICROBIOLOGY

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JULY, 2017

DECLARATION

I declare that the work in this thesis entitled ` MOLECULAR CHARACTERIZATION OF AFLATOXIGENIC MOULDS AND EFFECTS OF PROCESSING ON AFLATOXIN CONTAMINATION OF SOME WHEAT MILLS FROM NORTHERN NIGERIA ` has been carried out by me in the Department of Microbiology. The information derived from the literature has been duly acknowledged in the text and a list of references provided. No part of this thesis was previously presented for another degree at this or any other institution.

. _____ .	. _____ .	. _____ .
JOSEPH REUBEN WARTU	Signature	Date

CERTIFICATION

This thesis entitled "MOLECULAR CHARACTERIZATION OF AFLATOXIGENIC MOULDS AND EFFECTS OF PROCESSING ON AFLATOXIN CONTAMINATION OF SOME WHEAT MILLS FROM NORTHERN NIGERIA" by Joseph Reuben WARTU meets the regulations governing the award of the degree of Doctorate of Philosopy of Ahmadu Bello University, Zaria, and is approved for its contribution to knowledge and literary presentation.

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DEDICATION

This work is dedicated to my parents and children

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God bless us all.

ABSTRACT

Aflatoxins are carcinogenic and genotoxic secondary metabolites that represents serious health risk to both human and animals. Standard methods were used to determine total moulds viable count and some physicochemical parameters on in-process and major store samples. ELISA technique was employed to detect and quantify total aflatoxins from the samples. While yeast extract sucrose agar was modified with 0.3% cyclodextrin and 0.6% sodium desocholate to detect aflatoxigenic strains, multiplex PCR was used to amplify the key genes (*aflR-1, omt-A, ver-1, nor-1*) that code for enzymes involved in aflatoxin biosynthetic pathway. The nucleotide sequences obtained from *AFL2T* was used for phylogenetic studies. The mean moisture of In-process wheat products, such as wheat flour (13.90±0.37%) was significantly higher than that of major store samples (13.25±0.25%). In-process and major store samples had *Aspergillus flavus* as the most frequent occurring moulds (75.0 and 79.3%) respectively and least by *Aspergillus fumigatus* (3.0%). The predominant aflatoxigenic strain was *Aspergillus flavus* (37.7%) and 43.0% from in-process and major stores respectively. Bran had the highest percentage of aflatoxigenic moulds in both in-process (66.0%) and major stores (78.0%), while wheat flour had the least in-process (17.5%). The study had aflatoxin prevalence of 38, 21, 28, 39 and 89% for wheat, semolina, wheat flour, brown flour and bran respectively. As the extraction rate of wheat products such as wheat flour increased from 60 - 75%, the ash content and the total aflatoxin retention increases from 0.57 - 0.82%dm and 2.1- 65.9% respectively. The aflatoxin decontamination decreased from 98.0 - 34.1%. The effects of solid substrate fermentation on total aflatoxin contaminated wheat flour into leavened bread showed significant aflatoxin loss (55.2%) at pH 5.8 and 37^{o}C/120 minutes fermentation with 44.8% aflatoxin retention. Further aflatoxin loss was recorded (58.6 – 72.4%) as the baking temperature increases from 180 –

220^{o}C. Similar aflatoxin reduction after 48h of submerged fermentation of wheat-sorghum composite `Kunun-zaki` was 18.9 and 52.90% for refrigerated and ambient temperature storage respectively. Quadruplet PCR pattern was consistent at 1032, 797, 537 and 400 bp with all the aflatoxigenic strains indicating amplification of the four genes (*aflR-1, omt-A, ver-1* and *nor-1*) of the aflatoxin biosynthetic pathway, whereas the non-aflatoxigenic strains presented one, two and three banding patterns. The frequency of occurrence of aflatoxin biosynthetic gene in non aflatoxigenic strains were: *nor-1* (73.3%), *ver-1* (53.3%), *omt-A* (40.0%) and *aflR-1* (13.3%). Enough homology was found through alignment of *AFL2T* gene sequences with standard *aflR-1* of NCBI gene bank. The gene *AFL2T* nucleotide sequence generated dendogram produced two main clusters. All the twelve isolates were similar in their morphological characteristics, but genetically varied. The strains differentiated into groups and sub-group indicating general similarity. Some of the isolates varied greatly with visible mixtures of aflatoxigenic and non aflatoxigenic moulds and also with random mixture of in-process and store isolates with different genetic diversities at different leaf nodes within the two major clusters. Aflatoxigenic moulds counts and the total aflatoxin levels in some samples from this study could be a health threat to consumers.

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CHAPTER ONE

1.0 INTRODUCTION

1.1 Background of the Study

Aflatoxins are mycotoxins produced by certain strains of moulds as secondary metabolites that represent a serious risk for human and animal health (Noelia *et al.,* 2011; Dorwish *et al*., 2014). Aflatoxins are difuranocoumarin derivatives produced via a polyketide pathway (Yu *et al.,* 2004; Juan *et al*., 2008) by two fundamental aflatoxigenic strains; *Aspergillus flavus* and *Aspergillus parasiticus* (Klich *et al.,* 2000, Bennett and Klich, 2003; El-Khoury, 2011). Other very rare aflatoxin producers have also been reported (Horn, 2007). These two aflatoxigenic species have been reported to have different toxigenic profiles: *Aspergillus flavus* produce aflatoxin B_1 (excreted in breast milk as M_1), B_2, cyclopiazonic acid, aflatrem, 3-nitropropionic acid, sterigmatocystin, versicolorin A and aspertoxin, *Aspergillus parasiticus* produces aflatoxin B_1, B_2, G_1, G_2 and versicolorin A (Wilson *et al.,* 2002). Aflatoxin B_1 is usually the most predominant and the most toxic metabolite within this family and is also the most potent genotoxic agent and hepatocarcinogen to man. The International Agency for Research on Cancer (IARC) classified aflatoxin B_1 as a human group one carcinogen (IARC, 2002). Incidence of aflatoxin poisoning has been known primarily to occur through ingestion of contaminated foods or feeds and to some extent through skin contact. The diseases caused by aflatoxin consumption are loosely called aflatoxicosis. There are two types of aflatoxicosis; acute aflatoxicosis which results in death, and while chronic aflatoxicosis give rise to cancer, immune suppression, and other "slow" pathological conditions (Etzel, 2002). Aflatoxin contamination has been linked to increased mortality in farm animals and also significantly lowers the value of grains as an animal feed and as an export commodity (Aydin *et al.,* 2008).

In order to protect the consumers, the United Nation Protein Advisory Group has enacted tolerance maximum level of 30 µg/kg of aflatoxins in food supplements for undernourished children in developing countries, while the German Government permissible levels of aflatoxin in milk for infants is 50 ng/kg and 10 ng/kg for dietetics (Adegoke *et al.*, 2010). The European Union enacted tolerance level of not greater than 5 and 3 mg/kg for cereals and cereal products respectively (Commission of the European Communities, 2006) and maximum tolerable limits of 4 µg/kg for total Aflatoxins (B_1, B_2, G_1, G_2) and 2 µg/kg for AFB_1 respectively (European Commission, 2010). The National Agency for Food and Drug Administration and Control (NAFDAC) and the Standards Organization of Nigeria (SON) regulates the same aflatoxin level in unprocessed foods at maximum permissible level of 10 µg/kg in flours and consumable oils; it is 4 µg/kg in processed foods (Nigeria Industrial Standard, 2002).

Many researchers have specifically reported the occurrence of aflatoxigenic moulds and aflatoxins in cereals such as maize (Magan *et al*., 2011), wheat (Halt, 1994; Amra *et al*., 1996; El-Tawila, 2003; Cotty and Mellon, 2006; Norlia *et al*., 2011) a basic product in human diet and also in processed wheat flour (Gashgari *et al.*, 2010; Bhat and Fazal, 2011) and wheat flour products such as fermented bread (Chelae *et al*., 2003; Gumus *et al.*, 2009), macaroni (5.91 µg/kg), noodles (7.35 µg/kg) and spaghetti (9.12 µg/kg) (Iqbal *et al*., 2013).

Mould and aflatoxin contamination of wheat have been reported to take occur during periods of preharvest, processing, transportation and storage of the cereal (Bhat and Fasal, 2011) and such contaminants may be transferable during processing into finished products.

Wheat (*Triticum* sp *Link*) (Belderok *et al*., (2000) is a cereal grain which could be milled to produce wheat flour and other products like semolina (Herrera *et al.*, 2009), brown flour and an animal feed product called bran. Globally, wheat is the leading source of vegetable protein in

human food, having higher protein content than either maize (corn) or rice, and other major cereal flours. Wheat grain in its natural unrefined state features a host of important nutrients; vitamin B_1, B_2, B_3, E, folic acid, calcium, phosphorus, zinc, copper, iron, and fiber (Belderok *et al.,* 2000). Apart from carbohydrates, the mealy endosperm of wheat contains fats (1.5%) and very high proteins level (13-18%), albumins and globulins (Sramkováa, 2009). The major proteins of the gluten complex are glutenins and gliadins (Duarte *et al.,* 2010) .This is the protein that forms the gluten at dough making and this composition makes wheat a classical rich energy-yielding raw material. Wheat flour is a staple food used to make leavened, flat and steamed breads, biscuits, cookies, cakes, breakfast cereal, pasta, noodles, couscous (Gashgari *et al.,* 2010) and also used to produce fermented beer.

Wheat flour production involves a series of steps that may breed not only moulds but also bacteria. Such procedures include conditioning, and incubation. Conditioning is a procedure in milling which involves addition of calculated volumes of purified water to already pre-cleaned dry wholesome wheat. The wetted grains are then incubated in a large damp or incubation silos for 18-72h. This process raises the moisture content of the wheat grain from between 9 and 10 % to minimum of 15.0 %. Such changes in grain moisture contents of 1 – 2 % or more have been reported by Weidenbo¨ rner *et al.* (2000) to be sufficient to favour mould growth. Consequently This condition could lead to aflatoxin production.

Though aflatoxigenic moulds have been isolated and differentiated at morphological levels, this method requires mycological expertise and is usually time consuming. However, Polymerase chain reaction has now been a vital tool in fungal phylogenetics, where determining the DNA structure holds the promise of attaining true identity of the isolates in question. Polymerase chain reaction (PCR) technique has been used by many researchers (Gherbawy and Yaser, 2003;

Neissen, 2007; Cruz and Buttner, 2008; Gashgari *et al.,* 2010; Meng *et al.,* 2010; Alborch *et al*., 2012) to detect aflatoxigenic strains. This method focuses solely on amplifying conserved regions, such as *omt-A*, *var-1*, *nor-1* and a regulatory gene locus a*flR-1*, of the DNA. The *aflR-1* gene is known to regulate the structural genes positively at the level of transcription (Michael *et al*., 2005) during the biosynthesis of aflatoxins.

1.2 Statement of Research Problem

Cereals such as wheat amongst other commodities are naturally contaminated with aflatoxigenic moulds. Sometimes wheat and other crops become contaminated with aflatoxins in the field before harvest, where they are usually associated with drought stress (Diener *et al.,* 1987; Klich, 2002).The aflatoxigenic moulds render the cereal unsafe and unwholesome for consumption and processing. Thus such raw material could pass this contaminants into products processed from them. Furthermore wheat flours from contaminated raw materials are usually stored in houses under conditions that favour mould growth since most of the wheat flour storage houses are inadequate and poorly designed especially at the retail levels. The most important variables in wheat flour processing factories during storage are the moisture content of the wheat grains and that of the finished products such as wheat flour and the relative humidity of the surroundings (Wilson and Payne, 1994*;* Kelly, 2009). In line with this, the usual minimum 15% raw material damping moistures could be a suitable condition for breeding not only moulds but for other microorganisms such as bacteria. When cows consume aflatoxin-contaminated feed produced from wheat grains or wheat bran the animals metabolically biotransform aflatoxin B_1 into a hydroxylated form called aflatoxin M_1 (Van Egmond, 1989). The aflatoxin M_1 is then passed into cow milk to serve as an indirect reservoir of aflatoxin which can be a threat to consumers of such contaminated milk.

Recycling is a practice of returning floor wheat flour spillages and air filter materials back into the in-process milling streams. This practice serves as an inoculum of inestimable microbes of varying degrees into the milling system which may consequently, reconterminate the processing lines and thus exerts deleterious effects on human and animal consumers of such batch of wheat flour products.

1.3 Justification

Aflatoxin contaminations of foods and feeds have been a threat to health and international trade. Aflatoxin contamination leading to aflatoxicosis was first reported in 1960 from England, where about 100,000 turkeys and several other domestic birds died of aflatoxicosis (aflatoxin poisoning) after feeding on oil cake feed contaminated with aflatoxin, a highly potent mycotoxin produced (Kelly,2009). There have been several other documented cases of aflatoxicosis in both humans and animals; there was an outbreak of aflatoxicosis in western India in 1974 which resulted in 397 recognized cases and 106 deaths (Ananth and Farid, 2003). Similar cases was also experienced during the summer of 1987 in India, an outbreak of gastrointestinal disorder which affected thousands of residents of Srinagar and the surrounding areas in the Kashmir Valley after consuming wheat that was contaminated with both *Aspergillus* and *Fusarium* species (Anant and Farid, 2002). Also in Africa, Kenya 2004 several people were reported hospitalized and 125 victims died after eating maize contaminated with *Aspergillus flavus* (Anant and Farid, 2002). Similar mortality was recorded in Malawi, where 300 people died after eating aflatoxin contaminated cereal meal (Aziz-Baumgartner, 2005; Charles, 2007).

Processed wheat flour by national regulatory standards in Nigeria is date marked for three months before expiry. This could create a long time breeding or incubation period for

aflatoxigenic moulds to develop and produce toxins since the moisture of the wholesome wheat are usually raised from between 9 – 15.5% before milling.

Growth temperature requirements for *Aspergillus flavus* have been reported to be between 12 - 48 ºC, with optimum temperatures being between 25 - 42 ºC (Kelly, 2009). Coincidentally, the optimum temperature requirements for aflatoxin production have been reported to be between 24 - 35ºC when moisture content exceeds 10% with ventilation (Williams *et al*., 2004). These temperatures are a tenable in the wheat storage bins and especially damping bins due to grain respiration which lead to increased temperature in the wheat or grain storage bins. So there is need for continual in-process screening of wheat and wheat products in order to obtain wholesome raw materials and products. The result from this research could serve as a source of awareness and also as a baseline for national regulatory bodies in monitoring the spate of aflatoxins in wheat and products.

1.4 Aim and Objectives

1.4.1 Aim of the research

The aim of this research was to characterize aflatoxigenic isolates and to study the fate of aflatoxins during wheat processing into products.

1.4.2 Specific objectives

The specific objectives of this study were to:

1. determine some proximate composition on wheat and wheat products
2. isolate aflatoxigenic moulds from in-process wheat and major store wheat products.
3. detect and quantify total aflatoxin contents in wheat and wheat products

4. determine the fate of aflatoxins during wheat processing, fermentations and baking of wheat flour into dough and bread.
5. Molecularly characterize aflatoxigenic moulds from wheat and products

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1Moulds

Moulds are living organism that belongs to the fungal kingdom. While moulds have multicellular filamens referred to as hyphae, the counterpart (yeast) are fungi that usually exhibit single – celled growth and are referred to as being unicellular (Moore *et al*., 2011). There are common types of fungi that thrive in moist, warm conditions and are an important part of our ecosystems yet can be a nuisance. Moulds are heterotrophic and must gain nutrients from other organic substances. Moulds secrete enzymes that break down food substance into smaller organic molecules that can then be absorbed. Moulds have taxonomically diverse number of fungal species and the growth of hyphae could results in discoloration and a fuzzy appearance on food. Moulds have a network of tubular branching hyphae called a mycelium. The mycelium appears like very fine, fluffy white threads and is considered as a single organism. There may be cross-walls (septa) that delimit connected compartments along the hyphae, each containing one or multiple, genetically identical nuclei (Madigan and Martingo, 2005). At one end of the hyphe usually a structure called vesicle is formed on which conidia, also develop by differentiation (Bennet, 2010). The dusty texture of many moulds is caused by profuse production of asexual spores (Pitt and Hopkins, 2009). The mode of formation and shape of these spores is traditionally used to classify molds. Many of these spores are coloured, making the fungus much more obvious to morphological identification. In the past, most moulds were classified within the Deuteromycota, but they are considered to be microbes and do not form a specific taxonomic or phylogenetic group that can be found in the divisions Zygomycota and

Ascomycota (Hibbett *et al*., 2007). Moulds cause biodegradation of natural materials. They also play important roles in biotechnology and food science in the production of various foods, beverages, antibiotics, pharmaceuticals and enzymes. Some diseases of animals and humans can be caused by certain moulds: disease may result from allergic sensitivity to mould spores, from growth of pathogenic moulds (Empting, 2009) within the body, or from the effects of ingested or inhaled toxic secondary metabolite compounds referred to as mycotoxins produced by certain strains of moulds.

2.1.1 Types of moulds

Aspergillus was first catalogued in 1729 by Italian priest and biologist Pier Antonio Micheli. He was reminded of the shape of an *Aspergillum* (holy water sprinkler), from Latin spargere (to sprinkle), when he viewed the fungi under a microscope. Micheli then named the genus accordingly (Bennett, (2010). Today, *Aspergillum* is also the name of an asexual spore-forming structure common to all *Aspergillus* species. There are different types of moulds. One of the common genus is *Rhizopus* species, also known as black bread mould. Another well-known genus of mould is *Penicillium*, from which the antibiotic Penicillin was derived. Certain mycotoxigenic strains also produce ochratoxin A (Lee and Ryu, 2015). Penicillium can be found in a variety of places, including water-damaged structures as well as various foods. *Aspergillus* is a genus consisting of moulds species found in various climates worldwide. *Aspergillus* is a mould with some strains known to have several aflatoxigenic ability that are carcinogenic (Filazi and Sireli, 2013) and when found indoors, can cause severe respiratory problems. Another notable genus includes the *Fusarium* species with certain strains linked to the production of Fumonisins (Marin *et al*., 2013).

2.1.2 Multiplication and distribution of *Aspergillus* species

Aspergillus species are group of conidial fungi that reproduce asexually. Some of them, belonging the devision Ascomycota are known to have a teleomorph (sexual state). Members of the genus possess the ability to grow where a high osmotic concentration (high sugar and salt) exists (Gostincar *et al*., 2010; Zajc *et al*., 2012). Generally, fungi grow on carbon-rich substrates like monosaccharides such as glucose and polysaccharides such as amylose. High Growth of moulds was reported in grains under 30^{o}C and 35^{o}C incubation temperatures (Shehu and Bello, 2011). Although cereals in general when they are exposed to high humidity and temperature, this could subdue the gains to be susceptible to contamination by *Aspergillus* species. Fungi have a worldwide distribution, and grow in a wide range of habitats, including deserts, hyper saline environments, the deep sea, and soil (Okun *et al*., 2015), and in extremely low and high temperatures. *Aspergillus* species can be found in oxygen-rich environments, where they commonly grow as moulds on the surface of a substrate, as a result of the high oxygen tension. *Aspergillus* species are common contaminants of starchy foods (such as bread and potatoes), and grow in or on many plants and trees. *Aspergillus niger* is capable of growing on damp walls, as a major component of mildew. Many species of *Aspergillus* are oligotrophic and thus they are capable of growing in nutrient-depleted environments (Gostincar *et al*., 2011).

2.1.3 Medical and commercial importance of moulds

Moulds are ubiquitous, and the spores they produce can be air borne. Whenever the conidial heads mature, the spores are disbursed into the air where they form component of dust, household and workplaces. When mould spores are inhaled, they can present a health threat to humans in the form of allergic reactions and respiratory problems. Prolonged exposure may be particularly harmful. Symptoms caused by mould allergy are itchy eyes, chronic coughs,

headaches or migraines, difficulty in breathing, rashes, tiredness, sinus problems, nasal blockage and frequent sneezing. Moulds can also pose health hazards to humans and animals when they are consumed. Some species produce toxic secondary metabolites, collectively termed mycotoxins such as; aflatoxins, ochratoxins, fumonisins, trichothecenes, citrinin, and patulin (Ryan and Ray, 2004). Empting, (2009) also reported that exposure to high levels of mycotoxins can lead to neurological problems and in some cases, death. Species of *Aspergillus* are important medically and commercially as some species can cause infection in humans and other animals. Bozkurt *et al.* (2008) reported that more than sixty *Aspergillus* species are medically relevant pathogens. For humans, a range of diseases such as infection to the external ear, skin lesions, and ulcers classed as mycetomas have also been reported. Other species are important in commercial microbial fermentations. For example, alcoholic beverages such as Japanese sake are often made from rice or other starchy ingredients (manioc), rather than from grapes or malted barley. Not all yeasts of the genus *Saccharomyces*, can ferment these starches. However, koji mould such as *Aspergillus oryzae* has been used to first break down the starches into simpler sugars and the degradation of biodegradable plastic (Mabrouk and Sabry, 2001).

Aspergillus oryzae and *Aspergillus sojae*, have traditionally been used in the brewing industry for the production of fermented products (Tamang *et al.*, 2016).

Aspergillus niger has important hydrolytic capacities in α-amylase production and due to its tolerance of acidity (pH < 3), it selectively prevent the growth of bacterial in a culture medium (Djekrif-Dakhmouche *et al*., 2006). The organism is vital for the production of citric acid (Shami and Ehood, 2015).This organism accounts for over 99% of global citric acid production, or more than 1.4 million tons per year. *Aspergillus niger* is also commonly used for the production of

native and foreign enzymes (Oyewole *et al*., 2011), including glucose oxidase, lysozyme, and lactase. *Aspergillus niger* culture is more often grown as a submerged culture in a bioreactor. In this way, the most important parameters can be strictly controlled, and maximal productivity can be achieved. This process also makes it far easier to separate the enzyme of importance from the medium, and is therefore far more cost-effective.

Aspergillus terreus is commonly used in industry to produce important organic acids, enzymes and lovastatin a drug for lowering cholesterol. *Aspergillus terreus* is also capable of sexual reproduction when strains of opposite mating types are crossed under appropriate culture conditions *(*Arabatzis *et al*., 2013).

Filamentous fungi are suitable microorganisms for solid state fermentation (SSF), especially because their morphology allows them to colonize and penetrate the solid substrates (Rahardjo *et al.*, 2005). The fungal α-amylases are preferred over other microbial sources due to their more accepted generally recognized as safe status (Grupta *et al*., 2003).

The thermophilic fungus *Thermomyces lanuginosus* is thermostable and an excellent producer of amylase (Jensen *et al*., 2002; Kunamneni *et al.*, 2005). *Aspergillus oryzae* and *Aspergillus sojae*, species that have been widely used in Asian food fermentations such as soy sauce, miso, and sake, are closely related to aflatoxigenic *Aspergillus flavus* and *Aspergillus parasiticus*. Although these food fungi have never been shown to produce aflatoxin, they contain homologues of several aflatoxin biosynthesis pathway genes. Deletions and other genetic defects have also led to silencing of the aflatoxin pathway in both *Aspergills oryzae* and *Aspergillus sojae* (Takahashi *et al.*, 2002).

2.1.4 Pathogenic *Aspergillus* species

Some *Aspergillus* species cause serious diseases in humans and animals. Pathogenic species are *Aspergillus fumigatus* and *Aspergillus flavus (*Amaike and Keller, 2011), and which can contaminate foods such as wheat, maize and nuts. Some of the common species of *Aspergillus* causing allergic disease are *Aspergillus fumigatus* and *Aspergillus clavatus*. Other species are also important as agricultural pathogens (Agrios *et al.,* 2005). Certain strains of *Aspergillus* sp. produce secondary metabolites such as mycotoxins including aflatoxin on many grain crops, especially maize, wheat, nuts etc. Some variant of *Aspergillus* can cause neonatal infections (John *et al*., 2012). *Aspergillus fumigatus* has been the primary cause of pulmonary infections and can potentially become a rapidly necrotizing pneumonia with a potential to disseminate. *Aspergillus fumigatus* can be differentiated from other common mould infections because the organism takes on a mould form both in the environment and in the host unlike *Candida albicans* which is a dimorphic mould in the environment and yeast in the body.

2.1.5 Diseases caused by *Aspergillus* species

Aspergillus species are fungus that may cause major health threat to both human and animals. Aspergillosis is the groups of diseases caused by *Aspergillus* as a result of the organisms being a saprophytic to which humans are exposed daily in most parts of the world. The infection is initiated by inhalation of conidia, which are cleared quickly in a normal host but can cause invasive disease in immunocompromised individuals (Hohl and Feldmesser, 2007). Certain features empowers *Aspergillus species,* a ubiquitous pathogen such as; survival and growth in a wide range of environmental conditions, effective dispersal in the air, physical characteristics that allow conidia to reach the distal airways, and swift adaptability to the host environment.

The biology, pathogenesis, molecular biology, and virulence factors of the genus especially *Apergillus fumigatus* have been exhaustively reviewed (Dagenais and Keller, 2009). The most common subtype of among paranasal sinus infections associated with aspergillosis is *Aspergillus fumigatus* (Bozkurt *et al*., 20083). The symptoms include fever, cough, chest pain, or breathlessness, which also occur in many other illnesses, so diagnosis can be difficult. Usually, only patients with already weakened immune systems or who suffer other lung conditions are susceptible. Aspergillosis has also been reported in birds, and certain species of *Aspergilli* have been known to infect insects (CAST, 2003).

2.1.6 Grain contamination by moulds and conditions for aflatoxin production

Different moulds species may develop during grains storage in silos (Dovicicova *et al*., 2012; Ezekiel *et al*., 2012; Perrone *et al*., 2014). The contamination of grains before harvest has also been documented (Klich, 2007). The ability of these moulds to grow at different farms and storage conditions can lead to the production of unwanted secondary metabolites such as aflatoxins which consequently renders the quality of wheat grains unwholesome during storage (Magan *et al*., 2003). Fungi that produce aflatoxins can be classified into field and storage fungi based on their ecological requirements for growth. Field fungi are the moulds that invade the seeds on farm requiring high moisture (20-21%)(CAST, 2003) for growth such as *Fusarium*, *Cladasporium*, and *Alterneria* species. The moulds produce mycotoxin before harvest on the grains (Roige *et al.,* 2009). The second groups are moulds that contaminate the seeds during storage and are referred to as storage fungi (Magan *et al*., 2003; Roige *et al*., 2009). They storage fungi require moisture <18%, relative humidity of between 70 – 80% and temperature 20 – 35^{o}C with minimum temperature of 12^{o}C. These groups of moulds include *Aspergillus* sp and

Penicillium sp. Other important factors that affects wheat grains health is that the moulds on wheat grains influence reduction of seed germination rate (Doohan *et al*., 2003). Aflatoxigenic strains produce aflatoxins under stressful conditions such as temperature, moisture or aeration. Other conditions that influence moulds growths are; species, high temperature and moisture. Fungi generally grow at temperature range of between 20 – 30^{o}C. Some certain moulds grow at storage conditions of 13 –18% moisture (Novošinskas *et al*., 2005). Different mycotoxins have been produced by different moulds strains; Fumonisin, aflatoxin, ochratoxin, zearalenone and trichothecenes such as deoxynivalenol, T-2 toxin and nivalenol (Shepard, 2008). The important mycotoxins produced by *Aspergillus* species include aflatoxin B_1, B_2, G_1 and G_2, ochratoxin A, sterigmatocystin and cyclopiazonic acid. Aflatoxins are not produced by all strains of *Aspergillus* nor under all conditions favorable for mould growth. Strains of moulds that produce aflatoxins are referred to as aflatoxigenic strains. Aflatoxin production can be affected by carbon dioxide and oxygen concentrations (Taniwaki *et al*., 2009).

2.1.7 Aflatoxin profiles and implication of different species of moulds in foods

Aspergillus flavus and *Aspergillus parasiticus* are the major producers of aflatoxins (Gourama and Bullerman, 1995; Bennett and Klich 2003; Horn 2007; Gashgari *et al*., 2010; Philippe, 2014). Their aflatoxin profiles have also been elucidated. The aflatoxigenic strains of *Aspergillus flavus* and *Aspergillus parasiticus* have different toxigenic profiles. While the *Aspergillus flavus* produces only AFB_1 and AFB_2, the later *Aspergillus parasiticus* produces the four major types of aflatoxins; AFB_1, AFB_2 AFG_1 and AFG_2 (Davis *et al*., 1987; Diener *et al*., 1987; Van Egmond, 1989; Wilson *et al*. 2002). Almoammar *et al*., (2013) isolated aflatoxigenic strains of *Aspergillus niger*. *Penicillium* sp especially *Penicillium verrucosum* and *Penicillium nordicum.*

The later was also reported to produce Ochratoxin A (Bragulat *et al*., 2001; Duarte *et al*., 2010; Alborch *et al*., 2012). The International Agency for Research on Cancer (IARC) has classified aflatoxin B1 as a Group 1 carcinogen to humans (IARC, 2002). Implication of some moulds in food and feed has also been elucidated. *Fusarium* species especially *Fusarium graminearum* produces mycotoxin referred to as deoxynivalenol (DON), which is considered the most contaminant of wheat. The presence of DON and Zerealenone in cereals is often accompanied with other *Fusarium* toxins such as trichothecenes and fumonisin (Camelia and Lucian, 2014). The occurrence of these toxins in cereals and other foods has been recognized as a potential threat to human health (Bergamini *et al*., 2010; Al-Kahtani, 2014). *Rhizopus* sp produces acidic environment that is favourable to moulds and their presence could signify spoilage (Efiuvwevwere and Akano, 1997).

2.1.8 Differentiation of *Aspergillus flavus* from *Aspergillus parasiticus*

The differentiation of *Aspergillus flavus* and *Aspergillus parasiticus* is based on the descriptions and keys adopted by Rodrigues *et al*. (2009). One of the major separation feature is that *Aspergillus flavus* posses metulae and phialides (biseriate conidial head), while *Aspergillus parasiticus* has only phialides (strickly uniseriate conidial head) (Klich, 2002). The examination of a large number of *Aspergillus parasiticus* isolates have shown that up to 10% of conidial heads in *Aspergillus parasiticus* colony can have metulae and phialides (biseriate). Furthermore, not all *Aspergillus flavus* isolates consistently produce metulae (Klich and Pitt, 1988; Kozakieewcz, 1995). Conidial wall ornamentation is now regarded as the primary diagnostic character for separation of these two species. Scanning Electron Micoroscopy (SEM) micrographs clearly show these ornamentation differences for conidia of *Aspergillus flavus* have

relatively thin walls which are finely to moderately roughen and their shape can vary from spherical to elliptical. On the other hand, conidia of *Aspergillus parasiticus is* more spherical and noticeably echinulate or spinulose.

Laboratory culture media has also been a vital tool in the separation of *Aspergillus flavus* and *Aspergillus parasiticus* from other moulds. Quite few selected media been employed in differentiation: Aspergillus flavus and parasiticus agar (AFPA), coconut cream agar (CCA) and Czapek Dox agar (CZ). AFPA is a selective identification medium for the detection of *Aspergillus flavus* group strains (Pitt *et al*., 1983). The AFPA method distinguishes these species from other *Aspergillus* based on the development of orange colour on the reverse of the plates. The CCA is used to detect aflatoxin producer strains. The production of aflatoxin is detected by a blue fluorescence when exposed to a UV-light (Davis *et al*., 1987). When grown on CZ, the two species can also be separated. Colours of *Aspergillus flavus are* yellow-green and those of *Aspergillus parasiticus* are distinctly darker green, referred to as near Ivy green. Member of the *Aspergillus flavus* group are wide spread in most part of the world and they are capable of growing on, and producing aflatoxins in, a wide range of foods and feeds. However, not all strains of *Aspergillus flavus* produce aflatoxins (Cresio *et al*., 2008).

2.2 Aflatoxins

Aflatoxins are secondary metabolites of fungal origin produced by some *Aspergillus* species (Adejumo and Adejoro, 2014; Ezekiel and Sombie, 2014). *Aspergillus flavus* and *Aspergillus parasiticus* have been reported by many researchers (Klich *et al.*, 2000, Bennett and Klich, 2003; Horn, 2003; El-Khoury *et al*., 2011) to produce aflatoxins in substantial quantities. Also reported in rare cases are *Aspergillus niger, Aspergillus nominus* and *Aspergillus pseudotamari, Aspergillus nomius, Aspergillus tamarii, Aspergillus bombycis and Aspergillus ochraceoroseus*

(Erami *et al*., 2007). These moulds are widely distributed often referred to as being ubiquitous where they contaminate variety of crops such as nuts, cereals like wheat and wheat products. When environmental conditions and substrate are adequate these aflatoxigenic strains produce aflatoxins on crops. These toxins occur naturally, and are known to be carcinogenic, hepatotoxic, immunotoxic and teratogenic in humans and many animal species (IARC, 2002). Aflatoxigenic moulds and aflatoxins that contaminate cereals such as wheat have been published (Noelia *et al*., 2011) and also reported in fermented foods such as bread (Zuzana *et al*., 2014). Aflatoxin contaminated foods often lead to rejection of the food when the toxin levels are above recommended limits by national regulatory bodies (Williams *et al*., 2004).

Aflatoxins contaminate agricultural commodities before harvest and or during post-harvest conditions (Bhat and Fasal, 2011). Aflatoxin intoxication has been reported to be through ingestion, inhalation or when absorbed through the skin (Wagacha and Muthomi, 2008). The socio-economic status of majority of inhabitants of sub-Saharan Africa predisposes them to consumption of aflatoxins contaminated products either directly or at various points in the food chain. Factors that contribute to aflatoxin contamination of food and feed in Africa include environmental especially high humidity and temperatures which favours aflatoxigenic strains proliferation resulting in contamination of wheat, socio-economic and poor food manufacturing practices.

2.2.1 Types of aflatoxins and toxicity

There are more than 300 mycotoxins identified but, four major naturally occurring ones are mostly studied; Aflatoxin B_1 (AFB_1), Aflatoxin B_2 (AFB_2), Aflatoxin G_1 (AFG_1) and Aflatoxin G_2 (AFG_2) (Filazi and Sireli, 2013). Aflatoxin B_1 and B_2 were named because of the strong blue

fluorescence under ultraviolet light, hence the 'B', while Aflatoxin G_1 and G_2 are so named because of their greenish yellow fluorescence. The subscript designates relative chromatographic mobility during thin-layer chromatography, '1' and '2' indicate major and minor compounds respectively (Zain, 2011). In addition to the four major aflatoxins, there are two other metabolites; M_I and M_2. Aflatoxin M_1 and M_2 are metabolites of Aflatoxin B_1 and B_2 respectively. Reports from IARC, 2002; CAST, 2003 showed that these two metabolites could be present in milk, eggs or blood of animals that have been fed with AFB_1 and AFB_2 contaminated feeds. The order of toxicity in these aflatoxins were in the following order; AFB_1>AFG_1>AFB_2 >AFG_2. Aflatoxin B_2 and AFG_2 are typically nontoxic except their metabolic products in the form of AFB_1 and AFG_1 inside the cells (Kensler *et al*., 2011; Filazi and Sireli, 2013). Aflatoxin B_1 is genotoxic *in vitro* and *in vivo* (EFSA, 2007) and also the most toxic. It has been classified as Class 1 human carcinogen by the International Agency for Research on Cancer (IARC, 2002).

2.2.2 Health risk of aflatoxins to humans and animals

The mode of aflatoxin transmission is majorly through consumption of contaminated foods and feed. In developing countries the awareness about the effects of the toxin is low and the practice of determining the level of aflatoxins is scarce. In addition the set regulations by national regulatory bodies have been loosely monitored and due to the high level of allowable maximum limit of the toxin in food have greatly influenced the incidence of health risks to aflatoxins. Over four billion people in developing countries are repeatedly exposed to aflatoxins, contributing to greater than 40% of the disease burden in these countries (Schmalle III and Munkvold, 2015). Aflatoxin is the third-leading cause of cancer worldwide, with about 600,000 fresh cases each

year (WHO, 2006). Aflatoxin contamination has many consequences such as reduction of feed intake, increase liver cirrhosis (Kuniholm *et al*., 2008) and kidney weights of farm animals, as well as inducing immunosuppression (William *et al*., 2004) and growth impairment (Khlangwiset *et al*., 2011). The primary disease associated with the ingestion of aflatoxins is hepatocellular carcinoma (liver cancer) (Yang *et al*., 2012).

Aflatoxin may be passed to poultry products, such as meat or eggs which may lead to growth impairment in animals and humans. In cattle, increasing aflatoxin to levels such as 10, 26, 56.4, 81.1 and 108.5µg/kg has been shown to reduce feed intake depending on the dosage (Choudhary *et al*., 1998). Naveed *et al*. (2015) reported that the quality of milk produced by cattle fed on aflatoxin contaminated feed was also contaminated with the aflatoxins. Uriah *et al*. (2001) reported that the blood and semen in infertile men attending the University of Benin Teaching Hospital had aflatoxin levels that ranged from 700 - 1392ng/ml and 60 - l48ng/ml respectively and these values were significantly higher than the concentrations of the toxin in fertile men. Twenty-two out of the 60 samples (37%) of serum and semen of infertile men had aflatoxin B1 ranging from 60ng/ml to 148ng/ml. Onyemelukwe *et al*. (2012) found aflatoxins in both the sera and the blood of children with kwashiorkor. Furthermore, Oyelami *et al*. (1998) reported the presence of aflatoxins in the autopsy kidney specimens of 58% children with kwashiorkor at Obafemi Awolowo University Teaching Hospital, Ile-Ife. Higher aflatoxins were also frequently detected in sera and urine of patients with kwashiorkor, than healthy control within a similar age group (Hendrickse, 1983).

2.2.3 Economic impact of aflatoxins

Developing countries suffer mostly from impact of enforcement of mycotoxin regulation by European and international agencies in food crops. The economic losses to developing countries are variable and the data arising from such looses are not properly documented. Wu *et al*. (2011) reported that, the magnitude of the economic impacts of the health consequences associated with consumption of aflatoxin-contaminated foods in developing countries is not known due to lack of good data. The losses do not only arise from crop and livestock but also from costs associated with regulatory compliances (CRA, 2011). Bankole and Adebanjo (2003) reported that as a result of regulation, exports of agricultural products particularly groundnuts from developing countries had dropped considerably resulting in major economic losses to producing countries. Losses from rejected shipments and lower prices for inferior quality goods can devastate developing country export markets. In 2011, Argentina, China, India and South Africa experienced 37, 60, 136 and 12 rejections, respectively (Codex Alimentarius Commission, 2014). The World Bank predicted that, policy change by the EU will reduce by 64% imports of cereals, dried fruits and nuts from African countries like Chad, Egypt, Gambia, Mali, Nigeria, Senegal, South Africa, Sudan and Zimbabbwe, and thus cost African countries about US$670 million in trade loss per year (Bankole and Adebanjo, 2003).

2.3 Wheat processing and Fate of Aflatoxin during Wheat Flour Extraction and Aflatoxin Prevalence

The choice of wheat breed for wheat flour milling basically rest on the quality and quantity of gluten in the wheat cannel. Hard wheat has been the best choice for tprocessing because of its high quality gluten. Gluten is a protein containing glyadin and glutenin (Duarte *et al.,* 2010). This protein gives the wheat flour an elastic and extensible classical property that enables the wheat flour dough to have a good quality foam and sponge during fermentation and baking. The raw wheat is usually first of all screened to remove dust, chaffs, stones, broken wheat and other

impurities. To the wholesome wheat is then added calculated amount of water a condition referred to as damping or tempering inorder to raise the moisture content to 15.5%. The tempered wheat is then incubated in a closed silo for 48 – 72h depending on the quality of the wheat. The tempered wheat is then grinded in three stages as follows;

1. Break release: This is where the tempered wheat is punched using special grinders fitted with two oppositely rotating fluted break rollers. The product is then transported by a pneumatic system into a set of sieving nets (plant sieveters) with different sizes otherwise referred to as grading into different products.
2. Scratching: The punched grains flattens where the endosperm are now scratched off the bran (back covering of grains) using another set of special rollers called scratch rollers. The scratched endosperm is also graded.
3. Reduction: The endosperms are then reduced or grinded to various sizes that fit the products required using another set of specialized reduction rollers.

Mould contamination of wheat grains are majorly believed to be situated outside the bran (Radiana *et al.,* 2009). When aflatoxigenic strains are involved the equally secrete aflatoxins. Though the mycelia of the moulds are usually rooted into the grain tissue, the introduction of the aflatoxigenic moulds and the aflatoxins is strictly dependent on the degree of high scratching of bran to acquire more endosperm for flour production otherwise referred to as high extraction. Practically, the extraction rate is always in correlation with the level of bran retention in the flour product and thus also the total aflatoxins. High ash content in wheat flours is also accompanied by the presence of dusty bran introduced into the wheat flour due to over scraping of the wheat endosperm. Consequently, since the aflatoxigenic moulds strains and the aflatoxin are majorly situated outside the bran, hence their introduction into wheat products.

Other workers have reported the presence of aflatoxins in wheat and products. Abdallah *et al.* (1998) reported 25.6ppb aflatoxin B_1 from wheat flour in Malaysia. Halt (1994) reported 16.3ppb aflatoxin B1 from Croatia total aflatoxin in wheat (59%) and in wheat flour ranging 0.05 – 14.0 ppb in Turkey (Ayalewa *et al*., 2006).

2.4 Detection of Aflatoxigenic Moulds

2.4.1 Detection of aflatoxigenic moulds by culture dependent method

Aflatoxins have a native luminescence due to their oxygenated pentaherocyclic structure (Appendix VI). Analytical and microbiological methods for detection and quantification of aflatoxins are based on this feature. There are a number of microbiological methods that can be used to directly visualize aflatoxin-producing *Aspergillus* strains. Different additives have been used to increase the resolution of aflatoxin producing strains. The addition of 0.3% methyl-β-cyclodextrin or of a combination of methyl-β-cyclodextrin plus bile salts (0.6% Na-deoxycholate) (Odaeze *et al.,* 2003) enhances the natural fluorescence of aflatoxins, allowing detection of aflatoxigenic colonies. These aflatoxigenic colonies have been reported to present a beige ring surrounding the colonies. In addition, when the beige ring is examined under UV light (360nm), it exhibits blue fluorescence. There is also a reported situation where the aflatoxigenic strains in-vitro emits room temperature phosphorescence (RTP), when excited with ultraviolet rays and examined in the dark. The detection of aflatoxins by fluorescence of fungal colonies is not the ultimate because non-aflatoxigenic strains of *Aspergillus parasiticus* and *Aspergillus niger* fluoresce under Ultraviolet (UV) rays. This could be interpreted erroneously to be due to the presence of aflatoxins as case may be for true aflatoxigenic strains. A more diagnostic test for the presence of aflatoxins in fungal colonies is the room temperature phosphorescence of aflatoxins that lasts 0.5s after switching off the UV light (Rojas-Dura´n *et al*., 2007). While non-

aflatoxigenic Aspergilli do not phosphoresce, aflatoxigenic strains do. When aflatoxins are exposed to 365 nm ultraviolet light, AFB_1 and AFB_2 are activated, resulting in AFB2–8,9-oxide. This product then binds to DNA at the N7 position of guanine residues yielding 8,9-dihydro-2-(N7-guanyl)-3-hydroxyaflatoxin B2 . The structure of the AF-DNA photoadduct (Stark, 2007) is identical to the AF-DNA adduct that is formed *in vivo* from ingested AFB2 by cytochrome P450, mainly in the liver (Stark *et al*., 1979). Formation of AF-DNA adducts is considered as the initiating step of aflatoxin-induced carcinogenesis. Covalent DNA- and protein adducts are also responsible for acute aflatoxin toxicity.

2.4.2 Detection of aflatoxigenic moulds by multiplex polymerase chain reaction

The detection of aflatoxigenic moulds has earlier been through culturing the samples containing the moulds on suitable laboratory medium followed by identification of micro and macro morphological characteristics. This method however requires mycological expertise and is time consuming. With the advent of Polymerase Chain Reaction (PCR) technique, the identification of these aflatoxigenic moulds became easier and faster. The molecular technique is sensitive and highly specific. This technique employs amplifying genes that are involved in the aflatoxin biosynthetic pathway. Genetic studies suggest that the genes involved in the aflatoxin biosynthetic pathway are clustered (Bhatnagar *et al*., 2006) within conserved regions. Although there are at least 25 genes involved in the biosynthesis of aflatoxins and its regulation (Bhatnagar *et al*., 2006), primers pertaining to sequences of regulatory gene *aflR-1* and that of structural genes (*nor-1, ver-1* and *omt-A*) (Shapira *et al*., 1996; Geisen, 1996; Chen, 2002) have been used to detect and identify aflatoxigenic strains of *Aspergillus flavus* and *Aspergillus parasiticus* among isolated colonies, or in DNA extracts from food and feedstuffs. Thus the DNA of

Aspergillus is isolated and used as template for the amplification of genes involved in aflatoxin biosynthesis. Sequencing of the amplified fragments confirms the identity of aflatoxin biosynthetic genes. However, the mere presence of the genes reflects only the potential of the fungus to produce aflatoxin. Aflatoxin production depends on temperature, humidity, composition of the growth medium, growth phase, age of the culture and climatic changes (Peter and Jaime-Garcia, 2007). A recent application of reverse transcription polymerase chain reaction (RT–PCR) for the characterization of aflatoxigenic Aspergilli, relies on the presence of mRNAs pertaining to aflatoxin biosynthesis genes that can be expressed leading to the transcription and translation of the genes during the production of the toxin (Zhan *et al.,* 2004). Reverse transcription polymerase chain reaction (RT–PCR) is indicative of the presence of the aflatoxigenic fungus and of the aflatoxin biosynthetic enzymes (Zhou *et al.*, 2008). Multiplex RT–PCR containing 4–5 primer pairs of various combinations of *aflR-1, nor-1, ver-1* and *omt-A* have been used to detect toxigenic strains (Scherm *et al*., 2005; Degola *et al*., 2007). The regulatory gene *aflR -1* is a Transcription factor containing a zinc cluster DNA binding motif. That happens to be a positive regulator of the structural genes at transcription during the steps in aflatoxin biosynthetic pathway. The structural gene *omt-A* when expressed produces the enzyme O-methyltransferase where it steps in aflatoxin biosynthetic pathway through conversion of Sterigmatocystin to O-methylsterigmatocystin. In *Aspergillus flavus* and *Aspergillus parasiticus* the expression of the *aflD* (*nor-1*), a gene encoding an enzyme that catalyzes the conversion of the first stable aflatoxin biosynthesis intermediate, norsolorinic acid to averantin (Shapira *et al*., 1996) is a key structural gene in the biosynthetic pathway. Furthermore, *aflR-1* is a pathway regulatory gene coding for proteins shown to be involved in transcriptional activation of most of the structural genes (Cary *et al*., 2000). While aflatoxigenic strains always produce consistent

four banding patterns with the four key genes, non-aflatoxigenic strains lack one or more of the aflatoxin biosynthesis genes creating different band patterns with variable gene profiles (Chang *et al*., 1993; Geisen, 1996; Shapira *et al*., 1996; Bennett and Klich, 2003; Bhatnagar *et al*., 2006; Lee *et al*., 2006; Criseo *et al*., 2008). The non-aflatoxigenic *Aspergillus* species isolates would have multiple deletions or mutation of a gene or the entire aflatoxin gene cluster removed. Criseo *et al*. (2008) showed that there is a high level of genetic variability among non-aflatoxigenic isolates that require greater attention in order to design molecular experiment to distinguish true aflatoxigenic from non aflatoxigenic isolates.

Other PCR protocols are based on aflatoxin biosynthetic genes that are single copy. Multicopy genes such as ITS1 and ITS2 regions (Internal Transcribed Spacers or the rDNA) have been reported to be highly variable allowing discrimination among closely related species (Jurado *et al*., 2006). Other *Aspergillus* species producing mycotoxins have been successfully detected and identified using this method (Zhao *et al*., 2001). Such method has proved to be very efficient even at short incubation times and at low spore concentration of aflatoxigenic strains in wheat flour samples (Noelia *et al*., (2010).

2.4.3 Other aflatoxin detection methods

There are quite a number of aflatoxin detection methods that have been developed to determine aflatoxins in foods, such as; immunoassays techniques (Lippolis *et al*., 2006), chromatographic methods; Thin layer chromatography (TLC) (Stroker *et al*., 2000), Liquid Chromatography (LC), High-Performance Liquid Chromatography (HPLC) High-Performance liquid chromatography (HPLC) with fluorescence detection (Bacaloni *et al*., 2008). Recently, analytical methods have also been used which is based on clean-up with immunoaffinity column and HPLC with post

column derivatization and fluorescence detection. Semilarly, immunochemical methods have also been developed which are based principally on enzyme-linked immunosorbent assay (ELISA).

Fluorescent Polarization Immunoassay is another protocol involving a rapid assay that can detect mycotoxins (Maragos *et al*., 2002; Maragos and Kim, 2004; Lippolis *et al*., 2006; Thompson and Maragos, 2006). Small molecules surround a molecule of mycotoxins that are bound on a solid support could also be detected through polymerization of the surrounding molecules and washout of the mycotoxin through a process called Molecular Imprinting (Yu *et al*., 2007)

Biosensor arrays have been designed to perform simultaneous assays of many mycotoxins (Sapsford *et al*., 2006; Piermarini *et al*., 2007) with limit of detection of arrays are based on Fluorescent antibodies (Ngundi *et al*., 2005) . The arrays are regenerable (Ngundi *et al.,* 2006).

Electronic noses are sensors developed to identify volatile biomarker compounds emitted from grains. Electronic noses could differentiate between closely related *Penicillia* used in cheese production (Karlshøj *et al.,* 2007), and detect patulin in apples (Karlshøj *et al*., 2007).

Air is pumped from a container with a sample of grains. The volatiles are analyzed by an array of chemical sensors that operate at various temperatures. Every grain has its typical signature of volatiles. Every fungal contamination has its own signature of "off odors" (new patterns of volatiles deviating from the normal ones). The signature depends also on the presence of mycotoxins in the contaminated grains. For example, samples with normal odour have no detectable ochratoxin A and average DON contents of 26 ppb (range 0–80), whereas samples with off-odor had average OTA contents of 76 ppb, (range 0–934) and DON contents of 69 ppb (range 0–857) (Olsson *et al.,* 2002).

Odour Sensors applies single stranded (ssDNA) containing a fluorescent chromophore and dried onto a solid support can interact non-covalently with volatiles, which results in increase in fluorescence (White *et al.,* 2008). Volatile precursors of AFB_2, ochratoxin A and DON (Olsson *et al*., 2002) could be used to identify toxigenic fungi by such odour sensors.

2.5 Control of Aflatoxigenic Fungi and Aflatoxins

2.5.1 Moulds against aflatoxigenic strain

One of the recent advances in biological aflatoxin control is to use non aflatoxigenic strains of *Aspergillus* species to control aflatoxigenic strains (Chang and Hua, 2007; Yan-ni *et al*., 2008). Zuzana *et al*. (2014) used this ecofriendly method as well and demonstrated that non aflatoxigenic strains competitively inhibited toxigenic fungal strains from this protocol.

2.5.2 Biological control using competitive exclusion mechanism

Biological control management strategies have been developed that make use of competitive exclusion mechanism (Dorner and Cole, 2002). The principle is utilized by the field application of atoxigenic strains that out compete the toxigenic ones, thereby shifting the fungal population from toxigenic to atoxigenic. *Aspergillus flavus* strain AF36 is a fungal antagonist and is applied as a commercial biocontrol to cotton and corn to reduce aflatoxin exposure. AF36 was initially isolated in Arizona and was also found in Texas. It is grown on sterile seeds which serve as the carrier and a source of nutrients. Following application and colonization and in the presence of high moisture, AF36 grown seeds out compete aflatoxin-producing strains of *Aspergillus flavus*. Certain factors such as competition for space and nutrients, production of anti-mycotoxigenic metabolites by co-existing microorganisms, changes in pH of the substrates, or a combination of these factors may have been the driving force of biological control of aflatoxigenic fungi.

Dorner *et al*., (1999) reported a field application of non-toxigenic strains of *Aspergillus flavus* and *Aspergillus parasiticus* that reduced post-harvest aflatoxin contamination by 95.9%. A product, AflasafeTM from the International Institute of Tropical Agriculture (IITA), Nigeria has been pioneered to commercialize this technique (Probst *et al*., 2011). Certain bacteria have also been used to control afaltoxigenic moulds. Munibubazi and Bullerman (1998) reported 98% inhibition of aflatoxin production by *Aspergillus parasiticus* in cell free *Bacillus pumilus* and 53% inhibition of other mould growth.

2.5.3 Molecular technique for aflatoxin control

2.5.3.1 *Ribonucleic acid interference technology (RNAi)*

Foodborne illness caused by microorganisms is a serious threat to public health. There has been interest in exploring alternative means to control or reduce aflatoxin production**.** Ribonucleic acid interference technology (RNAi) has received much attention in biology. The principle behind RNAi is to abate specific messenger RNA (mRNA) species by inducing their degradation via cellular protein machinery; the RNA-induced silencing complex (Liu *et al*., 2002; Ahmed *et al*., 2011). Short-interfering double-stranded RNA (siRNA) is synthesized and introduced through common transfection methods into cells, where they serve to guide the RNA degradation machinery to the select target gene. Ribonucleic acid interference (RNAi) is an effective tool to investigate gene function, and may also be a useful tool to quench the expression of undesirable gene products (Ahmed *et al.,* 2011). Ribonucleic acid (RNA) silencing in filamentous fungi has been carried out using plasmid constructs expressing a hairpin dsRNA structure controlled by an inducible or constitutive promoter (Khatri *et al*., 2007). Liu *et al.,* (2002) demonstrated silencing of the cryptococcal *CAP59* and *ADE2* genes by double-stranded RNA homologous to these genes in the basidiomycetous yeast *Cryptococcus neoformans*. Application of siRNA-mediated

RNAi has also been reported in cultured cells from fungi. Double-strand of RNA (dsRNA) was also delivered directly into protoplasts of *Phytophthora infestans,* which belongs to the fungus-like Oomycetes. Ahmed *et al*. (2011) used RNA interference to silence one of the important structural genes in the aflatoxin biosynthesis pathway (*aflD* gene) in both *Aspergillus flavus* and *Aspergillus parasiticus* and to elucidate the function of this gene in aflatoxin production by direct delivery. The same study reported that all the three siRNAs designed to target *aflD* gene gave excellent levels of silencing. According to the report, transient gene silencing was observed at an early stage after 5 days of protoplast regeneration and hyphal growth. However, there was no changes in fungal growth observed between siRNA treated and untreated samples by the team. Thus according to their research the protoplasts have the ability to take up siRNAs from the medium during growth.

2.5.3.2 *Resistance genes encoding fungal growth inhibitors*

Gene is a unit of inheritance. Genetic resistance is another approach to molecular aflatoxin control. Certain gene confers resistance to microorganism. Resistance genes encoding fungal growth inhibitors in certain organism such as *Aspergillus flavus* and *Fusarium* species particularly Fusarium verticillioides have been reported (Hamiton, 2000; Munkvold, 2003).

2.5.4 Chemical control

2.5.4.1 *Application of fungicides and insecticides*

Fungicides are chemical compounds that are used to kill fungi, while insecticides used to kill insects. The application of fungicides and insecticides for the control and spread of aflatoxigenic isolates on crops have been reported (Munkvold, 2003). However, farmers have poor education

background on aflatoxins and its health implications in crops. Sometimes this often leads to misuse of pesticides as alternative aflatoxin control measure by the local people. A report from Bankole and Adebanjo, (2003) indicated high mortality among Nigerians as a result of consumption of cowpea treated with inappropriate pesticides. In 2008, 120 students of Government Girls' Secondary School, Dome, Gombe State were rushed to the hospital after consuming a meal of beans that was suspected to have been preserved with lindane (Gwary *et al.*, 2012). Similar reports in 2011 in Calabar showed that 112 people were hospitalized and 2 children confirmed dead, while 20 people were hospitalized in Oshogbo and 10 people died after eating a bean delicacy stored with pesticide (Gbadeyan, 2010).

2.5.4.2 *The use of free residual chlorine as disinfectant in wheat milling industry*

Chlorine has been mainly used for disinfection of water and certain house hold or mill wall, floor surfaces. The effects of the various free residual chlorine concentrations on baking qualities of bread have not been exploited.Though reduction of aqueous chlorine by organic materials have been reported (Kotula *et al*., 1997). However, the functional effects of chlorine on living cells have been documented to be on perhaps in part due to lethal DNA damage (Monokesh *et al*., 2013) and on the wheat flour starch granules. The chlorine concentrates on the starch granule surface making the starch aggregates at gas cell surface in batters .This leads to continuity of starch at the gel stage just before the foam breaks down to a sponge at baking. The administration of chlorine as a disinfectant in mills was earlier done through the gaseous form using the Nuchlo apparatus. Nichlo apparatus are sets of interlocking pipe lines through which the gas is pressured to reach all parts of the machineries for disinfection. The system could not be

sustained because of the pollution within the factory and the likely hazards associated with the chlorine exposure.

2.5.5 Detoxification and decontamination of mycotoxins

2.5.5.1 *Detoxification using microorganisms*

Detoxification is the systemic degradation of mycotoxins into less toxic products while, decontamination is a strategy that seeks to reduce or eliminate contamination by mycotoxigenic fungi. Earlier, it was demonstrated that *Agrobacterium-Rhizobium* strain E3-39 isolated from soil samples effectively converted deoxynivalenol (toxic) into 3- keto-deoxynivalenol (less toxic) under aerobic conditions (Shima *et al*., 1997). Later, Duvick *et al*. (2006) reported that the fungus *Expoliata spinnifera* was able to grow on fumonisin B1 as a sole of carbon source. The hydrolysis of fumonisin B_1 yields free tricarboxylic acid and aminopental, the intermediate aminopentol undergo oxidative deamination. In a separate study, Zhou *et al*. (2008) isolated a bacterium strain *Barpee* from soil samples enhanced with *Fusarium graminearum* infected maize. The strain showed strong DON transforming activity, transforming DON into two major products: a stereo-isomer of DON and 3-keto-DON, under aerobic conditions. A direct detoxification technique involves the use of enterosorption, which is based on the introduction of harmless phyllosilicate clay (Hydrated Sodium Calcium Alumino Silicates (HASCAS), which is widely used as an anticaking agent in animal feeds (Phillips, 1997). HASCAS is capable of tightly and selectively absorbing aflatoxins *in vitro* and *in vivo* (Bankole and Adebanjo, 2003).

2.5.5.2 *Detoxification and decontamination using botanicals*

Adejumo (2012) tested five botanicals against the maize mycotoxigenic fungus, *Fusarium verticillioides* and reported that *Piper guineense, Garcinia kola* and *Aframomum melegueta* had the potentials to be used as sustainable alternative biopesticides because they are cheaply available and effective. The essential oils and aqueous extracts of *Aframomum danielli* had been reported to reduce ochratoxin A by between 64 and 95% in spiked cocoa powder (Aroyeun and Adegoke, 2007). Although, ochratoxin molecule is stable, the authors stated that 40 to 90% of Ochratoaxin A got destroyed during roasting of coffee beans (Aroyeun and Adegoke, 2007).

2.5.5.3 *Mycotoxin detoxification and decontamination using irradiation*

Irradiation is also a useful tool in inactivating some mycotoxins. Herzallah *et al*. (2008) showed that exposure to sunlight (solar radiation), γ-radiation (^{60}Co), and microwave heating were efficient in decontaminating aflatoxin residues in poultry feeds. The effect of irradiation depends on the fungus, application dose, moisture content, composition of food and storage conditions (Kabak and Var, 2005; Aziz *et al*., 2006). There is a theoretical risk, however, of mycotoxins increasing after irradiation. This is because of higher radio-resistance of moulds and yeasts which results in a loss of competitive inhibition of mould and yeast growth. Any mould surviving under treatment with irradiation may be expected to grow more rapidly in the absence of competitors and eventually dominate the mycoflora (Temur and Tiryaki, 2013), although their potential to produce mycotoxins is the major concern.

2.5.6 Water activity (a_w) control

The available water present in grains for microbial growth called the water activity (a_w). Water activity is one of the serious problems in the storage of grains due to its ability to promote the

proliferation of aflatoxigenic moulds. The critical water content for safe storage corresponds to water activity (a_w) of about 0.7. Maintenance of grains below 0.7 a_w is an effective technique used throughout the world for controlling fungal spoilage and mycotoxin production in foods (Ariyo *et al*., 2013). A study on community-based intervention trial in Guinea, West Africa focused on thorough drying and proper storage of groundnuts in subsistence farm villages and achieved a 60% reduction in mean aflatoxin levels in intervention villages (Turner *et al*., 2005). Rapid drying of agricultural products to low moisture level creates less favourable conditions for fungal growth and proliferation, insect infestation and helps produce longer shelve life (Lanyasunya *et al*., 2005). In order to reduce or prevent the production of aflatoxins, the grains should be dried soon after harvest before storage.

Removal of broken grains, debris and other impurities through cleaning from previous harvest through sanitation measures have been suggested (Hell *et al*., (2000). Crops contaminated through insects feeding habits where they carry spores of aflatoxigenic fungi from plant surfaces could promote fungal growth (Hell *et al*., 2000; Avantaggio *et al*., 2002).

Irrigation and fertilization play an important role in the development of aflatoxins (CAC, 2003). When fertilizer and other soil conditioners are added to assure adequate soil pH and plant nutrition, aflatoxin development is reported to reduce (Munkvold, 2003). Early harvesting has also bee reported to reduce aflatoxin contamination of crops in the field (Rachaputi *et al*., 2002).

2.5.7 Education and awareness creation

The risks posed by mycotoxins are largely known to the scientific researchers and not to the general populace. The need to properly sensitize people about the menace, especially farmers, on the economic importance of mycotoxins and the dangers they may pose to human health has

been advocated (Bankole and Adebanjo, 2003). The authors advised that people who were trained and given orientation about mycotoxins should therefore be advised to train others by sharing their knowledge with them. The authors further said that this information flow could crate knowledge chain reaction that effectively creates an advantageous ripple effect. National Agency for Food and Drug Administration and Control (NAFDAC) and Standard Organization of Nigeria (SON) could partner with societies like Nigerian Institute of Food Science Technology (NIFST) and Mycotoxicology Society of Nigeria (MSN) on ways to educate the general public on mycotoxins. This can be carried out on media outlets such as television and radio stations, newspapers and on internet blogs and social media. Mycotoxicology Society of Nigeria (MSN) suggested collection of a database of predominant fungi and mycotoxins, establishment of a mycotoxin occurrence map to know the areas prone to mycotoxin contamination and the establishment of a permanent culture collection media for the control of hazards posed by mycotoxins.

2.5.8 Legislations

Certain legislations have been put in place by various countries for the control of aflatoxin contamination of food. In most countries, regulations are established to control the contaminants in foodstuffs and to protect health of consumers. These regulations include specific minimum and maximum limits for aflatoxin and several other contaminants for different foods. Sampling methods and performance criteria of analysis are usually spelt out in the regulation. The Codex Alimentarius Commission Standards for aflatoxin limits are 0.05µg/kg for milk and milk products, 20µg/kg for cereals, and 1750µg/kg for unprocessed wheat, oats and maize (EC, 2006). Other agricultural products such as beans are regulated at 4 to 5µg/kg, 10µg/kg in nuts such as

peanuts and almonds. Other mycotoxins such as fumonisins limit is <1000 µg/kg (CAST, 2003). The EU set a limit of 0.5µg/kg for OTA in raw cereals, with a tolerable weekly intake (TWI) of 0.12µg/kg, while maximum DON limits are set between 200µg/kg for processed cereal based foods and baby foods for infants and young children.

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Sampling point

3.1.1 Wheat flour mills

Only two standard wheat flour mills consented to this study out of the wheat flour mills in Northern Nigeria from Kaduna, Kaduna State coded (Mill A) and one from Katsina,Kastina State coded (Mill B). Two other batch wheat processors with codes (C) and (D) consented for this research to be carried out on their production lines. One of the batch wheat processor (C) domiciled within Kaduna metropolis and the other (D) from Katsina metropolis. The management of all the various companies gave approval for this research to follow up their milling protocols and the approval to collect maximum of 100g x 2 per sample per day.

3.1.2 Major distribution stores

Ten available wheat brands on sale by major distributors were given number codes. These coded brands were written each on a piece of paper and placed in a deep basket and mixed. Five brands were picked by simple random sampling from the list. The selected brands were then given new number codes.

3.2 Number of Samples

The number of samples used in this study was calculated using prevalence of 50% using the formular by Michael, (2005)

$$\text{Number of samples} = \frac{Z^2 pq}{I^2}$$

n= required sample size

Z= standard normal distribution at 95% confidence interval =1.96

I= allowable error =0.05

P= prevalence rate = 50.0 %

q= 1-prevelence

$$n = \frac{(1.96)^2 \times 0.50 \times 0.50}{(0.05)^2}$$

n=384.16

n=384

The samples was increased to 520 to archieve near equal representation from the different mills and major distribution stores

3.3 Collection of samples

3.3.1 Wheat

Samples of wheat (200g) were withdrawn from each lorry during discharge into the storage silos twice per day within five days for one and half months from four wheat flour mills = 240 samples.

3.3.2 In-process mill

One hundred grammes (100g) of raw wheat were also withdrawn. The milling protocols of each of the four selected wheat flour mills was followed individually and their samples collected from the screening section through the milling lines then along the finished products lines where assumed as critical control points for possible moulds development as follows; Sample spoons was first wrapped in aluminium foil and sterilized using hot air oven at 160^0C/1h. The sample spoon was allowed to cool. The sterile spoon was then used to withdraw aseptically 100g each duplicate of samples from the sample points below twice every week for one month x 4mills =

224 samples). The samples were transferred into sterile labeled low density cellophane bags and covered immediately.

The sampling points were as follows;

i. Precleaned wholesome raw wheat samples before tempering or damping (addition of water)
ii. Predamped 18-24h incubated wheat grains for first break milling
iii. Milling flour in-process line during production before bagging
iv. Bagging wheat flour
v. Bagging semolina
vi. Bagging brown flour
vii. Bagging bran

3.3.3 Major distribution stores

The five different wheat flour brands were collected from the major wheat flour distributor in the following manner. Collection of samples from the different selected wheat flour brands was obtained from new batch of trucks supplying the products into Kaduna once every week for three months (4samples x 2wk x 5brands x 3months = 120 samples) as follows; a metal sampler was sterilized by swapping with 70% ethanol and followed by flaming using blow touch. The sampler was then allowed to cool before use. A surface point of a sample sack was sterilized by cleaning a particular surface portion of the bag with methylated spirit and briefly allowed to air dry. The sample sack was pouched at the point sterilized using the sterile sampler and an approximately 100g each of wheat products was withdrawn in duplicate and poured into sterile

labeled low density cellophane bags. All the samples were transported immediately to Department of Microbiology laboratory for analysis.

Twenty five (25g) of each samples was withdrawn from each of the different samples immediately for microbial analysis and physicochemical analysis. Total aflatoxin was extracted from the same sample immediately and the extract used for determination and quantification of aflatoxins using ELISA.

3.4 Proximate Composition of Wheat and Wheat Products

3.4.1 Moisture content

The percentage moisture content of samples was determined as reported by Chaelae *et al. (*2002) and Adegoke *et al.,* (2010) as follows: Ten (10g) wheat and wheat flour samples was weighed in pre-weighed moisture plates and placed in a thermostatic oven at 105^{o}C temperature for one hour. The moisture was calculated based on weight difference and expressed as percentage.

$$\frac{W_2 - W_3 * 100}{W_1} = A$$

100- A = percentage moisture

W1= weight of sample (10g)

W2= weight of undried sample (10g) + petri dish

W3= weight of dried sample + petri dish

3.4.2 Ash content

The percentage ash content was determined following standard methods of the American Public Health Association APHA (1999). A porcelain crucible was washed and dried in an oven at 100^{o}C to a constant weight, cooled in a desiccator and weighed (W1). Two (2.0g) of finely ground wheat flour to approximately 200µm size sample at each determination was placed in the

crucible and reweighed (W_2). It was first charred and transferred into a muffle furnace at 550^{o}C. The sample was left in the furnace for eight hours to ensure proper ashing. The crucibles containing the ash was removed, cooled in a desiccator and reweighed (W_3). The percentage ash was computed from the calculation below on dry matter basis.

W_1 =weight of empty crucible

W_2=weight of crucible + sample

W_3=weight of crucible + ash

M_1= percentage moisture content of sample

$$A = \frac{W_3-W_1}{W_2-W_1} \times 100 = \text{percentage ash}$$

$$B = \frac{A\ .}{100-M_1} \times 100 = \text{percentage ash on dry matter basis}$$

3.4.3 Fat content

The fat content of the samples was obtained using Soxhlet apparatus according to AOAC. (1999). Firstly, a round bottom flask was cleaned, dried and antibumping granules put into it and the flask weighed. An aliquot of 10g of the wheat and wheat flour products was weighed and put into a clean extraction thimble. The extraction thimble containing the sample was placed inside the middle piece of the Soxhlet apparatus. The middle piece was fixed into a round bottom flask that was already filled to 2/3 of its volume with petroleum spirit with boiling point (40 - 60^{o}C) on the heating element of the Soxhlet apparatus. The cooling system was run and the electric unit put on. As the solvent boils it evaporates, passes up to the condenser containing cooling water, the vaporized petroleum spirit condenses back into the middle piece containing the sample thimble. The middle piece filled up with the condensed petroleum spirit, it dissolved the sample fat and siphoned back into the flask with the dissolved fats. The process was left to run for six

hours. The thimble was then removed and the solvent recovered. The fat at this stage was left in the flask. Finally the flask was dried in thermostatic oven at 90^{o}C and reweighed.

The percentage fats content was computed on dry matter bases as can be seen below;

W1 = weight of empty flask

W2 = weight of flask + fat

Q1 = quantity of sample

M1 = moisture of sample

$$\frac{W_2 - W_1}{Q_1} \times 100 = A \text{ (percentage fats as it is)}$$

$$\frac{A}{100 - M_1} \times 100 = \text{percentage fats on dry matter basis}$$

3.4.4 Free fatty acid content

The free fatty acid was determined according to the methods as reported by Manji *et al.,* (2006) as follows. Two (2.0g) of homogenized sample was accurately weighed into a conical flask. Then 1.0ml of 95% ethanol and phenolphthalein was added. This was titrated with 0.1M sodium hydroxide to pink colour that was persistent for thirty seconds. The percentage free fatty acid was then calculated using the formula below:

$$\% \text{ FFA} = \frac{\text{Titre x Chemical Factor x 100}}{\text{Weight (g) of Sample}}$$

Chemical factor for oleic acid = 0.028245

3.4.5 Crude protein content

3.4.5.1 *Nitrogen determination by microKjeldahl method*

The method consists of three basic steps:

1. Digestion of the sample in sulphuric acid with a catalyst, which results in conversion of nitrogen to ammonia
2. Distillation of the ammonia into a trapping solution and
3. Quantification of the ammonia by titration with a standard solution

3.4.5.2 *Digestion*

Accurately 10g of homogenized wheat flour sample was wrapped in a clean whatman no.1 filter paper and placed in Kjeldahl flask. Ten (10g) potassium sulphate and 0.2g copper sulphate was added together with antibumping agent. The flask was heated in an inclined position for one hour. Into the receiving flask, 20.0ml of standard sulphuric acid was added and diluted to 50.0ml. The flask was place on preheated burner and heated until white fumes clear bulb of flask, swirled gently, and continued heating for 90 min. The flask was cooled, cautiously 250 ml distilled water added and cooled to room temperature.

3.4.5.3 *Distillation*

Titration flask was prepared by adding appropriate volume accurately measured acid standard solution to amount of water so that condenser tip is immersed. Two (2) - 3 drops of antifoam agent (tributyl citrate) was added to digestion flask to reduce foaming. Another 0.5 - 1.0 g alundum granules was added. Slowly down side of flask, sufficient 45% sodium hydroxide solution (approximately 80 mL) was added to make mixture strongly alkali. It was not mixed

until after flask was connected to distillation apparatus otherwise ammonia could be lost. Immediately the flask was connected to distillation apparatus and to distill at about 7.5 boil rates (temperature set to bring 250 ml water at 25^{o}C to boil in 7.5 min) until at least 150 ml distillate is collected in titrating flask into 50ml of boric acid. The digestion flask was removed and the titrating flask from the unit and the condenser tube rinsed with distilled water as the flask is being removed.

3.4.5.4 *Titration*

Finally, the distillate was titrated with 0.05M H_2SO_4. However, 1ml 0.05M H_2SO_4 is equivalent to 0.0014g of N_2. For the blank, another separate empty clean whatman no. 1 filter paper was given same treatment (digestion, distillation and titration) as that of the sample. The percentage crude protein was calculated as follows:

$$\% N_2 = \frac{\text{Titre – blank titre x 0.0014 x 100}}{\text{Weight of sample (g)}}$$

% Crude protein = % N_2 x protein factor (flour)

= % N_2 x 5.7

3.4.6 Carbohydrate content

The carbohydrate concentration was estimated as reported by Amoo and Ascore (2006). The percentages of moisture, ash, crude fats, and crude protein were summed up and subtracted from 100 as illustrated below.

Percentage carbohydrate = 100 - (% moisture +%ash + %crude fat +%crude protein).

3.5 Cultural Characterization of Moulds

3.5.1 Isolation

Enumeration of moulds was carried out as reported by Wartu *et al.*, (2015). Sterile Yeast Extract Sucrose Agar (YES) plates were prepared based on manufacturer's instruction and allowed to cool to roughly 40 - 45^{o}C. The sterile media was then supplemented with 100ppm of chloramphenicol before pouring into sterile petri dishes and the mixture was allowed to set. Serial dilution of each sample was made and aliquots 0.1ml of the test samples was inoculated by spread plate methods using sterile bent glass rods on the sterile YES agar. All the inoculated culture media plates were aerobically incubated at 27^{o}C for 7days.The colonies were then counted using colony counter.

The dilution with plate counts of 10-300 colony forming unit (CFU) was used for enumeration, and the result expressed as colony forming unit per gram (CFU/g) of sample as follows.

$$\text{CFU/g} = \frac{\text{colony count x dilution factor}}{\text{Inoculum volume}}$$

3.5.2 Macroscopic method

Macroscopic identification was carried out using Czepadox agar as reported by Klich, (2002).

Colonies that showed dark-green colony morphology and rough conidia was tentatively identified as *Aspergillus parasiticus*, while, isolates with morphological characteristics of yellowish-green colonies and smooth conidia was identified as *Aspergillus flavus.* A green dense crenated colony with broom-like spore was identified as *Penicillium* sp**,** while white colonies with pink colours with characteristic polyphialides, as well as long, slender nonphialides were identified as *Fusarium* spp etc.

3.5.3 Microscopic method

Microscopic examination of cultures was carried out as reported by reported by Ellis, (2006). Briefly, a drop of lactophenol cotton blue was placed at the center of a clean microscope slide. The mould cultures was picked with a sterile shape pointed inoculation loop and placed inside the stain and covered with clean cover slip. The slide was mounted on the stage of the microscope and examined microscopically for identification using ×10 and × 40 objectives. The observed mould isolates were sent abroad for typing.

3.5.4 Polyphasic characterization of aflatoxigenic moulds

Aflatoxigenic moulds was cultured on Yeast Extract Sucrose Agar (YES) supplemented with 0.3% cyclodextrin and 0.6% Sodium Desoxycholate (YCSD) as reported by other authors (Ordaz, 2003.,Gashgari *et al.*,2010 and Alborch *et al.*,2012). Sterile YCSD plates were prepared by adding 0.3% cyclodetrin to YES mediumand sterilized at 121^0C for 15minutes. The modified medium was allowed to cool to roughly 40 - 45^oC before adding 0.6% Sodium desocholate. The mixture was poured into sterile plates and allowed to set. Serial dilution of each sample was made and aliquot (1.0ml) of the test samples was inoculated by spread methods using sterile bent glass rod on the sterile YCSD agar. The inoculated culture media plates were aerobically incubated at 27^oC for 3- 14 days. All colonies that form visible beige ring and blue fluorescent ring around the colonies under UV light at 365 nm were counted with the colony counter as aflatoxigenic moulds. The dilution with plate count of 10-300 colony forming unit (CFU) was used for enumeration, and the result expressed as colony forming unit per gram (CFU/g) of sample as earlier reported.

$$\text{CFU/g} = \frac{\text{colony count x dilution factor}}{\text{Inoculum volume}}$$

3.6 Detection and Quantification of Total Aflatoxin Content of Wheat and Wheat Products

3.6.1 Total aflatoxin extraction

Extraction of total aflatoxins from wheat and wheat products was carried out according to the manufacturers' instruction as follows:

Seventy (70%) methanol (70mls of absolute methanol + 30mls distilled water) was prepared and kept aside. Representative sample was ground to the particle size of coffee where 50% passes through a 200µm mesh Screen. Twenty (20g) ground portion of each of the samples were weighed, placed in a clean bottle with Cover and 100mls of the extraction solvent; 70% methanol was added to each. The samples were then mixed by gentle shaking for a minimum of two minutes. The particles were then allowed to settle and the filtered through no. 1 filter paper. The filtrate at this stage is ready for Total aflatoxin assay using ELISA.

3.6.2 Total aflatoxin assay

Total aflatoxins was detected and quantified following the Enzyme Linked Immunosorbent Assay (ELISA) kits based on manufacturers' instruction as follows:

All the reagents were removed from the refrigerator and were allowed to acclimatize to room temperature before use. One dilution well was placed in a micro well holder for each standard and sample to be tested. Then equal number of antibody coated micro titer wells in another micro well holder. Two hundred (200µl) of conjugate was dispensed into each dilution well. Using a new pipette tip for each, 100µl of each standard and sample was added to appropriate dilution well containing conjugate and mixed by priming at least three times. One hundred (100µl) of each content dilution well was then transferred to a corresponding antibody Coated micro titer well and incubated at room temperature for 15minutes. The content of the micro well was

decanted into a discard basin and the micro -wells washed by filling each with distilled water then the water was decanted into a discard basin. Wash was repeated five times. The micro wells were then taped face down on a layer of absorbent towel to remove residual water. Required volume of substrate reagent was measured (120µl/well) and placed in a separate container. Then 100µl was added to each micro well and incubated at room temperature for five minutes. The required stop solution (120µl/well) was placed in a separate container and 100µl was then added in the same sequence and at the same pace as substrate was added. The optical density (OD) of each micro well was read with a micro titer plate reader using a 450nm filter. Finally the optical density of each micro well was generated by the micro titer plate reader and was printed.

3.6.3 Total aflatoxin content of wheat and wheat products

A dose response curve was constructed using the unmodified OD values of the standards against the aflatoxin content of the following standards (0.0, 0.2, 0.5, 1.0, 2.0, and 4.0) that came along with the kits. Unknown total aflatoxin values of the optical densities measured were obtained by interpolation from the standard curve. However, the samples were diluted at a 5:1 ratio with 70% methanol, and the level of aflatoxin shown by the standard was multiplied by five in order to quantify the nano gramme of aflatoxin per gram of commodity (µg/kg).

3.6.4 Aflatoxin producing ability of aflatoxigenic Isolates

Representative aflatoxigenic isolates with beige ring on the modified yeast extract sucrose agar was cut out from the agar and was extracted with 70% methanol. ELISA was then used to detect and quantify the total aflatoxins produced by the individual species after 14 days on 0.3% m-β-cyclodetrin and 0.6% sodium Desocholate modified YES agar.

3.7 Determination of Fate of Aflatoxins

3.7.1 Distribution of aflatoxins into wheat products during wheat processing

Ten grams (10kg) of wholesome wheat used by the processors was collected from the screening section, damped with purified water and a known quantity of standard aflatoxin B_1 was spiked on it. The mixture was incubated at 27^oC for 48h. The incubated wheat was milled (compact China wheat mill with capacity of 350kg/h) into wheat flour, semolina and brown flour. The aflatoxin level in the finished products was determined by ELISA method. The aflatoxin percentage loose was obtained by difference between the aflatoxin levels detected in each of the wheat products minus aflatoxin content of the finished product divided by the aflatoxin level of the raw material and multiplied by 100.

3.7.2 Effects of different extraction rates on total aflatoxin during wheat processing

Forty kilograms (40kg) wholesome wheat was conditioned with purified water and incubated in a rubber drum for 48hour.The damped wheat is then divided into four portions. The first portion was milled at 75% extraction (75% wheat flour + 25% bran), 70%, while the third and forth portions was milled at 65% and 60% extractions respectively. Aflatoxins was determined from the raw wheat and wheat flour samples from each extraction and certain portion was taken immediately to National Research Institute for Chemical Technology, (NARICT), Zaria for some physicochemical analysis (moisture, protein, ash).

3.7.3 Reduction in the levels of aflatoxins during fermentation

Aflatoxin pre-determined contaminated wheat flour obtained from 3.7.1 above was fermented to

wheat flour bread as follows: Five hundred (500g) x 5 samples was used to make five different loaf of bread using automatic bread maker (Morphy Richards) as follows. Aliquots 300mls of clean water was poured into the pan of the bread maker and the following ingredients will be added in the following order; 5.5g of salt, 45g of sugar, 0.5g margarine. Then the first set of 500g of wheat flour (pH5.8) was added into the pan and lastly 5g of yeast was added to the flour in the pan. The pan was mounted back into its position in the bread maker. A programme for white flour bread was selected and the machine was allowed to run for 2h 30minutes to mix, and prove the dough before baking. One hundred (200g) of the fermented dough was aseptically withdrawn intermittently at 30minutes, 1, 1.5 and at 2h and weighed out and placed into a clean screw cap bottle containing 100mls of 70% methanol. The aflatoxin content of the dough was extracted and quantified using ELISA.Each aflatoxin value in 100g was multiplied by by five to get the aflatoxin content in 500g. The aflatoxin percentage loss was calculated by dividing the aflatoxin difference between the aflatoxin level of the wheat flour and the aflatoxin level detected in the baked bread divided by the aflatoxin level of the wheat flour multiplied by 100.

3.7.4 Reduction in aflatoxins levels during baking

Pre-determined aflatoxin contaminated wheat flour obtained from 3.7.1 above was fermented as described under 3.7.2 above and baked at 180 and 220^0C as follows. Five hundred grams (500g) x 5 samples with predetermined aflatoxin concentration were used to make five different loafs of bread using automatic bread maker (Murphy Richards) as follows. Three hundred milliliters (300mls) of clean water was poured into the pan of the bread maker and the following ingredients added in the following order; 5.5g salt, 45g sugar and 0.5g margarine. The first set of 500g of wheat flour was added into the pan and lastly 5g of yeast weighed out and added to the

flour in the pan. The pan was mounted back into its position in the bread maker. A programme for white flour bread was selected and the machine allowed running for 2h 30minutes to mix, and prove the dough before baking. Two hundred (200g) of the baked dough after baking and cooling was aseptically weighed out and placed into a clean screw cap bottle containing 200mls of 70% methanol. The aflatoxin content of the dough was extracted and quantified by ELISA method. The aflatoxin percentage loss was calculated by dividing the aflatoxin difference between the aflatoxin level of the wheat flour and the aflatoxin level detected in the baked bread divided by the aflatoxin level of the wheat flour multiplied by 100.

3.7.5 Reduction in aflatoxin levels during wheat – sorghum *bicolor* fermentation into ``kunun-Zaki``

3.7.5.1 *Preparation of composite wheat – sorghum bicolour for fermentation*

Wholesome wheat was blended 5, 7.5 and 10% with Sorghum bicolor to give a sample of (1kg) each for ``kunun-zaki`` production. Naturally spoiled, mouldy yellowish suspected aflatoxin contaminated cereals were sorted and also blended 1, 2, and 3% each with 100% Sorghum bicolor to obtain another separate sample of (1kg) each for ``Kunun-zaki`` production. The first blend of the wholesome cereal was constituted in duplicate. The second blend was spiked with standard aflatoxin B to serve as control.

3.7.5.2 ``*Kunun-Zaki*`` *production*

The medification of the method used by Egbere *et al*. (2008) was adopted for ``kunun-zaki`` production . An aliquot of 200 mls (20%) portion of the slurry was removed and sterilized spices (ginger, alligator pepper) added to it and kept separately. Then 150 mls of aseptically dried sweet potato was wet ground into a paste and added to the separately kept 20% slurry. The remaining

slurry (80%) portion was gelatinized by addition of boiling water while stirring until thick consistency was achieved. The liquefying agents were then added to the gel after cooling to approximately 30^{o}C. It is believed that this heat does not affect the activity of the alpha amylase in the malted cereal. The mixture was then stirred gently until liquefied or lost its consistency. Finally, the mixture was incubated at ambient temperature for 12h to ferment aerobically in an open stainless steel bioreactor. The finished product was then sieved using sterile muslin cloth. Sterilized sugar was added to taste and aseptically bottled and immediately used for physicochemical studies and aflatoxin determination.

3.8 Molecular Characterization of Aflatoxigenic Moulds using Polymerase Chain Reaction

3.8.1 DNA extraction from aflatoxigenic *Aspergillus* species

Deoxyribonucleic acid (DNA) was extracted from both the aflatoxigenic *Aspergillus* sp and non aflatoxigenic isolates as reported by obtained from this research according to the methods of Alborch *et al*. (2012). Briefly, Mycelia was picked using sterile inoculation loop and placed in eppendoff tube. The sample was ground with eppendoff pestle. Four hundred (400) µl lysis buffer was added followed by protinase K. The mixture was incubated at 68^{o}C for an hour with 20min. intermittent votexing. Then 400 µl phenol chloroform and was centrifuged at 13,000rpm/10min. The supernatant was pipetted out. The supernatant was added 400 µl of 100% ethanol and was span at 13,000rpm/15min. The mixture was incubated over night at -18^{o}C. The sample was removed and centrifuged at 14,00rpm/10min at 4^{o}C. One hundred (100) % ethanol was added and centrifuged 14,000ro/5min. The top layer was removed and placed in new eppendoff tube and 70% ethanol further added and centrifuged at 4^{o}C at 14,000rpm/2min. Finally the top layer contained the DNA was removed and air dried before use.

3.8.2 Polymerase chain reaction (PCR)

Polymerase Chain Reaction was carried out according to the method of Erami *et al.* (2007), to amplify four target fragments on *Aspergillus parasiticus and Aspergillus flavus* genomic DNA (Table 3.1). Polymerase Chain Reaction assays was performed in 50 µl of a reaction mixture that typically contain 100 ng of genomic DNA, deoxynucleoside triphosphates (200 µM), primers (1µM), and reaction buffer (10 mM Tris-HCl [pH 9.0], 50mM KCl, 1.5 mM $MgCl_2$, 0.1% Triton X-100, and 0.2 mg of gelatin per ml). The reaction mixture was heated to 95^0C for 10 min before 2.5µl of *Taq* DNA polymerase (Appligene, Pleasanton, Calif.) was added. A total of 30 PCR cycles (one cycle being 1 min at 94^0C for denaturation, 2 min at 65^0C for primer annealing, and 2 min at 72^0C for extension) and a 5-min final extension at 72^0C was run on a programmable DNA thermal cycler (NJ Research Inc., Watertown, Mass.). The PCR products were analyzed on electrophoresis on a 1% agarose gel stained with 1 µg of ethidium bromide.

Table 3.1: Primer Nucleotide Sequences for Key Aflatoxigenic Genes

Gene	Nucleatide Sequence	Amplification Target	Source
ver-	F 5` ATGTCGGATAATCACCGTTTAGATGGC-3`		
	R 5`CGAAAAGCGCCACCATCCACCCCAATG-3`	537bp	Scherm *et al*.,(2005).
omt-A-	F 5`GGCCCGGTTCCTTGGCTCCTAAGC-3`		
	R 5`CGCCCCAGTGAGACCCTTCCTCG-3`	797bp	Scherm *et al*., (2005).
aflR-	F 5`-TAT CTC CCC CCG GGC ATC TCC CGG-3`		
	R 5`-CCG TCA GAC AGC CAC TGG ACA CGG-3`	1032 bp	Scherm *et al*., (2005).
nor-	F 5`ACC GCT ACG CCG GCA CTC TCG GCAC-3`		
	R 5`GTT GGC CGC CAG CTT CGA CAC TCC G-3`	400-bp	Scherm *et al*., (2005).

3.9 Dioxyribonucleic Acid (DNA) Sequencing

Direct sequencing of PCR products was carried out to further confirm the PCR result since an internal positive control was used. Sequencing reactions were performed using an applied biosystems (ABI) Prism Sequencer™ (Perkin-Elmer) involving three basic steps.

3.9.1 Elution of DNA from the gel band:

The bands of *aflR-1* on electrophoretic gel was cut out and weighted in mg. Three hundred micrograms (300 µg) of gel binding (GB) buffer was added to each 100mg of the band. The mixture was heated at 50^0C for 10 minutes or until the gel is dissolved with inversion of the tube after every 2-3 minutes. Approximately equal volume of Isopropanol was added to the above suspended mixture (for Base pairs less than 500). They were mixed and transferred to binding columns. It was spun at 10,000 rpm for 1 minute and the content of the flow tube discarded (containing the gel) (while DNA would be bound to the binding column). To the binding column, 750µl of Protein extraction (PE) Buffer was added and allowed to stand for 5 minutes. It was spun at 13,000 rpm for 1 minute and the flow tube discarded. The empty tube was spun again rpm for 2 minutes. The content of the column was transferred to a new collecting tube and the column tube discarded. To this new collecting tube, 25µm ultrapure water was added and allowed to stand for 5 minutes. It was then spun at 13,000rpm for 1 minute.

3.9.2 Sequencing PCR product

Ten (10µl) of the above DNA was mixed with 2µl of reverse/or forward primer and 8µl of Dye terminator cycle (DTC) mixture added.The above mixture was put in a PCR machine and conditions set at 96^oC for 20 seconds, 50^oC for 20 seconds and 60^oC for 4 minutes for 30 cycles.

3.9.3 Sequencing clean-up

One hundred (100µl) of prewarm buffer (Pb) was added to the product of the above sequencing reaction and mixed by pipetting in and out. The entire reaction is transferred into the binding column and spun at 10,000rpm for 1 minute. The flow-trough was discarded and the collection tube re-used. Approximately 650µl of PE was added; the mixture allowed standing for 3 minutes and spun at 13,000rpm for 1 minute. 650µl of PE buffer and spun immediately at 13,000rpm for 1 minute. The empty column was spun at 13,000 rpm for 2 minutes. New collection tube was used. To the above reaction, 13µl of Sample Loading Solution (SLS) was added, allowed to stand for 5 minutes and spun at 13,000rpm for 1 minute. The elution step was repeated and the product was spun immediately. The above product was then loaded in the sequencing machine.

3.9.4 *AFL2T* sequence assembly

Sequences of the fragment (aflR-1) were assembled to yield the entire AFR2T gene sequence; the AFLR2T sequences of each strain were aligned using CLUSTAL X 1.83 (Thompson *et al*., 1997). All the sequences was visually assessed and optimized when necessary

3.10 *AFL2T* PCR Product and NCBI Blast Search for Homology

The sequenced *aflR-1* PCR products (*AFL2T*) were assembled using BioEdit software. The sequences were subjected to blast search. using specific *Aspergillus* species specific site; AspGD MultiGenome NCBI Search on the website http://www.aspergillusgenome.org/cgi-bin/compute/blast_clade.pl. The *AFL2T* sequences of each strain were aligned using CLUSTAL X 1.83 according to the methods of Rodrigues *et al.* (2009). The entire *aflR2T* gene sequences obtained in this study were aligned with existing data for known sequence from the gene bank to seek for enough homology or Compatibility.

3.11 Cluster Phylogenetic Relatedness of Isolates using Sequences of *AFL2T* PCR Products

The Phylogenetic analysis by Maximum Likelihood method based on Tamura-Nei model was adopted to generate a dendogramm. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The tree was drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 12 nucleotide sequences.

3.12 Data analysis

Data obtained was analysed using statistical package for social sciences (SPSS 7.0, 2013) to ascertain variation or association between the generated data from the various mills and major distribution stores. Analysis of variance was considered significant at $(P \leq 0.05)$.

CHAPTER FOUR

4.0 RESULTS

4.1 Proximate Composition

4.1.1 In-process wheat and wheat products

The mean±S.E.M. of the proximate composition of in-process wheat and wheat products is presented in Table 4.1. The highest moisture content (15.40±1.34%) was found in tempered wheat followed by semolina (13.60±0.27%), wheat flour (13.55±0.19%), brown flour (13.50±0.27%) and the least in bran (12.48±0.30%). The highest Ash values were found in wheat (1.29±0.63%) and the least in Semolina (0.56±0.13%). Protein content was highest in wheat (18.15±1.06$^{)}$ and least in bran (2.50±0.83%). Bran (0.19±0.002%) had the highest free fatty acid and by wheat flour (0.09±0.003%) the least. The proximate composition values were however not significant from each other.

4.1.2 Wheat products from major distribution stores

Table 4.2 shows the mean±S.E.M. of the some proximate composition of wheat products collected from major distributors. The mean moisture obtained in this study was highest (13.28±0.17%), in semolina and bran (12.33±0.27%) the least. There were no significant differences ($P \geq 0.05$) in the moisture content of samples collected from the major stores. Bran had the highest Ash content (1.17±0.13%), followed by brown flour (0.79±0.13%), and wheat flour 0.55±0.13% while semolina (0.52±0.14%) was the least and there was no significant difference ($P \geq 0.05$) in the ash content of the samples collected from the major stores. Semolina had the highest Protein content (16.33±1.11%), followed by wheat flour (15.15±1.06%) and brown flour (15.15±1.07%) and there was no significant difference ($P \geq 0.05$) between the

protein content of samples collected from the major stores. There was no significant difference ($P \geq 0.05$) in the fat content of the samples collected from the major stores and the highest amount was recorded in bran (0.76±0.17) and the least in semolina (0.60±0.17%). The free fatty acid was highest in bran (0.28±0.05%), followed by brown flour (0.15±0.02%) and was least in semolina (0.12±0.01%). Furthermore, there was no significant difference in the free fatty acid content of the samples collected from the major stores.

4.2 Mould Counts

4.2.1 In-process wheat samples and wheat products

Table 4.3 show the mould counts (logCFU/g) obtained from the contamination in-process wheat and wheat products. The mean moulds count was highest in bran (7.58±0.00) and least in wheat flour (2.36±0.001). Samples obtained from Mill B had the highest mould counts (4.79) and least by mill C (3.71). There was significant difference ($P \leq 0.05$) between the moulds counts of each samples collected from the various mills.

Table 4.1: Proximate Composition of In-process Wheat and Wheat Products Collected from some Wheat Mills in Northern Nigeria

Sample	Moisture (%)	Ash content (%)	Protein (%)	Fat (%)	free fatty acid (%)
Wheat	13.65±0.25[a]	1.29±0.63[b]	18.15±1.06[c]	1.34±0.16[d]	0.13±0.005[f]
Tempered Wheat	15.4 ± 1.34[a]	1.28±0.21[b]	18.11± 1.01[c]	1.31± 0.13[d]	0.10±0.22[f]
Wheat Flour	13.55±0.19[a]	0.71±0.14[b]	16.33± 0.11[c]	0.69±0.17[d]	0.09±0.003[f]
Brown flour	13.50±0.27[a]	0.79±0.13[b]	15.15± 0.07[c]	0.65±0.17[d]	0.12±0.004[f]
Semolina	13.60±0.27[a]	0.56±0.13[b]	16.30± 1.11[c]	0.58±0.17[d]	0.10±0.001[f]
bran	12.48±0.30[a]	1.25±0.60[b]	2.50± 0.83[d]	0.88±0.17[d]	0.19±0.002[f]
p-value	0.993	0.996	1.000	1.000	1.000

Mean values with same superscript within the same column are not significantly different

Table 4. 2: Proximate Composition of Wheat products Collected from Major Stores of Some Wheat Mills in Northern Nigeria

Wheat products	Moisture (%)	Ash content (%)	Protein (%)	Fat (%)	free fatty acid (%)
Wheat flour	13.25±0.25[a]	0.55±0.13[b]	15.15±1.06[d]	0.69±0.16[e]	0.13±0.003[f]
Semolina	13.28±0.17[a]	0.52±0.14[b]	16.33±1.11[d]	0.60±0.17[e]	0.12±0.001[f]
Brown flour	13.20±0.27[a]	0.79±0.13[b]	15.15±1.07[d]	0.63±0.17[e]	0.15±0.002[f]
Bran	12.33±0.27[a]	1.17±0.13[b]	3.30±0.11[d]	0.76±0.17[e]	0.28±0.005[f]
p-value	0.996	0.605	1.000	1.000	0.905

Mean values followed by same superscript within the same column are not significantly different

Table 4.3: Mould Counts (logCFU/g) of In-process Wheat and Wheat Products Samples Collected from Some Wheat Mills in Northern Nigeria

Mill	ww	TW	WF	SM	BF	BR
A	5.28±0.02^{c}	5.34±0.01^{c}	3.18±0.03^{c}	3.20±0.01^{c}	4.43±0.01^{g}	5.65±0.03^{c}
B	5.23±0.02^{c}	6.70±0.00^{e}	3.46±0.01^{d}	3.38±0.01^{e}	3.58±0.01^{d}	6.40±0.00^{a}
C	3.86±0.49^{b}	5.18±0.01^{b}	2.36±0.01^{b}	3.15±0.01^{b}	3.15±0.01^{b}	4.57±0.00^{a}
D	4.38±0.02^{b}	5.58±0.01^{d}	3.04±0.01^{d}	3.52±0.02^{c}	3.52±0.02^{e}	7.58±0.00^{h}
p-value	0.000*	0.000*	0.000*	0.000*	0.000*	0.013*

Mean values with different superscripts within the same column are significantly different ($P \leq 0.05\%$)

Key:

WW, wholesome wheat.

TW, tempered wheat,

WF, wheat flour,

BF, brown flour,

BR, bran.

4.2.2 Mould counts of wheat products from major stores

Major store samples had the highest moulds count in bran (7.77±0.02) and least in wheat flour (1.97±0.01). In addition, brand 5 had the highest moulds count (4.79) and least by brand 3 (3.93).There was significant difference ($p \leq 0.05$) between the moulds count of the different samples from the brands (Table 4.4).

4.3 Frequency of Occurrence of Fungi

4.3.1 Frequency of moulds species

The frequency (%) of moulds species isolated from wheat and wheat products during in-process and major stores is shown in Table 4.5. The most frequent moulds species from in-process and major stores was *Aspergillus flavus* (71.5 and 76.25%) respectively and least by *Aspergillus fumigatus* (3.0%). Bran had the highest mean moulds occurrence frequency (43.73 and 46.36%) from in-process and major stores respectively and least in wheat flour (19.82%) from in-process samples. Major store samples had significantly ($p \leq 0.05$) higher frequency of moulds isolates than in-process.

Table 4.4: Mould Counts (logCFU/g) of Wheat Products Collected from Major Stores (N=120)

Brand	WF	SM	BF	BR
1	3.57 ± 0.01^{g}	3.51 ± 0.00^{f}	3.51 ± 0.00^{c}	7.77 ± 0.02^{i}
2	4.61 ± 0.01^{i}	2.61 ± 0.00^{a}	2.61 ± 0.00^{a}	7.51 ± 0.00^{g}
3	2.64 ± 0.01^{c}	3.32 ± 0.02^{e}	3.32 ± 0.02^{d}	6.45 ± 0.00^{d}
4	1.97 ± 0.01^{a}	3.52 ± 0.02^{f}	3.52 ± 0.01^{e}	7.28 ± 0.00^{f}
5	3.67 ± 0.02^{a}	4.38 ± 0.00^{g}	4.38 ± 0.02^{f}	6.73 ± 0.00^{e}
P-value	0.000^{*}	0.000^{*}	0.000^{*}	0.013^{*}

Mean values with different superscripts within the same column are significantly different ($P \leq 0.05\%$)

Key:

WF, wheat flour,

BF, brown flour,

BR, bran.

Table 4.5: Frequency of Occurrence (%) of Moulds species isolated from Wheat and Wheat Products (In- process and Major Stores)

Fungi	In-process Samples						Major Store			
	WW	TW	WF	SM	BF	BR	WF	SM	BF	BR
Aspergillus flavus	78	82	51	40	95	93	75	50	83	97
Aspergillus parasiticus	60	67	43	31	69	76	67	42	75	83
Aspergillus niger	33	49	25	36	75	59	38	42	52	61
Rhizopus stolinifer	23	58	10	13	16	62	07	08	08	38
Penicillium chrysogenum	28	24	33	18	20	77	30	25	50	62
Fusarium moniliform	62	48	20	21	47	89	03	03	15	37
Aspergillus terrus	32	38	11	07	11	37	17	33	33	47
Saccharomycetes cerevisae	15	41	20	04	07	06	31	11	27	30
Rhizopus nigrican	04	38	01	05	09	18	03	01	03	33
Aspergillus fumigatus	02	04	02	01	03	05	01	01	04	10
Aspergillus tamari	05	01	02	02	04	07	02	03	08	12

Key:

WW, wholesome wheat

TW, tempered wheat,

WF, wheat flour,

BF, brown flour,

BR,bran.

4.3.2 Frequency of occurrence (%) of aflatoxigenic isolates

The frequency of occurrence (%) of aflatoxigenic strains isolated from wheat and wheat products from in-process and major stores is shown in Table 4.6. Three out of seven moulds species isolated had beige ring on the modified YES agar. The predominant aflatoxigenic strain isolated from in-process was *Aspergillus flavus* (37.7%) and (43.0%) from major stores, while *Aspergillus parasiticus* had 28.7% in-process and 33.8% from major stores. Least aflatoxigenic strain isolated was that of *Aspergillus niger* (03.0%) from in-process raw wheat. While bran had highest percentage of aflatoxigenic moulds for both in-process (66.0%) and major stores (78.0%), wheat flour had least percentage of aflatoxigenic strains from in-process (17.5%). Similarly, semolina had the least count (13.5%) from major stores.

4.4 Relative Density (%) of Moulds and Aflatoxigenic Isolates from Wheat Flour Improvers, Vitamin A Premix and Cassava flour.

Table 4.7 represents the relative density (%) of moulds and occurrence of aflatoxigenic strains (%) from wheat flour improvers, Vitamin A premix and cassava flour. Cassava flour significantly had higher percentage (79.0%) of aflatoxigenic moulds than alpha amylase and ascorbic acid premix (15.0%) improvers. Vitamin A premix had the least percentage occurrence (11.7%) of aflatoxigenic moulds. Certain moulds species such as *penicillium chrysogenum, fusarium* species and *Candida* species isolated from the improvers and vitamin A did not form beige ring on the modified YESA medium.

Table 4.6: Frequency (%) of Aflatoxigenic species Isolated from Wheat and Wheat Products In-process and Major Stores using Modified YES Medium (n=582)

Moulds	**In-process samples**						**Major Store Samples**			
	WW	TW	WF	SM	BF	BR	WF	SM	BF	BR
Aspergillus flavus	34	39	19	12	43	77	24	15	45	88
Aspergillus parasiticus	21	34	16	11	25	55	23	12	32	68
Aspergillus niger	03	-	-	-	-	-	-	-	-	-

Key:

WW, wholesome wheat

TW, tempered wheat

WF, wheat flour

SM, semolina.

BF, brown flour

BR, bran.

-, no beige ring

Table 4.7: Relative Density (%) of Moulds and Aflatoxigenic Strains (%) in Parenthesis from Wheat Flour Improvers, Vitamin A Premix and Cassava flour

Moulds	Vitamin A	Cassava flour	Wheat flour premix improvers α-amylase & ascorbic acid
Aspergillus spp	16.7(11.7)	83.3(79.0)	58.0(15.0)
Penicillium spp	16.7(-)	42.0(-)	25.0(-)
Fusarium spp	-	16.7(-)	-
Rhizopus spp	-	50.0(-)	25.0(-)
Candida spp	-	66.7(-)	16.7(-)

Key:

-, no fungi isolate

(-), no aflatoxigenic moulds isolate

4.5 Total Aflatoxin Content

4.5.1 Total aflatoxin content In-processed wheat and wheat products

The mean distribution of total aflatoxin level (µg/kg) in-process wheat into products is shown in Table 4.8. Bran presented highest mean aflatoxin value and least by semolina. Bran had the highest mean aflatoxin value (0.30±0.10 µg/kg) and semolina had the least (0.08±0.03). Mill B had the higher mean aflatoxin values and least in mill C. Mill A and B (conventional mills) aflatoxin ranges were higher than Mill C and D (batch mills). Bran significantly ($p \leq 0.05$) had higher aflatoxin ranges than wheat flour. There was also significant difference ($p \leq 0.05$) between the total aflatoxin values of wheat and wheat flour obtained from the different mills. Fifteen (18), 27, and 33% of the total aflatoxins obtained from in-process samples exceeded the NAFDAC 10µg/kg recommended limit for semolina, wheat flour, and brown flour respectively from the in-process samples.

Table 4.8: Total Aflatoxin Content (µg/kg) In-processed Wheat and Products

Mill	wheat	wheat flour	semolina	brown flour	bran
A	0.15±0.05[a]	0.12±0.03[a]	0.09±0.07[a]	0.18±0.07[a]	0.22±0.09[a]
B	0.16±0.70[a]	0.14±0.06[a]	0.13±0.04[a]	0.13±0.04[a]	0.30±0.10[a]
C	0.13±0.40[a]	0.08±0.03[a]	0.09±0.03[a]	0.15±0.06[a]	0.16±0.06[a]
D	0.14±0.07[a]	0.11±0.05[a]	0.08±0.03[a]	0.16±0.06[a]	0.18±0.08[a]
p-value	0.000[*]	0.000[*]	0.025[*]	0.011[*]	0.013[*]

Mean followed with different superscripts within the same column vary significantly ($P \leq 0.05\%$)

4.5.2 Total aflatoxin content in major store products

Table 4.9 shows the mean total aflatoxin level (µg/kg) content of the wheat product brands collected from major stores. Bran had the highest aflatoxin content (0.45±0.14) and the least was in semolina (0.09±0.01). All the brands had aflatoxin levels higher in bran followed by brown flour and least in semolina. Aflatoxin range of 0.5 – 73 µg/kg was recorded in this study from all the mills and the major stores. There was significant difference between the total aflatoxin values of the samples collected from the major stores. Twenty four percent (24.0), 29.0 and 45% of the total aflatoxins exceeded the NAFDAC 10µg/kg recommended limit for semolina, wheat flour, and brown flour respectively from the major store samples. Prevalence of thirty eight (38.0%) of the wheat samples collected from the processors was contaminated with aflatoxins. Likewise the prevalence of total aflatoxins obtained from this research was 21.0, 28.0, 39.0 and 89.0% for semolina, wheat flour, brown flour and bran respectively.

Table 4.9: Mean Total Aflatoxin Level (µg/kg) distribution Detected from Wheat Products Collected from Major Stores

Flour Brands	Wheat flour	Semolina	Brown flour	Bran
1	0.18±0.07[a]	0.12±0.02[a]	0.20±0.08[a]	0.30±0.10[b]
2	0.17±0.06[a]	0.15±0.04[a]	0.17±0.06[a]	0.45±0.14[b]
3	0.15±0.04[a]	0.11±0.03[a]	0.14±0.04[a]	0.28±0.-11[b]
4	0.14±0.05[a]	0.09±0.01[a]	0.18±0.08[a]	0.40±0.13[b]
5	0.16±0.06[a]	0.12±0.04[a]	0.16±0.07[a]	0.30±0.12[b]
p-value	0.030[*]	0.021[*]	0.0028[*]	0.034[*]

Mean followed with different superscripts within the same column vary significantly ($P \leq 0.05\%$)

4.6 Distribution and Fate of Aflatoxins

4.6.1 During different wheat processing extraction rates

Figure I shows the fate of total aflatoxin during endosperm extraction from contaminated wheat into wheat flour. At zero percent extraction, the wholesome America wheat *cultivar* presented ash content of 1.20%dm and spiked aflatoxin B_1 level of 44 µg/kg. At 60 % extraction, the percentage ash was at (0.57%dm) and the total aflatoxin retention was 2.1µg/kg and the decontamination was 98.0%. There was significant level of aflatoxin decontamination at 60% extraction. This work has shown that while the extraction rate increased from 60 - 75%, the ash content and the aflatoxin B_1 retention increased from 0.57 - 0.82%dm and 2.1- 65.9% respectively. Consequently, the aflatoxin decontamination decreased from 98.0 - 34.1% for the same extraction rate

4.6.2 Reduction of aflatoxin levels during fermentation of dough

Figure II presents the trends of total aflatoxin level during solid substrate fermentation and baking of bread. The unfermented dough had total aflatoxin content of 29.0 µg/kg. Following 30minutes of fermentation at 35±2^{o}C, the total aflatoxin level remained unchanged. After 60minutes of fermentation, the aflatoxin retention dropped to 26.0 µg/kg and the percentage aflatoxin loss was 10.3%. There was 34.5% aflatoxin loss after 90minutes incubation and the aflatoxin retention decreased to 19.0 µg/kg. There was significant aflatoxin loss (55.2%) after 120minutes fermentation giving 44.8% aflatoxin retention in wheat flour with moisture of 13.7% and pH 5.5. There was correlation between increase fermentation time of the dough and aflatoxin

loss. As the fermentation time increases, the percentage total aflatoxin loss increases and the percentage aflatoxin retention decreased.

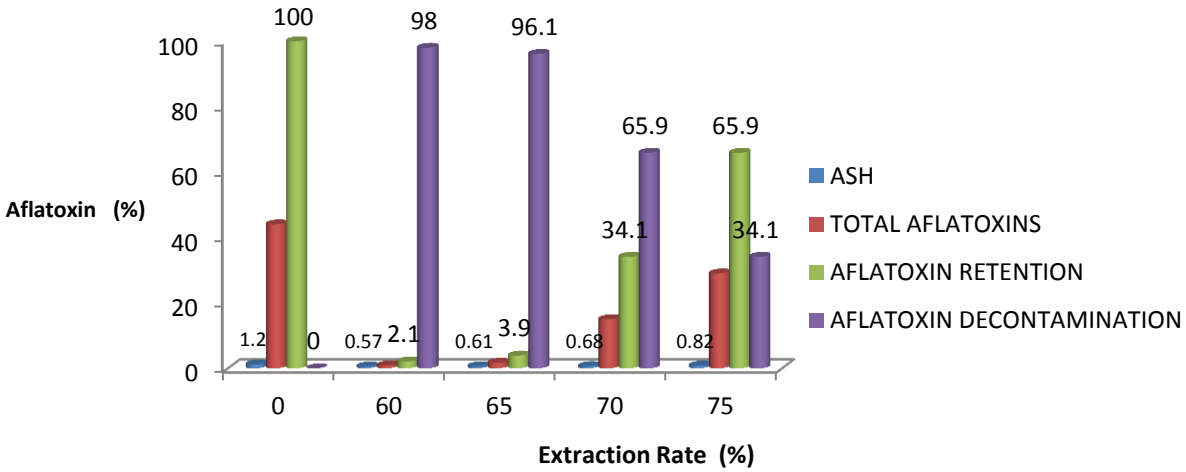


Fig. I: Fate of Total Aflatoxins during Endosperm Extraction from contaminated wheat into flour

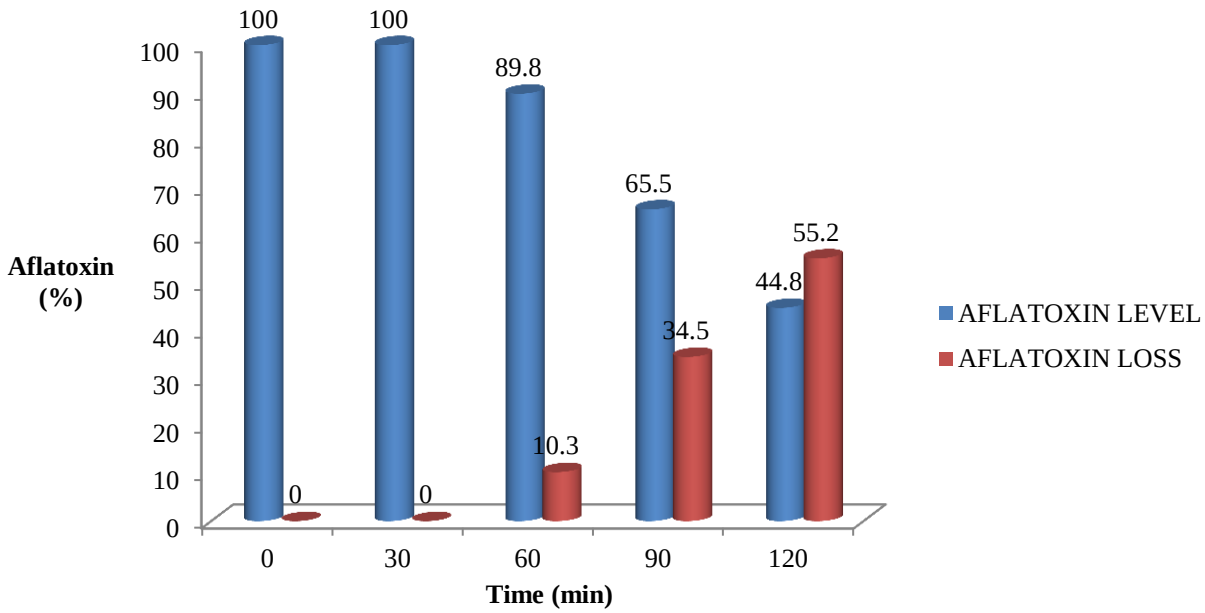


Fig. II: Trends in Total Aflatoxins during Fermentation of Wheat Flour Dough

4.6.3 Reduction of aflatoxins during baking

The baking profile of the fermented dough also showed positive correlation between the baking temperature and the percentage total aflatoxin loss (Figure III). As the baking temperature increases from 180 – 220^{o}C the total aflatoxin loss increased from 58.6 – 72.4%.

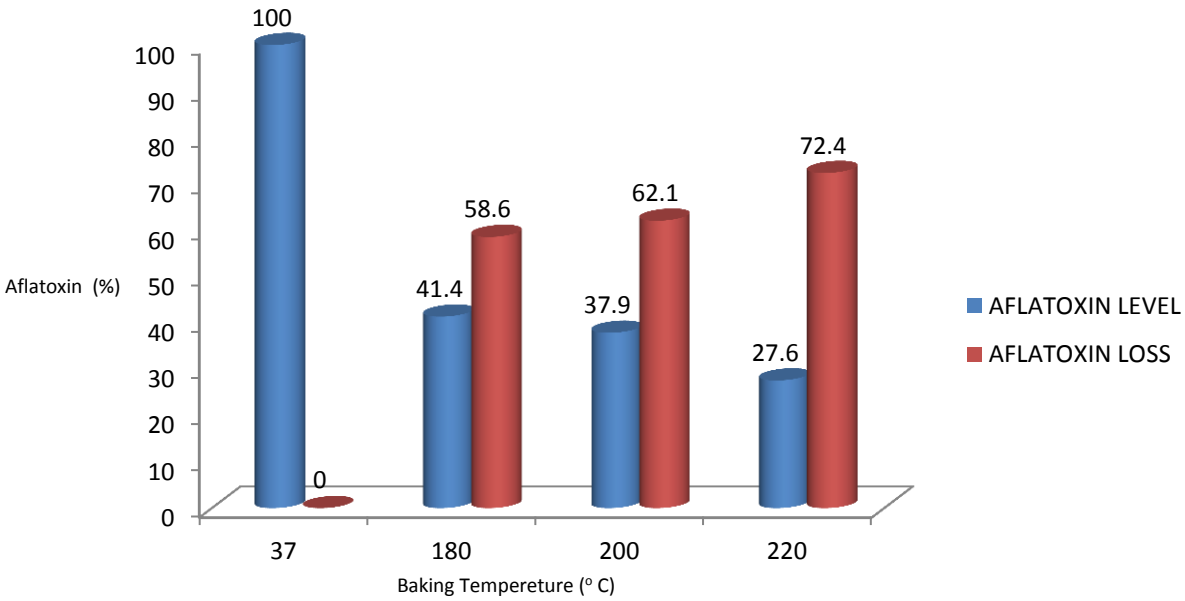


Fig. III: Total Aflatoxins Levels during Baking Wheat Flour Dough

3.6.4 Aflatoxin reduction during fermentation of wheat–Sorghum *bicolor* `kunun- zaki`

Table 4.10 shows the trends in total aflatoxins level in submerged liquid substrate fermentation of total aflatoxin contaminated wheat - sorghum composite and the total aflatoxin percentage loss shown in parenthesis. The slurry obtained after wet milling had more total aflatoxins compared to the other step total aflatoxin level. Conversely, the final product had lower total aflatoxin levels compared to the other stages. There was a correlation between the total aflatoxin levels of the spoilt-wholesome cereal and the various blends; as the percentage of the spoilt cereal increased from 1-3%, the level of total aflatoxins also increased. The slurry had higher total aflatoxin levels than the raw material flour. However, there was no significant difference in the level of aflatoxins in the different stages of kunun-zaki production steps.

4.7 Multiplex Polymerase Chain Reaction

4.7.1 Characterization of aflatoxigenic strains using multiplex polymerase chain reaction

Plate I shows the electrophoresis of the aflatoxigenic *Aspergillus flavus* and *Aspergillus parasiticus* isolates from wheat and wheat products. The Quadruplex PCR provided a confirmatory method for aflatoxigenic strains. The result revealed that applied conditions were set up perfectly, as though each primer formed sharp and distinct bands in its specific area. When the PCR was carried out and run on 1.5% agarose gel. The bands of fragments of the four sets of primers; *aflR-1, omt-A, ver-1 and nor-1* genes were visualized at 1032, 797, 537 and 400 bp, respectively. The quadruplet pattern was consistent with all the aflatoxigenic strains indicating

the presence of the four genes of the aflatoxin biosynthetic pathway. While the non aflatoxigenic strains had one, two and three banding patterns, aflatoxin had consistent four bands.

Table 4.10: Trends in total Aflatoxin Level during Submerged Barch Fermentation of Aflatoxin Contaminated Wheat – *Sorghum bicolor* Composite at 27^{o}C

	Total Aflatoxin (µg/kg) in Spoiled grain - wholesome sorghum blend			
Sample	1	2	3	WWW composite
Cereal blend	7.08	26.95	36.85	Nil
Slurry	8.00 *(2.50*)*	27.70*(2.71*)*	37.80 *(2.50*)*	Nil
Gel	6.90 *(13.75**)*	24.00 *(13.35**)*	33.20 *(12.17**)*	Nil
Fermented kunun-zaki	3.76 *(53.00**)*	13.50*(52.00**)*	17.5 *(53.70**)*	Nil

Values are mean of duplicate samples

Key,

*, percentage aflatoxin content higher than raw material aflatoxin

**, percentage aflatoxin loss.

1, 2, and 3, represents percentage of mouldy cereal to wholesome cereal blend

WWW, wholesome wheat-sorghum composite

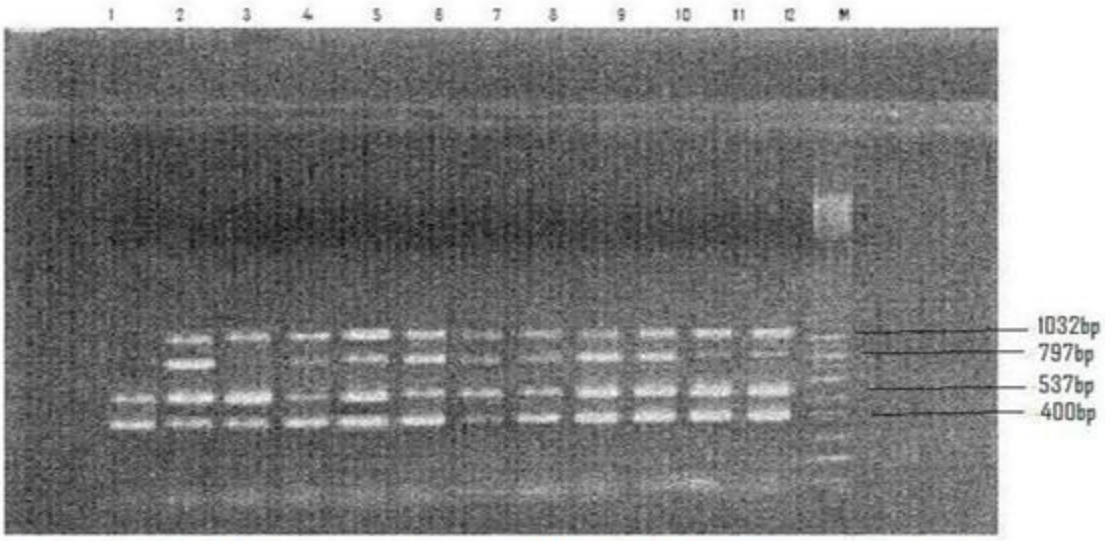


Plate I: Multiplex PCR Products Obtained using aflR-1, omt-1, ver-1 and nor-1 Genes from Aflatoxigenic Moulds

Key : Lane 2,4-12 are Aflatoxigenic Moulds. Lane 1 and 3 are Non Aflatoxigenic Moulds.Lane M, Molecular Size Ladder (Biolab 100bp Marker)

4.7.2 Characterization of non aflatoxigenic strains using multiplex polymerase chain reaction.

Plate II shows the electrophoresis of non aflatoxigenic *Aspergillus flavus* and *Aspergillus parasiticus* isolated from wheat and products. The non-aflatoxigenic strains presented one, two and three banding patterns with different profiles, while isolate 1, 2, 3, 8, 11 and 12 presented two bands each, isolate 3,4 and 7 presented one band. Both banding patterns had different profiles. The DNA amplifications of isolates 9 and 10 were that of an aflatoxigenic moulds used as control and had presenting four banding patterns each. Fifty (50.0%) of all the non-aflatoxigenic strains genes amplified in this study showed one profile, and two DNA banding patterns. On the same other, 66.7% of the two DNA amplification banding patterns showed varied profiles. Twenty percent (20.0%) of the non-aflatoxigenic strains showed one variable profile, and one DNA amplification banding pattern.

4.7.3 Frequency of occurrence of key genes in aflatoxin biosynthesis among non aflatoxigenic strains

Figure IV shows the frequency of occurrence of key genes in the aflatoxin biosynthesis thus indicating variability among the non aflatoxigenic strains. The genes from non aflatoxigenic strains showed that the *nor-1* gene had the highest frequency of occurrence (73.3%) followed by *ver-1* (53.3%), *omt-A* (40.0%) and the least was *aflR-1* (13.3%).

4.7.4 Sequencing of *aflR-1* PCR Product and NCBI Blast Search for Homology

Compatibility of the *AFL2T* gene was found to march entirely with standard *aflR-1* of NCBI Gene Bank resulting to alignment between the query sequence (*AFL2T*) and the standard *aflR-1* from standard NCBI data base. The hit list were edited and the organism on top of the hit list with highest identity positive (%) and their e-values was selected as the target strains as in (Table 4.12)

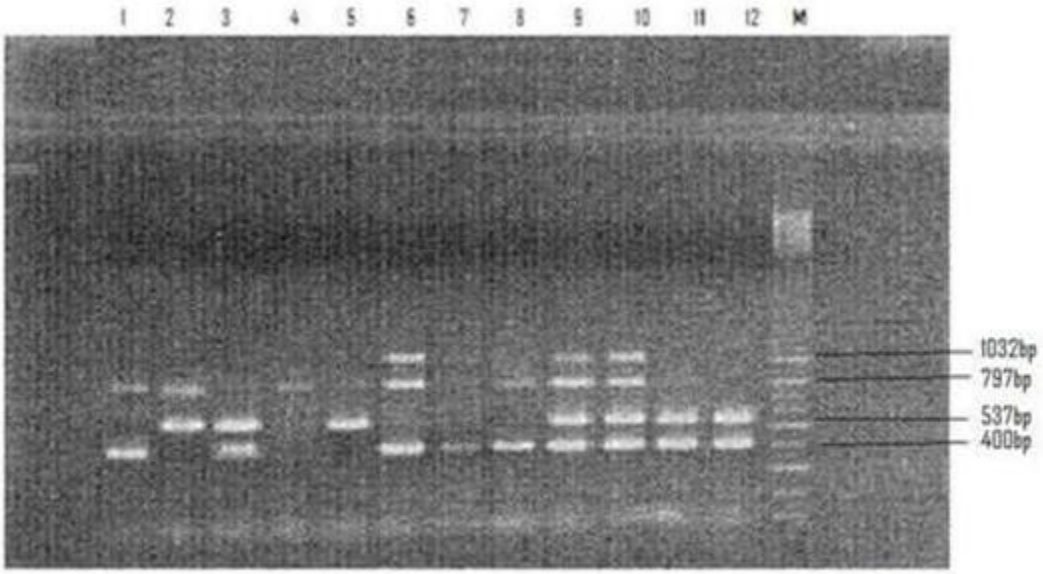


Plate II : Multiplex PCR Product Obtained using aflR-1, omt-1, ver-1 and nor-1 Genes from Non Aflatoxigenic Moulds

Key : Lane 1-12 are Isolate Codes (1-8, 11 and 12 are Non aflatoxigenic Moulds, whie Lane 9 and 10 are Aflatoxigenic Moulds). Lane M, Molecular Size Ladder (Biolab 100bp Marker)

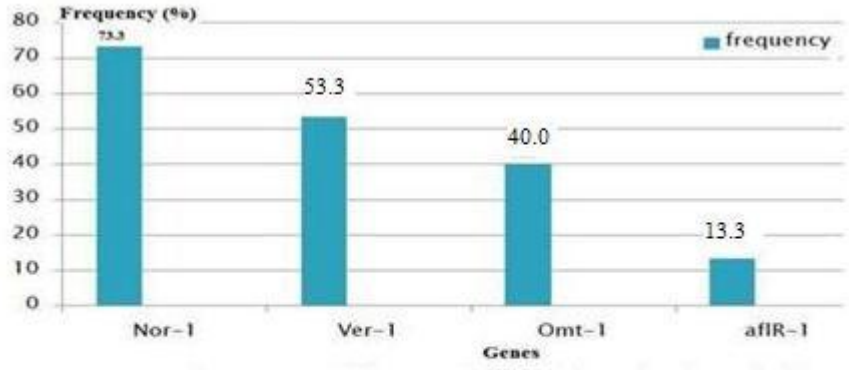


Fig. IV: Frequency of Occurance of Key Genes in Aflatoxin Biosynthesis Among Non Aflatoxigenic Moulds

Table 4.12: Blast Search Hit List of *AFL2T* query Sequences from Standard *aflR-1* NCBI Gene Bank

Microorganism	Accession number	Score (bits)	Identity positive (%)	e-value
Aspergillus flavus NRRL3357	DQ467939.1	572	98.8	0.3
Aspergillus flavus 07224	DQ467936.1	566	98.2	0.4
Aspergillus flavus AF13	DQ467941.1	566	98.2	0.4
Aspergillus flavus NRRL3357	DQ467938.1	572	98.5	0.2
Aspergillus flavus SK30	DQ467941.1	566	98.2	0.2
Aspergillus flavus NRRL3357	DQ467948.1	572	98.8	0.3
Aspergillus parasiticus 2999	DQ467949.1	566	98.2	0.1
Aspergillus flavus 56775	AF452809.1	572	98.5	0.1
Aspergillus parasiticus BN009-E	AF441436.1	572	98.5	0.2
Aspergillus parasiticus 2043	AF441438.1	572	98.5	0.2

4.7. 5 Cluster Phylogenetic Relatedness of Isolates using Sequences of *AFL2T* PCR Products

Figure V presents bootstrap dendogramm obtained from *AFL2T* sequences which illustrated the relatedness of the leaf nodes without making assumptions about ancestry of the twelve isolates. The result comprises of two main clusters with seven leaf nodes. The two main group clusters obtained with primer *AFL2T* exhibited genetic similarity. The two main clusters included ten aflatoxin producing isolates (M25, S33, M1, S4, M18, M39, M15, S19, M7 and S21) and two (S5 and M14) isolates that did not produce aflatoxins but had *AFL2T* from in-process wheat flour mills and major stores with a random distribution. All the sub clusters included a combination of aflatoxigenic isolates and one non-aflatoxigenic isolate. The first main group cluster included three strains; main group member M7 and leave node subgroup members M14 and S21. The second main cluster; comprise of nine strains assembled on five leave nodes; M25 and S5, M1 and S4, M18 and M39, M15 and S19, and a unpaired subgroup member S33. The first main group consisted of isolates from in-process wheat and three isolates from major stores (S5, S4 and S19). The bootstrap analysis of the twelve isolates presented 1.0 – 65.0% genetic similarity along the clade positions. While the second main group clusters; M25 and S5, M1 and S4, M18 and M39, M15 and S19 presented 9.0, 10.0, 9.0 and 65.0% genetic similarity respectively along

the clade positions. The first main cluster presented 2.0, 1.0, 9.0% genetic similarity between the first subgroup cluster (M25 and S5) and the single subgroup S33, second subgroup subcluster and the first subgroup cluster, third subgroup cluster with second subgroup cluster respectively. The second cluster recorded 25.0 and 63.0% genetic similarity between the subgroup cluster (M14 and S21) and between this group cluster and the subgroup (M7). There was random distribution of aflatoxigenic and non aflatoxigenic strains along the different leaf nodes within the two clusters.

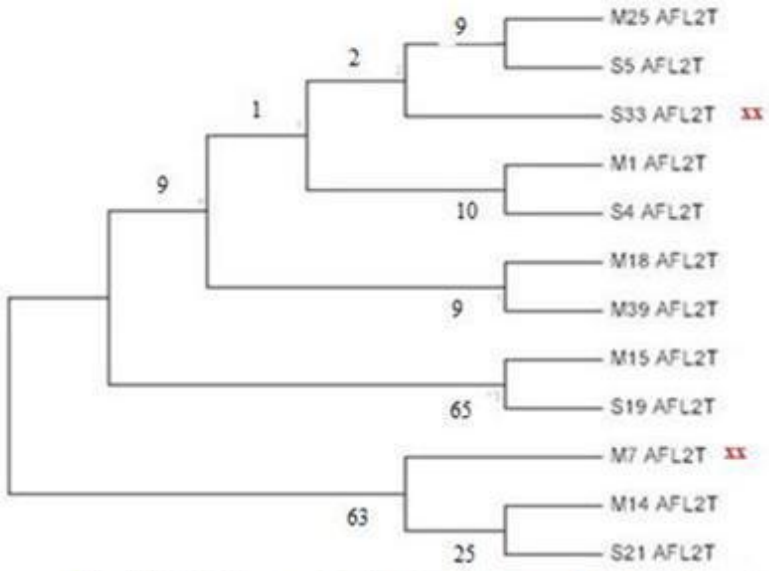


Fig. V: Neighbour - Joining Dendogram of AFL2T Gene Sequences from Aflatoxigenic A. flavus and A.parasiticus Isolated from In-process and Major Stores

CHAPTER FIVE

5.0 DISCUSSION

5.1 Proximate Composition on Samples

The proximate composition determined showed that there was significant increase of the moisture of the grains when tempered to 15.5% moisture content. The increase in moisture contents of the samples corroborate the report of Weidenbo¨ rner *et al.,* (2000) that 1 – 2% increase in moisture content favours the growth of fungi especially the tempered wheat from the in-process. The higher percentage free fatty acid reported in major store could be due to store storage which serve as an incubation period for the moulds to develop and hence their activity on the fatty acid resulting to the production of free acid than in the in-process.

5.2 Mould Counts from In-process and Major Stores

The samples had varying levels of mould contaminants which has implication on the safety of the wheat and products as a consequence of the tempering of the raw wheat. Tempering of the wheat could be responsible for the higher counts (Figure III) compared to the raw material moulds count due to favourable water activity, enhance moisture and the prolong incubation period (48-72 h) that favoured moulds growth. In addition, improperly clean silos could harbor huge amount of mouldy left over grains that could contaminate subsequent batches leading to increased mould counts. Similarly, the practice of covering the silos after wetting the grains might have contributed to mould growth of the wheat mills. Chang and Marakis (2013) stated that moulds growth are favoured when samples are incubated in closed containers at high moistures such as 12 and 14% and when temperature is between 25 - 30°C. This temperature range coincides with that of the storage silos in the conventional mills. The higher moulds count obtained from major stores than in-process samples from this study could be partly due to

contamination during transportation and storage of the wheat products before storage. The most critical control point of the aflatoxigenic isolates was largely at the raw wheat where the wheat is imported along with the toxigenic strains. The techniques of tempering and pneumatic system of transferring in-process materials may have also compounded the proliferation of the moulds within the processing system. Though there is a reduction of the aflatoxigenic isolates during processing of wheat products by the pneumatic system, the milled samples with already raised moisture content may be inoculated with toxigenic isolates in the factory before passage to the stores while may even serve as an incubation period for the moulds to proliferate thus the high count in major stores than in–process samples. In addition most of the moulds spores are randomly distributed by the pneumatic transport during processing and the spores thereafter grow to sporulate thus increasing the mould load in major stores. The high moulds counts. The high mould count obtained in this study (1.0×10^6CFU/g) corroborates the report of Riba *et al*. (2008) in Algeria who found fungal count of 2.8×10^2 CFU/g in wheat flour. Weidenborner *et al*. (2000) also reported fungal count from other countries $10^2 - 6.0 \times 10^3$CFU/g. The differences could be due to variations in country regulation and different levels of manufacturing practices by the processors. The high mean moulds count (5.3×10^5CFU/g) obtained in bran from this research could be due to the introduction of other mill floor wastes, poor handling since it is meant for animal consumption and hence the high moulds contamination. Moulds are mostly resident on the surfaces of grains (Schollenberger *et al*., 2002) and the moulds mycelia usually rooted into the offal tissue (Margit *et al*., 2002) thus it could be difficult to get rid of them.

Thirty eight (38%) of the moulds counted from in-process samples exceeded the NAFDAC 1.0×10^3CFU/g (NIS 396:2002) recommended level for wheat products such as wheat flour and semolina. Similarly, 42% of the major store samples exceeded the national regulation.

The occurrence of *Aspergillus* species as the dominant genus in both wheat and wheat products (Figure IV) agrees with the work of Gashgary *et al.* 2010; Al-Wadai *et al*. 2013). Al-Kahtani (2014) also reported the dominance *Alternaria* spp (68.96%) over *Aspergillus* sp (24.14%) in stored wheat from Saudi Arabia. Similarly, *Aspergillus flavus* had a higher percentage frequency when compared with other species of fungi isolated from wheat in Pakistan (Rashid *et al.*, (2010) and by Al-Wadai1 *et al*. (2013) in camel feeds, Somashekar *et al*. (2004) in food and feeds. This is contrast to the work of Rodriguez, (2009) who reported the dominance of *Aspergillus parasiticus*. These differences in the results could be due to geographical location (Ritter *et al*., 2011), environmental conditions such as temperature, moisture, carbondioxide and the substrate from which the moulds were isolated (Ordaz *et al.*,2003; Sardinas *et al*.,2011). The concomitant mould activities observed in this work can affect the affects the safety of wheat products and if aflatoxigenic strains are present, could produce secondary metabolites such as aflatoxins if the conditions are adequate. The aflatoxigenic fungi and aflatoxins in food had been reported: *Aspergillus flavus* and *Aspergillus parasiticus* had been reported to be the principal producers of aflatoxins (Gourama and Bullerman,1995; Gashgari *et al.*, 2010). The former produces aflatoxin B_1 while the later produces aflatoxin B_1, B_2, G_1, and G_2. Aflatoxin B_1 in particular has been classified as a group 1 carcinogen (1ARC, 2002).The work of Almoammar *et al.* (2013) also reported isolating aflatoxigenic strains of *Aspergillus niger* and *Penicillium* sp especially *Penicillium verrucosum* and *Penicillium nordicum* was reported by other researchers to produce Ochratoxins (Bragulat *et al.*, 2001; Duarte *et al.*, 2010; Alborch *et al.*, 2012). The International Agency for Research on Cancer (IARC) has classified Ochratoxin A under Group 2B as a possible carcinogen to humans (IARC, 2002). *Fusarium* species especially *Fusarium graminearum* which produces the mycotoxin referred to as deoxynivalenol (DON), is

considered the most predominant contaminant of wheat. The presence of DON and Zerealenone in cereals is often accompanied with other *Fusarium* toxins including trichothecenes and fumonisins (Camelia & Lucian, 2014) . The occurrence of these toxins in cereals and other foods has been recognized as a potential threat to human health (Bergamini *et al.,* 2010., Al-Kahtani *et al.,* 2014). The implication of the detection of certain moulds such as *Rhizopus* sp in this study in food stuff was also elucidated, an elevation of pH beyond the safety value of 4.6 making the environment more conducive for the growth of moulds (Efiuvwevwere and Akano, 1997). Considerable research has been documented on the growth of aflatoxigenic strains of *Aspergillus flavus* and *Aspergillus parasiticus* and the production of aflatoxins in-process wheat and products from other countries: Saudi Arabia (Al-Khatan *et al.,* (2014), Jeddah market (Gashgari, 2010), and Italy (Bergamini *et al*., 2010). The high percentage of aflatoxigenic fungi: *Aspergillus flavus, Aspergillus parasiticus* and *Aspergillus niger* respectively obtained in this research work reveled that aflatoxigenic fungi connote risks of aflatoxin contamination and this will ultimately affect the safety of this products and thus the need for strict surveillance and monitoring of wheat processing factories by national regulatory bodies. The prevalence of the toxigenic isolates and the aflatoxin contamination is most caused by unwholesome contamination of raw material such as wheat. Although, there is usually cleaning of the wheat before milling, but is no disinfection of the raw material with appropriate disinfectant before processing milling. More so, the long incubation period of samples under increased tempered moisture of the wheat could results to proliferation of the aflatoxigenic strains after milling and during storage. In addition, there could be low level of good manufacturing practice and an in adequate food security monitoring system in the industry by national regulatory bodies.

The absence of aflatoxigenic moulds from the various sealed premix were probably that conditions for aflatoxin production was not adequate probably due to insufficient oxygen rather carbondioxide instead which resulted from grain respiration. More so, the moisture of the premixes was very low < 10% and was kept air tight all the time and opened when required for use. Contrary to this however, premix samples that were opened accidentally during transportation were contaminated with aflatoxigenic moulds and aflatoxins ranged from 0.5 - 4.0ppm.

5.3 Aflatoxin Levels from In-process and Major Stores

Aflatoxigenic moulds and aflatoxins were detected in-process and major store wheat products. Some samples showed the presence of aflatoxigenic moulds but did not show sign of aflatoxins. Conversely, there were samples that contained aflatoxins but there were no aflatoxigenic moulds detected especially in major stores. The former could be due to the fact that the conditions for aflatoxin production may have been not adequate. The later may have been due to environmental conditions which was not favourable for the survival and growth of the toxigenic isolates but may have produced aflatoxins before entering death phase of growth. The incidence of aflatoxins (38.0%) in wheat used by the processors is lower than the (59%) reported by Giray *et al.*(2007). Conversely, The 0.5 – 73 µg/kg aflatoxin range obtained in this study is also higher than the (0.05 – 14.0 ppb) reported by Aydin *et al*. (2008) from Turkey. The prevalence of 28% aflatoxin from wheat flour in this study is higher than the 9% obtained by Ayalewa *et al*. (2006), the 25.6ppb aflatoxin B_1 from wheat flour in Malaysia (Abdallah *et al. (*1998) and the 16.3µg/kg reported by Halt (1994) aflatoxin B_1 from Croatia. These differences could be due to the different sources of the grains, the geographical location of the countries and differences in extraction rate of the various wheat flours from the various processors. In addition, the dynamics

of total aflatoxin distribution in wheat products arising from the homogenizing effects of the pneumatic system could also contribute to the moulds load and aflatoxin level observed in this research. The pneumatic system compresses unsterile air and uses same to transport products within the milling system. This unsterile air may contain aflatoxigenic strains that could contaminate the system and the entire products in-process. Consequently, aflatoxigenic strains produce aflatoxins in the products during storage and thus the higher moulds counts and aflatoxin levels in major store products than in-process samples. The higher total aflatoxin content of the bran could be due to the fact that the toxins are mostly situated outside the endosperm of the wheat and back covering of the offal as reported by Schollenberger *et al.* (2001). Aflatoxins are not distributed uniformly stored grains. In most cases, the toxin level varies from one seed to another. Moreover, it is possible that highly contaminated seeds could be next to uncontaminated seeds. Thus most of the toxins are distributed along with the brand into other wheat products depending on the level of contaminated offal in a particular product. This consequently, reduces wheat flour aflatoxin when the offal content are mostly low since wheat flour is chiefly from the wheat endosperm. However, this dynamics chiefly relays on the efficiency of the separation technique employed to obtain various products from wheat. Bran which is the wheat covering contains the highest aflatoxin prevalence (89.0 %). Similarly, brown flour contains next higher prevalence (39.0%) of aflatoxins and it hard the second higher dusty offal and the bases for the second high level of total aflatoxins obtained in this study.

5.4 Fate of Aflatoxin during Wheat Processing, Fermentation and baking

The high ash content in wheat is a reflection of the presence of the bran which when introduced into the wheat flour due to over scratching of the wheat endosperm from the bran leads to high extraction flours. Consequently, since the aflatoxigenic moulds strains and the aflatoxin are

majorly situated outside the bran (Radiana *et al.*, 2009), hence their introduction into high extraction wheat flours (Fig.I). This explains the correlation between extraction rate and aflatoxin retention; as the extraction rate decreased, the aflatoxin retention decreased with reducing ash content or vice versa. The golden safety rules in food manufacture and handling are to prevent contamination of food with harmful agents, prevent growth and survival of contaminants starting from farm level to table. The analysis of critical control points of aflatoxigenic strains and total aflatoxin occurrence in process samples identified hazards associated with different stages of the wheat processing. This study assessed the aflatoxigenic strains and total aflatoxin associated with different stages of the wheat processing and identified critical control points where control measures could be practicable. The raw material before tempering was the major source of contamination of aflatoxigenic strains in the wheat products. Despite the screening before milling, the tempering and incubation of the raw material breeds the moulds at the tempering silors. The aflatoxigenic strains and total aflatoxins on the grains are then homogenized in the wheat products. These two points are critical in the distribution and introduction of aflatoxigenic strains and aflatoxins in-process. Another point that was critical in the introduction of aflatoxins into the products was during the course of extraction of wheat products from wheat grain endosperm as explained earlier.

Based on the assessment of the effects of fermentation and baking on aflatoxins from this study, more than half of the aflatoxin content of the fermented dough was decontaminated and during fermentation (55.9%) (Figure II) and baking (77.4%) (Figure III). The 55.9% obtained from work is higher than the result obtained by Gumus *et al.* (2009) who reported 33.7% maximum fermentation of dough. Semilarly, 70.4% aflatoxin decontamination recorded during baking by the same author is less than the 77.4% recorded in this study. Another study by El-Tawila *et al.*

(2003) reported 41.17% total aflatoxin absorption after dough baking into bread. Though most of the products of wheat flour undergo baking (bread, cakes,) and frying (dough nuts, rolls), aflatoxins are very stable to heat (Ananth and Farid, 2002) in dry state up to their melting points, however, Ananth and Farid, (2003) documented that heating aflatoxin contaminated food in the presence of moisture, leads to destruction of aflatoxins over a period of time. This destruction leads to opening of the aflatoxin lactone ring with the possibility of decarboxylation and loss of the methoxy group from the aromatic ring at elevated temperatures. Such destruction occurs either with aflatoxin in oils or aqueous solution as would have been the case for submerged wheat-sorghum ``kunun-zaki` fermentation (Table 4.10). But ``kunun-zaki`` processing involves very brief heating by pouring boiled water into the large volume of ground wet milled cereal slurry with continuous stirring which immediately drops the temperature below $100^{o}$C. This drop in temperature and lack of continuous heating along ``kunun-zaki`` production protocol could probably be the reason why reduction of the natural total aflatoxin in such aqueous medium ``kunun-zaki`` beverage was not appreciably higher than the result obtained. The higher values of total aflatoxins obtained from the slurry than the raw material flour could be due to the effect of release of the toxin from the high surface area grain matrices of the slurry particles. The better aflatoxin absorption by room temperature fermented product was due to the most likely reaction of the acid produced during the fermentation process on the aflatoxins, which probably catalyzed addition of water across the double bound in the aflatoxin furan ring leading to conversions of the toxin to various adducts, such as acetoxy derivative as documented by Chaelae *et al.* (2002)**.** The detection of total aflatoxins across all the samples along the submerged ``kunun-zaki`` fermentation production steps depicts that aflatoxin contaminated raw materials will only serve as a source of aflatoxin inoculum into the finished product and hence

it's likely consequence of aflatoxicosis in the consumers. The mean 55.9% total aflatoxin absorption obtained from this research was a clear indication that kunun-zaki fermentation process partially eliminated the aflatoxins. Earlier report by Oluwafemi and Ikeowa (2005) showed 50% reduction of aflatoxin in contaminated maize used for pap (Ogi) production. Another study by El-Tawila *et al*. (2003) reported 41.17% total aflatoxin absorption after dough baking into bread. These are relatively solid cereal fermented products compared to liquid fermented kunun-zaki beverage. All the same, decontamination of aflatoxin from either solid or liquid substrate cereal based product such as kunun-zaki by fermentation proved to be in efficient. Generally, in most developing countries there is lack of aflatoxin control in agriculture based products and the lack of probably strict enforcement of aflatoxin standards by regulatory bodies at the ports and in the domestic market makes aflatoxin contaminated wheat products easily enter the consumption stream leading to the risk of adverse health risk. Largely, consumers are not aware of the problem.

5.5 Characterization of Aflatoxigenic Strains Using Multiplex Polymerase Chain Reaction

This study has showed that the detection of aflatoxigenic moulds using primers designed for the major aflatoxin regulatory biosynthetic pathway genes (*aflR, omt-A, ver-1* and *nor-1*) is more reliable as compared to the conventional plating techniques as some isolates not detected by the latter was detected by the former. The later technique has failed in some quarters specifically for isolate 7 that presented ELISA negative respond, a sign of no aflatoxin production in-vitro but presented positive PCR amplification response with the four biosynthetic pathway genes indicating potential for aflatoxin production. The inability of isolate 7 to produce aflatoxin in vitro could be due to either the conditions for aflatoxin production was not adequate or the level of aflatoxin produced was below detectable limit of ELISA. The failure in the expression of

regulartory gene *aflR-1* reported by Chang *et al.* (2002) could also result to inability of the isolate to produce aflatoxins. Farber *et al*. (1997) previously demonstrated that the *aflR-1* protein can bind the promoter region of aflatoxin synthesis gene and activate *aflR-1* gene expression. This explains the presence of *aflR-1* gene is vital in aflatoxin biosynthesis regulation. In addition, *w*henever *aflR-1* protein is absent or abnormal, Noorbacash *et al*. (2009) reported that this could give a clue to strong indication that such isolates cannot produce aflatoxins. In a separate study, Latha *et al*. (2008) also reported that the absence of *aflR-1* and *omt-1* genes confirms the presence of non aflatoxigenic isolates. Furthermore, environmental factors such as temperature, carbondioxide, water activity (Zhang *et al.*, 2014) could also affect the transcription of the genes which could lead to non production of aflatoxins. The complete four gene band patterns obtained in this study (Figure I) using multiplex PCR confirms the positive response for aflatoxin production by the isolates tested using conventional polymorphic beige ring. This key genes involved in the aflatoxin biosynthetic pathway form the basis for an accurate, sensitive and specific detection system, using PCR, for aflatoxigenic isolates in grains and products (Thompson *et al*., 1997; Somashekar *et al*., 2004; Gashgari *et al*., 2010). The quadruplex gene amplification patterns obtained in this study from aflatoxin producing isolates were also in line with earlier reports (Criseo *et al*., 2001; Latha *et al.,* 2008). The four genes (aflR, *omt-1*, *ver-1* and *nor-1*) are the major genes involve in the aflatoxin biosynthetic pathway. Many authors have reported that *AflR-1* gene encodes a positive regulator and an auto regulatory function gene that regulates the structural genes at transcription level (Chang *et al., 2002;* Yu *et al.*, 2004; Noorbacash *et al.,* 2009). Lee *et al*. (2006) suggested for safety reasons that *aflR* gene presence could be used to detect the aflatoxin production by aflatoxigenic strains because of its immense regulatory actions before transcription of the structural genes. All the other three genes (*Nor-1*,

ver-1, omt-1) used in this study were structural genes in cluster in aflatoxin biosynthetic pathway that code for key enzymes in production of aflatoxin (Yu *et al.,* 2004).

The inability of some isolates from Plate I and Plate II to amplify both the *aflR-1* and the structural genes confirms the report of Venden *et al*. (2001) who stated that *aflR-1* and the structural genes deleted strains of aflatoxigenic moulds turned out to be aflatoxin non-producers. The absence of the four genes and or one or more than one member of the four major genes involved in the aflatoxin biosynthetic pathway obtained from this study could be because the strains are non aflatoxigenic or they were aflatoxigenic but might have lost some gene through transgenic drift which was evident during the course of sub culturing in this work. Transgenic drift is the ability of an organism to lose certain genes from the genome. Loss of toxigenicity in *Aspergillus flavus* and *Aspergillus parasiticus* during sub-culturing was also reported by other workers (Bilgrami *et al*., 1988; Rodrigues *et al*., 2009). Similarly, lose of toxigenicity could also be due to point mutations exercised by the isolate that could lead to loss of either of the valuable genes that are involved in the biosynthetic pathway. Although *Aspergillus flavus* and *Aspergillus parasiticus* both contain aflatoxin gene cluster with genes in the same sequential order (Yu *et al*., 2004; Ehrlich *et al.,* 2005).

The different mills and major stores showed key genes variability within the non aflatoxigenic isolates. The variability of the non-aflatoxigenic moulds corroborates earlier (Geisen, 1996; Cary *et al*., 2000; Tominaga *et al*.,2006) who suggested that different types of mutations can be responsible for inactivation of the aflatoxin biosynthetic pathway of non aflatoxigenic moulds. This could be the reason behind the high aflatoxin biosynthetic gene variability obtained from non-aflatoxigenic moulds in this research. Due to the error of mutation created by transgenic drift, it is therefore paramount to use PCR method to probe directly aflatoxigenic isolates

through the genes involved in thier biosynthetic pathway by directly isolating the DNA from the wheat and flour rather than culturing before amplification. This technique could limit the chances of error due to transgenic drift.

Sequencing of the major aflatoxin biosynthetic *aflR-1* genes helped in the identification and differentiation of closely related moulds belonging to the same species and also those that produces the same secondary metabolite such as aflatoxins. Sequences obtained from this research were assembled to yield the entire *AFL2T* gene sequences. The sequences after they were blast using *Aspergillus* species specific site on NACBI data base, showed alignment between the query sequence and that of standard *aflR*-1 from NCBI data base indicated compatibility or homology of the *AFL2T* gene (flank DNA sense and antisense of the gene) with the standard *aflR-1* of NCBI Gene Bank.

The sequences of *AFL2T* obtained in this study were also used to generate dendrogram using the bootstrap similarity matrix. The sequences generated two main clusters in an attempt to resolve variability among strains. Based on the model on which the dendogramme was generated, isolates that clustered together showed closer relatedness while those that assemble on the same leave node indicated greater genetic relatedness. However, they vary along the clade positions. Assuming the dendogramme was rooted, one would have say that aflatoxigenic and non aflatoxigenic isolates that cluster together could have arisen from the same ancestral origin. The non aflatoxigenic isolates clustering with aflatoxigenic isolates might have been aflatoxigenic but might have lost one or more key genes due to mutation as earlier explained by other workers (Geisen, 1996; Cary *et al*., 2000; Venden *et al*., 2001; Tominaga *et al*., 2006). All the twelve isolates were similar in their morphological characteristics, but differed genetically. The isolates differentiated into sub-groups, while the others assembled together indicating closer relatedness

or general similarity compared to the sub-groups. Some of the isolates varied greatly with visible mixtures of aflatoxigenic and non aflatoxigenic moulds at different leaf nodes within the major clusters. Similarly, there was also random mixture of in-process and store isolates with different genetic diversities at different leaf node clade positions.

CHAPTER SIX

6.0 Conclusion and recommendation

6.1 Conclusion

The practice of tempering wheat and incubation before milling increased moisture of the raw wheat to a mean of 15.4% favoured moulds development during in-process and major stores. The critical control point of the aflatoxigenic strains was largely at the raw wheat samples where the wheat is imported along with the toxigenic strains.

While the mean moulds count (logCFU/g) obtained from in-process was highest in bran (7.58) and least in wheat flour (2.36), similarly, major store samples had in bran (7.77), and least in wheat flour (1.97). Both in – process and major store samples had *Aspergillus flavus* as the dominant moulds 75.0 and 79.3% respectively. The wheat product, bran presented highest moulds occurrence frequency (69.8%) and least in wheat flour (32.3%) from in-process samples. Furthermore, major store samples presented bran with highest frequency (83.8%) and least in semolina (33.8%). The predominant aflatoxigenic strain was *Aspergillus flavus* (37.7%) in-process and 43.0% from major stores.

The prevalence of total aflatoxins obtained from wheat and products were 38, 21, 28, 39 and 89% in wheat, semolina, wheat flour, brown flour and bran respectively.

This work has shown that as the extraction rate of wheat flour increases from 60 - 75%, the ash content and the total aflatoxin retention increases from 0.57 - 0.82%dm and 2.1- 65.9% respectively while the aflatoxin decontamination decreased from 98.0 - 34.1%. The effects of

solid substrate fermentation on total aflatoxin contaminated wheat flour into leavened bread showed significant aflatoxin loss (55.2%) at 37^0C/120 minutes fermentation with 44.8% retention and as the baking temperature of the dough increases from 180 – 220^{o}C at pH 5.8, the total aflatoxin loss further increased from 58.6 – 72.4%. Submerged fermentation of wheat-sorghum composite Kunun-zaki presented 18.9 and 52.90% aflatoxin decontamination in refrigerated and ambient temperature stored products respectively.

Quadruplet PCR pattern was consistent at 1032, 797, 537 and 400 bp with all the aflatoxigenic strains indicating the presence of the four genes of the aflatoxin biosynthetic pathway, whereas the non-aflatoxigenic strains presented one, two and three banding patterns with the genes; *aflR-1, omt-A, ver-1* and *nor-1* gene primers respectively.The frequency of occurrence of aflatoxin biosynthetic genes in non aflatoxigenic strains were : *nor-1* (73.3%), *ver-1* (53.3%), *omt-A* (40.0%) and *aflR-1* (13.3%). Enough homology (compatibility) was found through alignment of *AFL2T* gene sequences with standard *aflR-1* of NCBI gene bank. The gene *AFL2T* nucleotide sequence generated dendogram produced two main clusters. The dendogram generated two main clusters. The strains further differentiated into sub-group indicating general genetic relatedness. Some of the isolates varied greatly with visible mixtures of aflatoxigenic and non aflatoxigenic moulds and also with random mixture of in-process and store isolates with different genetic diversities at different leaf nodes.

6.2 Recommendation

1. Wheat aflatoxin content should be tightly controlled according to stipulated standard by national regulatory bodies before its use by millers.
2. Regulatory bodies should intensify effort in ensuring wholesome wheat products before they are allowed for circulation within citizen consumers.
3. Grain endosperm extraction rate should be part of laboratory routine quality assurance aflatoxin control in wheat processing factories to reduce the extent of aflatoxin contamination through bran into various products
4. Government should create awareness on the issue of aflatoxin contamination and its health consequence on consumers to its citizenry
5. Government should encourage research on aflatoxin that could lead to developing resistance seeds and control technique for Nigerians.
6. There is need for wheat processors to go into more research on disinfection of raw material before use.

REFERENCES

Abdallah,N.,Nawawi, A and Othman,I (1998).Survey of fungal counts and natural occurrence of aflatoxins in Malasian starch – based foods. *Mycopathology,* 143**(**53): 58-64

Adegoke, A.S., Akinyanju, J.A. and Olajide, L.E.(2010). Fermentation of aflatoxin contaminated white dent maize(zea maize). *Research Journal of Medical Sciences,* 4(3):111-115.

Adejumo, T.O. (2012). Evaluation of botanicals as biopesticides on the growth of *Fusarium verticillioides* causing rot diseases and fumonisin production of maize. *Journal of Microbiology Antimicrobial,* **4(**1):23-31.

Adejumo, T. O. and Adejoro, D. O. (2014). Incidence of aflatoxins, fumonisins, trichothecenes and ochratoxins in Nigerian foods and possible intervention strategies . *Food Science and Quality Management,* 31: 127-146

Adebayo, G.B., Otunola, G.A. and Ajao, T.A. (2010). Physico-chemical, Microbiological and sensory characteristics of `*kunu*` prepared from millet, maize and guinea corn and stored at selected temperature.Adv. *Journal of Food Science Technology,* **2(**1): 41-46.

Ahmed, M. Abdel-Hadi., Daniel, P. Caley, David., R. F. Carter and Naresh Magan (2011) Control of Aflatoxin Production of *Aspergillus flavus* and *Aspergillus parasiticus* Using RNA Silencing Technology by Targeting *aflD* (*nor-1*) Gene .*Toxins*, 3: 647-659; doi:10.3390/toxins3060647

Agrios, George N. (2005). Plant Pathology: Fifth Edition. Elsevier Academic Press. p. 922.

Alborch, L., M.R. Bragulat, G. Castellá., Abarca, M.L., Cabañes, F.J. (2012). Mycobiota and mycotoxin contamination of maize flours and popcorn kernels for human consumption commercialized in Spain. *Journal of food Microbiology*, **32**(1): 97-103

Al-Kahtani Muneera D.F. (2014).Isolation of fungi and mycotoxin extract from stored wheat and other grains imported in Saudi Arabia. *American Journal of Food Technology* **9(**7): 370 – 376

Al-Wadai, A.S., Al-Othman, M.R., Mahmoud,M.A. and Abd El-Aziz**1,** A.R.M. (2013). Molecular characterization of *Aspergillus flavus* and aflatoxin contamination of wheat grains from Saudi Arabia *Genetics and Molecular Research,* **12(**3): 3335-3352

Almoammar,Hassan., Ali H.Bahkali and Kamel A.Abd-Elsalem (2013). A polyphasic method for identification of aflatoxigenic Aspergillus species isolated from camel feeds. *Autralian Journal of Crop Science*, **7**(11): 1707 -1 713

Amaike, S and Keller,N.P. (2011). "*Aspergillus flavus*". Annual Review of Phytopathology, 49: 107–133. *doi*:*10.1146/annurev-phyto-072910-095221*

Amaoo,I.A.and Ascoore,F.P.(2006). Effects of processing the Nutrient composition and oil of peanuts(*Arachis hypogea*) seed flour. *Journal of chemical Society of Nigeria,* **31(**1&2): 1-5.

Amra, H.A., Mahmoud, S.A.Z., Taha,A.H and El-Azab,M.A.(1996).Destruction of aflatoxins B1 and G1 in bread making. *Mycotoxin Research* **12**(2):16-22

American Public Health Association (APHA) (1999).Compendium methods for the microbial examination of foods 2nd edition, Washington, D.C.

Ananth, S.B. and Farid, W.(2003)Importance of aflatoxins in human and livestock health. http://www.icrisat.org/aflatoxin/introduction.asp

Ananth ,S.B. and Farid, W.(2002). Importance of aflatoxins in human and livestock health. http://www.icrisat.org/aflatoxin/introduction.asp

Amyot, J. (1983). Social and Economic Aspects of Dryer Use for Paddy and Other Agricultural Produce in Thailand. Chulalongkorn University Social Research Institute and International Development Research Center

Arabatzis, M. and Velegraki, A. (2013). "Sexual reproduction in the opportunistic human pathogen *Aspergillus terreus".Mycologia,* **105** (1): 71–79. doi:10.3852/11-426

Ariyo, A.L., Peter, A.O., Abdulazeez, S.N. and Olatunde, O.H. (2013). Avian Mycotoxicosis in DevelopingCountries. In: H.A. Makun (Ed) Mycotoxin and Food Safety in Developing Countries. InTech, Rijeka ,Croatia, pp 93-119.

Aroyeun, S.O. and Adegoke, G.O. (2007). Reduction of ochratoxin A (OTA) in spiked cocoa powder and beverage using aqueous extracts and essential oils of Aframomum danielli. A*frican Journal of Biotechnology,* 6:612-616.

AOAC, (1999). Official Methods of Analysis of AOAC International, 6th ed., 5th revision; AOAC International Publishers: Gaithersburg, MA, USA,Volume 11.

Atanda, O., Makun, H.A., Ogara, I.M., Edema, M., Idahor, K.O., Eshiett, M.E. and

Oluwabamiwo, B.F. (2013).Fungal and mycotoxin contamination of Nigerian foods and feeds Countries. In: H.A. Makun (Ed) *Mycotoxin and Food Safety in Developing Countries.* InTech, Rijeka, Croatia pp3-38.

Avantaggio, G., Quaranta F., Desidero, E. and Visconti, A. (2002). Fumonisin contamination of maize hybridsvisibly damaged by Sesamia. *Journal of Science Food Agriculture*, 83: 13-18

Avrameas, S. (1969). "Coupling of enzymes to proteins with glutaraldehyde. Use of the conjugates for the detection of antigens and antibodies," *Immunochemistry*, 6(1):43–52 1

Ayelewa, A., Fehrmann,H., Lepschy,J., Beck.R.and Abate, D.(2006). Natural occurance of mycotoxins in staple cereals from Ethiophia.*Mycopathologia*, 162:57-63

Ayalewa, A (2010). Mycotoxins and surface and internal fungi of maize from Ethiopia. *African Journal of Food Agriculture Nutrition Development*, 10:4109-4123.

Aydin, A., Gunsen, U. and Demirel, S. (2008). Total aflatoxin, aflatoxin B_1 and ochratoxin a levels in Turkish wheat flour. *Journal of Food Drug Analysis*, 16:48–53.

Aziz-Baumgartner, (2005). Case-Control Study of an acute aflatoxicosis outbreak, Kenya 2004. *Environmental Health Perspectives*, 113:1779-1783

Aziz, N.H., Souzan, R.M. and Azza, A.S. (2006). Effect of gamma irradiation on the occurrence of pathogenic microorganism and nutritive value of four principal cereal grains. *Applied Radiation Isot* 64:1555-1562.

Bacaloni, A., Cavaliere, C., Cucci, F. and Patrizia, F. (2008). Determination of aflatoxins in hazelnuts by various sample preparation methods and liquid chromatographytandem mass spectrometry. *Journal of Chromatography A*, **1179**(2):182-189.

Bankole,S.A. and Adebanjo, A.(2003). Mycotoxin in West Africa: current situation and possibility of controlling it. *African journal of Biotechnology*, **2**(9):254-263

Bennett, J.W. and Klich,M.(2003).Mycotoxins. *Clinical Microbiology Reviews*, 16: 479-516

Bennett, J.W. (2010). "An Overview of the Genus *Aspergillus*" (PDF). Aspergillus*: Molecular Biology and Genomics*. Caister Academic Press. ISBN 978-1-904455-53-0.

Belderok, B., Mesdag, H., Donner, D.A. (2000). Bread-Making Quality of Wheat. Springer, New York

Bergamini, E.,Catellani,D.,Dallasta,C.,DAlavema,G,Dossena A.,Marchelli,R and Suman,M (2010). Fate of Fusarium mycotoxins in the cereal product supply chain: the deoxynivalenol (DON) case within industrial bread-making technology, **27(**5): 677-687

Bhat, M.Y. and Fasal, M. (2011). Effecs of *Aspergillus flavus* Metabolites on wheat Seed

Germination and Seedlings. *Arab Journal of Plant Protection, (*2), 139-140

Bhatnagar, D., Cary, J.W., Ehrlich, K., Yu, J., Cleveland, T.E. (2006) .Understanding the genetics of regulation of aflatoxin production and *Aspergillus flavus* development. *Mycopathologia,* 262: 255–266

Bilgrami, K.S., S.P Sinha and P. Jeswal. (1988). Loss of toxigenicity of *Aspergillus flavus* strains during subculturing—a genetic interpretation. *Curent Science,* 57: 551-552.

Bragulat,M.R.,Albarka, M.I.and Cananes, F.J.(2001).An easy Screening method for fungi producing ochratoxin A in pure culture.*International Journal of food Microbiology,* 71: 139- 144

Bozkurt, M.K., Ozçelik, T., Saydam, L. and Kutluay, L. (2008). "[A case of isolated aspergillosis of the maxillary sinus]". *Kulak burun bogaz ihtisas dergisi (in Turkish*), **18 (**1): 53–5. *PMID 18443405*.

Camelia and Lucian, (2014). Isolation and identification of mycotoxigenic fungi from wheat grains stored in bihor county. Analele Universităţii din Oradea, Fascicula: Ecotoxicologie, Zootehnie şi Tehnologii de Industrie Alimentară, *Vol.* XIII/A, 2014

Cary, J. W., K. C. Ehrlich, M. Wright, P.-K. Chang, and D. Bhatnagar (2000). Generation of aflR disruption mutants of Aspergillus parasiticus. *Applied Microbiology Biotechnology*, 53:680– 684.

Codex Alimentarius Commission (2014). Discussion paper on establishment of a maximum level for total aflatoxins in ready-to-eat peanuts and associated sampling plan. Joint FAO/WHO food standards committee on contaminants in food Mar 31 – Apr 4; The Hague, The Netherlands.

Codex Alimentarius Commission. (2003). Code of practice for the prevention and reduction of mycotoxin contamination in cereals, including annexes on ochratoxin a, zearalenone, fumonisins and tricothecenes. Available online at http://www.fao.org/fileadmin/user_upload/agns/pdf/CXS_051e.pdf.

Corn Refiners Association (CRA), (2011). Food Safety Information papers: Mycotoxins; USA. http://corn.org/wpcontent/uploads/2009/12/mycotoxins.pdf D

Council for Agricultural Science and Technology (CAST) (2003). Mycotoxins: risks in plant, animal, and human systems. *Ames, Iowa, USA*. Task Force Report. 139.

Chang, P.K., Cary, J.W., Bhatnagar, D., Cleveland, T.E., Bennett, J.W., Linz, J.E., Woloshuk , C.P. and Payne, G.A. (1993). Cloning of the Aspergillus parasiticus apa-2 gene associated with the regulation of aflatoxin biosynthesis. *Applied Environmental Microbiology,* 59: 3273–3279

Chang, P.K., J.W. Bennett and P.J. Cotty, (2002). Association of aflatoxin biosynthesis and sclerotial development in *Aspergillus parasiticus*. *Mycopathologia,* 153: 41-48.

Chang, P.K. and Hua, S. S. T. (2007). Nonaflatoxigenic *Aspergillus flavus* TX9-8 competitively prevents aflatoxin accumulation by A. *flavus* isolates of large and small sclerotial morphotypes. *International Journal of Food Microbiology,* 114: 275–279

Charles,M.(2007). Purging malawi's peanuts of deadly aflatoxin.SciDev.Net. http://www.Scidev. Net/en//features/purging-malawis-peanuts-of-deadly- aflatoxin.html

Chaelae, M.I., Jideani, I..J. and Abayeh, O.J.(2002).Occurrence of Aspergillus flavus and Aspergillus parasiticus in some ready to eat – foods sold in market places in Bauchi, Nigeria. *Nigerian Food Journal,* 20: 81-85

Chaelae,A.M.,Abayeh,O.J.and Jideani, (2003). Effects of cooking and fermentation on the fate of aflatoxin B1and B2 in contaminated millet and fresh cow milk. *Nigerian food Journal,* 21: 125-128

Chen, R.S., Tsay, J.G., Huang Y.F. and Chiou, R.Y. (2002). Polymerase chain reaction-mediated characterization of molds belonging to the Aspergillus flavus group and detection of *Aspergillus parasiticus* in peanut kernels by a multiplex polymerase chain reaction. *Journal of Food Protection*, 65: 840–844

Choudhary, P.L., Sharma, R.S., Borkhataria, V.N. and Desai, M.C. (1998). Effect of feeding aflatoxin B1 on feed consumption through naturally contaminated feeds. *International Journal of Animal Science,* 68: 400–401.

Criseo, G., Bagnara,A. and Bisignano ,G.(2001). Differentiation of aflatoxin-producing and non-producing strains of Aspergillus flavus group. *Letters Applied Microbiology,* 33: 291-295.

Criseo, G., Racco, C. and Romeo, O. (2008). High genetic variability in non-aflatoxigenic *A. flavus* strains by using Quadruplex. *International Journal of Food Microbiology*, **125**(3):341– 343. doi: 10.1016/j.ijfoodmicro.04.020.

Cotty, P.J. and Mellon, J.E.(2006). Ecology of aflatoxin producing fungi and biocontrol of aflatoxin contamination. *Mycotoxin research,* **22(**2):110-117

Cruz, Patricia and Mark P.Buttner (2008). Development and evaluation of real-time quantitative PCR assay for Aspergillus flavus. *Mycologia,* **100(**5): 683:690

Cummins, D.G., Hutto,G. and King, A. (eds). Impacts and Scientific Advances through Collaborative Research on Peanut CRSP, Griffin, GA. Pp. 117-130.

Dagenais, T.R. and Keller, N.P. (2009) Pathogenesis of *Aspergillus fumigatus* in invasive

aspergillosis. *Clinical Microbiology Review*, 22: 447–465

Darwish WS, Ikenaka Y, Nkayama SMM, Ishizuka M (2014). An overview of mycotoxin contamination of foods in Africa. *Journal of Vetnary Medical Science*, 76: 789-797.

Davis, N.D., Iver, S.K. and Diener, U.L. (1987).Improved Method of Screening for Aflatoxin with a Coconut Agar Medium, *Applied and Environmental Microbiology*, 53: 1593.

Duarte,S.C.,Pana., A. and Lino,C.M.(2010).A review on ochratoxin A occurrence and effects of processing of cereals and cereal derived food products. *Food Microbiology*, 27:187-198

Duvick, J., Rood, T.A., Maddox, J.R. and Gilliam, J. (2006). Advances in ochratoxin A biosynthesis. Book of Abstracts, International Conference on "Advances on genomics, biodiversity and rapid systems for detection of toxigenic fungi and mycotooxins" September 26-29, 2006. Monopoli (Bari), Italy Pp.39
Retrieved from http://www.Ispa.onr.n/mycoglobe-2006 [Accessed August 10, 2014].

Degola, F., Berni, E., Dall'Asta, C., Spotti, E., Marchelli, R., Ferrero, I., Restivo, F.M. (2007) A multiplex RT-PCR approach to detect aflatoxigenic strains of *Aspergillus flavus*. *Journal of Applied Microbiology*, 203: 409–427

Diener, U.L., Cole, R.J., Sanders, T.H., Payne, G.A., Lee, L.S. and Klich, M.A. (1987).Epidemio logy of aflatoxin formation by *Aspergillus flavus*. *Annual Review Phytopathology*, 25:249–270

Djekrif-Dakhmouche S., Gheribi-Aoulmi Z., Meraihi Z., Bennamoun, L.(2006). Application of a statistical design to the optimization of culture medium for α-amylase production by *Aspergillus niger* ATCC16404 grown on orange waste powder. *Journal of Food Process Engineering*, 73:190–197.

Doohan, F.M., J. Brennan and B.M. Cooke, (2003). Influence of climatic factors on Fusarium species pathogenic to cereals, *European Journal of Plant Pathology*, 109: 755-768.

Dovičičová, M, Tančinová, D, Labuda, R, Sulyok,M. (2012). *Aspergillus parasiticus* from Wheat grain of Slovak origin and its toxigenic potency. *Czech Journal of Food Science*, 30(5):483–487

Dorner, J.W., Cole, R.J., and Wicklow, D.T. (1999) Aflatoxin reduction in corn through field application of competitive fungi. *Journal of Food Protection*, 62:650-656.

Dorner, J.W. and Cole, R.J.(2002). Effect of application of nontoxigenic strains of *Aspergillus flavus* and *Aspergillus parasiticus* on subsequent aflatoxin contamination of peanuts in storage. *Journal of Stored Products Research*, **38**(4):329-339. [doi:10.1016/S0022-474X(01)00035-2]

Efiuvwevwere,B.J.O and Akano,O.(1997).Microbiological studies on a Nigerian Maize Product,Kwoka supplemented with soybeans. *Journal of Food Safety*, 17: 249-259

Egbere, O.J., Pam, K.V., Adesheyan, K.D., A`kadir, T., Oyero,S.K. (2008). Effects of pasteurization on survival partterns of microorganisms and vitamin retention in `kunun-zaki`. *African Journal of Biotechnolgy*, 8(23):6603-6607

El-Khoury,Andre.,Ali Atoui.,Toufic Roger Lteif.,Mireille Kallassy,and Ahmed Lebrihi.(2011).Differencition between *Aspergillus flavus* and *Aspergillus parasiticus* from pure culture and Aflatoxin-contaminated grapes using PCR-RFLP analysis of aflR-aflJ intergenic spacer. *Journal of food Science*, 76:247-253

Ellis, D. (2006).*Mycology on line*. The University of Adelaide. Australia.

El-Tawila,M.M.,Ibrahim,N.A.,Gomaa,N.F.,Omar,R.M. (2003).The effects of bread making steps on the degradation of aflatoxins produced as result of single inoculation with *Aspergillus flavus* and *Aspergillus parasiticus* and combined inoculation with *Aspergillus flavus* and *Aspergillus ochraceus*. *Journal of Egypt Health Association.* **78** (5-6): 373-386

Empting, L. D. (2009). "Neurologic and neuropsychiatric syndrome features of mold and mycotoxin exposure".*Toxicology and Industrial Health*. **25** (9-10): 577– 5 81 doi:10.1177/0748233709348393

Erami, M., Hashemi, S.J., Pourbakhsh, S.A., Shahsavandi, S., Mohammadi, S., Shooshtari, A.H., Jahanshiri, Z. (2007). Application of PCR on detection of aflatoxinogenic fungi. *Archives of Razi Institute*, **62**(2): 95-100

Ehrlich, K.C., Yu, J. and Cotty, P.J. (2005). Aflatoxin biosynthesis gene clusters and flanking regions. *Journal of Applied Microbiology*, 99:518–527

Etzel, R. A. (2002). Mycotoxins. *Journal of American Mededical Association,* 287: 425-427.

European Food Safety Authority (EFSA) (2007). Opinion of the scientific panel on contaminants in the food chain on a request from the commission related to the potential increase of consumer health risk by a possible increase of the existing maximum levels for aflatoxins in almonds, hazelnuts and pistachios and derived products. *The European Food Safety Authority Journal, 446: 1-127.*

European Comission (2006). Commission Regulation (EC) No 1881/2006 of 19 December 2006 Setting maximum levels for certain contaminants in foodstuffs (text with EEA relevance). *Off. Journal of European Union,* 364: 5-24.

Ezekiel, C.N, Kayode, FO, Fapohunda, SO, Olorunfemi, M.F, Kponi, B.T. (2012). Aflatoxigenic moulds and aflatoxins in street-vended snacks in Lagos, Nigeria. *Internet Journal of Food Safety*, 14: 83–88.

Ezekiel, C.N.1, and Sombie, J.I. (2014). Survey of aflatoxins and fungi in some commercial breakfast cereals and pastas retailed in Ogun State, *Nigeria Nature and Science*, **12**(6): 27-32

Farber, P., Geisen, R. and Holzapfel, W.H. (1997) Detection of afatoxinogenic fungi in figs by a PCR reaction. *International Journal of Food Microbiology,* (36): 215-220.

Filazi, A. and Sireli, U.T. (2013). Occurrence of Aflatoxins in Food. In: Mehdi Razzaghi-Abyaneh (Ed) *Aflatoxins - Recent Advances and Future Prospects*, InTech, pp143-170.

Gashgari, Rukaia M., Yassmin M. Shebany and Youssuf A. Gherbawy (2010). Molecular characterization of mycobiota and aflatoxin contamination of retail wheat flours from Jeddah markets. *Food borne pathogens and Disease,* **7**(9): 1047:1054

Gbadeyan, R.A. (2010). De-marketing of harmful products in Nigeria. *Pakistan Journal of Social Science,* **7**(6):449-455

Geisen, R. (1996) Multiplex polymerase chain reaction for the detection of potential aflatoxin and sterigmatocystin producing fungi. *Systematic and Applied Microbiology,* 19: 388-392.

Gherbawy, Y.A. and Yaser, M.(2003). Fungicides and some biological controller agents effects on the growth of Fusarium oxysporum causing wilt disease of paprika. *Archtecture Phytopathology of Plant Pro*tection, (36): 235–245.

Giray B., Girgin G,, Engin AB and Aydin S (2007). Aflatoxins levels in wheat samples consumed in some regions of Turkey. *Food Control,* (18): 23-29.

Gostinčar, C., Grube, M., De Hoog, S., Zalar, P., Gunde-Cimerman, N. (2010). "Extremotolerance in fungi: Evolution on the edge". *FEMS Microbiology Ecology*. **71** (1): 2–11. doi:10.1111/j.1574-6941.2009.00794.

Gostinčar, C.; Lenassi, M.; Gunde-Cimerman, N.; Plemenitaš, A. (2011). "Fungal Adaptation to Extremely High Salt Concentrations". *Advances in Applied Microbiology*. *Advances in Applied Microbiology*. **77**: 71–96. doi:10.1016/B978-0-12-387044-5.00003-0

Gupta, R., Gigras, P., Mohapatra, H., Goswami, V.K. and Chauhan, B. (2003). Microbial α-amylases: a biotechnological perspective. *Process Biochemistry*, (38): 1599–1616.

Gumus,T., Arici,M., Daglioglu,O., Velioglu, M.(2009). Fate of flatoxins during bread making *Journal of food Science and Technology, **46**(4):384-387.*

Gwary, O.M., Hati, S.S., Dimari, G.A. and Ogugbuaja, V.O. (2012). Pesticide residues in bean samples from Northeastern Nigeria. *ARPN Journal of Science Technology,* **2**(2): 79-84.

Halt, M. (1994). Aspergillus *flavus* and aflatoxin B1in flour production .*European Journal of Epidemiology*, **10**(5): 555:558

Hamiton, D. (2000). Toxic Fungus Threatens Health of Consumers. www.agnic.org/pmp/2000/ama0826.htm

Hell, K., Cardwell, K.F., Setamou, M. and Schulthess, F. (2000b) Influence of insect infestation on aflatoxin contamination of stored maize in four agroecological regions in Benin. *African Entomology*, 8:169-177

Hell, K., Setamou, M., Cardwell, K.F. and Poehling, H.-M. (2000a). The influence of storage practices on aflatoxin contamination in maize in four agro ecological zones in Benin, West Africa. *Journal of Stored Products Research* 36: 365-382.

Herrera, M.,Juan, T.,Estopanan, G., Arino, A. (2009).Comparison of deoxynivalenol, ochratoxin A and aflatoxin B1 levels in conventional and organic durum semolina and the effect of milling. *Journal of Food and Nutrition Research,* **48**(2): 92–99.

Herzallah, S., Alshawabkeh, K. and Al Fataftah, A.A. (2008). Aflatoxin decontamination of artificiallycontaminated feeds by sunlight, γ-Radiation, and microwave heating. *Journal of Applied Poultry Research*, 17:515–521

Hohl,T.M. and Feldmesser, M. (2007) *Aspergillus fumigatus*: principles of pathogenesis and host defense. *Eukaryot Cell*, 6: 1953–1963

Horn, B.W. (2003). Ecology and population biology of aflatoxigenic fungi in soil. *Journal of Toxicol-Toxin Review*, 22: 351–379.

Horn BW. (2007). Biodiversity of *Aspergillus* section *Flavi* in the United States: A review. *Food Additive Contaminatination*. 24:1088-1101.

Hendrickse, R.G. (1983). Aflatoxin and kwashiorkor: epidemiology and clinical studies in Sudanese children and findings in autopsy liver sample from Nigeria and South Africa. *Bulletin of Society Pathology Exotique*, 76: 559-566.

International Agency for Research on Cancer (IARC). (2002). Some traditional herbal medicines, some mycotoxins, naphthalene and styrene. *IARC Monogr Eval Carcinog Risks Hum, Scientific Publication*, **82** (82): 1–556.

Iqbal, S.Z., Asi M.R., Jinap S., Rashid, U. (2013). Detection of aflatoxins and zearalenone contamination in wheat derived products, *Food Control*, 35: 223–226

Jensen, B., Nebelong, P., Olsen, J. and Reeslev, M. (2002). Enzyme production in continuous cultivation by the thermophilic fungus, *Thermomyces lanuginosus*. *Biotechnology Letters*, 24: 41–45.

John, G., Gibbons, Anne Beauvais., Remi, Beau., Kriston, L., McGary,Jean-Paul Latgé And Antonis Rokas (2012). Global Transcriptome Changes Underlying Colony Growth in the Opportunistic Human Pathogen *Aspergillus fumigatus. Eukaryotic Cell,* **11**(1): 68–78. doi: 10.1128/EC.05102-11

Juan, C., Zinedine, A., Molto, J.C., Idrissi, L and Man, J. (2008). Aflatoxin levels in dried fruits and nuts from Rabat-Sale area, Morocco. *Food Control,* 19: 849-853.

Jurado, M., Vázquez, C., Marín, S., Sanchis, V. and González-Jaén, M.(T. 2006). PCR-based strategy to detect contamination with mycotoxigenic Fusarium species in maize. *Systemic Applied Microbiology,* 29: 681-689

Karlshøj, K., Nielsen, P.V. and Larsen, T.O. (2007). Differentiation of closely related fungi by electronic nose analysis. *Journal of Food Science,* 72: 287–292

Kelly,Wicks (2009). A preliminary review of aflatoxins and its implications on health and food production in Sana. *Journal of Science and Technology,* **27**(1): 17-27

Klich, M. A., E. J. Mullaney, C. B. Daly, and J. W. Cary. 2000. Molecular and physiological aspects of aflatoxin and sterigmatocystin biosynthesis by *Aspergillus tamarii* and *Aspergillus ochraceoroseus*. *Applied Microbiology Biotechnology*. 53:605-609.

Klich,M.A.(2002).Identification of common *Aspergillus* species. Centralbureau voor Schimmelcultures, Netherlands, ISBN-90-70351-46-3

Kensler, T.W., Roebuck, B.D., Wogan, G.N. and Groopman, J.D. (2011). Aflatoxin: A 50-Year odyssey of mechanistic and translational toxicology. *Toxicological Science,* **120(**1), 28-48.

Khlangwiset, P., Shephard, G. S., & Wu, F. (2011). Aflatoxins and growth impairment: A review. *Critical Reviews in Toxicology*, **41**(9), 740-755. doi: 10.3109/10408444.2011.575766

Kuniholm, M. H., Lesi, O. A., Mendy, M., Akano, A. O., Sam, O., Hall, A. J., Whittle, H., Bah, E., Goedert, J. J.,Hainaut, P., & Kirk, G. D. (2008). Aflatoxin exposure and viral hepatitis in the etiology of liver cirrhosis in the Gambia, West Africa. *Environmental Health Perspective,* **116**(11): 553-1557.

Kabak, B. and Var, I. (2005). Control of irradiation effects of mould and mycotoxin Development, *Food,* **30**(1):197-201.

Karlshøj, K, Nielsen, PV, Larsen, T.O. (2007a). Differentiation of closely related fungi by electronic nose analysis. *Journal of Food Science,* 72:287 – 292

Kelly,Wicks (2009). A preliminary review of aflatoxins and its implications on health and food production in Sana. *Journal of Science and Technology, Ghana,* **27**(1): 17-27

Khatri, M. and Rajam, M. V. (2007). Targeting polyamines of *Aspergillus nidulans* by siRNA specific to fungal ornithine decarboxylase gene. *Medical Mycology,* 45:211–220

Khoury,Andre.,Ali Atoui.,Toufic Roger Lteif., Mireille Kallassy and AhmedLebrihi.(2011). Differencition between *Aspergillus flavus* and *Aspergillus parasiticus* from pure culture and Aflatoxin-contaminated grapes using PCR-RFLP analysis of aflR-aflJ intergenic spacer. *Journal of food science*, 76: 247-253

Klich, M.A., Mullaney,E.J., Daly,C.B.and Cary, J.W.(2000). Molecular and physiological aspects of aflatoxin and sterigmatocystin biosynthesis by *Aspergillus tamarii* and *Aspergillus ochraceoroseus*. *Applied Microbiology Biotechnology*, 53:605-609

Klich, M.A. (2002). Identification of common *Aspergillus* species. Centralbureau voor Schimmelcultures, Netherlands, ISBN-90-70351-46-3

Klich, M.A.(2007). *Aspergillus flavus*: the major producer of aflatoxin. *Molecular Plant Pathology*, **8**(6):713-22. doi: 10.1111/j.1364-3703.2007.00436.x.

Klich, M.A. & Pitt, J.L. (1988). A laboratory guide to common *Aspergillus* species and their teleomorphs. North Ryde: Commonwealth Scientific and Industrial Research Organization (CSIRO)

Kotula, I. K., Kotula, A. W., Rose, B.E., Pierson,C.J. and M. Camp (1997). Reduction of aqueous chlorine by organic material. *Journal of Food Protection,* 60: 276-282.

Kozakiewicz,Z (1995) *in*: Fungal Identification Techniques, Proceedings from the Workshop in Barcelona, Spain, 5 to 8 April, EUR 16510, 183.

Kunamneni, A., Permaul, K. and Singh, S. (2005).Amylase production in solid state fermentation by the thermophilic fungus *Thermomyces lanuginosus. Journal Bioscience Bioengeniaring*, 100: 168–171

Lanyasunya, T.P., Wamae, L.W., Musa, H.H., Olowofeso, O. and Lokwaleput, I.K. (2005). The risk of mycotoxins contamination of dairy feed and milk on smallholder dairy farms in Kenya. *Pakistan Journal of Nutrition*, 4:162–169.

Latha, R., H.K. Manonmani and E.R. Rati, (2008). Multiplex PCR Assay for the Detection of Aflatoxigenic and Non-Aflatoxigenic *Aspergilli*. *Research Journal of Microbiology*, 3: 136-142.

Lee, C.Z., G.Y. Liou and G.F. Yuan. (2006).Comparison of aflR gene sequences of strains in *Aspergillus* section flavi. *Microbiology*, **152**(1): 161-170.

Lee, H.J. and Ryu, D.(2015). Significance of Ochratoxin A in Breakfast Cereals from the United States. *Journal of Agriculture Food Chemistry*, **63**(43): 9404-9409. doi:

10.1021/jf505674v. Epub 2015

Lippolis, V., Pascale, M. and Visconti, A. (2006). Optimization of a fluorescence polarization immunoassay for rapid quantification of deoxynivalenol in durum wheat-based products. *Journal of Food Prot*ection, 69**:** 2722–2729

Liu, H., Cottrell, T.R., Pierini, L.M., Goldman, W.E., Doering, T.L., (2002). RNA interference in the pathogenic fungus Cryptococcus neoformans. *Genetics*, 160: 463–470.

Luis Miguel Contreras-Medina, Alejandro Espinosa-Calderon, Carlos Duarte-Galvan, Arturo Alfonso Fernandez-Jaramillo, Rafael Francisco Muñoz-Huerta, Jesus Roberto Millan-Almaraz, Ramon Gerardo Guevara-Gonzalez and Irineo Torres-Pacheco, (2013). Characteristics of Mycotoxin Analysis Tools for Tomorrow, Aflatoxins - Recent Advances and Future Prospects, Prof. Mehdi Razzaghi-Abyaneh (Ed.), *InTechnology,* 289-313 DOI: 10.5772/51683

Mabrouk , M.M. and Sabry, S.A. (2001). Degradation of poly(3-hydroxybutyrate) and its copolymer poly(3-hydroxybutyrate-co-3-hydroxyvalerate) by a marine *Streptomyces* sp. SNG9. *Microbiology Research*, 156: 323-335.

Maliha, R., Samina, K. and Najma, A. (2010). Assessment of mycoflora and aflatoxin contamination of stored wheat grains. *International Food Research Journal*, 17: 71-81

Madigan ,M. and Martinko, J.(2005). *Brock Biology of Microorganisms* (11th ed.). Prentice Hall.

Manji ,A.J.,Aliyu,B.J.Nkafamiya,I.I. (2006).Degradation of groundnuts oil used for shallow frying. *Journal of chemical society of Nigeria,***31**(1&2): 22-26

Magan, N., Hope, R., Cairns, V., Aldred, D. (2003) Post-harvest fungal ecology: Impact of fungal growth and mycotoxin accumulation in stored grain. *European Journal Plant Pathology,* 109: 723-730.

Magan, N., Medina, A. and Aldred, A. (2011). Possible climate change effects on mycotoxin contamination of food crops pre- and post harvest. *Plant Pathology,* 60: 150-163.

Maragos, C.M., Jolley, M.E., Plattner, R.D. and Nasir, M.S. (2002). Fluorescence polarization as a means for determination of fumonisins in maize. *Journal of Agriculture Food Chemistry,* 49: 596–602

Maragos, C.M. and Kim, E.K. (2004). Detection of zearalenone and related metabolites by fluorescence polarization immunoassay. *Journal of Food Protection*, 67: 2039–2043

Marin, S., Ramos, A.J., Cano-Sancho, G.and Sanchis, V. (2013). Mycotoxins: occurrence, toxicology, and exposure assessment. *Food Chemistry Toxicology*, 60:218-237.

Meng, K., Wang, Y., Yang, P, (2010). Rapid detection and quantification of zearalenone-producing Fusarium species by targeting the zearalenone synthase gene PKS4. *Food Control,* 21: 207– 211.

Meyer, V, (2008).Genetic engineering of filamentous fungi Progress, obstacles and future trends. *Biotechnology Advances,* 26: 177–185

Michael J. Miller., Ludmila V. Roze ., Frances Trail3 and John E. Linz (2005). Role of *cis*-Acting Sites *NorL*, a TATA Box, and*AflR-1* in *nor-1* transcriptional activation in *Aspergillus parasiticus. Applied Environmental Microbiology,* **71(**3): 1539-1545 doi: 10.1128/AEM.71.3.1539-1545

Michael Thrusfield (2005). Vetenary epidemiology.Third edition, Blackwell publishing company,pp 232-233 ISBN-10:0-6320-6397-1

Mihoko, Tominaga.,Yun-Hae, Lee., Risa, Hayashi., Yuji Suzuki, Osamu Yamada, Kazutoshi Sakamoto, Kuniyasu Gotoh, and Osamu Akita (2006). Molecular Analysis of an Inactive Aflatoxin Biosynthesis Gene Cluster in *Aspergillus oryzae* RIB Strains. A*pplied and Environmental Microbiology*, **72(**1): 484–490

Moore, D., Robson, G.D.,Trinci, APJ (editors). (2011). *21st Century Guidebook to Fungi* (1st ed.). Cambridge University Press.

Monokesh, Kumer Sen., M.Abu Hena Mostafa Jamal and Shamima Nasrin, (2013).Sterilization factors affect seed germination and proliferation of *Achyranthes aspera* cultured in-vitro. *Environmental and Experimental Biology*, 11:119-123

Munkvold, G.P. (2003). Cultural and genetic approaches to managing mycotoxins in maize. *Annual Review Phytopathology,* 41: 99–116.

Munkvold, G., Hurburgh, C., Meyer, J., Loy ,D. and Robertson, A. (2005). Aflatoxins in corn. Iowa State University extension, File: *Pest Management* 2–5

Mustafa,A.,M.,Ahmed,El-Imam.,J.B.Ameh.,and I.O.Abdullahi (2012). Occurrence of fumonisins and deoxynivalenol in stored maize used in industrial productions in zaria, Nigeria. *African Journal of food Science,* 6(9): 249-252

Naveed, Aslam and Peter, C. Wynn (2015). Review Aflatoxin Contamination of the Milk Supply: A Pakistan Perspective. *Agriculture,* 5: 1172-1182; doi:10.3390/agriculture5041172

Ngundi MM, Shriver-Lake LC, Moore MH, Lassman ME, Ligler FS, Taitt CR (2005) Array biosensor for detection of ochratoxin A in cereals and beverages. *Analytical Chemistry*, 77: 248–254

Ngundi, M.M., Shriver-Lake, L.C., Moore, M.H., Ligler, F.S. and Taitt, C.R. (2006) Multiplexed

detection of mycotoxins in foods with a regenerable array. *Journal of Food Protection, (*69), 3047–3052

Niessen, L. (2007). PCR-based diagnosis and quantification of mycotoxin producing fungi. *International Journal of Food Microbioogy*, 119: 38–46.

Nigeria Industrial Standard (NIS) 396: (2002). The Standards Organization of Nigeria Standards for wheat flour.

Noelia,Sardinas.,Covadonga,Vazquez.,Jessica Gil-Serna.,Gonzalez-Jaen,M.Teresa.,Belen, Patino (2011). Specific detection and quantification of *Aspergillus flavus* and *Aspergillus parasiticus* in wheat flour by sybre green quantitative PCR. *International Journal of food Microbiology,* 145: 121-125

Noorbacash, R., R.B. Ahmed, S.A. Mortazavi, B. Forghani and N. Bahreini, (2009). PCR-based identification of afltoxigenic fungi associated with Iranian saffron. *Food Science and Biotechnology,* 18: 1038-1040.

Novošinskas H., Raila A., Zvicevičius E., Lugauskas A. (2005). Mycological state of premises for food storage and search of preventive safety measures, Botanica Lithuanica. Suppl. 7. P. 93–105.

Okun, D.O., Fathiya M. Khamis1., Geoffrey M. Muluvi., Joseph J. Ngeranwa1., Fidelis O. Ombura., Moses O. Yongo and Eucharia U. Kenya (2015). Distribution of indigenous Strains of atoxigenic and toxigenic *Aspergillus flavus and Aspergillus parasiticus* in maize and peanuts agro‑ecological zones of Kenya, *Agriculture & Food Securit,* 4:14 DOI 10.1186/s40066-015-0033-5

Olsson J, Bo¨rjesson T, Lundstedt T, Schnu¨rer J (2002) Detection and quantification of ochratoxin A and deoxynivalenol in barley grains by GC-MS and electronic nose, *International Journal Food Microbiology*, 72: 203–224

Oluwafemi,F. and Ikeowa,M.C.(2005). Fate of aflatoxin B1 during fermentation of maize into Ogi. *Nigerian Food Journal*, 23: 243-247

Ordaz, J. Jaimez., C.A. Fente, B.I. Va´zquez, C.M. Franco, A. Cepeda,(2003) "Development of a method for direct visual determination of aflatoxin production by colonies of the *Aspergillus flavus* group",.*International Journal of Food Microbiology,* 83: 219–225.

Onyemelukwe, G.C., Ogoina, D., Ibiam, G.E. and Ogbadu, G.H. (2012). Aflatoxins in body fluids and food of Nigerian children with Protein- Energy Malnutrition (PEM*). American Journal of Food, Agricultue Nutrition Development*, 12(5): 6553-6566.

Oyelami, O.A., Maxwell, S., Adelusola, K.A., Aladekoma, T.A. and Oyelese, A.O. (1998). Aflatoxins in autopsy kidney specimens from children in Nigeria. *Journal of Toxicology*

and Environmental Health, **55**(5): 317-323

Oyewole,O.A., Oyeleke, S.B., Dauda, B.E.N and Emiade,S. (2011). Production of Amylase and Protease Enzymes by *Aspergillus niger* and *Penicillium frequestans* Isolated from Abattoir Effluent. *Microbiology Journal*, 1: 174-180.

Pečivová , Pavlína., Vladimír Pavlínek and Jan Hrabě (2011). Changes of properties of wheat flour dough by combination L-ascorbic acid with reducing or oxidising agents. *Acta Chimica Slovaca*, 4(2): 108 – 117

Perrone, G., Haidukowski, M., Stea, G., Epifani, F.Bandyopadhyay, R, Leslie, J.F., Logrieco, A.(2014).Population structure and aflatoxin production by *Aspergillus* Sect. *Flavi* from maize in Nigeria and Ghana. *Food Microbiology*. doi:10.1016/j.fm.2013.12.005.

Peter J. Cotty & Ramon Jaime-Garcia (2007). Influences of climate on aflatoxin producing fungi and aflatoxin contamination. *International Journal of Food Microbiology*, 119: 109–115

Phillips, T.D. (1997). Detection and Decontamination of aflatoxincontaminated food products. In: J.H. Williams,

Philippe,V. (2014). Aflatoxins and safe storage. *Front Microbiology*, 5:158-165. doi: 10.3389/fmicb.2014.00158.

Piermarini, S., Michel L., Ammida, N.H.S., Palleschi, G. and Moscone D (2007) Electrochemical immunosensor array using a 96-well screen-printed microplate for aflatoxin B2 detection. *Biosens Bioelectron*, 22: 2434–2440

Pitt,J.I.Hocking,A.D. and Glenn, D.R.(1983).An improved medium for the detection of *Aspergillus flavus* and *Aspergillus parasiticus*. *Journnal of Applied Bacteriology*,54: 109-114.

Pitt, J.I. and Hopkins, A.D. (2009). *Fungi and Food Spoilage*. London: Springer. doi:10.1007/978-0- 387-92207-2_9

Pitt,J.L.,Samson,R.A. and Frisvad,J.C. (2000).In: integration of Modern Taxonomic Methods for Penicillium and Apergillus classification (R.A.Samson and J.L.Pitt,Eds.),A. Samson and J.I. Pitt, Eds.), Hardwood Academic Publishers, Reading, UK, 9-50 (2000). Hardwood Academic Publishers, Reading, UK, 9-50

Probst, C., Bandyopadhyay, R., Price, L.E. and Cotty, P.J. (2011). "Identification of Atoxigenic *Aspergillus flavus* Isolates to Reduce Aflatoxin Contamination of Maize in Kenya." *Plant Disease* 95: 212–218.

Rachaputi, N.R., Wright, G.C., Kroschi, S. (2002). Management practices to minimise pre-harvest aflatoxin contamination in Australian groundnuts. *Australian Journal of Experimental Agriculture* , 42: 595–605

Radiana Tamba-Berehoiu.,Popa Nicolae-Ciprian.,Popescu Stela., Cristea Stelica.,Culea Rodica and Tamba-Berehoiu Suzana,(2009).Distribution of some toxic contaminants in the milling products, during the milling process. *Romanian Biotechnological letters,* 15(3), 5281-5286

Rahardjo Y.S.P., Weber F.J., Haemers S., Tramper J., Rinzema A. (2005). Aerial mycelia of *Aspergillus oryzae* accelerate α-amylase production in a model solid-state fermentation system. *Enzyme Microbe Technology,* 36:900–902.

Rashid, Maliha., Samina ,Khalil., Najma, Ayub.,Waseem, Ahmed and Abdul Ghaffar Khan (2008).Categorization of A*spergillus flavus* and *Aspergillus parasiticus* isolates of stored wheat grains in to aflatoxinogenics and non-aflatoxinogenics. *Pakistan Journal of Botany*, 40(5): 2177-2192

Ryan, KJ and Ray, CG (2004). *Sherris Medical Microbiology* (4th ed.). McGraw Hill. pp. 633–8. ISBN 0-8385-8529-9.

Riba,A.,S.Mokrane.,F.Mathieu.,A.Lebrihi and N.S.abaou,(2008).Mycoflora and ochratoxin A producing strains of Aspergillus in Algerian wheat. *International Journal of Food Microbiology*, 122: 85-92

Ritter, A.C., Hoeeltz, M.,Noil,I.B.(2011)Toxigenic Potential of Aspergillus flavus tested in different culture conditions,*Cienc. Tecnol. Aliment.,Campinas,* **31**(3): 623-628

Rodrigues, Paula., Armando Venâncio., Zofia Kozakiewicz and Nelson Lima, (2009).A polyphasic approach to the identification of aflatoxigenic and non-aflatoxigenic strains of Aspergillus Section Flavi isolated from Portuguese almonds *International Journal of Food Microbiology*, 129: 187–193

Rojas-Durán, T. R., Fente, Vázquez. B. I., Franco, C. M., Sanz-Medel, A. & Cepeda, A. (2007). Study of a room temperature phosporescence phenomenon to allow the detection of aflatoxigenic strains in culture media. *International Journal of Food Microbiology*, **115**(2):149-158.

Roige Marcela Beatriz, Aranguren Sandra Mariela, Riccio Maria Belen, Pereyra Silvia, Soraci Alejandro Luis, Tapia Maria Ofelia, (2009). Mycobiota and mycotoxins in fermented feed wheat grains and corn grains in Southeastern Buenos Aires Province, Argentina, *Revista Iberoamericana de Micologia***, 26:04**

Sapsford KE, Taitt CR, Fertig S, Moore MH, Lassman ME, Maragos CM, Shriver-Lake LC (2006). Indirect competitive immunoassay for detection of aflatoxin B2 in corn and nut products using the array biosensor. *Biosens Bioelectron,* 22: 2298–2305

Shami E. A. Bakhiet and Ehood A. I. Al-Mokhtar , (2015). Production of Citric Acid by ‘ Aspergillus niger Using Sugarcane Molasses as Substrate. *Jordan Journal of Biological*

Sciences, **8**(3):211 - 215

Shehu,I.K. & Bello,M.T.(2011). Effect of Environmental Factors on the Growth of Aspergillus Species Associated with Stored Millet Grains in Sokoto. *Nigerian Journal of Basic and Applied Science,* **19**(2): 218-223

Shepard, G. S. (2008). Determination of mycotoxins in food. *Chemical Society Reviews*, 37: 2468- 2477.

Schollenberger, Margit., Helga,Terry Jara., Sybille Suchy., Drochner, W. and Muller, H.M. (2002). Fusarium toxin in wheat flour collected in an area in southwest Germany, *International Journal of of Food Microbiology*,**72(**1-2): 85-89

Scherm, B., Palomba, M., Serra, D., Marcello, A. and Migheli, Q. (2005) Detection of transcripts of the aflatoxin genes aflD, aflO, and aflP by reverse transcription-polymerase chain reaction allows differentiation of aflatoxin-producing and non-producing isolates of *Aspergillus flavus* and *Aspergillus parasiticus*. *International Journal Food Microbiol*ogy, 98: 201–210.

Schmalle III DG, Munkvold GP (2015). Mycotoxins in Crops: A threat to human and domestic animal health. DOI: 10.1094/PHI-I-2009-0715- 01

Shapira, R., Paster, N., Eyal, O., Menasherov, M., Mett, A. and Salomon, R. (1996) Detection of a¯atoxigenic molds in grains by PCR. *Applied and Environmental Microbiology*, 62: 3270-3273.

Shepard, G.S. (2009). Aflatoxin analysis at the beginning of the twenty-first century. *Analytical and bioanalytical Chemistry,* **395(**5): 1215-122

Shima, J. S., Takase, S., Takahashi, Y., Iwai, Y., Fujimoto, H., Yamazaki, M. and Ochi, K. **(**1997). Novel detoxification of the trichothecene mycotoxin deoxynivalenol by a soil bacterium isolated by enrichment culture. *Applied Environmental Microbiol*ogy, 63:3825-3830.

Somashekar, D.,Rati, E.R.and Chandrashekar, A. (2004). Isolation, enumeration and PCR characterization of fungi from food and feed samples in India. *Food Microbiology*, 20: 809 – 813

Sramkova Zuzana ., Edita Gregováb and Ernest Šturdík (2009). Chemical composition and nutritional quality of wheat grain. *Acta Chimica Slovaca. 2:1:115 – 138*

Stark AA, Essigmann JM, Demain AL, Skopek TR, Wogan GN (1979) Aflatoxin B2 Mutagenesis and adduct formation in Salmonella typhimurium. *Proc National Academic Scence, USA,* 76: 2343–2347

Stark,A.A.(2007).Molecular and biochemical mechanism of action of acute toxicity and carcinogenicity induced by by aflatoxin B_2 and of the chemoprevention of liver cancer.In:

Wilson CL.(ed) Microbial food Contamination. Boca-Raon CRC, pp227-246

Stroka, J., Van Otterdijk, R., & Anklam, E. (2000). Immunoaffinity column clean-up prior to thin-layer chromatography for the determination of aflatoxins in various food matrices. *Journal of Chromatography A*, **904**(2): 251-256.

Takahashi, T., P.K. Chang, K. Matsushima, J. Yu, K. Abe, D. Bhatnagar, T.E. Cleveland, Y.(2002). Koyama Non functionality of *Aspergillus sojae aflR* in a strain of *Aspergillus parasiticus* with a disrupted*aflR* gene. *Applied Environmental Microbiology*, 68: 3737–3743

Tamang, J.P., Watanabe, K. and Holzapfel, W.H. (2016). Review: Diversity of Microorganisms in Global Fermented Foods and Beverages. *Front Microbiology*, 7:377. doi: 10.3389/fmicb.2016.00377

Tamura, K. and Nei, M. (1993). Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. Molecular Biology and Evolution, 10:512-526

Taniwaki,M.H ., Hocking,A.D., Pitt,J.I. and Fleet,G.H.(2009). Growth and mycotoxin production by food spoilage fungi under high carbon dioxide and low oxygen atmospheres. *International Journal of Food Microbiology*, 132:100–108

Temur, C. and Tiryaki, O. (2013). Irradiation alone or combined with other alternative treatments to control postharvest diseases. *Assian Journal of Agricltural Research*, 8(5):421-434

Thompson, J.D., T.J. Gibson, F. Plewniak, F. Jeanmougin and D.G. Higgins, (1997). The CLUSTAL X windows interface: Flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acid Research*, 25: 4876-4882.

Thompson, V.S. and Maragos, C.M. (2006) Fibre-optic immunosensor for the detection of fumonisin B2. *Journal of Agricultural Food Chemistry*, (44), 2042–2046

Tominaga, Mihoko., Yun-Hae Lee., Risa Hayashi., Yuji Suzuki., Osamu Yamada., Kazutoshi Sakamoto., Kuniyasu Gotoh and Osamu Akita (2006). Molecular Analysis of an Inactive Aflatoxin Biosynthesis Gene Cluster in *Aspergillus oryzae* RIB Strains. *Applied and Environmental Microbiology*, 72: 484–490

Turner, P., Sylla, A., Gong, Y., Diallo, M., Sutcliffe, A., Hall, A. and Wild, C. (2005). Reduction of exposure to carcinogenic aflatoxins by postharvest intervention measures in West Africa: a communitybased intervention study. *Lancet*, 365:1950–1959.

Uriah, N., Ibeh, I.N. and Oluwafemi, F. (2001). A study on the impact of aflatoxin on human reproduction. *Assian Joural Reproductive Health*, **5(**1): 106-110.

Van Egmond, H. P. (1989). Aflatoxin M_1: occurrence, toxicity, regulation, p. 11-55. In H. P. Van Egmond (ed.), Mycotoxins in dairy products. Elsevier Applied Science, London.

Vanden, B.P., Pittet and H. Hajjaj. 2001. Aflatoxin genes and aflatoxigenic potential of Koji moulds. *Applied Microbiology and Biotechnology,* 57:192-199.

Wagacha, J.M., Muthomi, J.W. (2008). Mycotoxin problem in Africa: current status, implications to food safety and health and possible management strategies. *International Journal of Food Microbiology.* **124**(1S): 1-12.

Wartu, J.R., Whong ,C.M.Z., Abdullahi, I.O ., Ameh, J.B and Musa, B.J.(2015) Trends in total aflatoxins and nutritional impact of *Triticum* spp during fermentation of kunun-zaki; a Nigeria sorghum bicolor based non-alcoholic beverage. *International Journal of Life Science & pharma research,* 5:3: issn 2250-0480

Weidenbo¨ rner M, Wieczorek C, Appel S, (2000). Whole wheat and while wheat flour-the mycobiota and potential mycotoxins. *Journal of Food Microbiology.* 17:103–107

Williams, J.H., Phillips TD, Jolly PE, Stiles JK, Jolly CM, and Aggarwal D. (2004). Human aflatoxicosis in developing countries: a review of toxicology, exposure, potential health consequences, and interventions. *American Journal of Clinical Nutrition,* 80: 1106–1122.

Wilson, D. M., and G. A. Payne. 1994. Factors affecting Aspergillus flavus group infection and aflatoxin contamination of crops, p. 309-325. In D. L Eaton and J. D. Groopman (ed.), The toxicology of aflatoxins. Human health, veterinary and agricultural significance. Academic Press, San Diego, Calif.

Wilson, D. M., Mubatanhema, W. and Jurjevic, Z.(2002). Biology and ecology of mycotoxigenic *Aspergillus species* as related to economic and health concerns, p. 3-17. In J. W. deVries, M. W. Trucksess, and L. S. Jackson (ed.), Mycotoxins and food safety. Kluwer Academic Plenum Publications, Dordrecht, the Netherlands

Wilson, S. C., C. Wu., L. A. Andriychuk., J. M. Martin., T. L. Brasel., C. A. Jumper, and D. C. Straus (2005). Effect of Chlorine Dioxide Gas on Fungi and Mycotoxins Associated with Sick Building Syndrome. *Applied Environmental Microbiology,* **71(**9): 5399–5403.

White, J., Truesdell, K., Williams, L.B., AtKisson, M.S., Kauer, J.S. (2008). Solid-state, dye-labeled DNA detects volatile compounds in the vapor phase. PLoS Biol 6:e9. doi:20.2372/journal. pbio.0060009

World Health Organization (WHO) (2006). Mycotoxins in African foods: implications to food safety and health. AFRO Food Safety Newsletter. World Health Organization Food safety (FOS), www.afro.who.int/des.

World Health Statistics (WHO) (2008). WHO Press, Geneva, http://www.who.int/whosis/whostat/EN_WHS08_Full.pdf.

Wu, F., Narod, C., Tiongco, M. and Liu, Y. (2011). The health economics of aflatoxin: the global burden of disease. Available from: http://www.ifpri.org/sites/default/files/publications/aflacontrol_wp04.pd f accessed 28/01/2015.

Yan-ni Yin, Lei-yan Yan, Jin-hua Jiang, and Zhong-hua Ma(2008). Biological control of aflatoxin contamination of crops**.** *Journal of Zhejiang University Science B.* **9(**10): 787–792

Yang, J., Bai, F., Zhang, K., Bai ,S., Peng ,X., Ding, X., Li, Y., Zhang, J., Zhao, L.(2012). Effects of feeding corn naturally contaminated with aflatoxin B1 and B2 on hepatic functions of broilers. *Poultry Science*, **91(**11): 2792-2801. doi: 10.3382/ps.2012-02544.

Yu,J.,Chang,P.K.,Ehrlich,K.C.,Cary,J.W.,Bhatnagar,D.,Cleveland,T.E.,Payne,G.A.,Linz,J.E., Woloshuk.C.P.,Bennett,J.W.(2004).Clustered pathway genes in aflatoxin biosynthesis. *Applied Environmental Microbiology*, 70: 1253-1262

Yu, J.C., Hrdina, A., Mancini, C., Lai, E.P. (2007) Molecularly imprinted polypyrrole encapsulated carbon nanotubes in stainless steel frit for micro solid phase extraction of estrogenic compounds. *Journal Nanoscience Nanotechnology*, **7:** 3095–3203

Zain, M.E.(2011). Impact of mycotoxins on humans and animals. *Journal of Saudi Chemical Society*, **15**(2):129-144

Zajc, J.; Zalar, P.; Plemenitaš, A.; Gunde-Cimerman, N. (2012). "The Mycobiota of the Salterns". *Biology of Marine Fungi*. Progress in Molecular and Subcellular Biology. **53**. pp. 133–158. doi:10.1007/978-3-642-23342-5_7

Zhang, F., Guo, Z., Zhong, H., Wang, S., Yang, W., Liu, Y. and Wang, S. (2014). RNA-Seq-based transcriptome analysis of aflatoxigenic *Aspergillus flavus* in response to water activity.*Toxins*, 6: 3187-3207.

Zhao, J., Kong, F., Li, R., Wang, X., Wan, Z., Wang, D. (2001). Identification of *Aspergillus fumigatus* and related species by nested PCR targeting Ribosomal DNA internal transcribed spacer regions. *Journal Clinical Microbiology*, 39:2261-2266.

Zhao, Q., Ran, W., Wang, H., Li, X., Shen, Q., Shen, S., Xu, Y. (2013). Biocontrol of Fusarium wilt disease in muskmelon with *Bacillus subtilis* Y-IVI. BioControl, Amsterdam, *The Netherlands* **58**(2):283-292.

Zhou, T., He, J. and Gong, J. (2008). Microbial transformation of trichothecene mycotoxins. *World Mycology Journal*, 1: 23-30

Zheng, Z., Humphrey, C.W., King, R.S., Richard, J.L. (2005). Validation of an ELISA test kit for the detection of total aflatoxins in grain and grain products by comparison with HPLC. *Mycopathologia*

Zuzana Hruska, Kanniah Rajasekaran, Haibo Yao, Russell Kincaid, Dawn Darlington, Robert Brown, Deepak Bhatnagar, and Thomas E. Cleveland (2014). Co-inoculation of aflatoxigenic and non-aflatoxigenic strains of *Aspergillus flavus* to study fungal invasion, colonization, and competition in maize kernels. *Front Microbiology*, 5: 122. doi: 10.3389/fmicb.2014.00122

APPENDICES

Appendix I

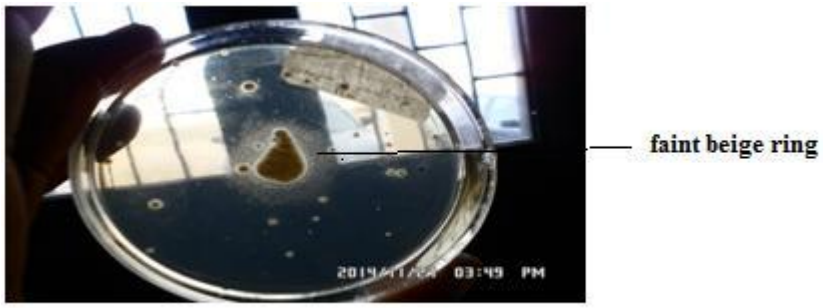


Plate III: 3 days Incubation of aflatoxigenic Strain

Appendix II

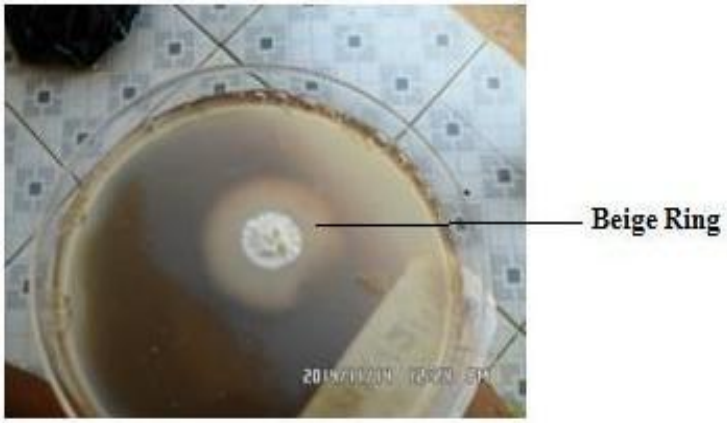


Plate IV: Aflatoxigenic Aspergillus flavus strain

Appendix III

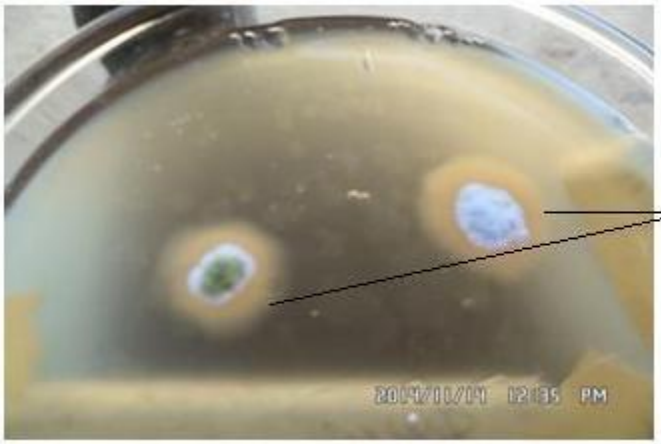


Plate V: Aflatoxigenic Aspergillus parasiticus

Appendix IV



Plate VI: Non Aflatoxigenic Strains (No Beige Ring)

Appendix V

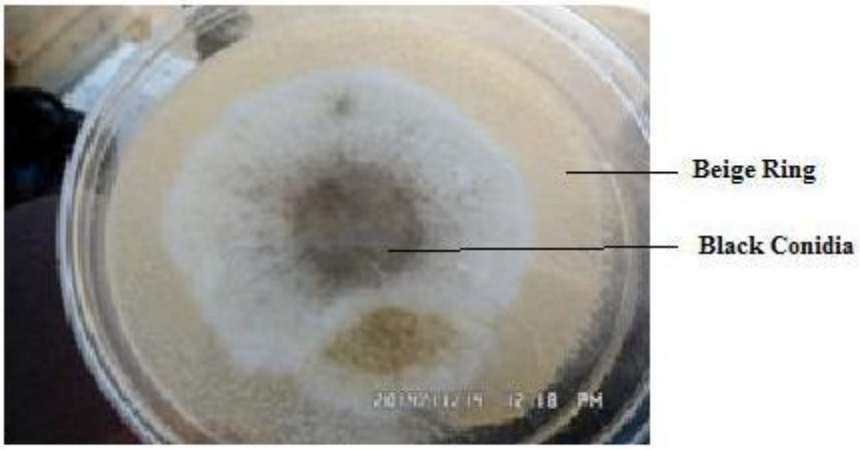
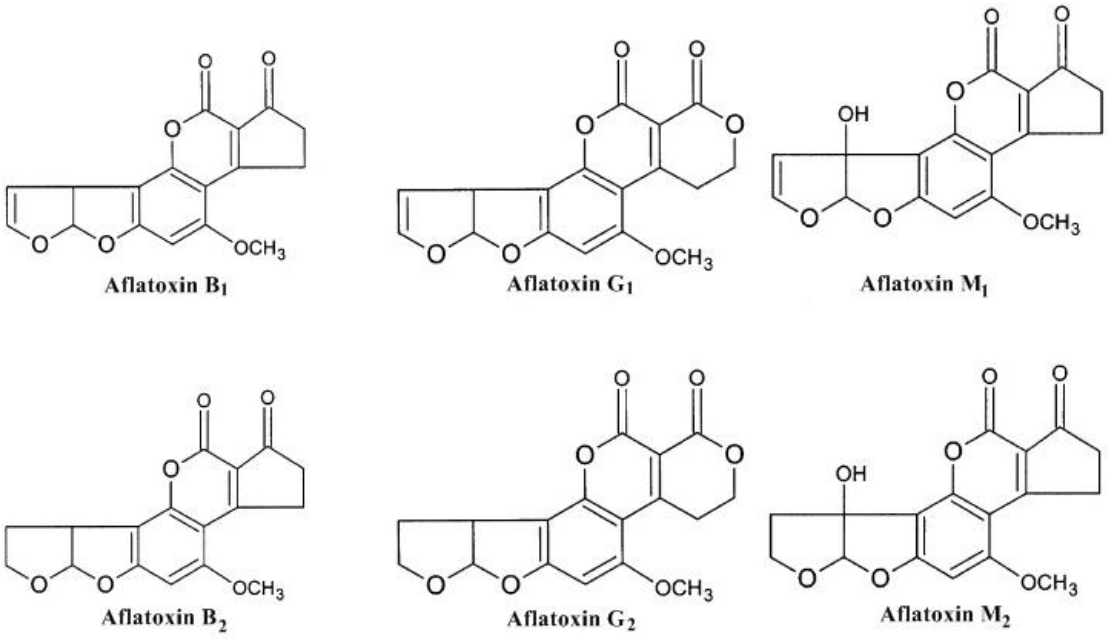


Plate VII: **Aflatoxigenic Aspergillus niger strain**

Appendix VI



Structure of Aflatoxins
Source : Zain *et al*.,2015