INCIDENCE OF MYCOTOXIGENIC FUNGI DURING PROCESSING AND STORAGE OF BAMBARA GROUNDNUT (*Vigna subterranea*) COMPOSITE FLOUR

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DECLARATION

I declare that the thesis herewith submitted to the Department of Biotechnology and Food Technology, Durban University of Technology for the award of Ph.D Degree in Food Science and Technology is my work and has not been previously submitted for a degree at any other University or Higher Institution of Education.

As the candidate's supervisors, we agree to the submission of this thesis.

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To God Almighty, whose grace has sustained me to complete this programme.

To the sweet memory of my late mothers, Madam Elizabeth Adenike Adesanya and Madam Elizabeth Mosunmola ‘Lagunju

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ABSTRACT

Fungal contamination of food commodities is a global food security challenge that impacts negatively on the health of consumers. Mycotoxins are produced as secondary metabolites by some pathogenic fungi and may contaminate agricultural products while on the field or during harvesting and storage. Processing operations and storage conditions of temperature and relative humidity have marked effect on the ability of fungal pathogens to grow and produce mycotoxins in agricultural food commodities. The consumption of mycotoxin-contaminated foods, even at low doses over a prolonged period of time, may have deleterious effects on health of consumers.

Bambara groundnut (*Vigna subterranea* (L.) Verdc) is an African legume gaining wide acceptance in various food applications due to its favourable nutritional composition, especially the high protein content. In several parts of Africa, it is used as a supplement in cereal-based foods, especially in weaning food for infants and young children. Bambara groundnut grows near or under the soil, which serves as inoculum of pathogenic fungi. Very little information is presently available on fungal and mycotoxin contamination of Bambara groundnut from Southern Africa. Hence, its safety for consumption from a mycological standpoint requires further studies.

To establish the profiling of fungal contaminants in food commodities consumed in Durban, South Africa, 110 samples of regularly consumed food samples which included rice (23), spices (38), maize and maize-derived products (32), and Bambara groundnut (17) were randomly collected over a period of five months from retail stores and open markets. The food samples were screened for fungal contamination using conventional and molecular methods. Fungal isolates were characterized following DNA extraction, polymerase chain reaction and sequencing. Using a modified QuEChERS method, the detection and quantification of mycotoxins in Bambara groundnut was performed via Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS), and isolation and detection of the causative pathogen was carried out. The effect of processing operations of milling, a combination of roasting and milling, and spontaneous fermentation on the survival of the natural fungal population of Bambara groundnut, and aflatoxin production under simulated tropical conditions of storage was further studied. Processed Bambara groundnut flour samples were stored at temperature of 30±1 °C and 85±2% relative humidity for 30 days,

and samples withdrawn at 5-day intervals for analyses, *i.e.*, fungal counts, aflatoxin accumulation and changes in water activity during storage.

Following the detection of aflatoxins in Bambara groundnut flour and the isolation of aflatoxigenic *Aspergillus flavus* in the seed, the effect of milling, roasting and milling, or lactic acid bacteria fermentation on the survival, growth and aflatoxin production of *A. flavus* in Bambara groundnut flour was studied. Irradiated seeds of Bambara groundnut were artificially inoculated with a 3-strain cocktail of *A. flavus* (2×10^6 spores/mL) and processed by milling, roasting at 140 °C for 20 min and milling. Slurries of irradiated Bambara groundnut flour were also inoculated with *A. flavus* spores and 1×10^8 CFU/mL inoculum of *Lactobacillus fermentum* or *Lactobacillus plantarum*. All inoculated samples were incubated at 25 °C for 96 h, samples withdrawn every 24 h were analyzed for viable *A. flavus* counts, changes in water activity during incubation, and aflatoxin production using Enzyme-linked Immunosorbent Assay (ELISA). Bambara groundnut flour samples fermented with lactic acid bacteria were further analyzed for pH, total titratable acidity, and viable lactic acid bacteria counts over the incubation period. The degradation of aflatoxin (AF) B_1 by both lactic acid bacteria was also studied. Slurries of irradiated Bambara groundnut flour were spiked with 5 µg/kg of aflatoxin B_1 (AFB_1) and the percentage reduction over the incubation period was determined using HPLC.

The survival, growth and aflatoxin production of *A. flavus* in Bambara groundnut and maize-composite flours as affected by milling, roasting and milling or lactic acid bacteria fermentation during storage was also studied. Processed and irradiated Bambara groundnut flour, maize flour and maize-bambara composite flour (70:30) were inoculated with 2×10^7 spores/ml of *A. flavus* and stored for up to 10 weeks at a temperature of 25±2 °C and relative humidity of 75±2%. Samples were withdrawn weekly and analyzed for viable populations of *A. flavus*, concentrations of aflatoxins B_1, B_2, G_1 and G_2, changes in pH and water activity over the storage period. The colonization of Bambara groundnut by *A. flavus* and the effects of fungal infection on the seed coat, storage cells and tissue structures were also studied. Irradiated Bambara groundnut seeds were artificially inoculated with spore suspension of aflatoxigenic *A. flavus* (2×10^6 spores/mL) and stored at a temperature of 25±2 °C and relative humidity of 75±2% for 14 days. Samples were withdrawn at 24 h intervals for 4 days, then at 7 and 14 days and examined using scanning electron microscopy (SEM) and transmission electron microscopy (TEM).

Various fungal genera were isolated from the food samples under study with *Aspergillus* (52.5%) and *Penicillium* (31.8%) as the dominant genera. All the 110 food samples were contaminated with more than one fungal species. *A. flavus* and other *Aspergilli*, *Penicillium citrinum* and *Fusarium oxysporum* were isolated from Bambara groundnut seeds. Aflatoxigenic *A. flavus* was isolated from Bambara groundnut seed, with a co-occurrence of Aflatoxin (AF) B_1 (0.13–6.90 µg/kg), AFB_2 (0.14–2.90 µg/kg), AFG_1 (1.38–4.60 µg/kg), and AFG_2 (0.15–1.00 µg/kg) in the flour. The fungal counts of the samples during storage significantly ($p \leq 0.05$) increased, irrespective of the processing method from 6.3 Log_{10} CFU/g in Bambara groundnut flour to 6.55 Log_{10} CFU/g in fermented Bambara groundnut flour. Aflatoxin concentration was affected markedly by the processing methods in Bambara groundnut flour (0.13 µg/kg) and fermented Bambara groundnut flour (0.43 µg/kg), aflatoxin was not detected in roasted Bambara groundnut flour.

The survival and growth of *A. flavus* was also markedly affected by lactic acid bacteria fermentation and roasting during incubation. Within 24 h of fermentation with *L. fermentum*, significant ($p \leq 0.05$) changes were recorded in viable population of *A. flavus* (6.30–5.59 Log_{10} CFU/mL), lactic acid bacteria count (8.54–13.03 Log_{10} CFU/mL), pH (6.19–4.12), total titratable acidity (0.77–1.87%) and a reduction by 89.2% in aflatoxin B_1 concentration. Similar significant changes were recorded in Bambara groundnut flour fermented with *L. plantarum*. *Aspergillus flavus* in the artificially contaminated seeds were completely eliminated by roasting. Aflatoxin production was not detected in Bambara groundnut flour samples over the incubation period.

During storage for 10 weeks, the population of *A. flavus* significantly ($p \leq 0.05$) decreased in roasted Bambara groundnut flour from 7.18 to 2.00 Log_{10} CFU/g. Similar significant ($p \leq 0.05$) decrease in *A. flavus* viable counts was recorded in fermented Bambara groundnut flour from 6.72 to 2.67 Log_{10} CFU/g, however after 7 weeks of storage and beyond, *A. flavus* was not detected. Significant ($p \leq 0.05$) decrease in aflatoxin B_1 (0.36–0.26 µg/kg) and aflatoxin G_1 (0.15–0.07 µg/kg) accumulation was also recorded in roasted Bambara groundnut flour. While *A. flavus* viable population significantly ($p \leq 0.05$) decreased in maize-Bambara composite flour from 6.90 to 6.72 Log_{10} CFU/g, aflatoxin B_1 accumulation significantly ($p \leq 0.05$) increased from 1.17 to 2.05 µg/kg. Microscopy studies showed that the seed coat of Bambara groundnut was rapidly colonized by *A. flavus* within 24 h of inoculation. The infection of internal tissues of the cotyledon was through the ruptured seed

coat, resulting in a disruption of the cellular architecture. Cell wall collapse, development of cavities in parenchymatous cells and ruptured storage cells resulted from *A. flavus* infection of the seed.

This study reports a high prevalence of fungal contamination in some food commodities consumed in Durban, South Africa. The isolation of live mycotoxin-producing fungi from the food commodities necessitates the need for regular routine checks to ensure the mycological safety of agricultural products offered for sale to consumers. The detection of aflatoxigenic *A. flavus* and aflatoxins in Bambara groundnut flour at levels above the maximum tolerable limits raises health concerns on its utilization in food applications, and in supplementary feeding for infants and young children. Although roasting was effective in degradation of aflatoxins in Bambara groundnut seeds, elimination of fungal contaminants was not achievable which resulted in continued production of aflatoxin during storage. Fermentation using *L. fermentum* or *L. plantarum* is effective in eliminating *A. flavus* and degrading AFB_1 in Bambara groundnut flour. Compositing Bambara groundnut with maize increased aflatoxin production in the flour. It is therefore necessary to implement legislation for aflatoxins in Bambara groundnut, and develop effective management practices during planting, harvesting and storage that will mitigate *A. flavus* infection in Bambara groundnut.

Key words: Bambara groundnut, Maize, *Aspergillus flavus*, Lactic acid bacteria, Aflatoxin, LC-MS/MS

PREFACE

The thesis is organised into eight chapters and presented as submitted for publication. Chapter One gives a general introduction to the thesis. Chapter Two provides a review of literature on Bambara groundnut composition, processing and utilization. It also presents a review on fungal and mycotoxin contamination of agricultural products, the mycotoxins of significance to human and animals, masked mycotoxins and general awareness of consumers to fungal contamination of foods. It further discusses the management and control of mycotoxins in agricultural products. Chapter Three presents the incidence of fungal contamination in Bambara groundnut and other food commodities in Durban, South Africa. Chapter Four deals with the detection of natural mycotoxins contaminating Bambara groundnut and the effects of processing methods on natural fungal and aflatoxin accumulation in Bambara groundnut flour. Chapter Five presents the study on the effect of processing methods on the survival, growth and aflatoxin production of *A. flavus* in Bambara groundnut. Chapter Six focuses on the behaviour of *A. flavus* in processed Bambara groundnut flour during storage. Chapter Seven reports the colonization and infection pattern of *A. flavus* in Bambara groundnut. Chapter Eight is a general discussion on the core findings of the research. Conclusions were drawn based on the findings obtained from the studies undertaken in this thesis and recommendations for future studies proposed.

TABLE OF CONTENTS

ABBREVIATIONS

AF:	Aflatoxin
AFPA:	*Aspergillus flavus parasiticus* agar
CIT:	Citrinin
CPA:	Cyclopiazonic acid
CTAB:	Cetyl-trimethyl ammonium bromide
CYA:	Czapex yeast extract agar
DNA:	Deoxyribonucleic acid
EDTA:	Ethylenediaminetetraacetic acid
ELISA:	Enzyme-linked Immunosorbent Assay
FB_1:	Fumonisin B_1
HPLC:	High Performance Liquid Chromatography
IAC:	Immuno affinity column
LAB:	Lactic acid bacteria
LC-MS/MS:	Liquid Chromatography Tandem Mass Spectrometry
MEA:	Malt extract agar
MRS:	De Man, Rogosa and Sharpe agar
NTD:	Neural tube defects
OTA:	Ochratoxin A
PCR:	Polymerase chain reaction
PDA:	Potato dextrose agar
PDAT:	Potato dextrose agar-Tartaric acid
QuEChERS:	Quick, Easy, Cheap, Effective, Rugged and Safe
SEM:	Scanning electron microscopy
TEM:	Transmission electron microscopy

PUBLICATIONS AND CONFERENCE PRESENTATIONS

PUBLICATIONS

- **Olagunju, O**., Mchunu, N., Venter, S., Guibert, B., Durand, N., Métayer, I., Montet, D., & Ijabadeniyi, O. (2018). Fungal contamination of food commodities in Durban, South Africa. ***Journal of Food Safety*, e12515** https://doi.org/10.1111/jfs.12515**.**

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CHAPTER ONE

1.1 Introduction

The utilization of agricultural food commodities faces many drawbacks which affect global food security. One such drawback is contamination by toxigenic molds, a group of fungi whose reproduction occurs with the release of tiny spores into the air (Bhat *et al.*, 2010; Ashiq, 2015). Mold contamination of agricultural products occurs while in the field, during processing, transportation or storage (Bhat *et al.*, 2010; da Rocha *et al.*, 2014). The lack of Good Agricultural Practices (GAP) and Good Manufacturing Practices (GMP) further facilitates the proliferation of fungal contaminants in food commodities.

Bambara groundnut (*Vigna subterranea* (L.) Verdc), a legume whose origin is Africa, is a rich source of protein and minerals (Murevanhema and Jideani, 2013). The high nutritional composition promotes its utilization in many food applications. Traditional processing of the seed includes dehulling, roasting or fermentation, and milling to obtain flour. The use of milk and flour obtained from Bambara groundnut is widely studied (Barimalaa *et al.*, 2005; Alozie *et al.*, 2009; Falade *et al.*, 2015; James *et al.*, 2017). Other studies also addressed the potential of Bambara groundnut as composite in home-made weaning foods for infants and young children (Ijarotimi and Olopade, 2009; Ijarotimi *et al.*, 2009). Bambara groundnut grows near or below the surface of the soil which serves as fungal inoculum (Guezlane-Tebibel *et al.*, 2013) .

Fungi are unicellular eukaryotes with multiple nuclei bound within a chitinous cell wall. Being heterotrophic in nature, they obtain nutrients from metabolism of solid substrates by the action of the enzymes they produce (Bhat *et al.*, 2010). They produce low molecular weight compounds generally referred to as 'secondary metabolites', some of which are referred to as 'mycotoxins' (Bhat *et al.*, 2010). Fungi belonging to *Aspergillus*, *Penicillium* and *Fusarium* genera produce mycotoxins which have significant effects on health of humans and animals (Ashiq, 2015). Some *Aspergillus* species produce aflatoxin B_1 (AFB_1), the most potent of all aflatoxins with carcinogenic, mutagenic, teratogenic and immunosuppressive activity on animals and humans (Golge *et al.*, 2013). Fumonisin B_1 (FB_1) produced by *Fusarium* species, and citrinin (CIT) produced by *Penicillium citrinum* have also been linked to other human diseases (Yogendrarajah *et al.*, 2014; Bordin *et al.*,

2015). However, presence of molds in foods does not usually correlate with mycotoxin production (Ashiq, 2015).

A majority of mycotoxins are chemical and heat stable and are not completely destroyed at normal temperature conditions adopted during food processing. With conventional cooking methods of frying, boiling or pasteurization, mycotoxin reduction is relatively low and as such, they are carried over into processed foods (Ashiq, 2015; Anfossi *et al.*, 2016). Various regulatory bodies have recommended maximum tolerable limits for most significant mycotoxins in foods destined for human consumption. The European Committee Regulations (ECR) has set limits of 4 µg/kg for total aflatoxins (AFB_1, AFG_1, AFB_2, and AFG_2) and 2 µg/kg for AFB_1 in cereals, peanuts, and dried fruits (European Commission, 2006). This is applicable for direct human consumption or where the food is used as an ingredient in food production. European Union recommends maximum tolerable limit of 0.1 µg/kg of aflatoxin for baby foods for infants and young children (European Commission, 2006).

Mold contamination and subsequent mycotoxin occurrence may become inevitable under certain environmental conditions (Abrunhosa *et al.*, 2016). Countries in Asia and Africa experience high temperature (26–39 °C) and high relative humidity (67–98%), conditions which support the growth of mycotoxigenic fungi on agricultural crops and consequently the production of mycotoxins on these crops (Sales and Yoshizawa, 2005). However, climate change due to global warming portends the prevalence of aflatoxigenic fungi and aflatoxin production in temperate regions where fungal contamination and toxin production rarely occur (Paterson and Lima, 2010; Baranyi *et al.*, 2014). Generally, fungal growth and mycotoxin accumulation in agricultural crops are affected by several factors which include fungal species potential, stress factors such as temperature and relative humidity, type of substrate, water activity, aeration and duration of fungal growth (Afsah-Hejri *et al.*, 2013; Ashiq, 2015).

Reports have documented the incidences of fungal and mycotoxin contamination in food commodities in different parts of the world (CDC, 2004; Atehnkeng *et al.*, 2008; BBC, 2010; Chilaka *et al.*, 2012; Mutiga *et al.*, 2014; Bertuzzi *et al.*, 2015; Chauhan *et al.*, 2016; Ezekiel *et al.*, 2016). Recent studies have reported contamination of complementary foods for infants and young children with aflatoxins. Alamu *et al.* (2018) evaluated the aflatoxin content of

two complementary foods, nshima and maize porridge, consumed by children (6 to 24 months) in Monze and Chipata districts of Zambia. High values of aflatoxin were detected in maize porridge (5.8 mg/100 g) and in nshima (3.8 mg/100 g). The concentration of aflatoxin in both complementary foods reported in this study, approximately 58, 000 and 38, 000 µg/kg are extremely high, and well over the 0.1 µg/kg level regulated for baby foods. The findings from this study further highlight the need for effective measures, which can be applied at household/rural levels to reduce aflatoxin contamination in various agricultural commodities used in the preparation of infant complementary foods. A similar study done in Ejura-Sekyedumase district in Ashanti Region of Ghana, revealed that 36 samples of 'weanimix', an infant complementary food prepared from groundnuts, beans and maize, tested positive for aflatoxin contamination. About 83.34% of the weanimix samples exceeded 20 ppb as recommended by the United States Food and Drug Administration (U.S. FDA) (Kumi *et al.*, 2014).

Tchana *et al.* (2010) reported the presence of aflatoxin B_1 in 35.5 and 45.5% of urine samples of children suffering from kwashiorkor and marasmic kwashiorkor in Cameroon. Aflatoxin B_1 was also detected in 3.9% of sera of liver cancer patients in the same study. Aflatoxin exposure early in life has been associated with impaired growth, particularly stunting, and predisposes young children to primary liver cancer (Tchana *et al.*, 2010; Kumi *et al.*, 2014). High consumption of cereal-legume complementary foods exposes young children to aflatoxins (Alamu *et al.*, 2018).

A major advantage of food processing is decontamination of toxic components of the food. Appropriate processing, as a preventive measure, has been used to control mold growth (Fasoyiro *et al.*, 2017). Various studies have reported the application of different food processing methods to reduce mold and mycotoxin contamination in foods. Matumba *et al.* (2009) reported significant reductions in AFB_1 concentrations during maize processing to obtain flour. Dehulling maize, soaking maize grits for 72 h and washing, soaking maize grit flour for 72 h and sun drying achieved about 29.3, 80.9, and 11.4% reductions respectively in AFB_1 concentration. Similarly, dehulling of maize recorded a decrease in AFB_1 concentration from 3.39–0.38 µg/kg, equivalent to about 88.8% reduction. Significant reductions were also recorded for aflatoxins B_2, G_1 and G_2 (Siwela *et al.*, 2005). Matumba *et al.* (2015a) also reported varying degrees of mycotoxin reduction by hand sorting, dehulling, floatation and a combination of the processes in shelled white maize. Roasting at

150 °C for 30 min reduced AFB_1 concentration in naturally contaminated whole pistachio kernels to about 63% of the initial level (Yazdanpanah *et al.*, 2005). During the production of doklu from maize, aflatoxins were not detected at the fermentation and cooking stages (Assohoun *et al.*, 2013).

Very little has been reported on fungal contamination in Bambara groundnut and its safety for consumption. There are few reports on isolation and characterization of the natural mycobiota of Bambara groundnut and possible occurrence of mycotoxins in Bambara groundnut flour. The application of processing methods to eliminate fungal infection and reduce mycotoxin contamination in the flour has not been reported. The survival, growth, and toxin production of the natural pathogen(s) of the seed as affected by processing and during storage has also not been investigated. The pattern of colonization and effect of fungal infestation on the seed has not been documented.

This study aims to isolate and characterize the filamentous fungi of Bambara groundnut using molecular methods of DNA extraction, polymerase chain reaction (PCR) and sequencing. It will investigate the possible co-occurrence of common grain-contaminating mycotoxins in Bambara groundnut flour using the QuEChERS method. It will further study the effect of milling, roasting and lactic acid bacteria fermentation on the growth and survival of the identified pathogenic fungi, and mycotoxin production using enzyme linked immunosorbent assay/chromatographic and spectrophotometric methods. The study will also investigate the behaviour of the identified pathogen(s) as affected by the processing methods during storage. It will elucidate the effect of compositing maize and Bambara groundnut flour on fungal growth and toxin production. Further, it will investigate the colonization of the identified pathogen in Bambara groundnut using scanning and transmission electron microscopy. Information generated from this study will contribute to mycotoxin research and legislation in Bambara groundnut and support several projects by non-governmental organizations to curb mycotoxins in cereals and legumes, so as to improve their utilization among households.

CHAPTER TWO

2.1 Literature review

2.2 Bambara groundnut origin and general description

Bambara groundnut (*Vigna subterranea* (L.) Verdc.) is a native African crop but whose cultivation also extends beyond Africa (Bamishaiye *et al.*, 2011). It can be traced to the Sahelian region of West Africa, and the name seems to have been derived from a tribe, Bambara who now live mainly in Mali (Nwanna *et al.*, 2005; Murevanhema and Jideani, 2013). The Bambara plant belongs to the *Leguminosae* family and yields valuable edible seeds, as with groundnut (*Arachis hypogea*) and cowpea (*Vigna unguiculata*) (Murevanhema and Jideani, 2013). Despite its importance in the daily diet of many Africans, growth of Bambara groundnut has been at a subsistence level and it is regarded as a 'famine culture' crop in semi-arid Africa especially by farmers (Bamishaiye *et al.*, 2011). It is reportedly grown primarily by the rural farming women; with about 10-40% of the yield sold and the rest for domestic consumption (Bamishaiye *et al.*, 2011). World-wide, annual production is about 330, 000 tons with Africa accounting for half of the production and Nigeria as a leading producer (Bamishaiye *et al.*, 2011). Cultivation of Bambara groundnut is practised for its edible seed which provides a major supply of dietary protein and calories. It is one of the five important protein sources for many Africans (Nwanna *et al.*, 2005; Murevanhema and Jideani, 2013).

Various factors militate against the efficient utilization of Bambara groundnut. It has poor dehulling and milling characteristics, it is hard to cook, has a strong beany flavour and contains antinutrients (Alozie *et al.*, 2009). Due to limited research, crop improvement and large scale cultivation remain neglected. In spite of these factors, a majority of farmers in Swaziland for example, consider Bambara groundnut as a profitable crop (Bamishaiye *et al.*, 2011). This can be attributed to the many benefits it holds over other legumes such as its resistance to droughts and insects, prolonged storage life and ability to yield reasonably well in poor soils (Murevanhema and Jideani, 2013). Under conditions that are too arid for other crops such as groundnut, maize and sorghum, Bambara groundnut is produced in higher yields. Fertilizers and chemicals are not needed in growing Bambara groundnut (Forsythe *et al.*, 2015). The nut is able to resist weevil attack and can be stored for long periods without loss due to the tough seed coat (Nwanna *et al.*, 2005). These characteristics have renewed interest in farming activities on Bambara groundnut in the arid savannah zone

(Murevanhema and Jideani, 2013). It also plays a part in international trade as it is exported to African markets in Western Europe and North America from Ghana, Burkina Faso, Cameroon, Zimbabwe and Madagascar (Forsythe *et al.*, 2015). This nut is known by different names in use in Africa, and other parts of the world where it is cultivated or consumed (Table 2.1).

Table 2.1 Common names of Bambara groundnut

Country	Language	Common name
Brazil	Portuguese	Mandubi d'Angola
Central Africa	Sangho	Njogo bean
Ghana	Ewe	Aboboi akyii
Kenya	Kiswahili	Njugu mawe
Madagascar	Malagasy	Pistache, &Voanjobory
Malawi	Nyanja	Nzama, & Njama
Nigeria	Hausa	Guijiya, & Kwaruru
	Ibo	Okpa
	Yoruba	Epa roro
Sierra Leone	Creole	Agbaroro
South Africa	Africaans	Jugoboon
	Tsonga	Kokane, Nyume, & Ndlowu
	Venda	Nduhu nwa tzidzimba
	Xhosa	Jugo
	Zulu	Indlubu
Sudan	Arabic	Fulabungawi
Zambia	Bemba	Juga bean, & Ntoyo
Zimbabwe	French	Voandzou, & pois d'Angole
	Ndebele	Indlubu
	Shona	Nyimo beans
Zwaziland	SiSwati	Tindlubu

Adapted from Bamishaiye *et al.*(2011); Murevanhema and Jideani (2013).

2.3 Bambara plant

The Bambara plant is an annual self-pollinated plant. The stem and tap root are well-developed, with numerous nitrogen- fixing nodules. The trifoliate leaves with long petioles and a green or purple base, are borne on the stem. Flowers are borne in a raceme on nodes

present on the stem (Bamishaiye *et al.*, 2011; Murevanhema and Jideani, 2013). The pods are large, round, and wrinkled; having single or sometimes multiple seeds. Similar to groundnut, the flower stalk bends downwards after fertilization, pushing the young developing pod into the soil or just above the ground to develop and mature (Figure 2.1) (Murevanhema and Jideani, 2013). Pods mature about four months after sowing and the plants are manually pulled out of the ground during harvest (Bamishaiye *et al.*, 2011).

Figure 2.1 (A) Bambara groundnut plant (B) Harvested Bambara groundnut seeds. Fleißner, 2006.

2.4 Bambara seed

According to Bamishaiye *et al.* (2011), Bambara groundnut seeds are smooth and very hard when dry with variations in the seed coat, size, and shape. Seed colour may range from white, cream, dark-brown, red, to black, while some seeds may be speckled or patterned with these colour combinations. Variations in seed shape has also been reported with some round or elliptical-shaped seeds. However, despite these variations, the nutritional composition do not differ greatly among varieties (Bamishaiye *et al.*, 2011). Figure 2.2 below presents some of Bambara groundnut varieties obtained in Southern Africa.

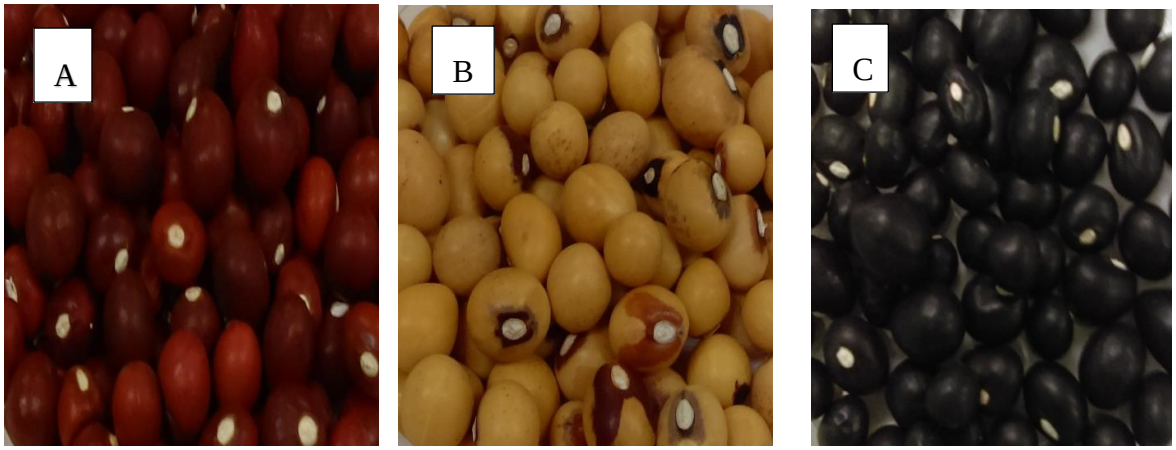

Figure 2.2 Varieties of Bambara groundnut. (A) Red (B) Cream (C) Black varieties.

Source: Olagunju O.F.

2.5 Nutritional composition of Bambara groundnut

The Bambara groundnut is a nutritious grain legume, providing essential nutrients necessary for growth and development. It is normally used to substitute animal protein in cases where household cannot afford such protein sources (Forsythe *et al.*, 2015). A comparison of the chemical composition of Bambara groundnut to that of peanut and cowpeas presented in Table 2.2 shows that its protein content is slightly lower than that of peanut but higher than cowpea. Bambara groundnut has low fat content, which promotes long storage with no evidence of spoilage. It also has high carbohydrate content, thus an excellent source of energy. The nut contains high amount of methionine, which promotes normal cell function and the production of another amino acid, cysteine. The seed is also a good source of calcium, iron and potassium (Murevanhema and Jideani, 2013). However, Bambara groundnut contains tannin found mainly in the seed coat, and low levels of trypsin inhibitor and phenolic compounds in the seed (Bamishaiye *et al.*, 2011). Although anti-nutritional factors in legumes reduce mineral bioavailability, Nwanna *et al.* (2005) reported that soaking and natural fermentation were effective in reducing tannin and phytate contents of Bambara groundnut.

Table 2.2 Comparison of the chemical composition of groundnut, cowpea, and Bambara groundnut

Legume	Common name	Moisture	Protein	Fat	Ash	Fibre	CHO
Arachis hypogaea[a]	Groundnut	10.99	25.12	46.06	2.23	7.56	7.77
Vigna unguiculata[b]	Cowpea	8.45	19.47	6.66	4.25	2.43	58.48
Vigna subterranea[c]	Bambara groundnut	11.45	24.67	6.0	3.70	3.55	59.80

Values are reported in % wet basis. CHO: Carbohydrate (determined by difference)

Adapted from [a]Olayinka *et al.*, 2015; [b]Oyeyinka *et al.*, 2018; [c]Kaptso *et al.*, 2015.

2.6 Processing and utilization of Bambara groundnut

Bambara groundnut is utilized in various forms. In Nigeria, 'okpa', a steamed gel is made from the flour (Barimalaa *et al.*, 2005). It is prepared by mixing Bambara groundnut paste with condiments, wrapped in banana leaves and boiled (Murevanhema and Jideani, 2013). 'Okpa' can keep for several days at room temperature without spoilage and this keeping quality may be attributed to the antimicrobial properties of polyphenols in banana leaves (Padam *et al.*, 2014; Singh *et al.*, 2018). The seed is fermented to produce a dawadawa-type condiment (Fadahunsi and Olubunmi, 2010; Ademiluyi and Oboh, 2011). Some tribes in Congo roast and pound the seed to extract oil (Murevanhema and Jideani, 2013). While immature, the seeds may be eaten fresh or grilled. Fresh pods may be made into a snack by boiling with salt and pepper. In Botswana, immature seeds are also boiled in pod, salted and consumed with or without maize seeds (Murevanhema and Jideani, 2013). Ripe, dried seeds are pounded into flour and used to make cakes and porridges (Bamishaiye *et al.*, 2011). The seeds harden when mature and require to be boiled before further preparation (Forsythe *et al.*, 2015). Roasted Bambara groundnuts are milled and made into a soup with or without condiments. Bambara groundnut flour is produced in Cote d'Ivoire while bread and cakes are made from the flour in Zambia and Botswana, respectively (Murevanhema and Jideani, 2013).

In some areas in Malawi, Bambara groundnut serves as an accompaniment to a traditional dish nsima, which is made from maize flour and water (Forsythe *et al.*, 2015). In South Africa, sekome also known as tihove or tshidzimba, is prepared by adding Bambara groundnut, peanut, or both to a meal of maize or millet, boiled to form a stiff dough, which is then salted and pounded into a ball. It keeps fresh for several days. Local white populations

in South Africa also use the dried beans for soup making (Bamishaiye *et al.*, 2011). In Ghana, fresh boiled and salted immature beans are eaten, fried cakes and balls are made from boiled and crushed dried seeds, which are then used in stew preparations (Bamishaiye *et al.*, 2011). Overnight-soaked beans, which are further boiled with or without capsicum pepper and salt are used to make porridge, and served with roasted and grated cassava or mashed, fried or ripe plantain (Bamishaiye *et al.*, 2011). In Kenya, green and dry beans are dehulled and cooked until very soft. When mashed, it is mixed with coconut juice, cooked and smoothened. It is served with maize meal porridge or rice. A popular children's meal is made from mashed boiled seeds or sometimes mixed with boiled sweet potatoes. Also, canned Bambara groundnut in tomato sauce and brine was reported to have been produced in the early 1960s in Ghana (Bamishaiye *et al.*, 2011).

Bambara groundnut can also be used in animal feed formulations. Fermentation with *Aspergillus niger, A flavus, Trichoderma* sp., *Yeast* sp., and *Penicillium* sp. was studied to improve its protein content and bioavailability as an ingredient in animal feedstuff (Nwanna *et al.*, 2005). The seeds are used to make chicken feed while the leaves, rich in nitrogen and phosphorus, are suitable for herbivores. In other parts of Africa, Bambara groundnuts are used for medicinal purposes. For example in Botswana, the seeds are used in traditional medicine (Bamishaiye *et al.*, 2011). In Kenya, diarrhoea is treated with water from boiled Bambara groundnut and maize. Leaves of Bambara groundnut and nyamrithi (*Lantana trifolia* L.) when pounded and mixed with water, is used as a pesticide on vegetable plantations and to spray on livestock for the prevention of ticks. Dried leaves pounded with traditional salt are used in the treatment of mouth disease in cattle (Bamishaiye *et al.*, 2011).

Bambara groundnut has been studied for use in various food applications. Milk obtained from Bambara groundnut and soybeans was fermented with *L. delbruieckii* subsp *bulgaricus* and *Streptococcus salivarus* subsp. *thermophilus* to produce plain yoghurt (Falade *et al.*, 2015). It is suggested as a supplement to cereal-based flour in the production of biscuits, bread, and weaning food for infants due to its ability to improve protein content and functional properties of the flour. Infant complementary food has been produced from sprouted and fermented millet and Bambara groundnut flour with improved functional properties (James *et al.*, 2017). Bread made from composite flour of Bambara groundnut and wheat flour showed higher crude protein content, crude fibre and ash contents (Alozie *et al.*, 2009).

2.7 Pests and diseases

Apart from weevil attack during storage, Bambara groundnut is relatively free of pests and diseases that plague other legumes. Hence, pesticides are hardly used during its cultivation. This resistance may also contribute to the ability of the plant to yield relatively well where other leguminous crops fail. However, damage may sometimes be caused by leaf hoppers such as *Hilda patruelis* and *Empoasca facialis*. The plant may be affected by *Fusarium* wilt caused by *Fusarium oxysporum,* powdery mildew and cercospora leaf spot. Root knot nematode (*Meloidogyne javanica*) attacks the roots in sandy soil while the pods are frequently attacked by termites during dry weather conditions (Bamishaiye *et al.*, 2011). The susceptibility of the plant to the action of these organisms portends the vulnerability of the seeds to fungal infection as damage by insects can contribute to fungal infection of agricultural crops.

2.8 Fungal and mycotoxin contamination

Bambara groundnut grows near or below the soil surface and can be contaminated by toxigenic fungi present in the soil (Guezlane-Tebibel *et al.*, 2013), which may lead to accumulation of mycotoxins in the seeds. However, very little information is available presently on fungal and mycotoxin contamination in Bambara groundnut. Kola (2003) studied the mycoflora of six bambara groundnut cultivars from South Africa. *Aspergillus* spp., *Chaetomium globosum* and *Fusarium* spp. were the major genera identified, while *Aspergillus flavus*, *Aspergillus niger*, *Fusarium oxysporum* and *F. solani* were isolated from the seeds. Fungal and mycotoxin presence in twenty-nine bambara groundnut samples collected from Limpopo, South Africa were investigated. *Aspergillus flavus*, *A. niger*, *Fusarium graminearum*, *F. verticilliodes*, *F. proliferatum*, *Penicillium roqueforti*, *P. citrinum* and other *Penicillium* spp. were isolated from the samples. Ten of the bambara groundnut samples were contaminated with aflatoxins, with concentrations ranging from 0.01 to 0.1 ppm (Shabangu, 2009).

2.9 Maize-Bambara groundnut composite

Maize (*Zea mays* L.) ranks third in cereal production worldwide after wheat and rice (Chilaka *et al.*, 2012). More than 4.5 billion people in 94 developing countries obtain more than 30% of their food calories from rice, wheat, and maize. Among these are about 900 million poor consumers whose preferred staple is maize (Shiferaw *et al.*, 2011). In some parts of West Africa, it may be consumed as frequently as three times daily. In Kenya, it has

an annual consumption per capita of 98 kg. In South Africa, it is cultivated widely and utilized by human and animals (Atehnkeng *et al.*, 2008; Chilaka *et al.*, 2012; Kilonzo *et al.*, 2014).

Maize is low in usable protein and its leucine blocks the absorption of niacin by the human body, resulting in protein deficiency (Shiferaw *et al.*, 2011). Cereals are deficient in total nitrogen and essential amino acids lysine and tryptophan and are used in combination with legumes, which are a rich source of tryptophan and lysine. The combination of cereals and legumes therefore provides a desirable pattern of essential amino acids (Alozie *et al.*, 2009).

Fungal contamination has also been reported in maize. Between 2008 and 2009, *F. graminearum, F. verticilliodes* and *F. subglutinans* were detected in maize samples in South Africa. Mycotoxin analysis detected moniliformin, type B trichothecenes [deoxynivalenol, nivalenol, 3-acetyl-deoxynivalenol (3-ADON) and 15-acetyl-deoxynivalenol (15-ADON)], zearalenone, fumonisins (FB_1 and FB_2) and beauvericin in maize samples collected throughout South Africa in the same period. Fumonisins have been reported as the most prevalent contaminants of South African maize for many years. From the maize samples collected in both years, 62 and 57% had detectable amounts of FB_1 and FB_2, respectively, with an average contamination of 1793 ng/g with FB_1 and 749 ng/g with FB_2 (Boutigny *et al.*, 2012).

Forty commercial maize samples were randomly collected from maize consignments of two commercial feed companies in KwaZulu Natal province of South Africa in 2010. Four fungal genera were isolated from the samples including *Fusarium, Aspergillus, Penicillium* and yeast. *Fusarium* spp., *F. verticillioides* and *F. proliferatum*, were most dominant with incidence rate of 88 and 73%, respectively. Fumonisin, with a range of 64 to 1035 ppb, was present in all the samples, with FB_1 (8–892 ppb), FB_2 (31–148 ppb) and FB_3 (0–242 ppb) (Chilaka *et al.*, 2012).

The use of Bambara groundnut to supplement maize flour in various food applications has been reported. Uvere *et al.* (2010) explored the use of other processed foods rich in calcium, iron, zinc and provitamin A to fortify maize-bambara groundnut complementary food. Bintu *et al.* (2015) studied the inclusion of Bambara groundnut to produce a cereal-legume complementary food with maize, roasted cowpea, and roasted groundnut. Mbata *et al.*

(2009) reported an increase in the protein content of Bambara groundnut fortified maize flour from 18.40 to 21.68% with an inclusion of 30% Bambara groundnut flour. Higher fat and ash contents were also noted in the maize-bambara groundnut composite flour.

Ijarotimi and Keshinro (2013) also formulated complementary foods from blends of fermented popcorn supplemented with Bambara groundnut (FPB) or African locust beans (*Parkia biglobosa*) (FPA). The inclusion of Bambara groundnut in FPB blend yielded higher values of protein content (20.87 g/100 g) compared to FPA (20.49 g/100 g) sample. Higher values of 1.15, 1.29, 5.28 and 4.41 mg/100 g were also reported for cysteine, methionine, lysine and isoleucine, respectively in FPB complementary food samples, while the FPA samples had values of 0.68, 0.84, 2.38, and 1.83 mg/100 g, respectively for the same amino acids. While various reports support the application of Bambara groundnut in cereal-legume blends, very little information is available on the fungal and mycotoxin contamination of maize-bambara groundnut composite flour. It is therefore necessary to investigate the mycological safety of maize-bambara groundnut composite flour.

2.10 Mold contamination of agricultural products

A major problem associated with the utilization of agricultural crops is contamination by toxigenic molds (Bhat *et al.*, 2010; Ashiq, 2015). Molds are ubiquitous organisms that grow almost everywhere (Matumba *et al.*, 2016). Given suitable eco-physiological conditions, they can grow and produce secondary metabolites including mycotoxins, on agricultural crops before harvesting, during storage and transport (Ashiq, 2015; Ji *et al.*, 2016). Mold damage during grain storage ranks second after damage caused by insect pests (Bhat *et al.*, 2010).

Fungal toxins, one of the most dangerous contaminants of food and feed have been detected in many food commodities worldwide (Bhat *et al.*, 2010). Mycotoxins affect cereals such as maize, wheat, oats, barley, rice; as well as seeds, nuts, beans, cassava, fodder and feed intended for animal consumption and equally compromise the quality of milk and other dairy products (Burger *et al.*, 2013; Ji *et al.*, 2016). However, not all molds are toxigenic and not all secondary metabolites from molds are toxic (Ji *et al.*, 2016). Similarly, not all toxic substances synthesized by fungi are mycotoxins, although all mycotoxins are metabolites of fungi (Ashiq, 2015). Molds belonging to *Aspergillus*, *Penicillium* and *Fusarium* also known as field fungi, are the producers of a majority of mycotoxins of health significance to humans

and animals (Ashiq, 2015). Mold contamination in foods does not always correlate with mycotoxin production (Ashiq, 2015).

Mold contamination and subsequent mycotoxin occurrence may become inevitable under certain environmental conditions (Abrunhosa *et al.*, 2016). For example, countries in Asia and Africa experience high temperature (26–39°C) and high relative humidity (67–98%) conditions, which support the growth of mycotoxigenic fungi on agricultural crops and consequently the production of mycotoxins on these crops (Sales and Yoshizawa, 2005). Fungal growth and the risk of mycotoxin contamination in agricultural products is further enhanced by poor harvesting practices, improper drying, handling, packaging, storage, and transportation (Bhat *et al.*, 2010). Direct contamination may be when the food or feed become infected by a toxigenic fungus and formation of mycotoxin occurs, while indirect contamination is when a previously contaminated food or product has been processed to eliminate the fungus but the mycotoxin remains in the final product (da Rocha *et al.*, 2014).

Growth and survival of pathogenic spoilage microorganisms on foods and food ingredients are influenced by storage temperature and relative humidity, amongst other factors. However, the requirements for optimal growth and toxin synthesis differ among fungi, even those within the same genus (Ashiq, 2015). Fungi grow between 10 and 40 °C, over a pH range of 4 to 8, and water activity level (a_w) above 0.70. Minimal water activity requirements vary for *A. flavus* (0.78–0.80), *A. fumigatus* (0.85–0.94), *A. parasiticus* (0.78–0.82,) and *Fusarium* spp (0.85–0.87) (Bhat *et al.*, 2010). Generally, field fungi require moisture content of 22–25% in a grain to grow and subsequently produce mycotoxins, while storage mycotoxin producers usually grow in grains with moisture content of 13–18% (equal to 70–90% relative humidity) (Afsah-Hejri *et al.*, 2013).

2.11 Mycotoxins in food and feed

Mycotoxin is derived from a Greek word '*mukes'* meaning fungi and a Latin word *toxicum* referring to poison (Bhat *et al.*, 2010). They are large molecules, heat stable and not significantly volatile, possibly carried over into processed foods due to their stability (Bhat *et al.*, 2010; Anfossi *et al.*, 2016). Their structures vary from simple heterocyclic rings to groups with molecular weights ranging from 50 to 500 Da (da Rocha *et al.*, 2014). Mycotoxins can be classified into four groups based on their biological origin and structure and they include polycetoacids, terpenes, cyclopeptides and nitrogenous metabolites (Afsah-

Hejri *et al.*, 2013). Figure 2.3 below shows the chemical structures of mycotoxins that are common in foods.

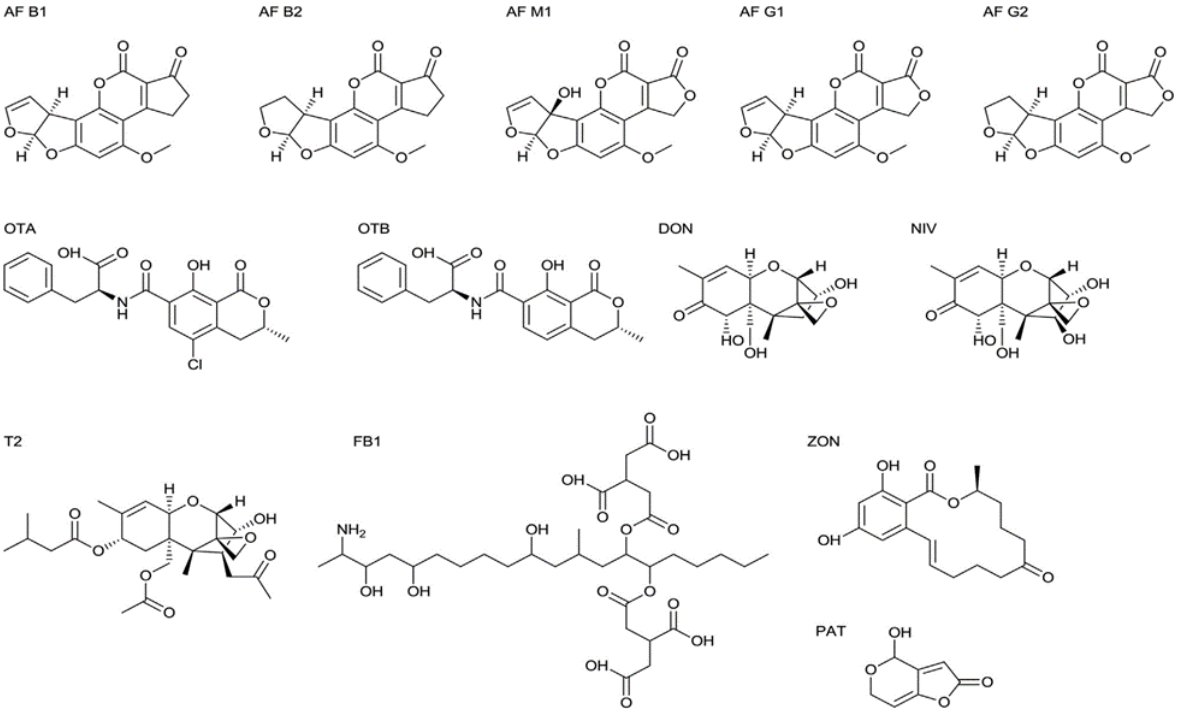

Figure 2.3 Commonly occurring mycotoxins in food. AF (Aflatoxin) B_1, B_2, M_1, G_1, G_2; OT (Ochratoxin) A, B; DON Deoxynivalenol; NIV Nivalenol; T2 T-2 toxin (Insariotoxin); FB_1 Fumonisin B_1; ZON Zearalenone; PAT Patulin.

Adapted from Turner *et al.*, 2015.

Contamination by mycotoxin-producing fungi results in wastage of massive amounts of food annually (Ashiq, 2015). The Food and Agriculture Organization of the United Nations has estimated that about 25% of the world's food crops are contaminated by molds and become affected by mycotoxins, translating to loss of billions of dollars annually (Bhat *et al.*, 2010). Mycotoxin contamination results in loss to crop producers and sellers who will be required to sell contaminated products at a cheaper rate or dispose such products in cases of severe contamination. It can also lead to loss of business, and product recall thereby adversely affecting international trade (Afsah-Hejri *et al.*, 2013). According to Marroquín-Cardona *et al.* (2014), about 4.5 billion persons in the developing countries are exposed to unchecked amounts of mycotoxins yearly. The ingestion in high quantities or over a long period of time of mycotoxins from mold-contaminated foods may become harmful (Marroquín-Cardona *et al.*, 2014).

Mycotoxins can be inhaled, ingested or absorbed through the skin (Afsah-Hejri *et al.*, 2013). Mycotoxicosis is a disease condition resulting from ingestion of food contaminated with mycotoxins and it has been linked to many human diseases. It may have chronic effects leading to cancer, immune suppression, and other 'slow' pathological conditions. Acute mycotoxicosis may result in death (Bennett and Klich, 2003). Aflatoxins produce clinical toxicosis, reduce resistance to diseases and interfere with vaccine induced immunity in poultry birds. Feeding livestock and poultry with aflatoxin contaminated feed can result in death, immune suppression and growth retardation (Fakruddin *et al.*, 2015). The type and dose of mycotoxin, extent of exposure, gender, age and health condition of the individual all determine the severity of mycotoxicosis (Afsah-Hejri *et al.*, 2013).

Mycotoxin production is influenced by fungal species potential, relative humidity, type of substrate, water activity, use of fungicides, duration of fungal growth, aeration, temperature, and storage conditions (Afsah-Hejri *et al.*, 2013; Ashiq, 2015). Toxin production is further enhanced by stress factors, which include shortage of water, insect infestation and attacks by other pests. (Ashiq, 2015).

2.12 Mycotoxins of significance to human and animal health

Several previous studies have been reported on the existence of mycotoxins in food systems. Researchers agree that there are about 400 confirmed existing mycotoxins (Bhat *et al.*, 2010; Abrunhosa *et al.*, 2016). Research underway also confirm that there are other various emerging toxins not yet studied extensively (Matumba *et al.*, 2016). Few of the mycotoxins discovered are a public health concern due to their high prevalence, teratogenic, carcinogenic, mutagenic, and immune suppression effects (Dalié *et al.*, 2010; Abrunhosa *et al.*, 2016; Ji *et al.*, 2016). Mycotoxins of concern are aflatoxins (AFs), ochratoxin A (OTA), trichothecenes (deoxynivalenol (DON) and T-2 toxin), zearalenone (ZEN), fumonisins (FBs) and patulin (PAT) (Dalié *et al.*, 2010; Afsah-Hejri *et al.*, 2013; Ashiq, 2015). This is due to their studied economic impact, frequency of occurrence in food systems and severity on humans and animals. These mycotoxins as well as ergot alkaloids produced mainly by fungi belonging to *Aspergillus, Penicillium, Fusarium* and *Claviceps* genera pose a threat to food safety (Abrunhosa *et al.*, 2016).

Mycotoxins are heat stable and chemically stable. Their stability contributes to their presence in processed foods. Since the regular processing methods do not eliminate mycotoxins and

they are often detected in processed food products, various regulatory bodies have set maximum tolerable limits for their presence in foods and feed. Table 2.3 below presents an overview of the worldwide legislation on mycotoxins.

Table 2.3 Overview of the worldwide legislation on mycotoxins

Mycotoxin	Commodity	Country	Maximum tolerable levels* (µg/kg**)**
AFs	Oil seeds, nuts, dried fruits, cereals, spices	EU	4–15* (2–12* for AFB1)
		Australia, Canada, Nigeria, New Zeland, South Africa,	(15 for AFB1)
		USA, Brazil	20
		India	30
AFM_1	Milk and infant formula	EU, Turkey, South Africa, Argentina, China, India, Kenya, Mexico, Uruguay, USA	0.25–0.0*5 0.5
		Brazil	0.5–5*
DON	Cereals, bakery products	EU	500–1750*
		Brazil	750–3000*
		Russia	700–1000
		Canada, China, India, Japan, USA[a]	1000
FMs	Maize	EU, Turkey, Norway Switzerland	800–4000*
		USA	2000–4000*
		Brazil	2000–5000*
OTA	Cereals, dried fruits, coffee, cocoa, wine, beer, grape juice, spices, liquor, rice, blood products	EU, Egypt	2–10*
		China, Kenya, Nigeria, Russia	5
		India	20
		Brazil	2–30*
		Uruguay	50
Patulin	Fruit juice, apple products	Brazil, China, EU, India, Japan, Kenya, Nigeria, Russia, South Africa, USA	50
T-2 and HT-2	Cereals	EU	Not permitted
		Russia	50-100*
ZON	Cereals, bakery products, maize oil	EU	75–400*
		Brazil	200–1000*
		China, Russia, Chile	200,000

*Depends on the commodity (lowest-highest MTL)

[a] Advisory level

Adapted from Anfossi *et al.*, 2016.

2.12.1 Aflatoxin

Toxigenic strains of *Aspergillus* species produce cancerous secondary metabolites in agricultural foodstuffs known as aflatoxins. Aflatoxins are difuranocoumarin molecules synthesized through the polyketide pathway (Wacoo *et al.*, 2014). They are very stable and can withstand conventional food processing (Ashiq, 2015). Aflatoxins have been isolated from various commodities including barley, wheat, sorghum pear millet, oilseeds, tree nuts, milk, and butter. Aflatoxins have been linked with carcinogenicity, mutagenicity, teratogenicity, and immune suppression in animals and humans (Abbas *et al.*, 2004). Previous research show that they are the most hazardous of all mycotoxins, causing damage to the liver (Ji *et al.*, 2016). Aflatoxins also influence aggravation of kwashiorkor and impaired childhood growth (Golge *et al.*, 2013). They can depress cell-mediated immunity and cause stunting in children (Ashiq, 2015). Evidence from studies indicates that aflatoxins increase the rate of progression from HIV infection to AIDS (Matumba *et al.*, 2016). Aflatoxins can bind with the DNA of cells and affect protein synthesis, and also contribute to thymic aplasia resulting in reduced cell immunity (da Rocha *et al.*, 2014). Consumption of food heavily contaminated with aflatoxin (> 20 ppb) has been linked to death (CDC, 2004).

Eighteen different aflatoxins have been identified, of which six are of concern in agricultural foodstuff and they include AFB_1, AFB_2, AFG_1, AFG_2, AM_1 and AM_2. Aflatoxin B_1 (AFB_1), AFG_1, AFB_2, and AFG_2, in order of decreasing toxicity, occur naturally (Matumba *et al.*, 2015b). The International Agency for Research on Cancer (IARC) has classified aflatoxins principally AFB_1, as Group 1 carcinogens (IARC, 1993). They exhibit molecular differences among one another; the B-group have a cyclopentane ring and exhibit blue fluorescence while the G-group have a lactone ring and exhibit yellow-green fluorescence under ultraviolet light. Aflatoxins M_1 and M_2 are hydroxylated products of the AFB_1 and AFB_2 respectively, and are found in milk, dairy products or meat (Wacoo *et al.*, 2014; Ashiq, 2015). Other derivatives of aflatoxin have been identified particularly as products of biotransformation of major metabolites in mammals and these are B_{2A}, G_{2A}, Q_1 and P_1. Other types of aflatoxin such as GM_1, GM_2, GM_{2A}, B_3, M_{2A} and parasitol have also been reported (Ashiq, 2015).

Aflatoxin B_1 is the most potent naturally formed carcinogen and is regarded as the strongest hepatocarcinogenic agent known (Anfossi *et al.*, 2016). It is also an immune suppressor, inflammation promoter and growth suppressor in animals and humans (Marroquín-Cardona *et al.*, 2014). About 75% contamination in food and feed can be traced to aflatoxin B_1 contamination. Aflatoxin B_1 is so far the only mycotoxin regulated by EU with limits ranging from 5 µg/kg for finished feed for dairy animals to 20 µg/kg for raw materials and other finished feed (Abrunhosa *et al.*, 2016). The toxicological effects are dependent on dose; at high doses, they are lethal and cause liver, myocardial and kidney tissue damage. At sub-lethal doses, chronic toxicity such as liver cirrhosis occur (Ashiq, 2015). The maximum acceptable levels of AFB_1 in cereals, peanuts, and dried fruits, either for direct human consumption or as an ingredient in foods are 4 µg/kg for total aflatoxins (AFB_1, AFG_1, AFB_2, & AFG_2) and 2 µg/kg for AFB_1 alone (European Commission, 2006).

When animals consume mycotoxin-contaminated feed, there is a carry-over effect of mycotoxin residues into the animal (cow) milk, meat, or eggs and can be consumed by humans (Abrunhosa *et al.*, 2016). Although rumen flora act as defence against mycotoxin by metabolizing some of them to less toxic compounds, the rumen barrier can be altered by diseases in the animal, a change in the diet or a very high concentration of mycotoxin in the feed (Flores-Flores *et al.*, 2015). This is of great concern as contaminated milk can be harmful to humans and especially to children who are more susceptible to action of toxic compounds.

2.12.1.1 Fungal species associated with aflatoxin production

Recent reports have suggested the production of aflatoxins by thirteen species belonging to three sections of the genus *Aspergillus*, *i.e*, Section *Flavi* (*A. arachidicola*, *A. bombycis*, *A. flavus*, *A. parvisclerotigenus*, *A. pseudotamari*, *A. minisclerotigenes*, *A. nomius* and *A. parasiticus*), *Nidulantes* (*Emericella astellata*, *E. venezuelensis* and *E. olivicola*) and *Ochraceorosei* (*A. ochraceoroseus* and *A. rambelli*) (Guezlane-Tebibel *et al.*, 2013). In dry conditions and elevated temperature, aflatoxins remain stable but can be metabolized into toxic derivatives such as epoxide, aflatoxin M_1 or M_2 in human and animals, and by microorganisms to less toxic derivatives such as B_{2a} (Ananthi *et al.*, 2016).

Aspergillus flavus is found in plants, animals, and insects. Based on physiological, morphological, and genetic criteria, two types of *A. flavus* exist and are designated as S- and

L-strains. Isolates of S-strain produce more aflatoxin than L-strain isolates. S-strain isolates produce many small sclerotia (< 400 µm in diameter) and fewer conidia than L-strain isolates (Garber and Cotty, 1997). Optimum temperatures for growth of *A. flavus* and aflatoxin production are 33 °C and 16–31 °C respectively; and optimum water activity for growth and aflatoxin production are 0.98 a_w and 0.95–0.99 a_w respectively (Seonyeong *et al.*, 2015). *A. flavus* normally produces aflatoxin B derivatives, while *A. parasiticus* produces both aflatoxins B and G derivatives (Matumba *et al.*, 2015b). However, some pathogenic strains of *A. flavus* have been shown to produce cyclopiazonic acid (CPA) as well as both B and G toxins. S-strains of *A. flavus* produce high amount of only B toxin or both B and G toxins, while the L-strains produce low amount of only B toxins (Pildain *et al.*, 2004). Both B and G aflatoxin derivatives are produced by other species that include *A. toxicarius, A. nomius, A. bombycis, A. parvisclerotigenus, A. minisclerotigenes,* and *A. arachidicola* (Matumba *et al.*, 2015b).

2.12.1.2 Aflatoxin contamination in foods

Foodstuffs such as groundnut and maize promote the growth of *A. flavus* (Egal *et al.*, 2005). Aflatoxin discovery dates to the 1960s when poultry and other farm animals died in the United Kingdom after the consumption of Brazilian peanut meal contaminated with *A. flavus*, a situation described as the Turkey 'X' disease outbreak. In Gujurat and Rajasthan, India in 1974, Krishnamachari *et al.* (1975), reported 106 deaths arising from 397 cases of hepatitis that resulted from the consumption of maize heavily contaminated with *A. flavus* and aflatoxin concentration between 6.25 and 15.6 µg/g. In Eastern Kenya, between 2004 and 2011, 40% deaths resulted from 477 cases of acute aflatoxicosis reported (Mutiga *et al.*, 2014). Among the 317 people who became ill after consuming maize contaminated with aflatoxin, 125 deaths occurred (CDC, 2004). In 2010, an outbreak of aflatoxicosis also due to contaminated maize killed at least one child (BBC, 2010; Mutiga *et al.*, 2014; Ashiq, 2015).

A study conducted by Gong *et al.* (2002) demonstrated a striking relationship between exposure of children to aflatoxin, and both stunting and malnutrition. Blood samples of 480 children (aged between nine months and five years) from Benin and Togo were analyzed to determine dietary exposure to aflatoxin. Results in that study revealed that aflatoxin-albumin adducts were detected in 99% of the samples. Children with stunted growth or low body weight had 30–40% higher mean aflatoxin-albumin concentrations. The presence of AFM_1

in dairy products has been reported in Portugal. In a report documented by Abrunhosa *et al.* (2016), 52% of dairy products examined were contaminated with AFM_1, 68% had levels of AFM_1 ranging from < 0.005 to 0.08 µg/kg with 9% of the samples having values higher than the legal limit of 0.05 µg/kg in milk. Sampled yoghurt and cheese (6 %) had AFM_1 values above this legal limit set for milk.

'Kutukutu' is a fermented paste consumed in the Northern part of Cameroun by adults as breakfast and as complimentary food for young children. A study by Roger *et al.* (2015) reported that twenty-nine samples of 'kutukutu' collected in Maroua, Ngaoundere and Garoua of North Cameroun were all contaminated with AFB_1 with the highest mean contamination level recording in Maroua (2.3 ±0.5 ppb), followed by Ngaoundere (1.3±0.4 ppb). The values recorded in 'kutukutu' in Maroua are higher than the EU minimum tolerable limit of 2 ppb.

Four different samples of peanuts (*Arachis hypogaea*) originating from China and marketed in Khraissia, Bab Ezzouar, Bordj El Kiffan and Kouba in Algiers (Algeria) were analyzed for aflatoxin concentration. Aflatoxin was detected in all the samples, with concentrations ranging from 0.71 to 25.50 µg/kg. AFB_1 was detected in all the four samples, and concentrations ranged from 0.29 to 20.50 µg/kg, with the highest concentration of 20.50 µg/kg found in peanut marketed in Bab Ezzouar region. AFG_1 was present in peanuts from all the four regions with concentrations ranging from 0.19 to 4.83 µg/kg. For AFG_2, peanuts marketed in all the regions except Kouba contained the toxin, at concentrations ranging from 0.17 to 0.20 µg/kg (Guezlane-Tebibel *et al.*, 2013).

Abrunhosa *et al.* (2016) also reported incidences of aflatoxin contamination in different food commodities in Portugal. Among the dried figs analysed, 82% were contaminated with aflatoxin with a maximum concentration of 159.4 µg/kg, well above the 4 µg/kg allowed in Europe for dried fruits. For spices, concentration ranged from 0.4 to 58.0 µg/kg, 10 samples exceeded the EU limit (5 µg/kg) in 64% of the contaminated samples. Pistachios were contaminated with aflatoxin levels of up to 360.7 µg/kg. Incidence of aflatoxins in peanut did not exceed 18% but the maximum concentration detected was 902.4 µg/kg.

2.12.2 Ochratoxin A

Ochratoxins are a group of mycotoxins produced as secondary metabolites primarily by *A. ochraceus* and *Penicillium verrucosum* (Bhat, 2010), as well as some isolates of *A. carbonarius*, *A. niger*, *Neopetromyces* spp., and *Petromyces* spp (Ashiq, 2015). Significant in this group are OTA, OTB, OTC, methyl ester of OTA and ethyl ester of OTB. Ochratoxin A (OTA) is the most common and is resistant to heat and affects protein, DNA and RNA synthesis in the body (Ekici *et al.*, 2016). Ochratoxin A, a potent nephrotoxin, is produced at optimum temperature and water activity of 0.98 a_w and 25 to 30 °C. Ochratoxin A is also teratogenic and immunotoxic, and has been classified as a probable human carcinogen (IARC, 1993). Toxicity of OTA is exhibited through various mechanisms. For example, in the phenylalanine-tRNA-catalysing reaction, it inhibits synthesis of protein by competing with phenylalanine and the DNA adducts formed interfere with DNA repair system and control system of the cell cycle, thereby initiating carcinogenesis (Ashiq, 2015).

Ochratoxin A contaminates various commodities such as corn, coffee, wine, dried fruits, cocoa, beans, spices, cereal grains and rice (Ashiq, 2015). It has been found in oats, barley, wheat and other products consumed by human and animals (da Rocha *et al.*, 2014). European Union regulatory limits for OTA differ according to food type. A limit of 5 µg/kg is regulated in unprocessed cereals, 3 µg/kg in cereal-based processed foods, 10 µg/kg in coffee, and raisins and maximum of 15 µg/kg in spices. In a study conducted in Portugal, OTA was detected in 59% of the 601 samples of cereals and cereal based products analyzed, at concentrations ranging from 0.02 to 7.97 µg/kg with only four samples having values above the legal limits. Concentrations of OTA found in 73% of 421 coffee beans samples, ranged from 0.2 to 30.1 µg/kg with only four samples exceeding the EU legal limit. (Abrunhosa *et al.*, 2016).

2.12.3 Fumonisin

Fumonisins are recently discovered mycotoxins that are nephrotoxic and hepatotoxic, known to possess high cancer-inducing properties. Fumonisins cause neural tube defects in experimental animal species and their presence in corn grains has been reportedly linked with oesophageal cancer in Transkei region of Southern Africa, in China and in north-eastern Italy (da Rocha *et al.*, 2014; Ashiq, 2015). Fumonisin has immunosuppressive ability and has been classified as a group 2B carcinogen (possibly in humans) according to the International Agency for Research on Cancer (IARC, 1993). The fumonisin group of

mycotoxins comprises 16 compounds referred to as B_1 (FB_1, FB_2, FB_3 and FB_4), A_1, A_2, A_3, AK_1, C_1, C_3, C_4, P_1, P_2, P_3, PH_1a and PH_1b. The fumonisin B_1, a diester of propane 1,2,3-tricarballylic acid and 2-amino-12, 16 dimethyl-3,5,10,14,15-pentahydroxycosane, is the most extensively studied (da Rocha *et al.*, 2014). Fumonisins are produced by some *Fusarium* species (Bhat *et al.*, 2010). *Fusarium proliferatum*, *F. verticillioides* (previously classified as *F. moniliforme*) are the primary producers of fumonisins; as well as *F. nygamai* (Ashiq, 2015). Other producers are *F. polyphialidicum*, *F. anthophilum*, *F. dlamini*, *F. napiforme*, *F. subglutinans* and *F. oxysporum* (da Rocha *et al.*, 2014). *Aspergillus niger* has also been reported to produce fumonisins, especially FB_2 (Ashiq, 2015; Abrunhosa *et al.*, 2016).

Fumonisins have been found to contaminate beer, rice, corn, herbal tea, bovine milk, medicinal plants, figs and other agricultural commodities (Bhat *et al.*, 2010; Ashiq, 2015). Bordin *et al.* (2015) reported the occurrence of FB_1 in corn meal, corn flour, popcorn, cornflakes and polenta. In preliminary evaluation reports, human daily intake of fumonisin has been recommended to be below 1 µg/kg body weight/day (Bhat *et al.*, 2010).

2.12.4 Zearalenone

Fusarium species, especially *F. graminearum* and *F. culmorum* produce zearalenone (ZEN) as a secondary metabolite (Ashiq, 2015). Zearalenone has worldwide contamination record for various cereals and animal feeds. It is hepatotoxic, hematotoxic, genotoxic and immunotoxic. Also referred to as mycoestrogen, it has ability to bind to estrogen receptors and significantly affect the reproductive system and may cause severe reproductive disorders. It is linked to early onset of puberty in young children and is a potential stimulator of human breast tumorigenesis (Ashiq, 2015). In swine, zearalenone exposure may lead to vaginal and/or rectal prolapse, vulvovaginitis, disrupted conception, abortion and infertility (Ji *et al.*, 2016). Regular monitoring, therefore, is required to regulate the presence of this fungal toxin in foods and feeds and reduce its harmful effects on human and animal health.

2.12.5 Deoxynivalenol

Deoxynivalenol (DON) is a trichothecene commonly found in rye, barley and wheat grains (da Rocha *et al.*, 2014; Ji *et al.*, 2016). It is produced majorly by *F. graminearum* and inhibits the synthesis of RNA, DNA, and protein. In experimental animals, it causes genotoxicity, cytotoxicity, teratogenicity, and induces foetal skeletal deformities. Ingestion of DON-

contaminated foods by animals at higher doses causes vomiting, diarrhoea and feed refusal leading to severe weight loss, damage to the hematopoietic system and immune dysregulation (Ji *et al.*, 2016). At small doses, in pigs and other animals, its ingestion causes nausea and feed refusal (da Rocha *et al.*, 2014; Ji *et al.*, 2016). Deoxynivalenol is very heat stable, although its concentration can be reduced by boiling in water (Ashiq, 2015).

2.12.6 Patulin

Patulin was first isolated as an antimicrobial compound from *P. patulum* now known as *P. griseofulvum*. It is also produced by *A. clavatus*, *A. giganteus* and *A. terreus* (da Rocha *et al.*, 2014). Initially used to treat common cold and skin infections, its toxicity to animals and plants was discovered during the 1960s which led to its re-classification as a true mycotoxin (da Rocha *et al.*, 2014). *Penicillium expansum*, a natural and most efficient producer of patulin, causes 'blue mold' in apple, pear, cherry, and other fruits (da Rocha *et al.*, 2014).

2.12.7 Ergot alkaloids

Ergot alkaloids are indole alkaloids derived from a tetracyclic ergoline ring system and include the clavines and lysergic acid alkaloids. Ergoline forms the basic structure of clavines while lysergic acid alkaloids include ergotamine and ergine (Bennett and Klich, 2003). These compounds are produced in the sclerotia of species belonging to the genus *Claviceps* which cause diseases in grass plants (da Rocha *et al.*, 2014). The consumption of cereals infected with ergot sclerotia leads to ergotism or otherwise called St. Anthony's fire. Ergotism can be gangrenous, affecting blood supply to the extremities of the body or convulsive and affect the central nervous system (Bennett and Klich, 2003; da Rocha *et al.*, 2014). In susceptible animals such as cattle, ovine species, pigs and birds, noticeable symptoms of ergotism include abortion, convulsions, gangrene, suppression of lactation, hypersensitivity and ataxia (da Rocha *et al.*, 2014). Incidences of human ergotism due to bread made from contaminated flour was recorded in Europe in the Middle Ages (Bennett and Klich, 2003). In modern times, human ergotism is less common due to modern methods of grain cleaning and processing in mills leaving very low levels of the toxin. Ergot alkaloids are also heat labile and are destroyed during bread making (da Rocha *et al.*, 2014).

2.13 Masked mycotoxins

In food commodities contaminated with mycotoxins, the native toxins may have structurally related compounds, produced by plant metabolism or by food processing, and co-exist together with them. These are called masked or conjugated mycotoxins (Galaverna *et al.*,

2009). Plant enzymes, especially enzymes involved in detoxification processes are catalysts of chemical transformations that generate masked mycotoxins (Berthiller *et al.*, 2013). They are not detected by conventional analytical techniques as they exhibit changes in physicochemical properties of their molecules which influence their chromatographic behaviour (Berthiller *et al.*, 2013). In the digestive tracts of animals, these masked mycotoxins undergo hydrolysis to their corresponding precursors that may or may not exert similar toxic effects as their parent compounds (Galaverna *et al.*, 2009).

Masked mycotoxins comprise the extractable conjugated variety which are detectable by appropriate analytical methods, and the bound (non-extractable) variety which are not directly accessible and require liberation from the matrix by chemical or enzymatic treatment before analysis. Plant metabolites have been identified for deoxynivalenol, nivalenol, fusarenon-X, T-2 toxin, HT-2 toxin, zearalenone, ochratoxin A, destruxins and fusaric acid (Berthiller *et al.*, 2013). Zearalenone-14-β-D-glucopyranoside (Z14G) and deoxynivalenol-3-β-D-glucopyranoside (D3G) have been detected in naturally contaminated cereals such as wheat, barley, and maize. Fusaric acids methylamide has been shown to occur in infected vegetables. Bound fumonisin has been reported in raw maize and cereal-derived foods (Berthiller *et al.*, 2013).

Although there is a sparse information on the recovery of masked mycotoxins in Bambara groundnut, the detection of masked mycotoxins in maize raises concerns on its utilization in maize-bambara composite flour. Since masked mycotoxins may be generated during plant metabolism or food processing and may or may not exert similar harmful effects as their parent compounds, further studies to identify masked mycotoxins in maize-bambara composite flour may be vital in the control of mycotoxin contamination in the seed. Figure 2.4 below presents some of the structurally elucidated masked mycotoxins.

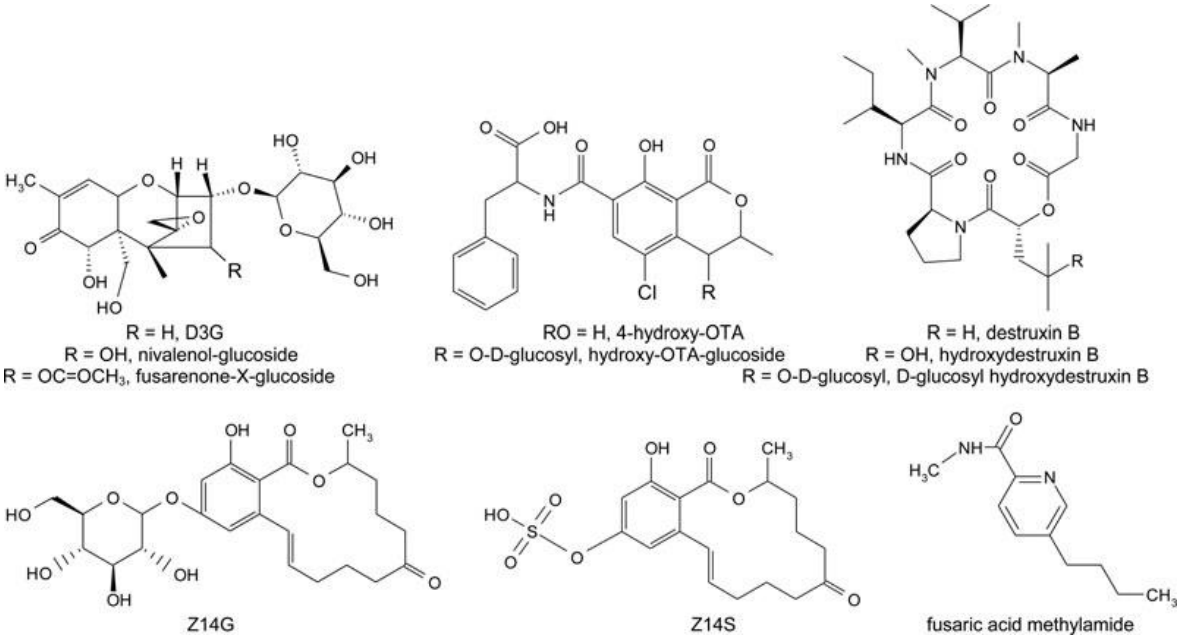

Figure 2.4 Structurally elucidated masked mycotoxins.

Berthiller *et al.* (2013).

2.14 Consumer awareness and attitudes to mycotoxin contamination in foods

Despite the high incidence of mycotoxicosis outbreaks, some consumers still hold very little awareness on the dangers of consuming mold and mycotoxin contaminated foods. Matumba *et al.* (2016) investigated on the subject among members of the general public in Malawi and reported low knowledge of molds across the sample population. Of the 805 respondents, 58.6% were male and 41.4% female that were either farmers, medical personnel, teachers, agricultural staff or engaged in other professions. Among the respondents, only 2.8 and 11.0% were familiar with the terms "mycotoxin" and "aflatoxin" respectively, 12% did not consider moldy foods to be dangerous, 10% were of the opinion feedstuffs severely colonized by molds were not dangerous to livestock while 9% held contrary belief that toxin could affect animal products such as meat, milk and egg when consumed by livestock.

In a similar study, Adekoya *et al.* (2017) appraised the attitudes, practices and knowledge of fungal colonization of foodstuffs among fermented food sellers from Southwest Nigeria. Amongst 86 respondents of which 98% were female, 11% had no formal education while 61% had primary education. A majority of the respondents (98%) could not link fungi to mycotoxin contamination and the perceived associated health risks. The level of education of the respondents had a significant but slightly positive influence on their apprehension of fungi and mycotoxin contamination.

Ezekiel *et al.* (2013) also studied the level of consumer awareness about the risk of aflatoxin contamination in peanut cake- 'kulikuli', a major relished snack in Nigeria. A total of 329 respondents from Lagos, Ogun, Oyo, Niger and Kaduna states of Nigeria participated in the study. Most of the consumers (64%) had low literacy levels and 85% of the consumers were not aware of the risk of aflatoxin contamination of vended peanut cake. The consumers' knowledge of food safety information had significant positive influence on their awareness.

The awareness of mycotoxin contamination among subsistence farmers in Southern African Development Community (SADC) region was studied with 260 randomly sampled households in Rungwe district. Of the participants, 58.5% had the perception that fungi could not produce harmful substances or mycotoxins. Infected maize was hardly thrown away but graded for use as food, for brewing beer or as animal feed (Mboya and Kolanisi, 2014). It is evident from these studies that a wide knowledge gap exists, which requires the need for public awareness trainings on mycotoxin mitigation.

2.15 Management and control of mycotoxins in food commodities

In the control or prevention of mycotoxins in food commodities, the fungal strain and food commodity determine the effectiveness of any method employed (Ashiq, 2015). Preventive measures at pre-harvest include the use of mold-resistant varieties such as transgenic plants, which possess antifungal or anti-mycotoxin compounds. Lytic synthetic peptides in transgenic plants inhibit toxigenic molds (Ashiq, 2015). Proper agronomic practices can also prevent mycotoxin contamination. These include crop rotation, avoiding overcrowding of plants, monitoring of soil pH and mineral deficiency, avoiding planting crops at periods of high temperature and water stress, and use of harvesting techniques that will minimize mechanical damage to grains (Siwela *et al.*, 2005; Ashiq, 2015). Timely management guided by predictive models which can predict the growth of toxigenic molds and mycotoxin contamination, forecast optimum harvest and antifungal application time may limit mycotoxin contamination (Ashiq, 2015).

Postharvest strategies aimed at mycotoxin control focus on drying and cleaning. These include drying of commodities to water levels too low to favour growth of fungi (Ashiq, 2015). Dry, clean, insect-free containers should be used in transporting commodities (Ashiq, 2015). During storage, cooling and aeration systems are necessary to regulate temperature, humidity and gas atmosphere (Siwela *et al.*, 2005). Storage facilities should protect

commodities from rainwater and ground water drainage. The use of appropriate anti-fungal agents and pesticides to protect against rodents, insects and fungal growth is recommended (Ashiq, 2015). Essential oils are plant compounds with antimicrobial and antioxidant activities. Being volatile and biodegradable in nature, they may be used as fumigants for stored food commodities (Prakash *et al.*, 2015). Regular check for any rise in temperature and moisture levels that could support mold growth should be implemented (Ashiq, 2015).

2.15.1 Chemical methods

The use of chemical compounds in the degradation of mycotoxins has not been entirely effective. While some success have been noted, for example, the use of extracts and powders of various spices, herbs, and essential oils against aflatoxin-producing fungi and inhibition of aflatoxin formation (Kachouri *et al.*, 2014), the application of some chemicals may have detrimental effects on the nutritional values of the foods in which they are used. The use of ammonia and hydrochloric acid, although may achieve efficient detoxification, may also compromise the nutritional values of the treated commodities (Ashiq, 2015). Another limitation to the application of chemical methods in mycotoxin control is the development of resistance to chemicals and some preservatives by some molds. For example, some *Penicilli* can grow in the presence of potassium sorbate, while others are able to degrade sorbate (Dalié *et al.*, 2010).

2.15.2 Processing operations

While pre-processing operations such as sorting, trimming and cleaning may reduce mycotoxin contamination of food commodities, these operations may not achieve complete elimination of the toxins in food (Bullerman and Bianchini, 2007). During milling for example, mycotoxins undergo re-distribution and concentration in certain mill fractions, particularly in the germ and bran fractions during dry milling. In wet milling, as in the case of corn, mycotoxins are not destroyed but may be dissolved in the steep water or evenly distributed in the processed by-products (Bullerman and Bianchini, 2007). Although pre-processing operations may be limited in their effectiveness to control mycotoxin contamination, some processing operations such as roasting may however, achieve considerable reduction of mycotoxin in food commodities. Darko *et al.* (2018) reported that partial roasting and blanching reduced aflatoxin production in peanut by above 72% during storage. Roasting at 150 °C for 30 min also degraded AFB_1 and AFB_2 by 66 and 63%, respectively, in naturally contaminated pistachio kernels (Yazdanpanah *et al.*, 2005).

Roasting therefore, may present an effective alternative in the control of fungal and mycotoxin contamination in Bambara groundnut.

2.15.3 Biological control

Various physical methods such as microwave heating, electronic eye sorting, UV irradiation, solar irradiation, solvent extraction, adsorption, ozone gas, gamma rays and floatation have been applied to control mycotoxins in foods. These methods have had limited efficiency based on safety issues, losses in nutritional values and palatability of feeds, cost implications and limited efficacy with different mycotoxins (Ashiq, 2015; Ji *et al.*, 2016). Consequently, there is need to seek for an effective, specific, feasible and environmentally sound decontamination technique (Ji *et al.*, 2016).

Bio-preservation is a method of preserving food products that is based on the principle of employing the use of one organism to control another. In recent years, it has received much attention for control of spoilage in foods (Dalié *et al.*, 2010). Biological control can be achieved using non-toxigenic strains of *A. flavus* and *A. parasiticus* in the soil of growing crops, by inoculating grains with conidia suspension or applied directly to the seedling or soil surface before planting. The naturally occurring toxigenic strains are then competitively eliminated by the non-toxigenic strain (Ashiq, 2015). Lactic acid bacteria (*Lactobacillus* spp.) can also inhibit mold development and consequently, the production of mycotoxins (Kachouri *et al.*, 2014).

2.15.3.1 Lactic acid bacteria as biocontrol agents

Lactic acid bacteria (LAB) are naturally present in food systems and are part of the human diet. They are divided into four genera: *Lactococcus*, *Lactobacillus*, *Leuconostoc* and *Pediococcus*; and are found in dairy products, meat and meat-derived products, and cereal products (Dalié *et al.*, 2010). They are traditionally used in food and feed as preservative agents to prevent spoilage and extend shelf life. They are certified safe for use in food applications and many species have been granted the status of Generally Regarded as Safe (GRAS) and Qualified Presumption of Safety (QPS) by the Food and Agriculture Organization of the United Nations and the European Food Safety Authority, respectively (Ananthi *et al.*, 2016). Lactic acid bacteria have found main use in fermented products and as starter cultures in the brewing industry. At present, they are incorporated into biofilms and encapsulation process (Kachouri *et al.*, 2014).

The use of LAB in the control of *A. flavus* and AFB_1 in foods are gradually being employed due to the ability of LAB to produce antimicrobial compounds such as lactic acid, acetic acid and bacteriocin. Lactic acid bacteria also produce hydrogen peroxide and compete with the fungi for nutrients. These mechanisms help in the inhibition of fungal growth (Roger *et al.*, 2015). Bacteriocins are antimicrobial proteins synthesized by *Lactococcus lactis* subsp. *lactis*, *Lactococcus lactis* subsp. *cremoris*, *Lactococcus lactis* subsp. *diacetylactis*, *L. acidophilus, L. plantarum* and *L. curvatus*, which act only against closely taxonomically-related bacteria (Kachouri *et al.*, 2014).

2.15.3.2 Antifungal metabolites produced by lactic acid bacteria

Several low-molecular-weight antifungal compounds have been isolated from bacterial cultures and they include organic acids, phenolic compounds, hydrogen peroxide, reuterin, hydroxyl fatty acids and proteinaceous compounds. Organic acids such as lactic acid and acetic acid are primary products of LAB fermentation of carbohydrates and are used for food preservation. These acids in their hydrophobic un-dissociated forms diffuse through membrane of target organisms, reduce cytoplasmic pH and stop metabolic activities (Dalié *et al.*, 2010).

The acids may neutralize the electrochemical potential of the plasmic membrane and increase its permeability. This condition of bacteriostasis culminates in the death of vulnerable organisms, including molds (Dalié *et al.*, 2010). Acetic acid has been reported to be more effective than lactic acid due to its high dissociation constant and is by far the best inhibitor of mold growth. Phenyl lactic acid also has antifungal activity (Dalié *et al.*, 2010).

Hydroxylated fatty acid compounds are produced by some LAB which not only improve sensory quality of fermented products but also present a broad inhibitory spectrum and are efficient against molds and yeasts e.g. caproic acid isolated from *L. sanfrancisco* CB1 (Dalié *et al.*, 2010). Most LAB produce hydrogen peroxide in the presence of oxygen but are unable to produce catalase to degrade the hydrogen peroxide. It therefore accumulates and causes oxidation of the liquid membrane and cellular proteins of the target organism. Under anaerobic conditions, *L. reuteri, L. brevis, L. buchneri, L. collinoides* and *L. coryniformis* ferment glycerol and produce reuterin. In the target organism reuterin can suppress ribonuclease activity, the main enzyme involved in DNA biosynthesis. Reuterin has been

reported to inhibit the growth of *Aspergillus* and *Fusarium*. Lactic acid bacteria cultures that produce reuterin have increased antifungal activity with glycerol (Dalié *et al.*, 2010).

2.15.3.3 Lactic acid bacteria-mycotoxin interactions

Mechanism of removal of mycotoxin by LAB involves physical process of binding and inhibition of mycotoxin accumulation. At its exponential growth phase, LAB produce a low molecular weight metabolite, which is capable of inhibiting mold growth and mycotoxin accumulation (Dalié *et al.*, 2010). The efficiency of aflatoxin B_1 binding *in vitro* and *in vivo* by several LAB is dependent on the bacterial strains; with aflatoxins B_2, G_1, and G_2 having less sensitivity to the binding process. The ability of LAB to bind aflatoxin B_1 *in vitro* is a reversible, fast (occurring within a minute), and strain- and dose- dependent process. Other organisms such as *Saccharomyces cerevisae* possess the potential to bind aflatoxin B_1 (Dalié *et al.*, 2010).

Six (6) isolates of LAB (*L. brevis* G11, *L. brevis* G25, *L. buchneri* M11, *L. cellobiosus* M41, *L. fermentum* N33 and *L. fermentum* N25) were used to demonstrate the inhibition of the growth of *A. flavus* M15 and aflatoxin in 'kutukutu', a maize-based fermented paste consumed in Northern Cameroun. After 24 hours of fermentation, *A. flavus* M15 growth was completely inhibited by *L. buchneri* M11, *L. brevis* G11 and *L. cellobiosus* M41. After 120 hours, a 64.2 and 63% reduction in AFB_1 was observed in 'kutukutu' fermented with *L. buchneri* M11 and *L. brevis* G25, respectively (Roger *et al.*, 2015).

The action of three LAB isolated from curd, *S. lactis, S. cremoris* and *L. acidophilus*, against toxigenic strains of *A. flavus* isolated from grapes and coconut from Madurai, a local market in Tamilnadu, India was reported by Ananthi *et al.* (2016). The strains were able to degrade AFB_1 within 24 hours of incubation and achieved 54-94% reduction after fermentation. Total AFB_1 reduction was most effective by *S. lactis*, followed by *S. cremoris* and *L. acidophilus*. In another study by Kachouri *et al.* (2014), naturally contaminated olives harvested in Tunisia for 2009 and 2010 season were inoculated with *L. plantarum* and stored for sixteen days at ambient temperature. That study revealed a reduction in AFB_1 level from 11 to 5.9 µg/kg at day 0, with a complete removal of AFB_1 during storage. Given the effectiveness of LAB to control fungal and mycotoxin accumulation in several food commodities as reported, its application can be exploited in Bambara groundnut flour as a mycotoxin decontamination strategy.

2.16 Conclusion

The high protein content of Bambara groundnut justifies its use as a supplement in cereal-based foods. The development and maturition of the Bambara groundnut pod just above the soil or underneath the soil surface, with the soil serving as a source of inoculum of pathogenic fungi, suggests a possible fungal contamination of the seeds and mycotoxin production during storage. Very limited information is available on the natural mycoflora and mycotoxin contamination of Bambara groundnut, hence safety of the nuts for consumption requires further study. Reports of mycotoxicosis, especially in developing countries where Bambara groundnut is commonly used in cereal-based foods for complementary feeding of infants and young children have been documented. Since Bambara groundnut also plays a role in international trade, it becomes important to ascertain its safety for consumption. This study will provide information required for intervention strategies that will assist farmers and food processors in the prevention and management of mycotoxin contamination in Bambara groundnut.

2.17 Aim, hypotheses, and objectives

2.17.1 Aim

To study the survival and colonization of mycotoxigenic fungi, and aflatoxin profiling in Bambara groundnut (*Vigna subterranea* (L.) Verdc) and its maize composite flour as affected by processing methods and during storage.

2.17.2 Hypotheses

This study is based on testing the following hypotheses:

1) Bambara groundnut from Southern Africa like other agricultural commodities, can become susceptible to mold and mycotoxin contamination. Given suitable temperature and humidity conditions, molds have the ability to grow and produce mycotoxins on agricultural crops, while in the field and/or during storage (Ji *et al.*, 2016).

2) Processing methods of dehulling, milling, roasting and lactic acid bacteria fermentation can affect the growth, survival, and mycotoxin production of mycotoxigenic fungi in Bambara groundnut, and during storage. Mold growth may be controlled by adequate processing and storage (Fasoyiro *et al.*, 2017). Lactic acid bacteria can contribute to inhibition of molds development and consequently, production of mycotoxins (Kachouri *et al.*, 2014).

3) Fungal infection in Bambara groundnut can lead to adverse changes in seed coat and internal cellular architecture. Achar *et al*. (2009) reported tissue distortion in peanut infected with *A. flavus.*

2.17.3 Objectives

The objectives of this study include:

1) To isolate and characterize mycotoxigenic fungi in Bambara groundnut, maize and maize-derived products, and other food commodities in Durban, South Africa;
2) To determine the effect of processing on the survival and mycotoxin production of the identified pathogenic fungi in Bambara groundnut and maize composite;
3) To determine the growth, behaviour, and aflatoxin production of identified pathogenic fungi in processed Bambara groundnut and its maize-composite flour during storage; and
4) To study the mode of infection of identified mycotoxigenic fungi on Bambara groundnut with Scanning Electron Microscopy and Transmission Electron Microscopy.

CHAPTER THREE

Fungal contamination of food commodities in Durban, South Africa

Abstract

Fungal contamination of food commodities is an increasing threat to the health of consumers. Pathogenic fungi produce mycotoxins, which have genotoxic and carcinogenic effects on consumers. This study aimed at investigating the occurrence of natural fungal contaminants in food commodities consumed in Durban, South Africa. A total of 110 samples including Bambara groundnut, maize and maize-derived products, rice, and spices were collected from open markets and retail stores and screened for fungal contaminants using conventional and molecular methods. Fungal DNA was amplified using polymerase chain reaction and amplicons were sequenced for identification. A total of 179 isolates belonging mainly to the fungal genera including *Aspergillus*, *Penicillium*, and *Fusarium* were recovered. *Aspergillus* (52.5%) and *Penicillium* (31.8%) were the most prevalent genera, contaminating 85.5 and 51.8% of the total samples analyzed, respectively. Bambara groundnut (100%), spices (89.5%), rice (86.9%), and maize and maize-derived products (71.9%) were contaminated by *Aspergillus* sp. Bambara groundnut (64.7%) and spices (44.7%) were contaminated by *A. flavus*. *Penicillium citrinum* was recovered from spices (71.1%), Bambara groundnut (61.7%), rice (34.8%), and maize and maize-derived products (31.3%). The isolation of mycotoxigenic fungi from these food samples is a concern to health of consumers, as some of them are known producers of their respective mycotoxins.

Key words: Bambara groundnut, Maize, Markets, Fungi, DNA, PCR

3.1 Introduction

Agricultural products grown in tropical areas, characterized by high temperature and relative humidity conditions, are susceptible to fungal contamination. The lack of Good Agricultural Practices (GAP) and Good Manufacturing Practices (GMP) further facilitates the proliferation of fungal contaminants. Some filamentous fungi growing in foods produce mycotoxins, which are toxic to humans and domesticated animals and therefore pose a significant threat to global food security. *Aspergillus, Penicillium,* and *Fusarium* are naturally occurring fungal genera in ambient air, soil and crops (Rodríguez *et al.*, 2015). Several species of these fungi have been isolated from food commodities while in the field, during harvest, transportation, and storage. Some *Aspergillus* species produce aflatoxin B_1

(AFB_1), the most potent of all aflatoxins with carcinogenic, mutagenic, teratogenic and immunosuppressive activity on animals and humans (Golge *et al.*, 2013). Fumonisin B_1 (FB_1) is produced by *Fusarium* species occurring mainly in corn and corn products as well as several other foods worldwide. Consumption of foods contaminated by FB_1 has been linked to human esophageal cancer and increased neural tube defects (NTD). This toxin has been classified as a possible human carcinogen (Bordin *et al.*, 2015). *Penicillium citrinum*, a member of the *Penicillium* genus, produces a nephrotoxin, citrinin (CIT) (Yogendrarajah *et al.*, 2014).

Food spoilage by microorganisms is governed by factors including water activity, hydrogen ion concentration, temperature (processing as well as storage), gas tension (specifically for oxygen and carbon dioxide), consistency, nutrient status, specific solute effects and preservatives (Pitt and Hocking, 1997). Improper processing, poor harvesting techniques, drying, packaging and transport activities increase the risk of fungal growth and mycotoxin production (Afsah-Hejri *et al.*, 2013). Foods contaminated by molds may contain mycotoxins, and their ingestion in high quantities or over a long period of time may cause harm (Marroquín-Cardona *et al.*, 2014). Mycotoxins commonly found in cereal grains are stable compounds, which are not destroyed during most processing operations (Bullerman and Bianchini, 2007). Due to their stability, they are carried over into processed foods (Anfossi *et al.*, 2016). Microorganisms, including human pathogens, are able to survive drying processes (Beuchat *et al.*, 2013). Molds capable of producing aflatoxins, ochratoxins, fumonisins, as well as other mycotoxins have been detected in a wide range of dried foods, including corn, rice, spices, coffee, cocoa, peanuts, tree nuts, and dried fruits. Mold-infested legumes, grains, and spices stored in humid environments pose a serious risk to consumers (Beuchat *et al.*, 2013).

Maize (*Zea mays*) is the third largest cereal produced after wheat and rice. It is the most widely cultivated cereal in South Africa and is utilized commonly as human food and animal feed (Chilaka *et al.*, 2012). It is considered to be one of the best agricultural commodities, however frequently infected by *A. flavus* that is subsequently accompanied by the production of attendant mycotoxins (Chauhan *et al.*, 2016). Rice (*Oryza sativa*) is a staple food for over half of the global population and provides 20% of the dietary energy supply globally. During the past decades, rice production increased from 518 million tons in 1990 to 599 million tons by 2000 and 701 million tons in 2010. The major genera of fungi found on rice are

Aspergillus, Penicillium, Fusarium, Alternaria, Mucor, Rhizopus. Trichoderma, Curvularia, Helminthosporium and *Cladosporium* (Seonyeong *et al.*, 2015). Spices are indispensable agricultural commodities in the art of cuisine (Yogendrarajah *et al.*, 2014). Despite being used in small quantities in foods, they are among the most versatile and widely used ingredients in food preparation and processing throughout the world (Kong *et al.*, 2010). Like other agricultural products, spices may be contaminated by a wide range of toxigenic fungi including *A. flavus*, *Alternaria alternate*, as well as *Penicillium* species (Kong *et al.*, 2014). Bambara groundnut (*Vigna subterranea*) on the other hand, is an underutilized legume cultivated in South Africa. The seeds are low in fat but are a good source of fiber, protein, and an essential sulfur-containing amino acid, methionine. Bambara groundnut is believed to be ranked third grain legume after groundnut (*Arachis hypogaea*) and cowpea (*Vigna unguiculata*). Like groundnut, the pods develop and mature in the soil or just above the ground (Murevanhema and Jideani, 2013), with the soil serving as a source of inoculum of pathogenic fungi (Guezlane-Tebibel *et al.*, 2013). The high protein content of Bambara groundnut signifies its use as a supplement in cereal-based foods. However, limited information on its mycological safety is available.

Several reports have documented the isolation of filamentous fungi in food matrices (Chilaka *et al.*, 2012; Hammami *et al.*, 2014; Egbuta *et al.*, 2015; Chauhan *et al.*, 2016; Jogee *et al.*, 2017). However, there is a lack of information on the natural occurrence of filamentous fungi in these food commodities in Durban, South Africa. Appropriate periodic monitoring is essential to ensure safety along the food supply chain and reduce health concerns for consumers. Therefore, this study aims to identify the natural fungal contaminants of rice, maize and maize-derived products, spices, and Bambara groundnut, obtained from retail stores and open markets destined for human consumption; and to study the extent of contamination by these fungi.

3.2 Materials and Methods

3.2.1 Sample collection and preparation

A total of 110 samples of food commodities including Bambara groundnut (17), rice (23), spices (38), and maize and maize-derived products (32) were randomly collected from open markets and retail stores in Durban, South Africa over a period of five months (February to June 2016). The collected maize and maize-derived products included maize grits, ready-to-eat breakfast cereals, maize meal/porridge, and popcorn. The spices included black pepper,

white pepper, chili, turmeric, coriander, cinnamon, cumin, basil, cloves, and curry powder. The rice samples included yellow and brown rice (long- and short -grain). Cream, maroon, purple and mixed varieties of Bambara groundnuts were collected. All the food commodities weighed between 100–200 g and were destined for human consumption. They were collected in the original packaging materials into sterile sample bags, sealed and were taken immediately to the laboratory. The grains and seed samples were ground for 60 s using a sterile blender and the ground samples stored at 4 °C till further analysis.

3.2.2 Fungal enumeration

One gram of each thoroughly mixed ground sample was weighed and diluted using 9 mL sterile Ringer's solution. The suspension was vortexed and serially diluted in sterile Ringer's solution. One millilitre each from dilutions 10^{-4}, 10^{-5}, and 10^{-6} was plated onto potato dextrose agar (PDA; pH 3.5) and selective identification medium *Aspergillus flavus parasiticus* agar (AFPA) (Oxoid, England) supplemented with chloramphenicol. Both media were sterilized at 121 °C for 15 min and cooled to 40–45 °C in a water bath before use. Plates were incubated at 28 °C for 5–7 days. Fungal colonies present in each plate were counted using a colony counter (UVP, UK), and the number of fungal colonies per gram of sample (CFU/g) was calculated as:

$$\boldsymbol{CFU/g = \frac{Number\ of\ colonies\ x\ reciprocal\ of\ dilution\ factor}{Plating\ volume\ (1\ mL)}}$$

3.2.3 Fungal screening and identification

Following incubation, individual colonies from each of the plates were sub-cultured on solidified PDA, Malt extract agar (MEA) and Czapex yeast extract agar (CYA) in sterile Petri-dishes. The Petri-dishes were incubated at 28 °C for 5–7 days and thereafter examined for identification. For microscopic identification, mycelium of the isolated fungi was placed on a glass slide, stained using lactophenol cotton blue (Merck, Germany), covered with cover slip, and examined under the microscope for observation of mycelial structure and fruiting bodies. Isolates were classified using standard nomenclature (Pitt and Hocking, 1997; Klich, 2002; Samson and Varga, 2007). Macroscopic classification of isolates was based on the color of aerial parts of the mycelium, the presence of pigmentation at the base of the mycelium, and surface texture of the conidia on various agars. Fifty-two of the 179 fungal isolates were selected based on their cultural and morphological characteristics for further molecular analyses and were used as a reference to identify the remaining fungal isolates.

Representative isolates were transported by air to CIRAD, Montpellier, France, for molecular identification.

3.2.4 Molecular identification

3.2.4.1 DNA extraction

The DNA was extracted using the protocol described by Moslem *et al.* (2010), with modifications. Isolates were sub-cultured on PDA (Biokar Diagnostics, France) plates and incubated at 28 °C for 4–5 days. Fungal spores were collected by overlaying 50 µL of Triton X-100 (VWR Prolabo, France) on 1 cm^2 of growth. The viscous suspension was added to a 1.5 mL microcentrifuge tube (Eppendorf) containing 500 µL of freshly prepared Cetyl-trimethyl ammonium bromide (CTAB) buffer (100 µL of β-mercaptoethanol and 20 mL CTAB) and glass beads (Sigma Life Science, USA). CTAB solution was prepared by adding 100 mL 1 M Tris pH 8, 280 mL 5 M NaCl, 40 mL 0.5 M EDTA and 20 g CTAB. This mixture was homogenized, and the volume was made up to 1 L using distilled water. The microcentrifuge tubes were initially agitated for 2 min (speed 5–6) using the Disruptor Genie (Scientific Industries, USA), then incubated in a water bath (Gallenkamp) at 65 °C for 15 min, agitated again for 1 min, and finally incubated at 65 °C for 15 min. Chloroform and isoamylic alcohol in the ratio of 24:1 (500 µL) was added to each tube, mixed uniformly, and centrifuged at 17 000 *g* for 5 min. The aqueous phase was transferred to fresh tubes. To these tubes, 64 µL of 3 M sodium acetate and 233 µL Isopropanol were added and mixed. The tubes were centrifuged at 21,000 *g* for 5 min to precipitate the DNA. The DNA pellets were washed with 500 µL of (70%) glacial ethanol and dried under a fume-hood for several hours. The pellets were re-suspended in 50 µL of molecular water and homogenized. DNA was quantified by measuring its absorbance at 260 nm (A_{260}) using a Nano-Drop spectrophotometer (BioSpec, Shimadzu), and stored at 4 °C till further use.

3.2.4.2 Polymerase chain reaction and sequencing of amplicons

The fungal genomic DNA was amplified using polymerase chain reaction (PCR) to obtain an approximately 260-bp fragment of the 28s rRNA gene. PCR was performed in tubes containing 48 µL of standard PCR mix, 10 µL each primer (U1 5´-GTGAAATTGTTGAAAGGGAA–3´ and U2 5´- GACTCCTTGGTCCGTGTT–3´), 0.25 µL Taq polymerase (Promega, Madison W1 USA) and 2 µL of the extracted DNA. Amplification was performed in a thermal cycler (Bio-Rad Laboratories, USA) programmed for 30 cycles of denaturation at 94 °C for 45 s (initialization at 94 °C for 3 min), annealing

at 50 °C for 50 s, polymerization at 72 °C for 5 min and a final extension at 72 °C for 5 min. To evaluate the quality of the amplicons, 5 µL aliquots of the PCR products were analyzed by electrophoresis in 2% (w/v) standard agarose gel (Eurobio, France) with 1X buffer Tris Acetate-EDTA. A 100 bp DNA Marker ladder (Promega, Madison, W1 USA) was used as the standard. Gels were electrophoresed at 100 V for 30 min, then observed and photographed using a CCD camera and Gel Smart system software (ClaraVision, France). The PCR products were stored at 4 °C till sequencing. Amplicons were sequenced by GATC Biotech (Konstanz, Germany).

3.2.5 Statistical analysis

The fungi were enumerated in three sets for each sample. Mean CFU/g of samples was calculated using SPSS for Windows version 24.0 (IBM Corporation, New York, USA). DNA base sequences were compared with the GenBank databases of the National Centre for Biotechnology Information. The GenBank was searched using BLAST program to determine the closest known relatives of the partial 28s rRNA gene sequences.

3.3 Results and discussion

The percentage of fungal recovery from the samples is shown in Table 3.1. A total of 179 isolates were recovered from the food samples, of which 94 belonged to genus *Aspergillus* and 57 belonged to genus *Penicillium*. Figure 3.1 shows the incidence of the fungal genera, with *Aspergillus* (52.5%) and *Penicillium* (31.8%) as the dominant genera, followed by *Fusarium* (5.0%) and other genera (10.7%) which included *Purpureocillium*, *Paecilomyces*, *Debaryomyces*, *Byssochlamys* and *Epicoccum*. Mold counts recorded in rice and spices from retail stores were similar to those from open markets. However, Bambara groundnut, maize and maize-derived products from open markets had a slightly higher mold count than samples from retail stores (Figure 3.2). The fungal contamination by species belonging to the *Aspergillus* and *Penicillium* genera was recorded in all the samples, both from the retail stores and open markets (Figures 3.3–3.6). *Fusarium* spp. were observed to contaminate Bambara groundnuts, maize and maize derived products from open markets; and rice, spices, maize and maize-derived products from retails stores (Figures 3.3–3.5). The fungal population of samples from retail stores and open markets differed irregularly among samples. For example, rice samples from the retail stores showed lower fungal incidence and population compared to the same sample from open markets. In contrast, spices from the retail stores were more heavily contaminated than those from the open markets (Table 3.3).

Rice, spices, maize and maize-derived products, and Bambara groundnut were contaminated with more than one fungal species (Table 3.4).

Table 3.1 Percentage of fungal genera recovered from food commodities obtained from retail stores and open markets in Durban, South Africa

Genera	Number of isolates	Percentage (%)
Aspergillus	94	52.5
Penicillium	57	31.8
Fusarium	9	5.0
Purpureocillium	9	5.0
Debaryomyces	2	1.1
Epicoccum	1	0.6
Byssochlamys	3	1.7
Paecilomyces	4	2.2

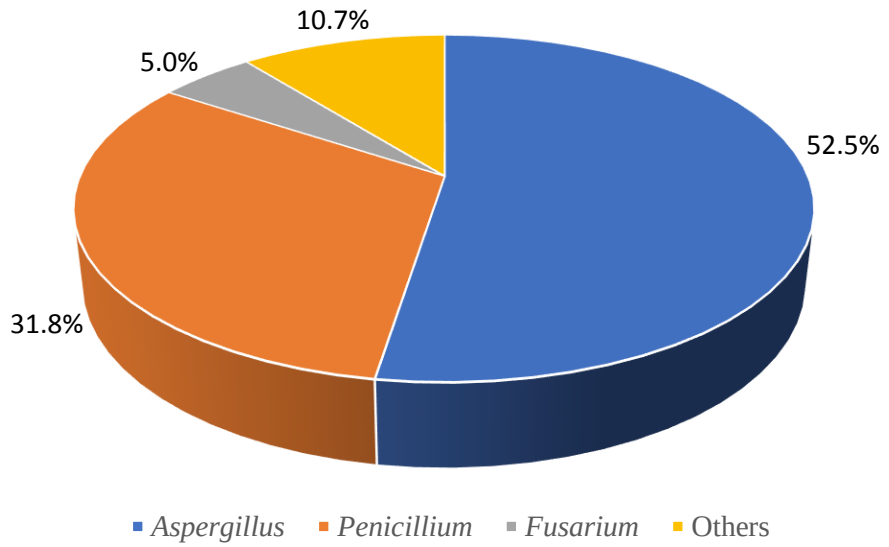

Figure 3.1 The incidence of identified isolates at genus level, recovered from food commodities obtained from retail stores and open markets in Durban, South Africa.

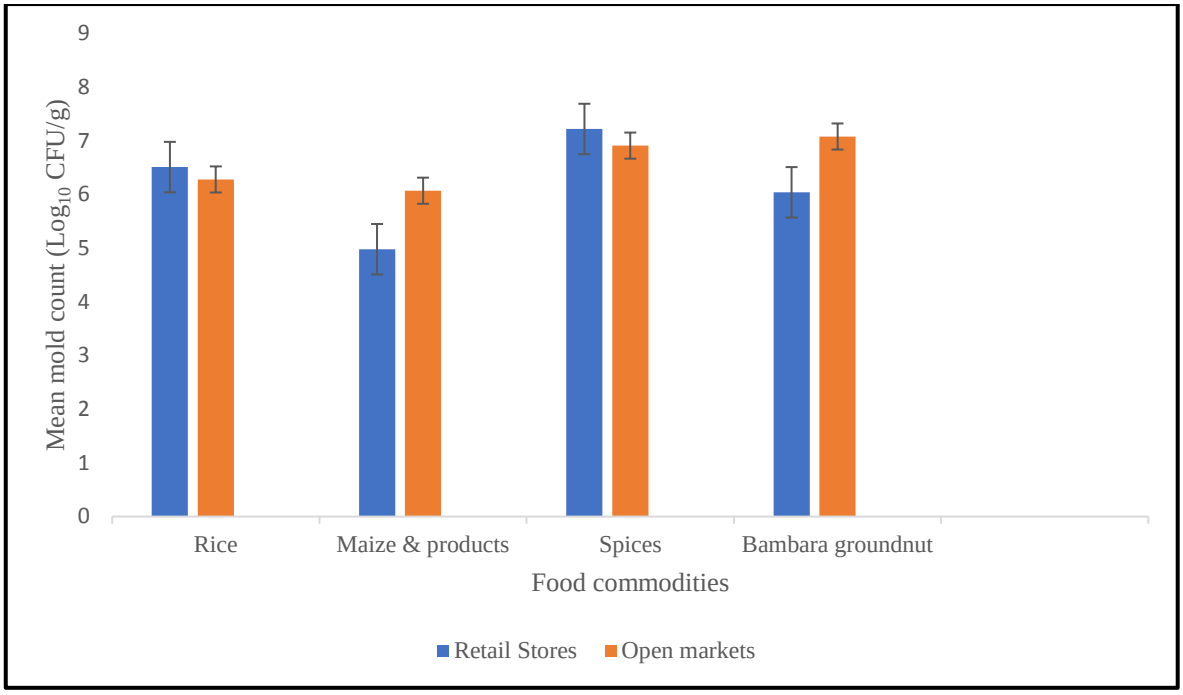

Figure 3.2 Mean mold count (Log_{10} CFU/g) in samples from retail stores and open markets in Durban, South Africa.

3.3.1 Incidence of different fungal genera contaminating maize and maize-derived products

The genus *Aspergillus* was recorded in 71.9% of the maize and maize-derived products, representing 73.9 and 66.7% of samples from retail stores and open markets, respectively (Figure 3.3). *Aspergillus niger* was found to contaminate 71.9% of the samples from both retail stores and open markets, while incidence of *A. flavus* (9.3%) was detected only in samples from the open markets. *Aspergillus wentii* was isolated from only maize and maize-derived products collected from retail stores, representing 6.3% incidence rate in all samples. The genus *Penicillium* was found in 30.4% samples from retail stores and 44.4% samples from open markets. *Penicillium citrinum* was detected in 31.3% of maize and maize-derived products obtained from both retail stores and open markets. *Fusarium nygamai* was detected in 8.2 and 22.2% of the maize and maize-derived products obtained from both retail stores and open markets, respectively. *Fusarium incarnatum*, which was detected in 3.1% of the samples, was found only in samples from retail stores. The fungi *Purpureocillium lilacinum* and *Epicoccum sorghi*, respectively, contaminated 6.3 and 3.1% of samples from the open

market and retail stores. There were slight differences in the average mold counts of maize and maize-derived products obtained from both sources (Figure 3.2).

Aspergillus niger and *A. flavus* were the major fungal contaminants isolated from the maize and maize-derived products. A study conducted to determine the occurrence of *Aspergillus* sp. in Malaysian foods used for human consumption reported that *Aspergillus niger* was dominant in corn-based foods (Reddy *et al.*, 2011). Fungi belonging to the genera *Aspergillus*, *Fusarium, Penicillium, Trichoderma* and *Macrophomina* were isolated from maize samples collected during a survey in the maize production areas from three agro-ecological zones in Nigeria (Atehnkeng *et al.*, 2008). *Aspergillus* spp. were the most prevalent fungi identified. In this study, *A. flavus* was isolated only from the samples from open markets. This could be attributed to improper handling and storage practices. Accordingly, food products sold in the open market are usually stored or displayed in inappropriate conditions, exposing them to dust and other environmental pollutants for longer periods. These conditions may facilitate mold growth and mycotoxin production (Reddy *et al.*, 2011). *Fusarium*, *Aspergillus, Penicillium,* and several genera of yeasts have been isolated from commercial maize samples in South Africa. Consumption of mycotoxin-contaminated maize has been linked to human esophageal cancer in the Transkei region in South Africa and China, and upper gastrointestinal tract cancer in Northern Italy (Chilaka *et al.*, 2012). *Aspergillus* was more prevalent with an incidence of 75% in maize samples collected from the Dilla region of Ethiopia (Chauhan *et al.*, 2016). Other genera identified were *Fusarium* (11%), *Penicillium* (8%) and *Trichoderma* (6%). Tournas and Niazi (2017) reported the isolation of *Fusarium* sp. and *A. flavus* in cornmeal and popcorn purchased from local markets in Washington, DC.

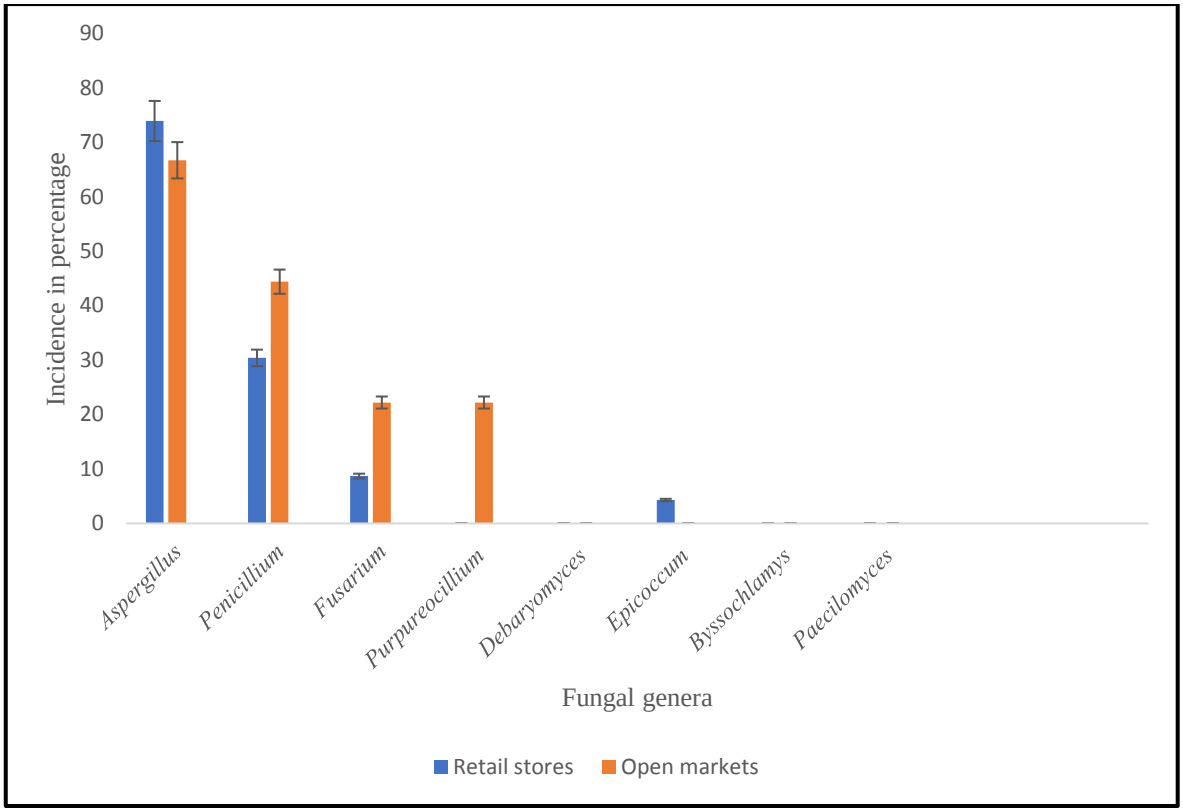

Figure 3.3 The incidence (in percentage) of different fungal genera contaminating maize and maize-derived products from retail stores and open markets in Durban, South Africa.

3.3.2 Incidence of different fungal genera contaminating rice samples

Rice samples analyzed in this study were mainly contaminated by *Aspergillus* genera, with an incidence rate of 86% recorded. High incidences of contamination were recorded in samples from retail stores (91.7%) as well as those from open markets (81.8%) (Figure 3.4). Rice samples from the open markets showed low contamination rate of *A. flavus* (8.7%) and *Aspergillus terreus* (4.3%). *Aspergillus niger* was detected in 82.6% of all samples analyzed (Tables 3.3). Other members of the genera were also present in 56.5% of the analyzed samples. The genus *Penicillium* was observed to contaminate 34.8% of all the rice samples analyzed. Samples from the open markets showed a higher rate of contamination (63.6%) than samples from retail stores (8.3%). *Penicillium citrinum* was isolated with 34.8% of the rice samples containing this fungus, but only for those obtained from open markets. Contamination with *Purpureocillium lilacinum* (4.3%) was recorded in rice samples from the retail stores only, while *Fusarium incarnatum* was detected in 4.3% of the samples from open markets but not for those from retail stores (Table 3.3). Average mold counts in samples

from both sources were similar (Figure 3.2). In a research conducted by Egbuta *et al.* (2015), rice samples were observed to be contaminated by species belonging to *Aspergillus*, *Penicillium*, *Fusarium*, *Alternaria*, *Rhizopus*, and *Cladosporium*, with *A. flavus* as the dominant species. Variation in fungal genera contaminating samples from the markets and stores was also reported. The high incidence of *A. flavus*, *A. niger* and *Penicillium citrinum* in rice samples raises some health concerns among consumers as these organisms are known producers of aflatoxin, ochratoxin A (OTA), and citrinin (CIT), respectively. Aflatoxins have been described to have genotoxic and carcinogenic properties (Golge *et al.*, 2013). Citrinin, produced by *Penicillium citrinum*, is a mycotoxin with nephrotoxic activity. It is proposed to have synergistic effects with OTA to cause Balkan Endemic Nephropathy in humans (Bertuzzi *et al.*, 2015). OTA has nephrotoxic, hepatotoxic, genotoxic, immunosuppressive, and carcinogenic effects, as demonstrated on animal models (Geremew *et al.*, 2016).

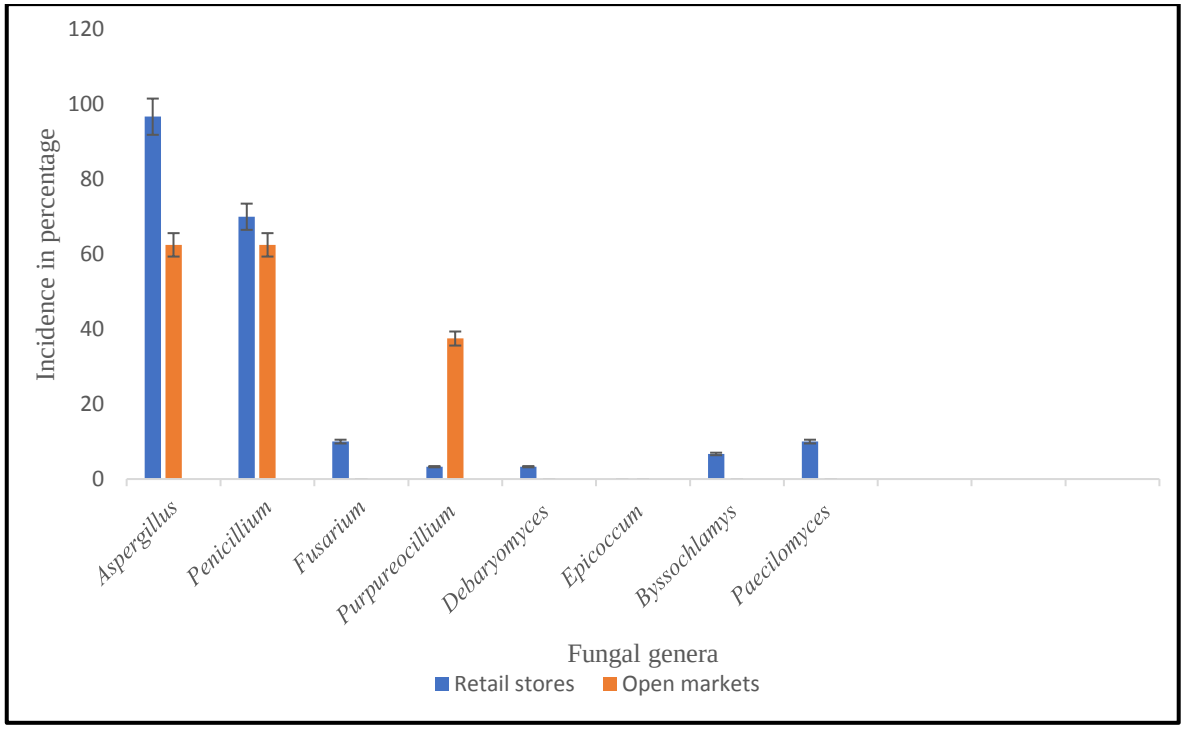

Figure 3.4 The incidence (in percentage) of different fungal genera contaminating rice samples from retail stores and open markets in Durban, South Africa.

Table 3.2 . Mean counts (Log_{10} CFU/g) of fungal species recovered from rice, maize and products, spices and Bambara groundnut from retail stores and open markets in Durban, South Africa

	Mean counts (Log_{10} CFU/g) of fungi in food commodities from retail stores and open markets							
	Retail stores				Open markets			
Fungi	Rice	Maize & products	Spices	Bambara nuts	Rice	Maize & products	Spices	Bambara nuts
A. flavus	-	6.78	6.86	6.87	6.74	-	6.89	6.92
A. niger	4.27	6.49	6.77	6.36	6.27	6.62	6.79	6.57
A. ochraceus	-	-	6.30	-	-	-	-	-
A. tamarii	-	-	6.89	-	-	-	-	6.30
A terreus	-	-	5.14	-	6.30	-	6.36	-
A. wentii	-	6.43	-	-	-	-	-	-
Other *Aspergilli*	6.36	6.64	6.47	6.30	6.55	6.41	6.51	6.32
P. citrinum	-	6.11	6.30	-	6.36	6.30	6.23	6.34
P. minioluteum	-	-	6.00	-	-	-	-	-
P. oxalicum	-	-	6.30	6.30	-	-	-	-
Other *Penicilli*	-	6.50	6.43	-	6.20	6.32	-	6.34
F. nygamai	-	6.07	6.14	-	-	6.07	-	-
F. oxysporum	-	-	6.94	-	-	-	-	5.69
F. incarnatum	-	6.30	-	-	6.30	-	-	-
Others								
Purpureocillium lilacinum	5.80	-	6.36	6.32	-	6.11	6.25	6.34
Debaryomyces hansenii	-	-	5.41	-	-	-	-	6.11
Epicoccum sorghi	-	6.32	-	-	-	-	-	-
Byssochlamys spectabilis	-	-	5.17	-	-	-	-	6.30
Paecilomyces spp	-	-	6.30	-	-	-	-	6.43

Table 3.3 Incidence (%) of fungal species recovered from rice, maize and products, spices and Bambara groundnut obtained from retail stores and open markets in Durban, South Africa

Incidence (%) of fungi in different food commodities				
Fungi	Rice	Maize and products	Spices	Bambara nuts
A. flavus	8.7	9.4	44.7	64.7
A. niger	82.6	71.9	76.3	100
A. ochraceus	-	-	5.3	-
A. tamarii	-	-	5.3	5.9
A terreus	4.3	-	2.6	-
A. wentii	-	6.25	-	-
Other *Aspergilli*	56.5	21.9	47.4	52.9
P. citrinum	34.8	31.3	71.1	64.7
P. minioluteum	-	-	2.6	-
P. oxalicum	-	-	2.6	5.9
Other *Penicilli*	4.3	9.4	26.3	29.4
F. nygamai	-	12.5	2.6	-
F. oxysporum	-	-	5.3	5.9
F. incarnatum	4.3	3.1	-	-
Others				
Purpureocillium lilacinum	4.3	6.3	10.5	11.8
Debaryomyces hansenii	-	-	2.6	5.9
Epicoccum sorghi	-	3.1	-	-
Byssochlamys spectabilis	-	-	5.3	5.9
Paecilomyces spp	-	-	7.9	5.9

Table 3.4 The frequency of contamination by different fungal genera in food commodities obtained from retail stores and open markets in Durban, South Africa

	Number of samples contaminated			
Genus	Rice	Maize and products	Spices	Bambara nuts
Aspergillus	20	23	34	17
Penicillium	8	11	26	12
Fusarium	1	4	3	1
Purpureocillium	1	2	4	2
Debaryomyces	-	-	1	1
Epicoccum	-	1	-	-
Byssochlamys	-	-	2	1
Paecilomyces	-	-	3	1

3.3.3 Incidence of different fungal genera contaminating spice samples

Mycological analyses showed that all spices analyzed were contaminated with various fungal genera, including *Aspergillus* (89.5%), *Penicillium* (68.4%), *Fusarium* (7.9%), *Purpureocillium* (10.5%) and other filamentous fungi (Figure 3.5). Spices obtained from retail stores showed a higher incidence of contamination by *Aspergillus* sp. (96.7%) compared to samples from the open markets (62.5%). *A. flavus* (44.7%) and *A. niger* (76.3%) were the dominant fungal species isolated from samples from both sources. *A. ochraceus* (5.3%) and *A. tamarii* (5.3%) were only recovered less frequently from retail stores' samples, while *A. terreus* (2.6%) was a rare contaminant of samples from both sources (Table 3.3). *Penicillium* spp. were observed to contaminate 70% of spices from retail stores and 62.5% of spices from the open markets. *Penicillium citrinum* was found to contaminate 71.1% of all spice samples. *Penicillium minioluteum* and *Penicillium oxalicum* (2.6% each) were detected in samples from retail stores only. The other *Penicillium* species were recorded in 23.6% of the spices. *Fusarium nygamai* (2.6%) and *Fusarium oxysporum* (5.3%) were found to contaminate spices from retail stores only. Similarly, *Paecilomyces* species (10%), *Debaryomyces hansenii* (3.3%), and *Byssochylamys* sp. (6.7%) were recorded in samples from retail stores only. *Purpureocillium lilacinum* had a higher incidence in samples from open markets (37.5%) than samples from retail stores (3.3%). The average mold counts of samples from both sources were not remarkably different (Figure 3.2).

A majority of filamentous fungi was isolated from the spices analyzed, with a higher contamination of spices observed in retail stores than for open markets. In a study conducted in Malaysia by Reddy *et al.* (2011), data obtained revealed the presence of *A. flavus* and *A. niger* in all spice samples analyzed, while *A. tamarii* was found in chili. *A. tamarii* and *A. terreus* were also found to contaminate pepper samples in Sri Lanka (Yogendrarajah *et al.*, 2014). Vyhnánek *et al.* (2017) also reported the presence of *A. fumigatus, A. terreus,* and *Fusarium* sp. in samples of sweet and hot paprika. Yogendrarajah *et al.* (2014) reported significantly lower counts of *Aspergillus* spp. in pepper samples from open markets when compared to retail stores. Gnonlonfin *et al.* (2013) reported the detection of *Aspergillus, Penicillium,* and other genera in dried and milled spices collected in southern Benin and Togo. *A. flavus* was isolated from all the samples. *A. ochraceus* and *A. niger* produce OTA in warm and tropical climates (Nganou *et al.*, 2014; Prelle *et al.*, 2014). OTA is known to have nephrotoxic, hepatotoxic, immune-toxic, and teratogenic properties, and classified in the group 2B (possible human carcinogen) by the International Agency for Research on Cancer (IARC, 1993). In this study, *Penicillium citrinum* had a higher incidence in spice samples from retail stores than those from open markets. This may be attributed to the higher humidity in the pack. Hammami *et al.* (2014) suggested that packaging may cause variation in factors such as humidity and aeration that affect fungal population and growth rate.

Although part of the culinary art in South Africa, spices' requirements are largely met by import from the spice-producing countries or major players in the spices trade, such as Singapore, Indonesia India, Viet Nam, and Malaysia (Vlok and Olivier, 2003). Like other agricultural products, spices may be contaminated by a wide range of toxigenic fungi including *A. flavus, Alternaria alternate* as well as *Penicillium* spp. from soil or plants during growth, harvest, processing, storage, and transportation (Kong *et al.*, 2014). Fungal proliferation and mycotoxin production are supported by high temperature, rainfall, and relative humidity conditions in countries where spices are mainly cultivated and processed. Extended drying periods, poor handling conditions, and simple production processes expose spices to microbial contamination from farm -to-fork (Yogendrarajah *et al.*, 2014).

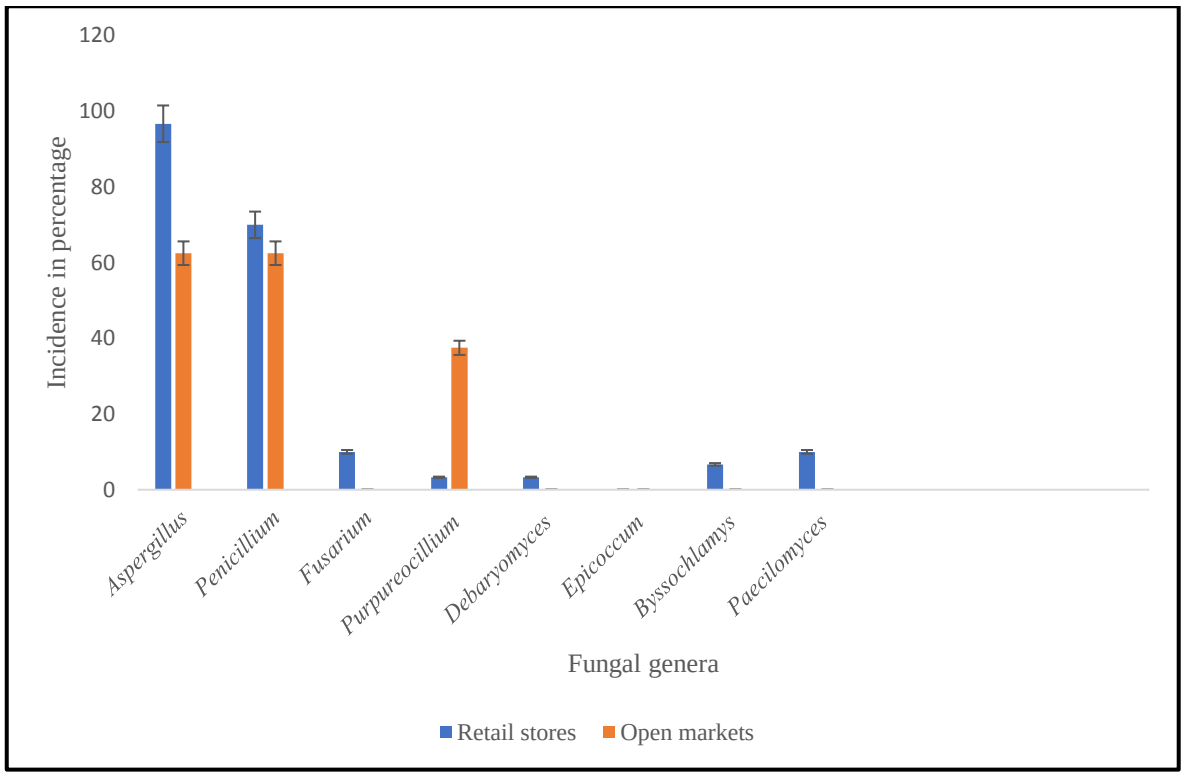

Figure 3.5 The incidence (in percentage) of different fungal genera contaminating spices from retail stores and open markets in Durban, South Africa.

3.3.4 Incidence of different fungal genera contaminating Bambara groundnut

Bambara groundnut samples analyzed in this study showed contamination by a wide range of filamentous fungal genera, including *Aspergillus*, *Penicillium*, *Fusarium*, *Purpureocillium*, *Paecilomyces*, *Debaryomyces*, and *Byssochlamys*. *Aspergillus* (100%) and *Penicillium* (70.6%) were the dominant genera, while *Fusarium* sp. (5.9%), *Paecilomyces* sp. (5.9%) and *Byssochlamys* sp. (5.9%) were rarely occurring genera (Figure 3.6). *A. flavus* and *A. niger* were observed to contaminate Bambara groundnut from both retail stores and open markets with incidences of 64.7 and 100% respectively (Table 3.3). Contamination by *A. tamarii* was recorded in Bambara groundnut collected from the open markets with an incidence of 5.9%. The presence of *Penicillium citrinum* (64.7%) and *Penicillium oxalicum* (5.9%) were observed only in Bambara groundnuts from the open markets and retail stores, respectively. *Purpureocillium lilacinum* was isolated from samples from both sources, contaminating 11.8% of all the samples. *Debaryomyces hansenii* and *Fusarium oxysporum* (6.3% each) were isolated from Bambara groundnut from the open markets only. Average mold count slightly differed between the samples from the two sources. Bambara groundnut

samples from the open markets showed slightly higher fungal contamination than those from the retail stores (Figure 3.2).

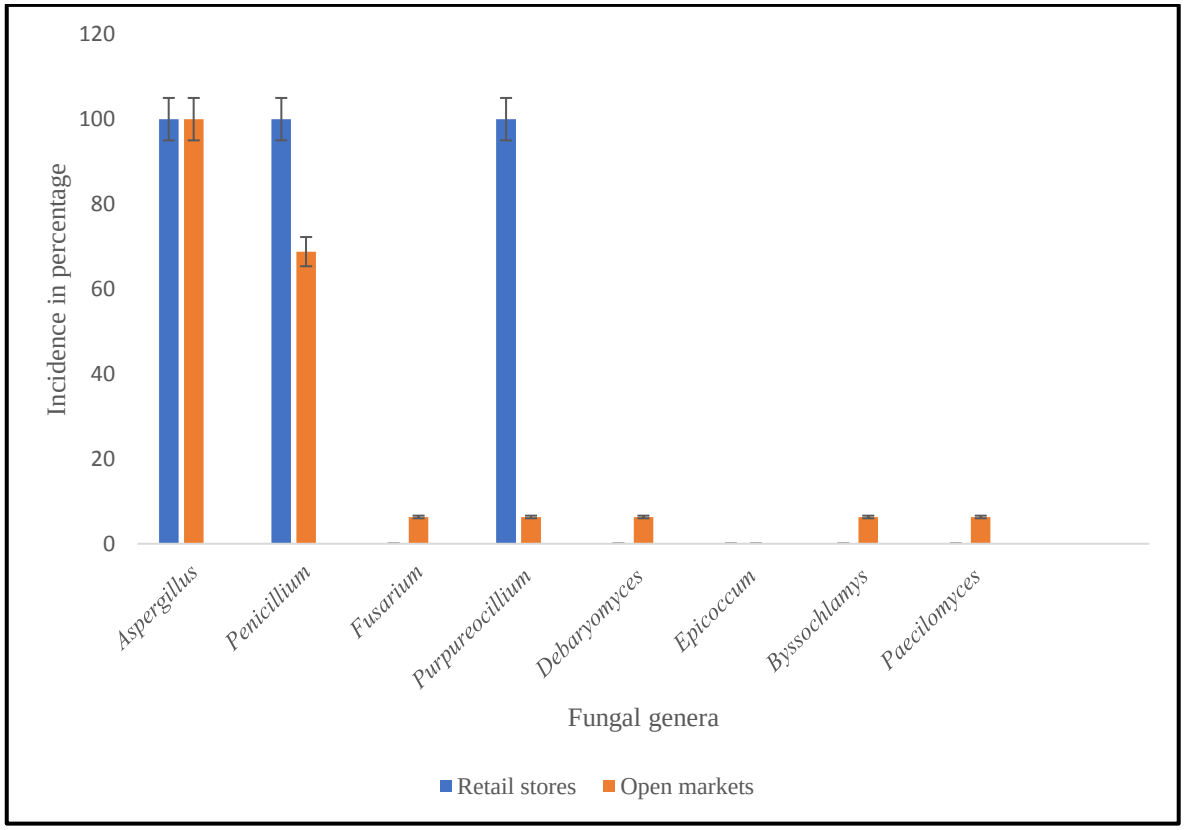

Figure 3.6 The incidence (in percentage) of different fungal genera contaminating Bambara groundnut obtained from retail stores and open markets in Durban, South Africa.

In a study conducted by Shabangu (2009), *A. flavus*, *A. niger*, *Penicillium citrinum*, and other fungi were isolated from maize and Bambara groundnut collected from the Limpopo province of South Africa. Kola (2003) also reported the presence of *A. flavus* and *A. niger* on differently coloured seeds of Bambara groundnut. This study also reported high incidence of contamination of Bambara groundnut by *A. flavus*, *A. niger* and *Penicillium citrinum*, among others. The isolated fungi are associated with the production of toxic metabolites, including aflatoxin and ochratoxin.

Few researchers have worked on the mycobiota of Bambara groundnut, and its safety for consumption from a mycological viewpoint has not been extensively studied. The isolation of mycotoxigenic fungi from Bambara groundnut raises some concerns on the health of consumers, especially in developing countries where the nut is commonly used in cereal-

based foods as a supplementary food, especially for growing children. The exposure to mycotoxin-contaminated foods at a very young age, through supplementary foods contributes to the problems in the growth of infants and young children in Africa (Achaglinkame *et al.*, 2017). A study conducted by Gong *et al.* (2002) demonstrated a striking relationship between exposure of children to aflatoxin, with both stunting of growth and malnutrition. Blood samples from 480 children (aged between nine months and five years) from Benin and Togo were analyzed to determine dietary exposure to aflatoxin. Aflatoxin-albumin adducts were detected in 99% of the samples. Children with stunted growth or low body weight had 30–40% higher mean aflatoxin-albumin concentrations.

3.4 Conclusion

Fungal screening of the food commodities showed the presence of pathogenic fungi in samples from retails stores as well as open markets. Contamination of these food commodities by various fungal species, particularly by known producers of mycotoxins, raises serious health concerns. There is need to raise awareness on the utilization of Bambara groundnut, especially for infant supplementary feeding. Further research to determine the potential mycotoxin contamination of Bambara groundnut from Southern Africa, as well as the development of mitigatory measures, is being carried out at our laboratories.

CHAPTER FOUR

Effect of milling, fermentation or roasting on water activity, fungal growth, and aflatoxin contamination of Bambara groundnut (*Vigna subterranea* (L.) Verdc)

Abstract

Bambara groundnut (*Vigna subterranea* (L.) Verdc) is an underutilized African legume gaining wide acceptance in various food applications due to its high protein content, which allows its incorporation as a supplement in cereal-based foods. Bambara groundnut grows near or under the soil, which serves as inoculum of pathogenic fungi and very little information is available on the fungal and mycotoxin contamination of Bambara groundnut from Southern Africa. Bambara groundnut flour (BGF) was analyzed for the presence of grain-contaminating mycotoxins, namely aflatoxin, beauvericin, citrinin, fumonisin, ochratoxin, trichothecenes and zearalenone, using a modified QuEChERS method. The quantification of the mycotoxins was performed via Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS). *Aspergillus flavus* isolates from Bambara groundnut were identified using conventional and molecular methods. Bambara groundnut flour obtained by milling, milling and roasting, and spontaneous fermentation were stored at simulated tropical conditions of 30±1 °C and 85±2 % relative humidity for 30 days. The effect of processing on the fungal counts, water activity and aflatoxin (AF) production over the storage period was determined. Aflatoxin-producing *A. flavus* was isolated from Bambara groundnut seed, with a co-occurrence of Aflatoxins (AFs), *i.e.*, AFB_1 (0.13–6.90 µg/kg), AFB_2 (0.14–2.90 µg/kg), AFG_1 (1.38–4.60 µg/kg), and AFG_2 (0.15–1.00 µg/kg) in the flour. There was significant ($p \leq 0.05$) increase in water activity of roasted Bambara groundnut flour (RBGF) (0.09–0.95), fermented Bambara groundnut flour (FBGF) (0.34–1.02), and BGF (0.42–0.89) over storage period. The fungal counts of all the samples significantly ($p \leq 0.05$) increased with increasing storage period, irrespective of the processing method. Roasting at 140 °C for 20 min achieved a complete degradation of AFs present in the seeds. The detection of aflatoxigenic *A. flavus* and AFs in BGF raises health concerns on its utilization as supplement in cereal-based foods, especially for infants and young children.

Key words: Bambara groundnut, Milling, Roasting, Fermentation, Aflatoxin, *Aspergillus flavus*

4.1 Introduction

Bambara groundnut (*Vigna subterranea* (L.) Verdc) is an underutilized, indigenous African leguminous crop grown for human consumption. In Africa, it is ranked third in importance

after groundnut (*Arachis hypogaea*) and cowpea (*Vigna unguiculata*) (Mubaiwa *et al.*, 2017). The seed contains carbohydrate (58.3%), protein (23.7%), and fat (4.3%) with a relatively higher methionine content than other commonly consumed legumes (Oyeyinka *et al.*, 2018). Its high protein content makes it one of the five important protein sources for many Africans (Murevanhema and Jideani, 2013). The dry seeds are roasted while freshly harvested seeds may be consumed boiled. It is commonly milled into flour and consumed in different forms (Alozie *et al.*, 2009). In developing countries, Bambara groundnut is utilized as a supplement for cereal-based flour in the production of biscuits, bread, and weaning food for infants due to its ability to improve protein content and functional properties of the flour (Okafor *et al.*, 2015; James *et al.*, 2017; Ogunmuyiwa *et al.*, 2017). Cereals are deficient in total nitrogen and essential amino acids lysine and tryptophan, and are used in combination with legumes which are rich sources of tryptophan and lysine to provide a desirable pattern of essential amino acids comparable to or higher than the reference protein (Alozie *et al.*, 2009). Like groundnut, the pods of Bambara groundnut develop and mature in the soil or just above the ground (Murevanhema and Jideani, 2013), with the soil serving as source of pathogenic fungi (Guezlane-Tebibel *et al.*, 2013). However, little has been reported on the mycobiota and mycological safety of this legume.

Molds are ubiquitous organisms with ability to infect almost all agricultural products while in the field, during or after harvest because of their tolerance to low water activity (Dall'Asta and Berthiller, 2015). Certain molds produce mycotoxins as toxic metabolites, which are chemical and heat stable and can be carried over into processed foods (Anfossi *et al.*, 2016). *Aspergillus*, *Penicillium*, and *Fusarium* species have been isolated from food commodities while in the field, during processing, transportation, and storage due to their ever presence in ambient atmosphere, soil and natural vegetation (Bhat *et al.*, 2010; Rodríguez *et al.*, 2015). Some *Aspergillus* species produce AFB_1, the most potent of all AFs with carcinogenic, mutagenic, teratogenic and immunosuppressive activity on animals and humans (Golge *et al.*, 2013). Aflatoxins exist in many tropical and subtropical regions of the world and many crops and agricultural commodities are susceptible to AF contamination (Zheng *et al.*, 2015). Human diseases including cancer and childhood stunting may result from prolonged exposure to low amounts of various mycotoxins (Misihairabgwi *et al.*, 2017). Hotter areas experience higher incidence of fungal spoilage and food commodities in the areas with high temperature and humidity are susceptible to harmful mycotoxin contamination (Ashiq, 2015).

Appropriate processing, storage and handling of food products are some preventive measures used to control mold growth (Fasoyiro *et al.*, 2017). The effect of processing on the natural fungal contaminants of Bambara groundnut and their toxins has not been reported, hence its safety for consumption from a mycological standpoint still requires further study. The objectives of this work were to 1) study the mycotoxin profile of Bambara groundnut; and 2) determine the effect of processing on water activity, survival of the fungal contaminants and AF concentration in BGF stored under simulated tropical conditions.

4.2 Materials and Methods

4.2.1 Sample collection and extraction

Cream-colored variety of Bambara groundnut was obtained from Southern Africa. The seeds were cleaned by hand picking of foreign matter. Cleaned seeds were dehulled, milled into flour (< I mm mesh size) and stored at 4 °C before analysis. The extraction of the BGF was carried out using a modified QuEChERS method, according to Polish Standard PN-EN 15562:2008 (Polish Committee for Standardization, 2008). The sample (5 g) was mixed with 20 mL of an extraction solvent (acetonitrile/water/acetic acid: 50/50/1) in a 50-mL centrifuge tube and vigorously shaken by hand for 1 min. After agitation, 8 g of $MgSO_4$ and 2 g NaCl were added to allow phase separation. The supernatant was diluted with mobile phase A (0.5% acetic acid in water), followed by the addition of the internal standard mix.

4.2.2 Mycotoxin analyses

Following extraction, the quantification of the various mycotoxins present in BGF was determined using ultra-high pressure liquid chromatography system, with pump model LC 20 AD and autosampler SIL 20 A XR (Shimadzu, Kyoto, Japan) interfaced with a mass spectrometer (Shimadzu, Kyoto, Japan). LC separation was performed using Phonemenex Kinetex XB Column C_{18} (50 mm x 2 mm; 2.6 µm particles) at 50 °C. The injection volume was 50 µL and mobile phase was pumped at a flow rate of 0.4 mL min^{-1}. Mobile phase composition was (A) 0.5% acetic acid in water and (B) 0.5% acetic acid in Isopropanol (1:1, v/v). A mobile phase gradient programme was started at 90% A (0.01 min), 45% A at 1.5 min, 15 % A at 3.5 min, 20% A at 4 min, 98% A at 4.01 min and finally 98% A at 11 min. The MS instrument was used in electron spray (ESI) positive and negative mode for all analytes. The two most abundant product ions generated from each precursor ion were chosen as the MRM transitions of each analyte.

4.2.3 Surface disinfection and isolation of fungal species

The procedure described by Pitt and Hocking (1997) was used with modifications. Whole Bambara groundnut seeds were immersed in 0.4 % chlorine solution for 2 min, with continuous stirring using sterile forceps. The seeds were rinsed in sterile water for 1 min, cut into small pieces and kept for drying in a laminar air flow. Under the laminar flow, the pieces (6–8 per plate) were fixed on solidified agar plates of potato dextrose agar supplemented with 10% tartaric acid **(**PDAT; pH 3.5). Plates were sealed with parafilm and incubated upright at 30 °C for 6 days. The percentage contamination in the seeds was calculated as:

$$\boldsymbol{Percentage\ infection} = \frac{\boldsymbol{Number\ of\ infected\ seeds\ \ x\ 100}}{\boldsymbol{Number\ of\ seeds\ plated}}$$

4.2.4 Fungal screening and microscopic examination for *Aspergilli*

Following incubation, the individual colonies from each of the plates were sub-cultured. Using a sterile wire loop, the colonies were picked and transferred onto petri dishes containing solidified PDA and Malt extract agar (MEA). Petri dishes were incubated at 25 °C for 5 days and colonies examined under a microscope for species belonging to *Aspergillus* genera. Mycelium of isolated fungi was placed on glass lens stained with lactophenol cotton blue (Merck, Germany), covered with cover slip and examined for mycelium structure and fruiting bodies. Isolates were classified using standard nomenclature for *Aspergillus* species (Klich, 2002). Macroscopic classification of isolates was based on color of the aerial parts of the mycelium, pigmentation at the base of the mycelium, and surface texture of the conidia on the different agar. The molecular identification of isolates was carried out in CIRAD, Montpellier, France.

4.2.5 Molecular identification

4.2.5.1 DNA extraction

The DNA was extracted using the protocol described by Olagunju *et al.* (2018b). Isolates were sub-cultured on PDA (Biokar Diagnostics, Beauvais, France) plates and incubated at 28 °C for 4–5 days. Fungal spores were collected by overlaying 50 µL of Triton X-100 (VWR Prolabo, France) on 1 cm^2 of growth. The spore suspension was added to a 1.5 mL microcentrifuge tube (Eppendorf) containing 500 µL of freshly prepared Cetyl-trimethyl ammonium bromide (CTAB) buffer (100 µL of β-mercaptoethanol and 20 mL CTAB) and glass beads (Sigma Life Science, USA). CTAB solution was prepared by adding 100 mL 1 M Tris pH 8, 280 mL 5M NaCl, 40 mL 0.5 M EDTA and 20 g CTAB. This mixture was homogenized, and the volume made up to 1 L using distilled water. The microcentrifuge

tubes were initially agitated for 2 min (speed 5–6) using the Disruptor Genie (Scientific Industries, USA), then incubated in a water bath (Gallenkamp, UK) at 65 °C for 15 min, agitated again for 1 min, and finally incubated at 65 °C for 15 min. Chloroform and isoamylic alcohol in the ratio of 24:1 (500 µL) was added to each tube, mixed uniformly, and centrifuged at 17 000 *g* for 5 min. The aqueous phase was transferred to fresh tubes. To these tubes, 64 µL of 3 M sodium acetate and 233 µL Isopropanol were added and mixed. The tubes were centrifuged at 21,000 *g* for 5 min to precipitate the DNA. The DNA pellets were washed with 500 µL of (70%) glacial ethanol and air dried under a fume hood for several hours. The pellets were re-suspended in 50 µL of molecular water and homogenized. DNA was quantified by measuring its absorbance at 260 nm (A_{260}) using a Nano-Drop spectrophotometer (BioSpec, Shimadzu, Kyoto, Japan), and stored at 4 °C till further use.

4.2.5.2 Polymerase chain reaction and sequencing of amplicons

The fungal genomic DNA was amplified using Polymerase Chain Reaction (PCR) to obtain an approximately 260-bp fragment of the 28S rRNA gene. PCR was performed in tubes containing 48 µL of standard PCR mix, 10 µL each primer (U1 5´-GTGAAATTGTTGAAAGGGAA–3´ and U2 5´- GACTCCTTGGTCCGTGTT–3´), 0.25 µL Taq polymerase (Promega, Madison W1 USA) and 2 µL of the extracted DNA. Amplification was performed in a thermal cycler (Bioer, China) programmed for 30 cycles of denaturation at 94 °C for 45 s (initialization at 94 °C for 3 min), annealing at 50 °C for 50 s, polymerization at 72 °C for 5 min and a final extension at 72 °C for 5 min. To evaluate the quality of the amplicons, 5 µL aliquots of the PCR products were analyzed by electrophoresis in 2% (w/v) standard agarose gel (Eurobio, France) with 1X buffer Tris Acetate-EDTA. A 100 bp DNA Marker ladder (Promega, Madison, W1 USA) was used as the standard. Gels were electrophoresed at 100 V for 30 min, then observed and photographed using a CCD camera and Gel Smart system software (ClaraVision, France). The PCR products were stored at 4 °C till sequencing. Amplicons were sequenced by GATC Biotech (Konstanz, Germany).

4.2.6 Toxigenicity of *Aspergillus flavus* isolated from Bambara groundnut

Isolates of *A. flavus* obtained from Bambara groundnut were tested for their ability to produce AF using cultural method as described by Abbas *et al.* (2004). Isolates showing orange underside on *Aspergillus flavus parasiticus* agar (AFPA) were sub-cultured on PDA at 25 °C for 5 days. The plates were inverted and few drops of 25% ammonia solution (Merck, South Africa) were placed on the inside of the lids.

4.2.7 Processing and storage of Bambara groundnut

4.2.7.1 Milling

Bambara groundnut seeds were cleaned by handpicking to remove foreign materials. Seeds were toasted at 85 °C for 3 h in a convection oven, cooled and cracked manually to remove seed coats (Alozie *et al.*, 2009). Dehulled seeds were milled to flour using a blender (Waring, USA) and sieved through a 1 mm sieve. Flour was packed in polyethylene bags and stored at 4 °C till further use.

4.2.7.2 Roasting

Whole Bambara groundnut seeds were toasted and dehulled as described in section 4.2.7.1. Dehulled seeds were roasted at 140 °C for 20 min using a laboratory roaster (PROBAT-Werke, Germany) (Okafor *et al.*, 2015). Roasted and cooled seeds were milled to flour and stored at 4 °C till further use.

4.2.7.3 Fermentation

Fermentation was carried out as reported by Fasoyiro *et al.* (2016) with modifications. Cleaned whole Bambara groundnut seeds were soaked in distilled water (1/3; w/v) and allowed to ferment at room temperature for 3 days. After fermentation, the water was decanted, and seeds washed using distilled water and dehulled by pressing between the clean fingers. Cotyledons were washed in several rinses of distilled water and dried at 65 °C for 8 h using a convection oven. Seeds were cooled, milled to flour, packed in polyethylene bags, and stored at 4 °C till further use.

4.2.7.4 Storage tests

Twenty-five grams (25 g) of each processed BGF flour was aseptically transferred to sterile petri dishes and stored at simulated tropical conditions of 30±1 °C temperature and 85±2% relative humidity in a sterile climatic oven (Binder, USA) for 30 days. Samples were withdrawn at 5-day intervals over the storage period for analysis.

4.2.8 Determination of water activity

Measurements of water activity of processed and stored samples were carried out as described by Fasoyiro *et al.* (2016) with modifications, using an Aqua Lab 4TE water activity meter (Decagon Inc., USA) that had initially been calibrated with NaCl (a_w 0.753). About 5 g from each of well mixed flour sample was spread evenly in the sample container

and measured. Readings were carried out at 25 °C. Average of duplicate determinations was recorded.

4.2.9 Determination of fungal counts in Bambara groundnut flour during storage

The sample (10 g) was mixed with 90 mL sterile Ringer's solution and pummelled using a laboratory stomacher (Stomacher Lab-Blender 400) for 2 min and serially diluted in sterile Ringer's solution to 10^{-6}. The stock sample and all serially diluted samples (0.1 mL) were spread-plated on potato dextrose agar (PDAT; pH 3.5) plates. Agar had already been sterilized at 121 °C for 15 min and cooled to 40–45 °C using a water bath before dispensing in plates. Plates were sealed with parafilm, inverted, and incubated at 25 °C for 5 days. Following incubation, fungal colonies present in each plate was determined using a colony counter (UVP, UK) and the number of fungal colonies per gram of sample (CFU/g) calculated as:

$$\boldsymbol{CFU/g = \frac{Number\ of\ colonies\ x\ reciprocal\ of\ dilution\ factor}{Plating\ volume\ (0.1\ mL)}}$$

4.2.10 Aflatoxin extraction and quantification in processed and stored Bambara groundnut flour

The sample (25 g) was homogenized with 50 mL methanol-water (80:20; v/v) and 5 g of NaCl at high speed for 2 min with a blender (Waring, France). The extract was centrifuged at 6000 rpm for 10 min. Two mL of the filtrate was diluted with 18 mL of PBS buffer. Ten mL of this diluted sample was passed through an immunoaffinity column (IAC) (Aflaprep, R-Biopharm). The IAC was washed twice with 10 mL of PBS each time before being eluted with 2 mL methanol. The eluting fraction was then evaporated and 1 mL of methanol-water (50/50; v/v) was added. The obtained fraction was collected into a glass bottle and the quantification of AFs determined via LC-MS/MS as described in section 4.2.2.

4.2.11 Statistical analysis

All experiments were conducted in duplicate. Statistical analysis was carried out using SPSS version 24.0 for Windows (IBM Corporation, New York, USA). Data was analyzed using One-way ANOVA and means were compared using Fischer's Least Significant Difference Test ($p<0.05$). DNA base sequences were compared with those in the GenBank databases of the National Centre for Biotechnology Information. The GenBank was searched using BLAST program to determine the closest known relatives of the partial 28s rRNA gene sequences.

4.3 Results and discussion

4.3.1 Multi-mycotoxin analysis of Bambara groundnut flour

Seven grain-contaminating toxins were tested for their possible presence in BGF (Table 4.1). Among the mycotoxins, AFs (AFB_1, AFB_2, AFG_1, and AFG_2) were detected. Aflatoxin B derivatives are produced by *A. flavus* while *A. parasiticus* produce both aflatoxin B and G derivatives. Some pathogenic strains of *A. flavus* have been shown to produce both B and G aflatoxins. S-strains of *A. flavus* produce high amount of only B aflatoxin or both B and G aflatoxins, while the L-strains produce low amount of only B aflatoxins (Pildain *et al.*, 2004).

Table 4.1 Analysis of mycotoxins of Bambara groundnut flour

Mycotoxin	*Concentration (µg/kg)
Aflatoxin	
AFB_1	0.13–6.90
AFB_2	0.14–2.90
AFG_1	1.38–4.60
AFG_2	0.15–1.00
Beauvericin	ND
Citrinin	ND
Fumonisin	ND
Ochratoxin	ND
Trichothecenes	ND
Zearalenone	ND

Limits of detection and quantification for aflatoxin were 0.01 and 0.05µg/kg, respectively

ND, not detected

*minimum - maximum concentration detected

Aspergillus flavus and other *Aspergilli* have previously been isolated from Bambara groundnut (Olagunju *et al.*, 2018b). Other species that produce both AF derivatives include *A. toxicarius*, *A. nomius*, *A. bombycis*, *A. parvisclerotigenus*, *A. minisclerotigenes*, and *A. arachidicola* (Matumba *et al.*, 2015b). The detection of G aflatoxins in BGF suggests possible presence of these fungi or other unidentified producers. Mycotoxin presence in food commodities may occur without the detection of the associated fungi and vice versa (Sulyok *et al.*, 2010). The concentration of AFs in BGF reported in this study exceeds the maximum levels recommended by EU (European Commission, 2006) of 2 µg/kg for AFB_1 and 4 µg/kg for total AF in groundnuts and nuts, and 0.1 µg/kg for baby foods for infants and young children. The utilization of BGF to supplement cereal-based foods especially weaning foods for infants and young children, requires further studies on prevention and detoxification of the seeds.

4.3.2 Fungal contamination of Bambara groundnut

The different fungal species contaminating the Bambara groundnut under study are shown in Figure 4.1. Macroscopic and microscopic classification identified fungi belonging to the *Aspergillus*, *Penicillium*, and *Fusarium* genera. Contamination by these fungal genera has previously been reported in Bambara groundnut obtained from retail stores and open markets in Durban, South Africa (Olagunju *et al.*, 2018b). A twenty-five-percentage contamination was recorded in the seeds. Aflatoxigenic *A. flavus* were detected among the *A. flavus* strains isolated from the Bambara groundnut seeds. The toxin-producing strains were identified by a color change of the underside of colonies due to ammonium hydroxide vapour (Figure 4.2).

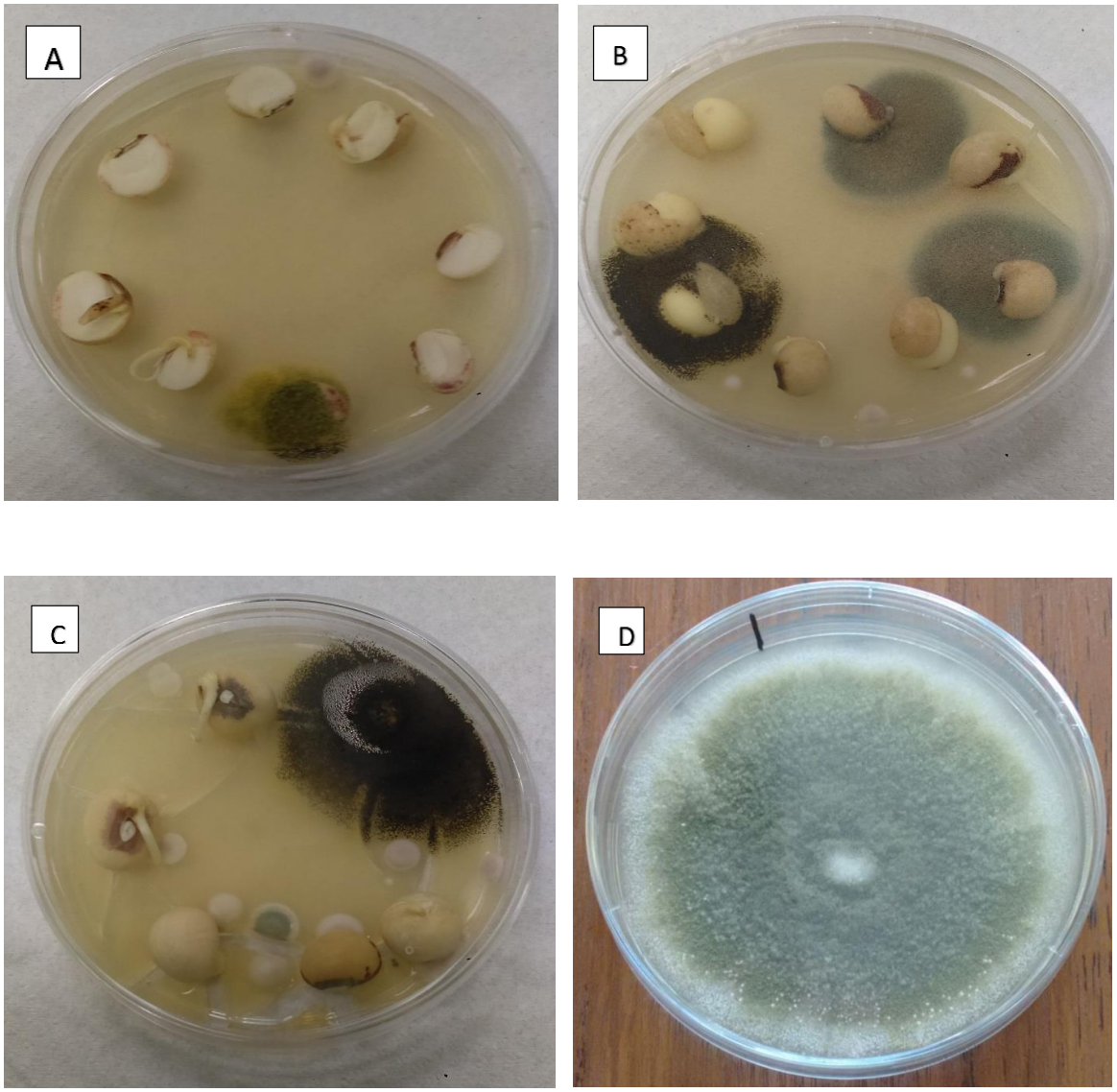

Figure 4.1 (A)-(C) Fungal species contaminating Bambara groundnut fixed on PDAT plates incubated at 30 °C for 6 days. (D) *Aspergillus flavus* isolate from Bambara groundnut grown on PDA plate incubated at 25 °C for 5 days.

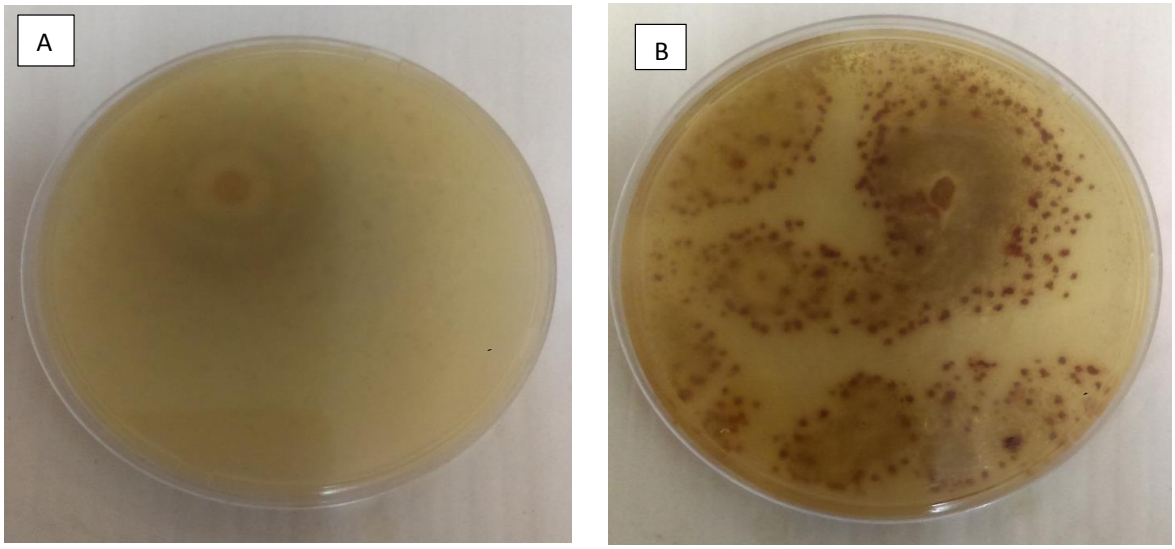

Figure 4.2 (A) Non-toxigenic (B) aflatoxigenic *Aspergillus flavus* isolates from Bambara groundnut on PDA plates incubated at 29 °C for 5 days. Colour change induced by ammonium hydroxide vapour observable on underside of colonies producing aflatoxin.

4.3.3 Effect of processing on water activity of flours during storage

The change in water activity of BGF, FBGF, and RBGF is presented in Figure 4.3. There was significant ($p \leq 0.05$) increase in the values obtained for RGBF (0.09–0.95), FBGF (0.34–1.02), and BGF (0.42–0.89) over the storage period. RBGF had a significantly ($p \leq 0.05$) lower water activity at day 0 when compared to BGF and FBGF. This can be attributed to the high temperature applied during roasting. There was significant ($p \leq 0.05$) increase in water activity of all the samples as storage progressed. All the samples had visible fungal growth at day 10 of storage with water activity values ranging from 0.79 to 0.95. FBGF had a significantly ($p \leq 0.05$) higher water activity values at days 15 and 20, slightly higher than the absolute value (1.0). At days 15 and 20, RBGF had a significantly ($p \leq 0.05$) higher water activity values of 0.86, and 0.95, respectively than those of BGF (0.80 and 0.89, respectively).

Water activity, usually measured within the range of 0–1.0, is useful to predict the amount of water available for biochemical processes and for the growth of molds, yeasts and bacteria in foods (Fasoyiro *et al.*, 2017). Foods with high water activity (up to 0.65) are prone to fungal proliferation that could be accompanied by mycotoxin contamination. The increase in water activity during storage reported in this study can be attributed to continuous metabolism of the bacterial and fungal species contaminating BGF and possible absorption

of humidity from the ambience. BGF provides suitable nutrients to support the growth of bacteria and molds, making the flour susceptible to fungal degradation during storage. The natural fungal species contaminating BGF can also survive these processing conditions and continue to grow during storage. An increase in water activity of flour samples during storage has also been reported by Fasoyiro *et al.* (2016).

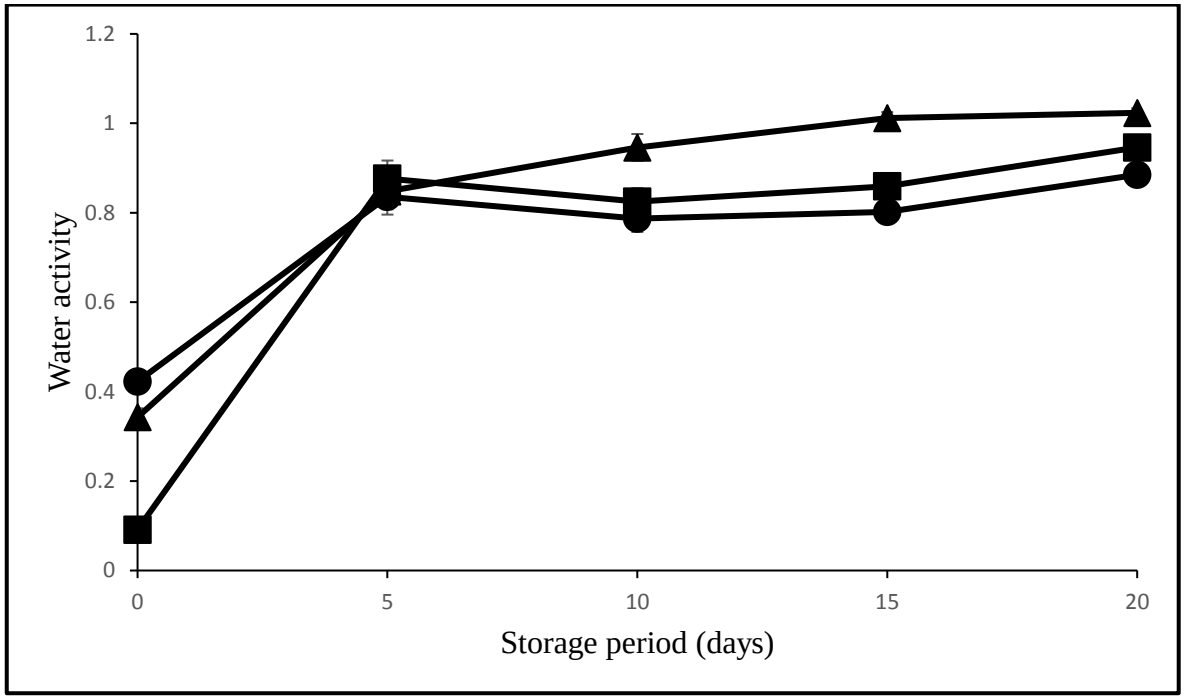

Figure 4.3 Water activity of Bambara groundnut flour (●), Fermented Bambara groundnut flour (▲) and Roasted Bambara groundnut flour (■) during storage at 30±1 °C and 85±2% relative humidity. Error bars indicate standard deviation (N=2).

4.3.4 Effect of processing on fungal counts of flours during storage

The effect of processing on the fungal counts of the flour samples is shown in Figure 4.4. The fungal count in all processed samples at day 0 of storage was significantly ($p \leq 0.05$) different and ranged from 6.3 Log_{10} CFU/g in BGF to 6.55 Log_{10} CFU/g in FBGF. The fungal counts of all the samples significantly ($p \leq 0.05$) increased over the storage period, irrespective of the processing method. At day 20, FBGF had a significantly ($p \leq 0.05$) higher mold count (7.34 Log_{10} CFU/g) than RGBF (7.30 Log_{10} CFU/g) and BGF (7.27 Log_{10} CFU/g). This increase can be attributed to favorable growth conditions provided by the fermented flour. *Bacillus* spp. involved in fermentation of legumes produce several enzymes including amylase, fructofuranosidase, glucosidase and galactinase, which degrade carbohydrates into simple sugars used as energy source by the microorganisms (Olagunju *et al.*, 2018c). Roasting conditions employed in this study did not eliminate the fungal

contaminants in BGF. However, roasting at 200 °C for 30 min has been reported to eliminate fungal contaminants in coffee beans (Barcelo *et al.*, 2017). Similar roasting conditions may achieve elimination but also compromise the sensory attributes of the flour. Barcelo and Barcelo (2018) also reported the absence of fungal contaminants in contaminated roasted coffee beans.

Microbial contamination of crops is influenced by factors that relate to the environment in which grains are grown, handled and processed such as rainfall, drought, humidity, temperature, sunlight, frost, soil conditions, wind, insect, bird and rodent activity, storage and handling, moisture control, etc. (Temba *et al.*, 2017). Water activity, temperature, pH, atmospheric condition, substrate, interaction among species, and time influence the growth of molds and accumulation of mycotoxins in food and feedstuff (Lahouar *et al.*, 2016). Temba *et al.* (2017) reported an increase in total fungal counts of maize-groundnut composite flour during storage. Fasoyiro *et al.* (2017) also reported an increase in fungal counts of maize-pigeon pea flour mixtures from 1.69–2.31 Log_{10} CFU/g at day 0 to 2.48–3.79 Log_{10} CFU/g after 24 weeks of storage. Fungal and bacterial contamination of flours is influenced by water activity in the flours. The higher the water activity, the higher the level of contamination and spoilage. However, this relationship varies from species to species (Temba *et al.*, 2017).

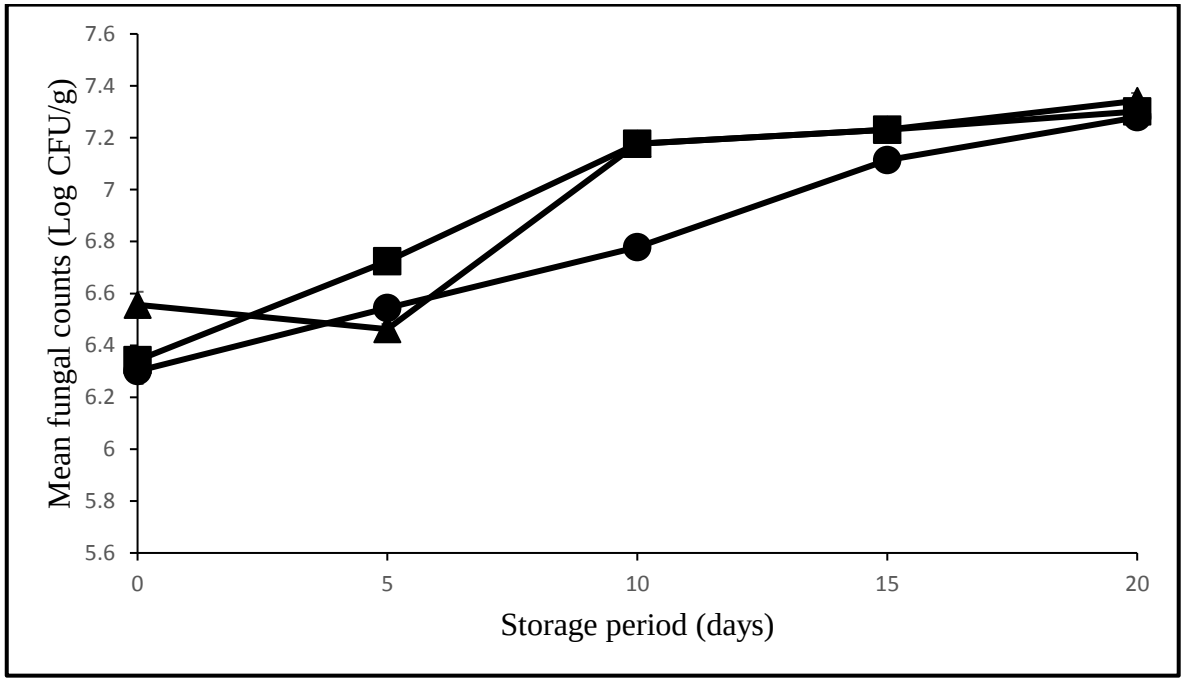

Figure 4.4 Mean fungal count of Bambara groundnut flour (●), Fermented Bambara groundnut flour (▲) and Roasted Bambara groundnut flour (■) during storage at 30±1 °C and 85±2 % relative humidity. Error bars indicate standard deviation (N=2).

4.3.5 Effect of processing on aflatoxin production in flours during storage

The concentration of AFs detected in the processed samples during storage is presented in Table 4.2. A co-occurrence of aflatoxins (AFB_1, AFB_2, AFG_1, and AFG_2) was recorded in the samples, with higher concentrations of AFB_1 noted. Aflatoxin production in the samples during storage was affected markedly by processing method employed. At day 0, there was significant ($p \leq 0.05$) difference in the concentration of AFB_1 detected in BGF (0.13 µg/kg) and FBGF (0.43 µg/kg), with aflatoxin not detected in RBGF. This implies that roasting at 140 °C for 20 min degraded the AFs present in Bambara groundnut seed but did not achieve complete elimination of the fungal contaminants present in the seeds which continued to produce AF during storage. The concentration of all the AFs in FBGF during storage significantly ($p \leq 0.05$) differed with those recovered from BGF and RBGF. This suggests that spontaneously fermented Bambara groundnut flour provides a rich substrate for fungal proliferation and AF production. Darko *et al.* (2018) reported that partial roasting and blanching reduced AF production in peanut by above 72% during storage. Roasting at 150 °C for 30 min also degraded AFB_1 and AFB_2 by 66 and 63% respectively, in naturally contaminated pistachio kernels (Yazdanpanah *et al.*, 2005).

Table 4.2 Occurrence of aflatoxins in processed Bambara groundnut during storage (Values are given in µg/kg)

Toxins	Day 0			Day 5			Day 10		
	BGF	FBGF	RBGF	BGF	FBGF	RBGF	BGF	FBGF	RBGF
AFG_2	ND	ND	ND	ND	0.05^{a} (0.01)	0.05^{a} (0.01)	ND	0.05^{a} (0.01)	ND
AFG_1	ND	ND	ND	ND	ND	0.75^{a} (0.02)	ND	80.19^{a} (0.03)	ND
AFB_2	ND	0.08^{a} (0.03)	ND	ND	0.05^{a} (0.01)	0.06^{a} (0.01)	ND	1.01^{a} (0.01)	ND
AFB_1	0.13^{b} (0.01)	0.43^{a} (0.10)	ND	0.05^{c} (0.00)	0.16^{b} (0.02)	1.07^{a} (0.02)	ND	88.67^{a} (0.03)	0.05^{b} (0.01)
Toxins	Day 15			Day 20			Day 30		
	BGF	FBGF	RBGF	BGF	FGBF	RBGF	BGF	FBGF	RBGF
AFG_2	ND	0.16^{a} (0.02)	ND	ND	0.98^{a} (0.01)	ND	-	-	-
AFG_1	0.28^{a} (0.02)	0.05^{b} (0.01)	ND	0.09^{b} (0.01)	9.36^{a} (0.02)	0.09^{b} (0.01)	-	-	-
AFB_2	0.05^{b} (0.01)	0.08^{a} (0.01)	ND	ND	29.05^{a} (0.00)	ND	-	-	-
AFB_1	0.38^{b} (0.01)	133.55^{a}(0.06)	ND	0.19^{b} (0.01)	65.56^{a} (0.00)	0.11^{c} (0.03)	-	-	-

Values represent mean (standard deviation) of duplicate determinations. Values with different superscripts along the row are significantly different ($p \leq 0.05$). Limits of detection and quantification are 0.01 and 0.05µg/kg, respectively. AFG_2-Aflatoxin G_2, AFG_1-Aflatoxin G_1, AFB_2-Aflatoxin B_2, AFB_1-Aflatoxin B_1, BGF- Bambara groundnut flour, FBGF- Fermented Bambara groundnut flour, RGBF- Roasted Bambara groundnut flour
ND, not detected.
-, not determined.

4.4 Conclusion

Bambara groundnut flour is susceptible to fungal growth and AF contamination under conditions of storage reported. Dehulling, milling and roasting had varied effects on water activity, fungal count, AF production and on the overall stability of BGF flour during storage but did not eliminate fungal and AF contamination. The detection of aflatoxigenic *A. flavus* and AFs in BGF flour raises health concerns on its utilization as a supplement in cereal-based foods, especially for infants and young children. Studies on prevention such as training of farmers and improvement in drying conditions, effective fungal elimination, and detoxification methods as well as legislation on AF levels in Bambara groundnut are required.

CHAPTER FIVE

Effect of milling, lactic acid bacteria fermentation and roasting on survival, growth and aflatoxin production of *Aspergillus flavus* in Bambara groundnut (*Vigna subterranea* (L.) Verdc.) and maize composite flour

Abstract

Bambara groundnut (*Vigna subterranea* (L.) Verdc.) is susceptible to contamination by aflatoxigenic *Aspergillus flavus*. The study investigated the effect of processing methods of milling, lactic acid bacteria (LAB) fermentation and roasting on the survival, growth and aflatoxin (AF) production of *A. flavus* in Bambara groundnut and its maize composite flour. The degradation of aflatoxin B_1 (AFB_1) by LAB was also studied. Irradiated seeds of Bambara groundnut, maize and composite were artificially inoculated with a 3-strain cocktail of *A. flavus* (2×10^6 spores/mL) and processed by milling and roasting at 140 °C for 20 min. Slurries of irradiated Bambara groundnut flour (BGF) were also inoculated with *A. flavus* spores and 1×10^8 CFU/mL inoculum of *Lactobacillus fermentum* or *Lactobacillus plantarum*. All inoculated samples were incubated at 25 °C for 96 h. Samples were withdrawn every 24 h and analyzed for *A. flavus* count, water activity and AF concentration using Enzyme-linked Immunosorbent Assay (ELISA). Samples fermented with LAB were further analyzed for LAB counts, pH, total titratable acidity (TTA) and AFB_1 reduction. *Aspergillus flavus* was able to survive and grow in the milled samples with a significant ($p \leq 0.05$) increase in population in BGF (7.15 Log_{10} CFU/g). The population of *A. flavus* significantly ($p \leq 0.05$) decreased in maize flour (MZF) (6.02 Log_{10} CFU/g) and maize-bambara groundnut composite flour (MBCF) (4.10 Log_{10} CFU/g) during incubation, while roasting eliminated all fungal spores and mycelia. Aflatoxin production was not detected in the processed BGFs under the experimental conditions. Growth of *A. flavus* was eliminated within 24 h of incubation with both LAB. Significant ($p \leq 0.05$) percentage reduction of AFB_1 was recorded by *L. fermentum* (89.2%) and *L. plantarum* (75%). A decrease in pH from 6.21 to 4.08, increase in TTA from 0.77 to 1.87%, and increase in LAB population from 8.54 to 13.41 Log_{10} CFU/mL was recorded during fermentation. Roasting and fermentation using *L. fermentum* and *L. plantarum* were effective in the decontamination of BGF.

Key words: Bambara groundnut, Maize, Lactic acid bacteria, ELISA, Aflatoxin, pH

5.1 Introduction

Bambara groundnut (*Vigna subterranea* (L.) Verdc.) is grown in Africa for its edible seed, which provides a major supply of dietary protein and calories, and it is one of the five

important protein sources for many Africans (Nwanna *et al.*, 2005; Murevanhema and Jideani, 2013). As a member of the *Leguminous* family, Bambara groundnut provides tryptophan and lysine, which are limiting in cereal. It is often composited with maize and other cereal grains to obtain a desirable pattern of essential amino acids (Alozie *et al.*, 2009). A major, yet widely neglected problem associated with the utilization of agricultural crops is contamination by toxigenic molds (Bhat *et al.*, 2010; Ashiq, 2015). Given suitable temperature and humidity conditions, molds have the ability to grow and produce mycotoxins on agricultural crops while in the field and/or during storage (Ji *et al.*, 2016). Mycotoxins are toxigenic secondary metabolites of some fungi and the contamination by mycotoxin-producing fungi results in wastage of massive amount of foods annually (Ashiq, 2015). Major mycotoxins of public health concern are produced by fungi belonging to *Aspergillus*, *Penicillium*, *Fusarium* and *Claviceps* (Abrunhosa *et al.*, 2016).

Processing of agricultural products involves the conversion of raw materials into semi-finished or finished products. Food processing contributes to increased shelf life, stability of colour and flavour, improved bio-accessibility of nutrients, increased economic value and facilitates the preparation of raw food ingredients (Decker *et al.*, 2014). A major benefit of food processing is the reduction or elimination of pathogenic microorganisms such as molds and bacteria. Milling, roasting, cooking, frying are various food processing operations that may affect mycotoxins in food commodities (Bullerman and Bianchini, 2007). Lactic acid bacteria belonging to the genera *Lactococcus*, *Lactobacillus, Leuconostoc* and *Pediococcus*, are naturally present in food systems and for centuries, have been a part of the human diet (Dalié *et al.*, 2010). They are traditionally used in food and feed as preservative agents to prevent spoilage and extend shelf life (Ananthi *et al.*, 2016). The use of LAB in the control of *A. flavus* and AFB_1 in foods are gradually being employed due to the ability of LAB to produce antimicrobial compounds and compete with the fungi for nutrients. These mechanisms help in the inhibition of fungal growth (Roger *et al.*, 2015). Various studies have reported the antifungal and mycotoxin degradation abilities of LAB (Ali and Mustafa, 2009; Omemu, 2011; Zuo *et al.*, 2012; Kachouri *et al.*, 2014; Roger *et al.*, 2015)**.**

The isolation of fungal contaminants, and the effect of milling, spontaneous fermentation or roasting on the fungal and mycotoxin contamination of Bambara groundnut under simulated tropical conditions of storage have been reported in Chapters Three and Four. Fungi belonging to the genera *Aspergillus*, *Penicillium* and *Fusarium* were isolated from Bambara groundnut. Roasting completely degraded aflatoxin B and G derivatives in naturally

contaminated Bambara groundnut. Despite the incidence of other fungal genera in Bambara groundnut, its flour was contaminated with only B and G aflatoxin derivatives. Mold contamination in foods does not usually correlate with mycotoxin production (Ashiq, 2015). Aflatoxigenic *A. flavus* had previously been isolated from Bambara groundnut (Olagunju *et al.*, 2018a). The objective of this study, therefore, was to determine the effect of milling, roasting and LAB fermentation on survival, growth and AF production of *A. flavus* in Bambara groundnut and maize composite flour.

5.2 Materials and methods

5.2.1 Materials

Cream-colored variety of Bambara groundnut (20 kg) was purchased from Mbare Msika market, Harare, Zimbabwe. Seeds were transported to Food Technology laboratory, Durban University of Technology where they were cleaned by handpicking of dirts and other foreign matter and stored at 4 °C until use. Maize grits (10 kg) used in the study were obtained from a retail store in Durban, South Africa.

5.2.2 Processing and treatment of Bambara groundnut and maize

Cleaned Bambara groundnut seeds were soaked in distilled water (1:5; w/v) for 12 h. The water was decanted, and seeds rinsed 2–3 times with distilled water. Whole cotyledons obtained by pressing soaked seeds between clean fingers were washed with excess distilled water and dried at 50 °C to a constant weight using a convection oven. A portion of the dried and cooled seeds was milled to flour using a blender (Kenwood, China) to pass through a 710 µm test sieve (Universal Laboratory, 128921). Maize grits were also dried in a like manner. A portion of the grits was also milled to flour as described above. Dried seeds and flour samples of Bambara groundnut and maize weighing 2 kg each were packed in 270 x 250 mm double seal tufflock bags (Tuffy Brands, South Africa) and subjected to irradiation (20 kGy; Gamwave Pty, Isipingo, South Africa) before use in experiments. The absence of live fungi and bacteria was confirmed on the irradiated samples by spread-plating 0.1 mL of 10^{-1} dilution on Potato dextrose agar (PDA) and Tryptic soy broth (TSB), respectively.

5.2.3 Lactic acid bacteria strains and preparation of inocula

Lyophilized cells of *L. fermentum* ATCC 9338 was purchased from Anatech Analytical Laboratory, Guateng, South Africa, while *L. plantarum* that had previously been characterized was obtained from the culture bank of the Department of Biotechnology and Food Technology, Durban University of Technology. *L. fermentum* was initially homogenized in 5 mL MRS broth. Both strains were streaked on solidified MRS agar plates.

MRS agar was prepared by dissolving 66 g of the powder in 1 L distilled water. Tween 80 (I mL) was added and the suspension heated with continuous stirring until completely dissolved. Agar preparation was then autoclaved at 121 °C for 15 min, cooled to 45 °C and poured into sterile petri plates.

Streaked plates were incubated at 37 °C for 24 h under anaerobic conditions. Fully established colonies of both bacteria strains were grown in sterile MRS broth without agitation at 37 °C for 16 h. Cell suspension was centrifuged at 4000 x *g* for 10 min and the supernatant was discarded. Pellets were washed twice with 10 mL phosphate buffered saline (PBS) (Sigma, USA), prepared by dissolving 1 tablet in 200 mL distilled water, and the solution sterilized at 121 °C for 15 min. The concentration of viable cells was adjusted at 1 x 10^8 CFU/mL using a spectrophotometer set at wavelength of 625 nm, corresponding to McFarland standard tube No. 0.5 with an absorbance value ranging from 0.08 to 0.10 (Khan *et al.*, 2012; Arullappan *et al.*, 2014) . Cell suspension was stored at 4 °C until use.

5.2.4 *Aspergillus flavus* strains and preparation of inocula

Aspergillus flavus ATCC 9643 purchased from Anatech Analytical Laboratory, Guateng, South Africa, and *A. flavus* BGC 01 and BGC 02 were used in the preparation of a 3-strain cocktail. *Aspergillus flavus* BGC 01 and BGC 02 had previously been isolated from Bambara groundnut and characterized (Olagunju *et al.*, 2018b). The abilities of the selected strains to produce aflatoxin had previously been confirmed. *Aspergillus flavus* ATCC 9643 was grown on Saboraud dextrose agar at 25 °C for 7 days according to supplier's instructions. Strains BGC 01 and BGC 02 were grown on potato dextrose agar supplemented with 10% tartaric acid (PDAT; pH 3.5) slants at 25 °C for 7 days. To harvest the spores, 20 mL of sterile distilled water containing 0.02% Tween 80 (Sigma-Aldrich, Switzerland) was deposited on the mat surface and gently rubbed with a sterile loop. The suspension was filtered through several layers of sterile cheese cloth. The total viable spores were determined using a hemocytometer and the concentration of viable spores/mL was calculated. The suspension was centrifuged at 8000 x *g* for 10 min and spores re-suspended in sterile distilled water to give a concentration of 2 x 10^6 spores/mL. A three-strain cocktail was prepared by combining suspensions from each of the three strains (2 x 10^6 spores per strain).

5.2.5 Rehydration and inoculation of Bambara and maize seeds

Irradiated Bambara groundnut and maize seeds were rehydrated to a moisture content of 18%, optimum moisture content required for fungal growth and AF production by storage fungi (Afsah-Hejri *et al.*, 2013). Appropriate volume of sterile water was added to the seed

in a sterile container with cover and allowed to stand at room temperature for 24 h under a laminar flow with intermittent shaking. Volume of water added was calculated according to Corrales *et al.* (2017) as

$$\boldsymbol{Water\ (g) = Wx\ \frac{Md - Mi}{100 - Md}}$$

Where

W is the weight of seed before rehydration;

Md the final moisture content (%); and

Mi the initial moisture content (%)

5.2.6 Processing of inoculated Bambara and maize seed and flour

5.2.6.1 Milling

Under a laminar flow, 30 g of rehydrated seeds of Bambara groundnut, maize or maize-bambara groundnut composite (70:30) in sterile petri plates was spot-inoculated with 100 µL of *A. flavus* cocktail at five randomly selected locations and milled for 60 s using a sterile blender (Kenwood, China). Milled samples were transferred into sterile petri plates, sealed with parafilm, and incubated at 25 °C for 96 h. Samples were withdrawn at 24 h intervals and analyzed for *A. flavus* count, water activity and AF concentration using an ELISA test kit (Max Signal, Bioo Scientific Corporation, Austin, USA).

5.2.6.2 Roasting

Irradiated and rehydrated Bambara groundnut (30 g) in sterile petri plate were spot inoculated with 100 µL of *A. flavus* cocktail under a laminar flow as described in section 5.2.6.1. Inoculated samples were roasted at 140 °C for 20 min (Okafor *et al.*, 2015). Roasted samples were milled to flour as described in section 5.2.6.1. Roasted and milled samples were transferred into sterile plates, sealed with parafilm and incubated at 25 °C for 96 h. Samples withdrawn at 24 h intervals were analysed for *A. flavus* count, water activity and AF concentration using ELISA test kit.

5.2.6.3 Fermentation

Fermentation of Bambara groundnut flour was carried out according to the method described by Obinna-Echem *et al.* (2015) with modifications. Under a laminar flow, irradiated BGF weighing 30 g was thoroughly mixed with 60 mL sterile distilled water in 100 mL sterile plastic containers (Lichron, Durban, South Africa). The slurry was inoculated with 1 mL of *L. fermentum* or *L. plantarum* (1 x 10^8 CFU/mL) and 100 µL of 2 x 10^6 spores/mL of *A. flavus* cocktail and mixed uniformly. Inoculated slurry was covered and sealed with

parafilm, incubated at 25 °C for 96 h without agitation and samples withdrawn at 24 h intervals for analysis (Roger *et al.*, 2015). At each sampling time, pH, LAB and *A. flavus* counts were determined. The remaining slurry after analysis was dried at 40 °C using a sterile air oven, packed in polyethylene bags, and stored at -20 °C till further analyses. Incubator was disinfected before use in the experiment. Bambara groundnut slurry inoculated with only *A. flavus* cocktail served as control sample.

5.2.7 Aflatoxin B_1 degradation by lactic acid bacteria

Under a laminar flow, irradiated BGF (30 g) in sterile sample bottle was mixed uniformly with 60 mL of sterile water. A pure solution of AFB_1 (5 µg/kg) was added to the slurry with 1 mL of *L. fermentum* or *L. plantarum* (1 x 10^8 CFU/mL). The preparation was gently mixed, covered, sealed with parafilm and incubated at 37 °C for 96 h (Roger *et al.*, 2015). Sample withdrawn every 24 h was dried at 40 °C using a convection oven, packed in polyethylene bags, and stored at -20 °C till determination of AF concentration.

5.2.8 Determination of water activity

Measurements of the water activity of the flour samples was carried out by using a Lab Swift water activity meter (Novasina AG CH8853 Lachen, Switzerland) that had been initially calibrated (Fasoyiro *et al.*, 2016). About 3 g from each well mixed flour sample was spread evenly in the sample container and measured. Readings were carried out at 25 °C. Average of triplicate determinations was recorded.

5.2.9 Determination of pH

The pH of Bambara groundnut fermented slurries and control sample was determined using a pH meter (Jenway, UK) that was initially calibrated using buffers 4 and 7. One milliliter (1 mL) of the slurry was mixed uniformly with 10 mL sterile distilled water and pH of the mixture was determined (Obinna-Echem *et al.*, 2015). Readings were carried out at room temperature and average of triplicate determinations was recorded.

5.2.10 Determination of total titratable acidity

The TTA of *L. fermentum* and *L. plantarum* fermented BGF was carried out according to AOAC (1990) with modifications. Two gram of flour sample was uniformly mixed with 20 mL distilled water and the mixture was filtered on Whatman filter paper (no 4). The filtrate (10 mL) was titrated against 0.1 N NaOH to first persistent pink colouration using phenolphthalein as indicator. Titre value was recorded, and TTA calculated as percentage of lactic acid by weight (1 mL 0.1 N NaOH = 0.009 g lactic acid).

5.2.11 *Aspergillus flavus* counts

Each flour sample (1 g) and fermented slurry (1 mL) was mixed with 9 mL sterile Ringer's solution, vortexed and serially diluted in sterile Ringer's solution to 10^{-6}. The stock sample and all serially diluted samples (0.1 mL) were spread plated onto Potato dextrose agar (PDAT; pH 3.5) plates prepared as described in section 3.2.2 of Chapter Three. Plates were sealed with parafilm, inverted, and incubated at 25 °C for 2 days (Roger *et al.*, 2015). Following incubation, fungal colonies present in each plate were counted using a colony counter (UVP, UK) and the number of fungal colonies per gram (CFU/g) or per milliliter of sample (CFU/mL) calculated as

$$\boldsymbol{CFU/g} = \frac{\boldsymbol{Number\ of\ colonies\ x\ reciprocal\ of\ dilution\ factor}}{\boldsymbol{Plating\ volume\ (0.1\ mL)}}$$

5.2.12 Lactic acid bacteria count

The growth of *L. fermentum* or *L. plantarum* during the fermentation period was determined at each sampling time. One (1) mL of the fermenting slurry was serially diluted in 9 mL sterile PBS. Dilutions 10^{-10}, 10^{-11}, and 10^{-12} and the stock solution (0.1 mL) were spread-plated on MRS agar plates prepared as described in section 5.2.3. The plates were incubated at 37 °C for 48 h. Following incubation, CFU/mL of LAB was determined by manual counting in plates showing 30–300 colonies (Obinna-Echem *et al.*, 2015).

5.2.13 Determination of total aflatoxin concentration using ELISA

Total AF concentration was determined according to the ELISA test kit protocol. Each flour sample was uniformly mixed. To 5 g of the mixed sample was added 25 mL of 70% methanol and the mixture vortexed for 3 min. The supernatant was filtered, and 1 mL of the filtrate was diluted with 1 mL of distilled water. The diluted sample (50 µL) was used in the test.

Aflatoxin B_1 standards provided in the kit were added in duplicate into different wells in order of increasing concentration. Each diluted sample (50 µL) was also added in duplicate into different wells. To each well was added 50 µL of HRP-Conjugated Antibody and 50 µL of Antibody. The plate was gently rocked for 1 min manually, and then incubated for 10 min at room temperature. After incubation, the plate was washed thrice with 250 µL of 1X Wash solution pipetted into each of the wells. After the last wash, the plate was dried by inverting and gently tapping it on dry paper towels. Immediately after, 100 µL of TMB substrate was added to each well and the plate gently rocked for 1 min, then left to incubate again for 5 min at room temperature. After incubation, 100 µL of Stop Buffer was added to each well

to stop enzyme reaction. The plate was read immediately on a plate reader (Thermo Fisher Scientific Oy, Vantaa, Finland) at 450 nm wavelength using Multiskan GO version 101.10. Aflatoxin B_1 standard curve was constructed by plotting the mean relative absorbance (%) of each standard against its concentration in ng/mL on a logarithmic curve. The mean relative absorbance value of each sample was used to determine the concentration of AFB_1 (ng/mL) present in the sample from the standard curve.

$$\textbf{\textit{Relative absorbance}}\ (\%) = \frac{\textbf{\textit{absorbance standard (sample) x 100}}}{\textbf{\textit{absorbance zero standard}}}$$

5.2.14 Measurement of aflatoxin B_1 degradation

The sample (25 g) was homogenized with 50 mL methanol-water (80:20; v/v) and 5 g of NaCl at high speed for 2 min with a blender (Waring, France). The extract was centrifuged at 6000 rpm for 10 min. Two mL of the filtrate was diluted with 18 mL of PBS buffer. Ten mL of this diluted sample was passed through an immunoaffinity column (IAC) (Aflaprep, R-Biopharm). The IAC was washed twice with 10 mL of PBS each time before being eluted with 2 mL methanol. The eluting fraction was then evaporated and 1 mL of methanol-water (50/50; v/v) was added. The obtained fraction was collected into a glass bottle, identified and quantified using HPLC with spectrofluorescence detection (Shimadzu RF 20A, Japan) after post column derivatization with electrochemical system (Kobra Cell™ R. Biopharm Rhône Ltd, Glasgow, UK). Fluorescence detector for AFs detection was set at excitation and emission wavelengths of 365 and 435 nm, respectively. The mobile phase was water-methanol (55/45; v/v), 119 mg of potassium bromide and 350 µL of nitric acid. Aflatoxin standard solutions were used for the construction of a five-point calibration curve of peak areas versus concentration (ng/mL). The operating conditions were as follows: injection volume of 100 µL of sample and standard solutions; C_{18} reverse-phase HPLC column, Uptisphere type, ODS, 5 µm particle size, 5 ODB, 250 x 4.6 mm, with identical pre-column, thermostatically controlled at 40 °C; isocratic flow rate of 0.8 mL/min. The limits of detection (LoD) and quantification (LoQ) of AFs were 0.01 and 0.05 µg/kg, respectively. The contents were calculated from a calibration curve established with AFs (TSL-108, Biopharm Rhône Ltd, Glasgow, UK).

5.2.15 Statistical analysis

Experiments were conducted in triplicates. Statistical analysis was carried out using SPSS version 24.0 for Windows (IBM Corporation, New York, USA). Data was analyzed using

One-way ANOVA and means were compared using Fischer's Least Significant Difference Test ($p<0.05$).

5.3 Results and discussion

5.3.1 Survival and growth of *Aspergillus flavus* and change in water activity during processing

5.3.1.1 Milling

Aspergillus flavus survived and continued to grow in BGF, MZF and MBCF after milling (Figures 5.1–5.3). The initial population of *A. flavus* in the flour samples was 6.30 Log_{10} CFU/g. After 96 h of incubation, the population of *A. flavus* significantly ($p\leq0.05$) increased in BGF (7.15 Log_{10} CFU/g), while a significant ($p\leq0.05$) decrease was recorded in MZF (6.02 Log_{10} CFU/g) and MBCF (4.10 Log_{10} CFU/g). A reduction in water activity of the flour samples was recorded during incubation and being significantly ($p\leq0.05$) different among all the samples tested. These findings show that milling did not eliminate the proliferation of *A. flavus* in Bambara groundnut and its maize composite flour. Bambara groundnut flour also provided a richer substrate for *A. flavus* growth than MZF or MBCF. The presence of molds including *Aspergillus*, was detected in 96% of flour samples obtained after wheat conditioning and milling (Berghofer *et al.*, 2003).

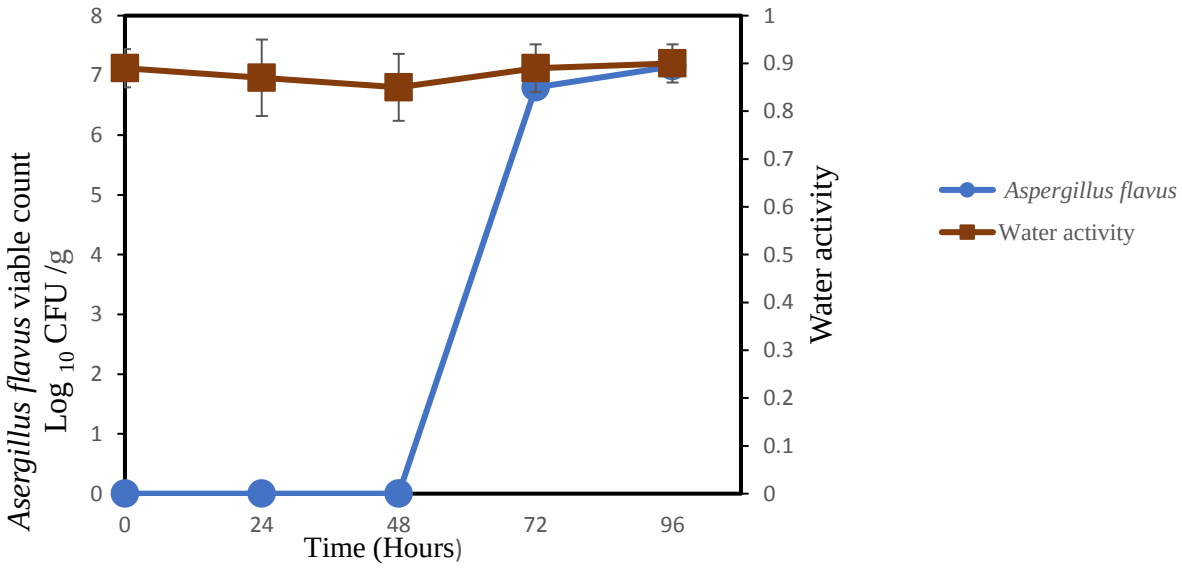

Figure 5.1 *Aspergillus flavus* viable count and water activity in Bambara groundnut flour incubated at 25 °C for 96 h.

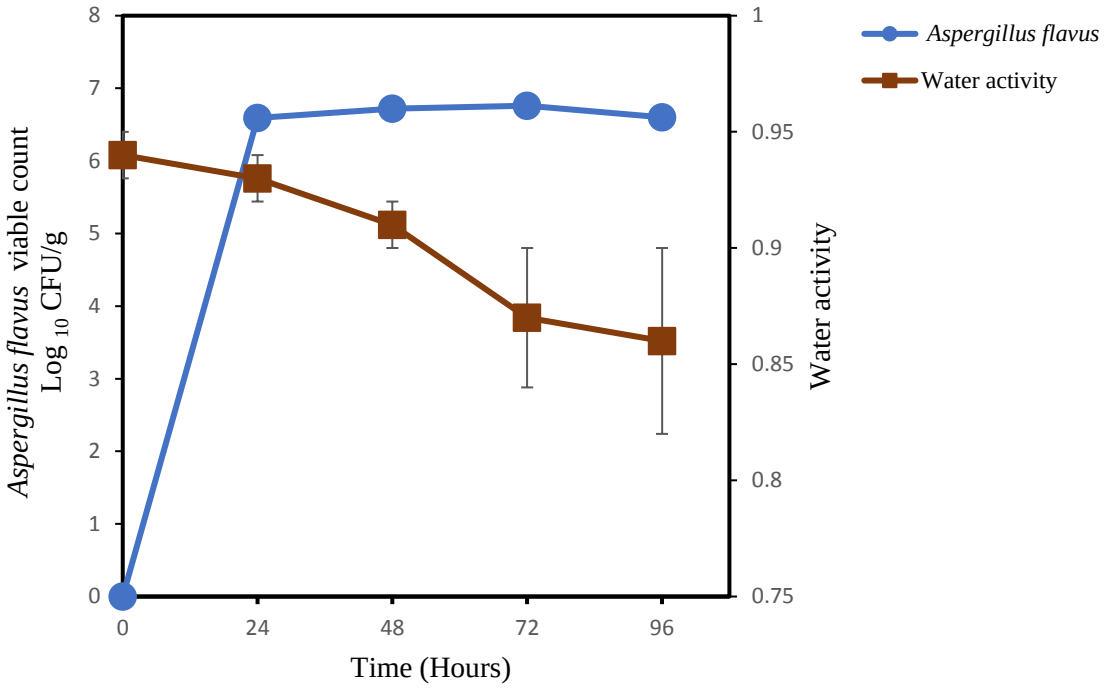

Figure 5.2 *Aspergillus flavus* viable count and water activity in maize flour incubated at 25 °C for 96 h.

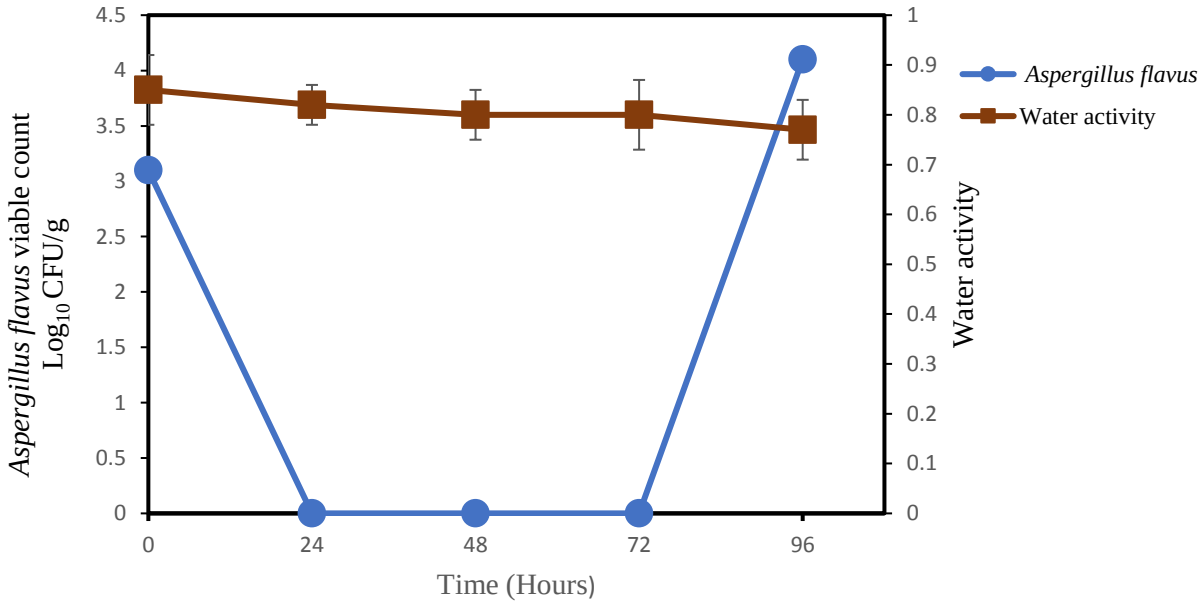

Figure 5.3 *Aspergillus flavus* viable count and water activity in maize-bambara composite flour incubated at 25 °C for 96 h.

5.3.1.2 Lactic acid bacteria fermentation

Population of *A. flavus* decreased within 24 h of fermentation to 5.59 Log_{10} CFU/mL and 5.73 Log_{10} CFU/mL in BGF fermented with *L. fermentum* and *L. plantarum*, respectively. *Lactobacillus fermentum* showed an increase in population from 8.54 to 13.37 Log_{10} CFU/mL from 0 to 24 h of fermentation followed by a decrease after 72 h to 12.25 Log_{10} CFU/mL. A further increase to 13.03 Log_{10} CFU/mL was observed after 96 h (Figure 5.4). The population of *L. plantarum* progressively increased with fermentation period from 8.5–13.41 Log_{10} CFU/mL (Figure 5.5). A complete elimination of fungal spores was recorded after 24 h and throughout the fermentation period, while the population of *A. flavus* in the control sample increased up to 72 h (Figure 5.6).

Lactococcus and *Lactobacillus* genera of LAB have been studied for their antifungal properties (Roger *et al.*, 2015). Various mechanisms are suggested in the inhibition of fungal growth by LAB and relate to the yield of organic acids, production of antagonistic compounds and competition for nutrients (Kachouri *et al.*, 2014). Ogunbanwo *et al.* (2004) reported the production of lactic acid, hydrogen peroxide, di-acetyl and bacteriocin in LAB. *Lactobacillus plantarum* is also reported to produce phenyllactic acid and 4-hydroxyphenyllactic acid (Lavermicocca *et al.*, 2000). The mechanism of fungal inhibition may be explained by the diffusion of acids into the membrane of target organisms, thus cytoplasmic pH is reduced, and metabolic activities stop (Dalié *et al.*, 2010). The electrochemical potential of the plasmic membrane may also be neutralized, and its permeability increased by the acids, leading to death of the vulnerable organism. In the absence of oxygen, hydrogen peroxide produced by LAB may not be degraded, and its accumulation may result in oxidation of liquid membrane and cellular protein of the susceptible organism (Dalié *et al.*, 2010). The findings from this study agree with several other reports. Roger *et al.* (2015) reported a complete inhibition of *A. flavus* growth after 24 h of fermentation with *L. buchneri* M11, *L. brevis* G11 and *L. cellobiosus* M41, while the *A. flavus* control sample exhibited an increase in the number of spores during incubation. In the same study, an increase in the growth of *L. fermentum* N25 and *L. fermentum* N33 was recorded until 120 h of fermentation with a growth range of 3.3–6.3 Log_{10} CFU/g. Kachouri *et al.* (2014) also reported mold reduction by 86.3% in olives inoculated with *L. plantarum* during storage, while *L. casei* CGMCC1.62 also showed some antifungal activity against *A. flavus* in liquid medium (Zuo *et al.*, 2012). Mold population including *A. flavus* decreased at 0 h from 6.8 to 3.7 Log_{10} CFU/g after 12 h of fermentation of *Ogi* (fermented corn paste)

without any further detection throughout the fermentation period, while the population of LAB increased at 0 h from 4.65 to 7.0 Log_{10} CFU/g at 48 h of fermentation (steeping) (Omemu, 2011).

The complete elimination of fungal spores after 24 h of fermentation as reported in this study can be attributed to the production of antifungal metabolites within this period. In a study by Sathe *et al.* (2007), antifungal activity was initially noted in *L. plantarum* at 12 h when the culture was in the early logarithmic phase of growth. At late logarithmic phase (36 h), maximum antifungal titre was recorded and remained high over the next 12 h until the culture entered the stationary phase. This decline during the stationary phase was attributed to a conversion to other metabolites or a degradation by autolytic enzymes.

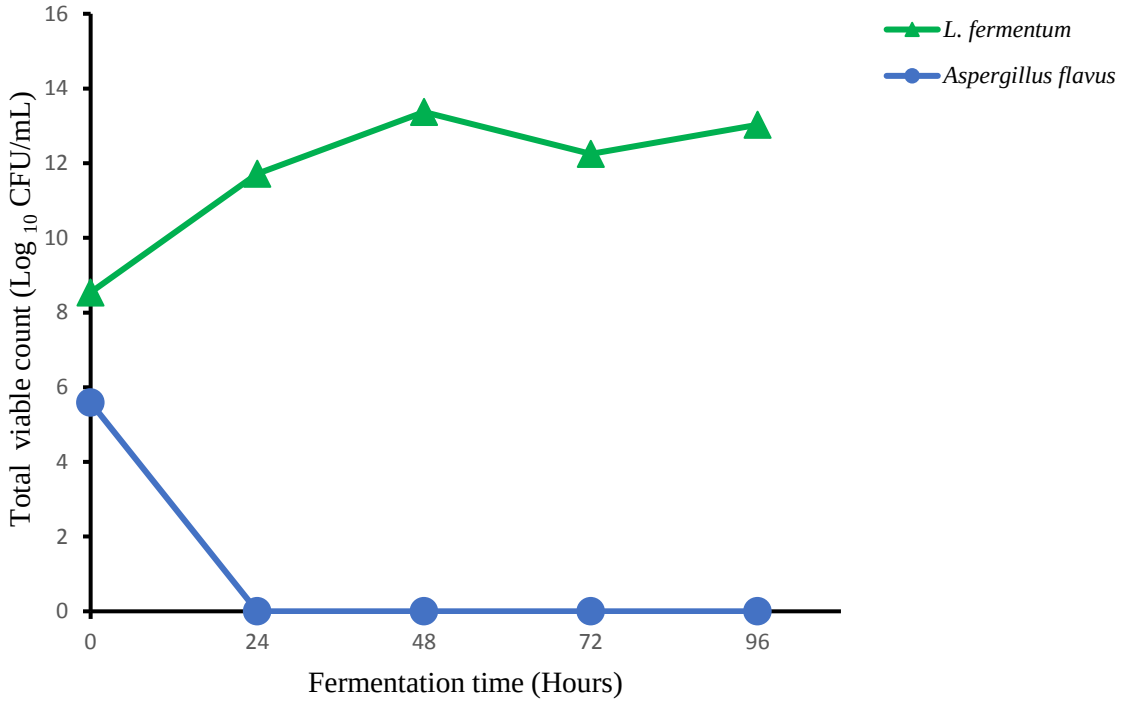

Figure 5.4 *Lactobacillus fermentum* and *Aspergillus flavus* viable counts in Bambara groundnut flour during fermentation at 25 °C for 96 h.

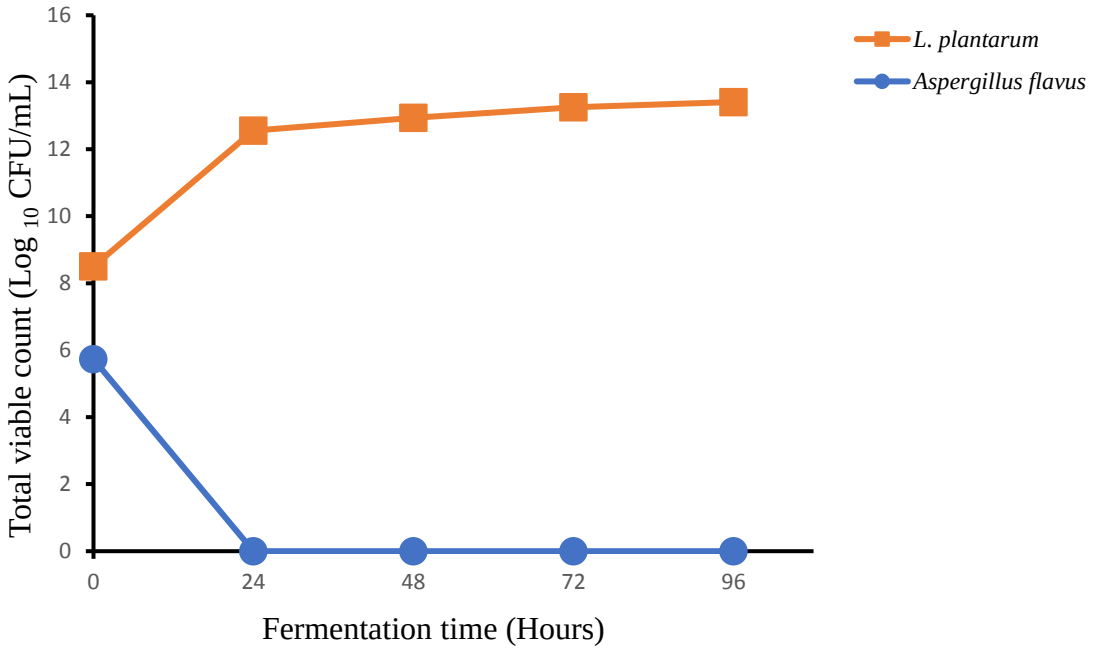

Figure 5.5 *Lactobacillus plantarum* and *Aspergillus flavus* viable counts in Bambara groundnut flour during fermentation at 25 °C for 96 h.

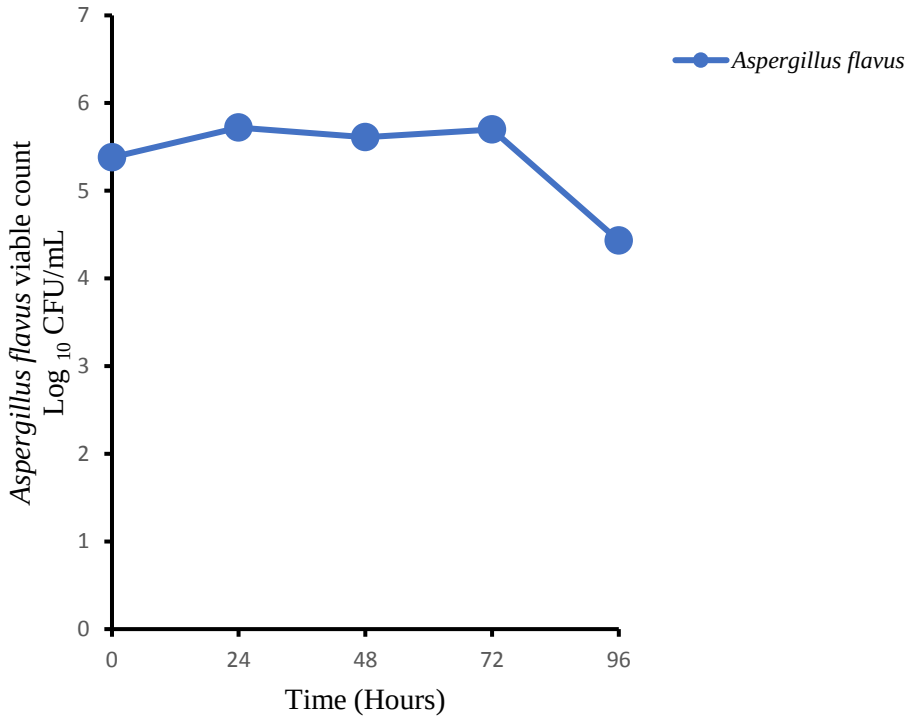

Figure 5.6 *Aspergillus flavus* viable count in Bambara groundnut flour incubated at 25 °C for 96 h.

5.3.1.3 Roasting

Aspergillus flavus spores were eliminated by roasting at 140 °C for 20 min in artificially inoculated Bambara groundnut seeds, without any fungal growth noted in the flour samples over the period of incubation. Water activity of the seeds was reduced to 0.76 a_w after roasting without significant ($p \geq 0.05$) increase in the values recorded for the flour samples over the 96 h of incubation (Figure 5.7). Nkwonta *et al.* (2015) reported complete prevention of fungal presence in non-stored walnuts at early maturity toasted at 200 °C for 60 min, and attributed it to exposure to high temperature conditions, which resulted in the destruction of the spore deposits on the nut shells. Barcelo *et al.* (2017) also reported complete elimination of fungal contaminants in coffee beans treated to medium roasting at 200°C for 30 min. Drying by heat treatment or forced air at 50–70°C can lead to death of toxigenic fungi (Tolosa *et al.*, 2013). In section 4.3.4 of Chapter Four, fungal spores were not eliminated in naturally contaminated Bambara groundnut seed roasted under similar conditions, which led to visible fungal growth in the flour during storage. The discrepancy in the reports can be explained by the ineffectiveness of heat treatment to penetrate and eliminate viable fungi present in the internal tissues of the seeds.

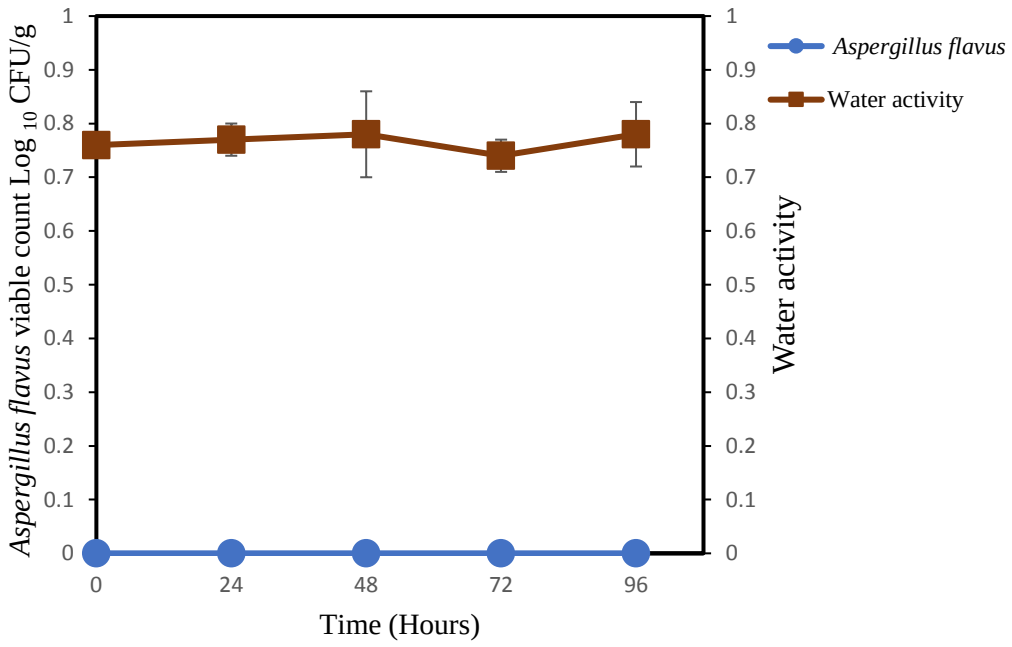

Figure 5.7 *Aspergillus flavus* viable count and change in water activity in roasted Bambara groundnut flour incubated at 25 °C for 96 h.

5.3.2 Total aflatoxin production by *Aspergillus flavus* during processing of Bambara groundnut and maize composite flour

Total AF concentration in BGF, RBGF, MZF and MBCF treated with *A. flavus* over the incubation period as determined by ELISA are presented in Table 5.1. *Aspergillus flavus* spores were eliminated in roasted flour sample at day 0, and in LAB fermented samples after 24 h. This may explain the non-detection of AF, a secondary metabolite of the fungus in the LAB fermented samples. The growth of *A. flavus* in BGF, MBCF and BGF treated with *A. flavus* recorded at different times in the stored samples equally did not produce any detectable quantities of AF. Mold contamination in foods may not correlate with mycotoxin production (Ashiq, 2015). Further to this, two strains of *A. flavus* exist based on physiological, morphological, and genetic criteria, and are designated as S- and L-strains. Isolates of S-strain produce more aflatoxin than L-strain isolates while many L-strain isolates produce little or no aflatoxin (Garber and Cotty, 1997). However, AF was detected in MZF at measurable levels of 4.04 and 2.67 ng/mL, after 24 and 72 h, respectively. Aflatoxin production might have equally occurred at other times but at levels below the detection limit of 2.5 ng/g provided by the assay kit. Many foodstuffs support the growth of *A. flavus*, particularly groundnut and maize (Egal *et al.*, 2005). Rapid growth of *A. flavus* in the MZF may also explain the measurable AF concentration in the samples.

Table 5.1 Total aflatoxin concentration (ng/mL) in Bambara groundnut and maize composite flour

Day	BGF	RBGF	MZF	MBCF
0	-	-	-	-
1	ND	ND	4.04^{a}±0.01	ND
2	ND	ND	ND	ND
3	ND	ND	2.67^{b}±0.02	ND
4	ND	ND	ND	ND

-, not determined; ND, not detected; Limit of detection is 2.5 ng/g; BGF-Bambara groundnut flour; RBGF-Roasted Bambara groundnut flour; MZF-Maize flour; MBCF-Maize-bambara composite flour.
Values represent mean ±standard deviation (N=3). Means with same letter within the column are not significantly different ($p \geq 0.05$).

5.3.3 Change in pH and total titratable acidity in Bambara groundnut flour during fermentation

A decrease in pH was observed in FBGF from 6.19 to 4.12, and from 6.21 to 4.08 from 0 to 96 h with *L. fermentum* and *L. plantarum*, respectively (Figure 5.8). The observed decrease in pH was significantly ($p \leq 0.5$) different over the fermentation period in both LAB fermented samples. Concurrent with pH reduction was an increase in TTA of the samples,

measured as the percentage of lactic acid produced by both LAB (Figure 5.9). Bambara groundnut flour fermented with *L. fermentum* recorded an increase in TTA from 0.77 to 1.87% over the 96 h fermentation, while a similar increase from 1.26 to 1.85% was recorded in BGF fermented with *L. plantarum* over the same period. The control sample inoculated with only spores of *A. flavus*, exhibited only a significant ($p \leq 0.5$) decrease in pH after 48 h from 6.22 to 6.05, and finally to 4.82 after 96 h.

Lactic acid bacteria produce acetic acid and lactic acid during hydrolysis of carbohydrate (Roger *et al.*, 2015), as part of mechanism to inhibit fungal growth and aid preservation of food commodities. The decrease in pH can be attributed to an increased production of lactic acid during fermentation as has been reported elsewhere. A significant decrease in pH and increase in TTA was recorded as fermentation progressed during the production of *Akamu*, a maize infant complimentary food with *L. plantarum* (Obinna-Echem *et al.*, 2015). Omemu (2011) reported a decrease in pH, and an increase in TTA from 0.1 to 0.4% during the fermentation of *Og*i. Ali and Mustafa (2009) also reported a reduction in pH from 4.33 to 3.47 after 6 h of fermentation of sorghum flour (variety dabar). The decrease in pH observed in the control sample may be attributed to the hydrolytic action of fungal enzymes on the substrate. The decrease in pH had a positive influence on the growth of LAB over time in this study as recorded by the increase in population. Lactic acid bacteria are able to multiply at weak pH because of their acidophilic nature (Roger *et al.*, 2015), with the genus *Lactobacillus* as the most aciduric of all (Ogunbanwo *et al.*, 2004). The decrease in pH reported by Ali and Mustafa (2009) was accompanied with an increase in the population of *Lactobacillus* from 8.31×10^7 to 8.20×10^8 CFU/g after 4 h. Obinna-Echem *et al.* (2015) also reported an increase in microbial growth concomitant with pH decrease.

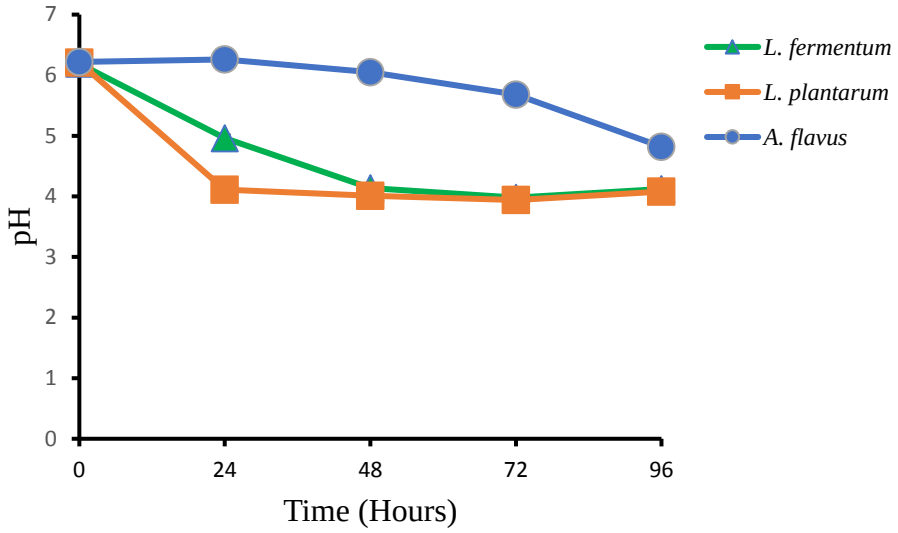

Figure 5.8 pH of *Lactobacillus fermentum*, *Lactobacillus plantarum*, and *Aspergillus flavus* (control) fermented Bambara groundnut flour incubated at 25 °C for 96 h.

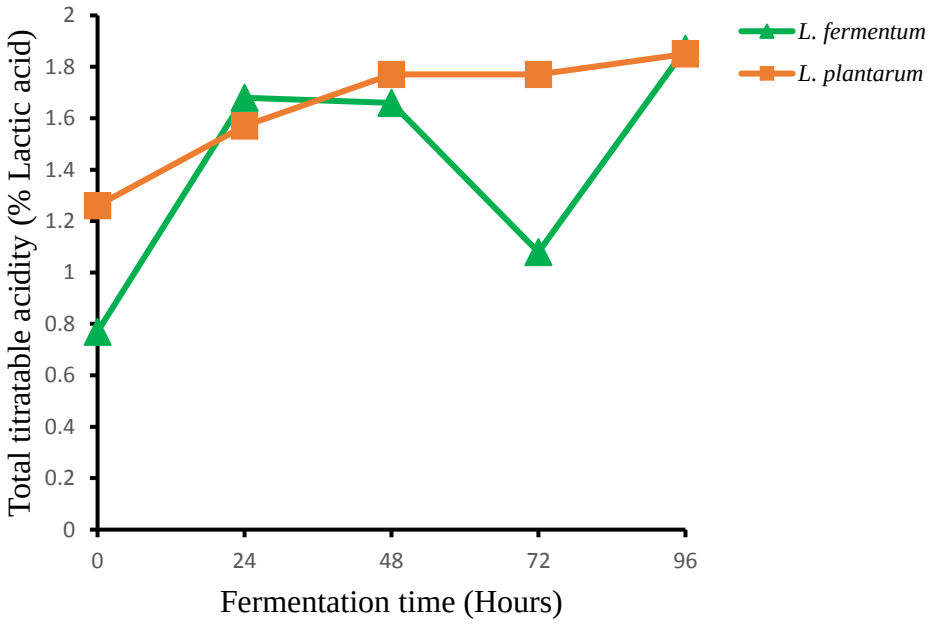

Figure 5.9 Total titratable acidity (% lactic acid) of Bambara groundnut flour fermented with *Lactobacillus fermentum* and *Lactobacillus plantarum* at 25 °C for 96 h.

5.3.4 Aflatoxin B_1 reduction by *Lactobacillus fermentum* and *Lactobacillus plantarum*

The percentage reduction of AFB_1 in BGF by both *L. fermentum* and *L. plantarum* are shown in Figure 5.10. The initial concentration of AFB_1 in the fermenting slurries was 5 µg/kg. At 0 h of fermentation, a decrease to 0.54 µg/kg (89.2%) was recorded in BGF fermented with *L. fermentum*. Similarly, the concentration of AFB_1 in *L. plantarum* fermented sample decreased to 1.25 µg/kg (75%) at 0 h. A significant ($p \leq 0.5$) decrease was observed in the percentage reductions over the 96 h of fermentation with both LAB, with a final decrease of 50.4 and 55% by *L. fermentum* and *L. plantarum*, respectively.

Various studies have reported the ability of LAB to reduce mycotoxins. However, the mechanism of AF removal by LAB remains unclear. It has been suggested to depend on fractions of the cell wall skeletons of LAB, which are able to bind with mutagens through non-covalent bonds (Roger *et al.*, 2015). This theory has been supported by other researchers. According to Bueno *et al.* (2007), *in vitro* removal of AFB_1 by LAB occurs via adhesion to cell wall components rather than covalent binding or metabolic degradation, and probably involves weak Van der Waals bonds, hydrogen bonds or hydrophobic interactions. The cell wall of LAB such as *Leuconostoc* and *Streptococcus* are able to bind mutagens, which include amino acid pyrolysates and heterocyclic amino acids produced during cooking (Dalié *et al.*, 2010). Another theory suggests that LAB fermentation opens the AFB_1 lactone ring resulting in its complete detoxification. A decrease in pH has also been hypothesized to contribute to the removal of AFB_1 (Roger *et al.*, 2015).

The reduction in AFB_1 concentration by LAB reported in this study has also been reported elsewhere. Assohoun *et al.* (2013) reported a reduction in AFB_1 concentration (about 80%) and a decrease in pH from 5.4 to 2.2 at 48 h during the processing of *Doklu*, a fermented maize-based food. Roger *et al.* (2015) similarly reported a decrease in concentration of AFB_1 in 'Kutukutu' fermented with *L. fermentum* N 33 (21.8%), *L. brevis* G 25 (19.1%) after 24 h of incubation, and with *L. buchneri* M11 (64.2%) and *L. brevis* G25 (63%) after 120 h. Similar reduction in pH was reported during the same study. Kachouri *et al.* (2014) reported a decrease in AFB_1 level from 11 to 5.9 µg/kg at day 0 in naturally contaminated olives inoculated with *L. plantarum*. The amount of AFB_1 removed by LAB is toxin- and bacteria concentration-dependent, it is a fast (no more than 1 min) and reversible process without chemical modification of AFB_1 (Bueno *et al.*, 2007). The speed and reversibility of the

reaction may account for 75–89.2% reduction of AFB_1 by both LAB at 0 h, and the significant differences in percentage reduction during the incubation period.

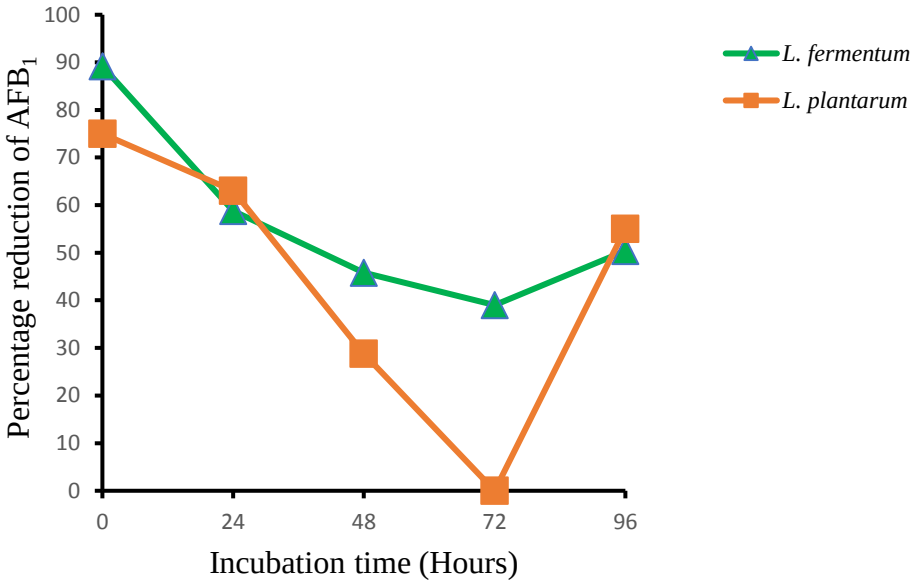

Figure 5.10 Percentage reduction of AFB_1 by *Lactobacillus fermentum* and *Lactobacillus plantarum* in Bambara groundnut flour incubated at 25 °C for 96 h.

5.4 Conclusion

Food processing operations such as milling, roasting, and LAB fermentation had varied effects on the survival and aflatoxin production of *A. flavus* in Bambara groundnut and maize composite flour. Milling did not prevent *A. flavus* growth in the flour samples, although AF production was not detected under the experimental conditions. Roasting eliminated spores of *A. flavus* on Bambara groundnut and consequently, prevented AF production. Lactic acid bacteria achieved complete inhibition of *A. flavus* growth and a significant reduction of AFB_1 within 24 h of incubation. Compositing Bambara groundnut with maize similarly had no detectable effect on survival and AF production of *A. flavus* under the experimental conditions. Bambara groundnut flour can be decontaminated by roasting and fermentation using *L. fermentum* or *L. plantarum.* Similar study over an extended period of storage is suggested to understand more clearly the behaviour of *A. flavus* in Bambara groundnut and its maize composite flour.

CHAPTER SIX

Growth and aflatoxin production of *Aspergillus flavus* in Bambara groundnut (*Vigna subterranea* (L.) Verdc) as affected by milling, fermentation or roasting during storage

Abstract

Fungal contamination and mycotoxin accumulation in agricultural products are influenced markedly by processing and storage conditions. This study aimed at determining the growth of *Aspergillus flavus* and aflatoxin (AF) production in Bambara groundnut flour (BGF) processed by milling, lactic acid bacteria (LAB) (*Lactobacillus plantarum*) fermentation or roasting at 140 °C for 20 min and stored for up to 10 weeks at 25±2 °C and 75±2% relative humidity. It also studied the behaviour of *A. flavus* in maize-bambara composite flour (MBCF). Processed and irradiated flour samples were inoculated with 2 x 10^7 spores/ml of *A. flavus* and stored. Samples were withdrawn weekly and analysed for viable populations of *A. flavus*, concentrations of AFB_1, AFB_2, AFG_1 and AFG_2 using HPLC-Fluorescence detection method, and changes in water activity values. *Aspergillus flavus* population significantly ($p \leq 0.05$) decreased in roasted Bambara groundnut flour (RBGF) (7.18–2.00 Log_{10} CFU/g) over the storage period, and in fermented Bambara groundnut flour (FBGF) (6.72–2.67 Log_{10} CFU/g) after 7 weeks of storage and beyond was not detected. Significant ($p \leq 0.05$) decrease in AFB_1 (0.36–0.26 µg/kg) and AFG_1 (0.15–0.07 µg/kg) production was also recorded in RBGF over the storage period. Conversely, AFB_1 concentration in the composite flour significantly ($p \leq 0.05$) increased from 1.17 to 2.05 µg/kg over the storage period. Lactic acid bacteria fermentation, roasting and compositing markedly influenced the growth of *A. flavus* and AF production in Bambara groundnut and maize flours during storage.

Key words: *Aspergillus flavus*, Bambara groundnut, Maize, HPLC, Aflatoxin, *Lactobacillus plantarum*

6.1 Introduction

Contamination of agricultural crops by mycotoxigenic fungi and their toxic metabolites is a worldwide food security challenge. Mycotoxigenic fungi are often present in the soil or air and contaminate food commodities while in the field, during processing, transportation or storage (Bhat *et al.*, 2010; da Rocha *et al.*, 2014). The ability of food pathogens to survive and produce toxins during storage is governed by various ecological and environmental conditions, particularly the nature of the substrate, temperature and relative humidity (Afsah-

Hejri *et al.*, 2013; Ashiq, 2015). The use of different processing methods to reduce fungal growth and mycotoxin accumulation in food matrices are documented (Yazdanpanah *et al.*, 2005; Roger *et al.*, 2015; Barcelo *et al.*, 2017). Fungal growth and toxin production in food commodities during storage occurs much rapidly in regions with higher temperature and relative humidity conditions (Sales and Yoshizawa, 2005; Ashiq, 2015). However, climate change due to global warming portends the prevalence of aflatoxigenic fungi and AF production in temperate regions where incidences of fungal contamination and toxin production rarely occur (Paterson and Lima, 2010; Baranyi *et al.*, 2014).

Bambara groundnut is an African legume with a high protein content of 24.67% (Murevanhema and Jideani, 2013; Kaptso *et al.*, 2015). It also provides a source of vitamins and minerals (Forsythe *et al.*, 2015), usually incorporated into cereal flours to improve the protein content and flour properties to be utilized in several food applications. Bambara groundnut from Southern Africa is susceptible to *A. flavus* contamination (Kola, 2003; Shabangu, 2009; Olagunju *et al.*, 2018b) and accumulation of AF in the flour during storage in the tropics has been reported (Olagunju *et al.*, 2018a). However, further studies are required to understand the behaviour of *A. flavus* in BGF as affected by processing methods under simulated temperate conditions of storage. To study the effect of compositing maize and BGF on *A. flavus* growth and AF production is also of importance. The objective of the study, therefore, was to investigate the growth and aflatoxigenic ability of *A. flavus* in BGF as affected by milling, fermentation or roasting during storage under simulated temperate conditions.

6.2 Materials and methods

6.2.1 Materials

Cream variety of Bambara groundnut obtained from Southern Africa was used throughout the experiment. Dirts including shells, stones and other extraneous substances were removed by handpicking. Maize seeds used in the experiment were obtained from a retail store in Durban.

6.2.2 Preparation and treatment of flours

Cleaned seeds of Bambara groundnut were dehulled and dried to a constant weight as described in section 5.2.2 of Chapter Five. Maize seeds were also dried in a like manner before use. The dried seeds were divided into three portions and processed by milling, roasting or fermentation.

6.2.2.1 Milling

A portion of Bambara groundnut seeds (2 kg) and maize (2.5 kg) were milled using a blender, to pass through a 710 µm sieve (Universal Laboratory Test Sieve, 128921). Flour was packed in double-seal Tuff lock bags and subjected to gamma irradiation (20 kGy) to eliminate all viable fungi or bacteria present.

6.2.2.2 Fermentation

About 2 kg of already irradiated flour sample was fermented using *L. plantarum* obtained from the culture collection of the Department of Biotechnology and Food Technology and grown as reported in section 5.2.3 of Chapter Five. Cell suspension was adjusted to 8.0 Log_{10} CFU/mL using a McFarland standard tube 0.05 as described in section 5.2.3 of Chapter Five. Under a laminar flow chamber, 30 g BGF was uniformly mixed with 60 mL sterile water in 100 mL sterile sample container and inoculated with 1 mL (1 x 10^8 CFU/mL) spore suspension of *L. plantarum*. Inoculated slurry was incubated at 30 °C for 72 h. After fermentation, slurries were dried at 40 °C to a constant weight using a sterile convection oven. The dried mass was milled to flour using a sterile blender under a laminar flow. Fermented flour was packed in a sealed polyethylene bag and stored at 4 °C until use.

6.2.2.3 Roasting

Dehulled and dried Bambara groundnut seed weighing about 2 kg was roasted at 140 °C for 20 min and milled into flour as described in section 6.2.2.1. Roasted flour packed in Tuff lock bag was also subjected to gamma irradiation (20 kGy) and stored at 4 °C before use. The absence of viable cells of fungi and bacteria in all gamma irradiated Bambara groundnut and maize flours was confirmed by plating 1 mL of stock solution using PDA and Tryptic Soy Broth (TSB).

6.2.3 *Aspergillus flavus* strains and inoculum preparation

Aspergillus flavus strains BG01 and BG02 isolated from Bambara groundnut and identified by sequencing, and ATCC 9346 obtained from Anatech Analytical Laboratory, Guateng, South Africa were cultured as described in section 5.2.4 of Chapter Five. Spores were harvested and a 3-strain cocktail of *A. flavus* was prepared using the method reported in the same section. Total viable cells were determined from a mixture of 100 µL of spore suspension and 100 µL of Lactophenol blue (Merck, Germany). The cell suspension was viewed under the microscope (Nikon, Eclipse E200, China) using a hemocytometer. Cell suspension was adjusted at 2 x 10^7 spores/mL and stored at 4 °C until use in the experiment.

6.2.4 Inoculation and storage of flour samples

Thirty grams each of BGF, FBGF, RBGF, maize flour (MZF) and MBCF (70:30) was aseptically weighed into sterile petri plates under a laminar flow chamber. Each flour sample was then spot-inoculated with 100 µL of *A. flavus* suspension at 5 randomly selected positions. All samples were stored at 25±2 °C and 75±2% relative humidity for a period of 10 weeks. Samples were withdrawn weekly and analyzed for *A. flavus* counts, water activity and AFs production. Duplicate samples were prepared for each treatment and the whole experiment was repeated twice.

6.2.5 Determination of growth of *Aspergillus flavus* during storage

At each sampling period, 10 g of each flour sample was mixed with 90 mL sterile Ringer's solution and pummelled in a stomacher for 2 min. The homogenate was then transferred into a sterile sample bottle and 1 mL serially diluted in sterile Ringer's solution to 10^{-5}. One milliliter (1 mL) of the stock solution and all dilutions were plated on PDAT (pH 3.5). Plates were stored at 25 °C for 5 days. The number of colonies on each plate after incubation was determined using a colony counter, CFU/g of sample calculated, and results expressed in Log_{10} CFU/g as reported in section 5.2.11 of Chapter Five.

6.2.6 Determination of water activity

Water activity of the samples was measured as described in section 4.2.8 of Chapter Four using a calibrated Lab Swift water activity meter (Novasina AG CH8853 Lachen, Switzerland)**.** Readings were taken at room temperature and average of duplicate determinations was recorded.

6.2.7 Determination of pH

The pH of fermented Bambara groundnut flour samples during storage was determined at each sampling time. The pH meter (Jenway, UK) was initially calibrated using suitable buffers and readings were carried out at room temperature as described in section 5.2.9 of Chapter Five. Average of duplicate determinations was recorded.

6.2.8 Determination of aflatoxin production during storage

The concentration of AFs in stored samples was determined following the protocol already reported in section 5.2.14 of Chapter Five. Each sample (25 g) was homogenized with 50 mL methanol-water (80:20, v/v) and 5 g of NaCl at high speed for 2 min with a blender (Waring, France). The extract was centrifuged at 6000 rpm for 10 min and 2 mL of the supernatant diluted with 18 mL of PBS buffer. Ten mL of this diluted sample was passed

through an immunoaffinity (IAC) column (Aflaprep, R-Biopharm). The IAC was washed twice with 10 mL of PBS each time before being eluted with 2 mL methanol. The eluting fraction was then evaporated and 1 mL of methanol-water (50/50, v/v) added. The obtained fraction was collected into a glass bottle, identified and quantified using HPLC with spectrofluorescence detection (Shimadzu RF 20A, Japan) after post-column derivatization with electrochemical system (Kobra Cell™ R. Biopharm Rhône Ltd, Glasgow, UK). Fluorescence detector for AFs detection was set at excitation and emission wavelengths of 365 and 435 nm, respectively. The mobile phase used consisted of water-methanol (55/45, v/v), 119 mg of potassium bromide and 350 µL of nitric acid. Aflatoxin standard solutions were used for the construction of a five-point calibration curve of peak areas versus concentrations (ng/mL). The operating conditions were as follows: injection volume of 100 µL of sample and standard solutions; C_{18} reverse-phase HPLC column, Uptisphere type, ODS, 5 µm particle size, 5 ODB, 250 x 4.6 mm, with identical pre-column, thermostatically controlled at 40 °C; and an isocratic flow rate of 0.8 mL/min. The detection and quantification limits of AFs were 0.01 and 0.05 µg/kg, respectively. The contents were calculated from a calibration curve established with AFs (TSL-108, Biopharm Rhône Ltd, Glasgow, UK).

6.2.9 Statistical analysis

All experiments were conducted in duplicates. Statistical analysis was carried out using SPSS version 24.0 for Windows (IBM Corporation, New York, USA). Data were analyzed using One-way ANOVA. The Fischer's Least Significant Difference Test ($p \leq 0.05$) was used to compare means.

6.3 Results and discussion

6.3.1 Growth of *Aspergillus flavus* in Bambara groundnut flour as affected by processing method during storage

Table 6.1 shows the growth and survival of *A. flavus* in inoculated BGF, FBGF and RBGF stored at 25±2 °C and 75±2% relative humidity for up to 10 weeks. The initial populations of *A. flavus* in BGF, FBGF, and RBGF were 6.72–7.18 Log_{10} CFU/g of flour sample. The population of *A. flavus* recovered in BGF (6.85 Log_{10} CFU/g), RBGF (5.76 Log_{10} CFU/g), and FBGF (4.07 Log_{10} CFU/g) decreased significantly ($p \leq 0.05$) during storage for one week. A significant ($p \leq 0.05$) decrease in the population of *A. flavus* from 6.72 Log_{10} CFU/g to 4.07 Log_{10} CFU/g recorded in FBGF after one week of inoculation and storage suggests that growth of the fungus was not well supported by FBGF. This may be attributed to the acidity

(pH 4.01) of the flour sample due to *L. plantarum* fermentation (Table 6.1). Fermentation of carbohydrates by LAB leads to the production of lactic acid and acetic acid, which have the ability to stop metabolic activities in susceptible organisms by reducing cytoplasmic pH (Dalié *et al.*, 2010). In section 5.3.1.2 of Chapter Five, *A. flavus* spores were eliminated after 24 h in BGF fermented using *L. fermentum* and *L. plantarum*. Significant differences were observed when determining the population of *A. flavus* recovered from BGF, FBGF, and RBGF up till 7 weeks of storage with higher recovery of *A. flavus* in BGF than other substrates. Growth of *A. flavus* was not detected in FBGF after inoculation and storage for 7 weeks, and over the remaining storage period. The population of *A. flavus* in RBGF significantly ($p \leq 0.05$) decreased from 7.18 Log_{10} CFU/g at the start of the experiment to 2.00 Log_{10} CFU/g after storage for 10 weeks.

The introduction of an organism into a new environment can either lead to its survival or death. In a bid to survive, the organism becomes adapted to a two-stage process involving changes in the cell's thermodynamics and alteration of metabolic activities, enzyme synthesis and enzyme activity, during cell growth (Lenovich, 1987:121). Nutrient status of a substrate and other factors such as pH, water activity, temperature of processing and storage affect the growth and survival of fungi in foods. According to Pitt & Hocking (1997:10), fungal metabolism is best suited to substrates high in carbohydrates. The higher population of *A. flavus* recovered in BGF when compared to that of FBGF and RBGF over the storage period suggests that BGF provides a more favourable substrate to support growth of *A. flavus*. The survival of a microorganism in a low water activity environment is influenced by factors such as water-binding properties, pH, nutritive potential, E_h, and the presence of antimicrobial compounds (Lenovich, 1987:123). Growth and survival of *A. flavus* was significantly ($p \leq 0.05$) decreased in *L. plantarum* -FBGF over the storage period. The decrease in fungal population can be attributed to the presence of antifungal compounds in the flour. Such antifungal compounds, phenyllactic acid and 4-hydroxyphenyllactic acid, produced by *L. plantarum* have been reported (Lavermicocca *et al.*, 2000).

Table 6.1 pH and viable counts of *Aspergillus flavus* (Log_{10} CFU/g) in processed Bambara groundnut flour during storage

	Viable counts			pH
Period (weeks)	BGF	FBGF	RBGF	FBGF
0	B 6.94^{c} (0.51)	C 6.72^{d} (0.83)	A 7.18^{a} (0.61)	4.11^{d} (0.06)
1	A 6.85^{d} (0.36)	C 4.07^{e} (0.21)	B 5.76^{h} (0.89)	4.01^{d} (0.01)
2	A 7.24^{a} (0.97)	B 6.85^{a} (0.36)	B 6.86^{c} (0.56)	4.02^{d} (0.07)
3	C 6.16^{g} (0.39)	B 6.76^{c} (0.66)	A 6.92^{b} (0.24)	4.12^{d} (0.00)
4	A 5.93^{h} (0.85)	C 4.02^{f} (0.71)	B 5.01^{i} (0.83)	4.09^{d} (0.07)
5	A 6.82^{e} (0.85)	B 6.78^{b} (0.60)	C 5.93^{g} (0.19)	4.09^{d} (0.03)
6	A 6.85^{d} (0.21)	ND	B 6.80^{d} (0.75)	4.07^{d} (0.06)
7	A 6.85^{d} (0.71)	C 2.67^{g} (0.71)	B 6.74^{e} (0.70)	4.17^{d} (0.08)
8	A 7.08^{b} (0.07)	ND	B 6.30^{f} (0.02)	4.46^{c} (0.10)
9	A 6.76^{f} (0.14)	ND	B 3.20^{j} (0.14)	4.76^{b} (0.12)
10	A 6.83^{e} (0.71)	ND	B 2.00^{k} (0.06)	6.50^{a} (0.01)

Values represent mean (standard deviation) of duplicate determinations. Means followed by the same superscripts within the column are not significantly different ($p \geq 0.05$). Means preceded by the same uppercase letter along the row are not significantly different ($p \geq 0.05$). BGF- Bambara groundnut flour, FBGF- Fermented Bambara groundnut flour, RGBF- Roasted Bambara groundnut flour.
ND, not detected.

6.3.2 Aflatoxin production by *Aspergillus flavus* in Bambara groundnut flour as affected by processing method during storage.

Tables 6.3–6.6 show the ability of *A. flavus* to produce AF in processed and inoculated BGF samples during storage. Aflatoxin B_1, B_2, G_1, and G_2 were detected in all the flour samples over the storage period. Aflatoxin production by *A. flavus* in the flour samples was significantly ($p \leq 0.05$) different and markedly affected by storage temperature and such processing operations as fermentation and roasting. The concentrations of AFB_1 detected in BGF, FBGF, and RBGF over the storage period was higher than the concentrations of AFB_2, AFG_1 and AFG_2 in all the inoculated flour samples. The concentration of AFG_1 determined was higher than those of AFB_2 and AFG_2 in BGF, FBGF, and RBGF over the storage period. Accumulation of B and G AFs changed significantly in the flour samples during storage. Over the storage period, AFB_1 level was significantly reduced in BGF (2.86–0.24 µg/kg), FBGF (2.71–0.70 µg/kg), and RBGF (0.36–0.26 µg/kg). A similar reduction in AFG_1 concentration was noted in BGF (1.38–0.11 µg/kg), FBGF (1.18–0.10 µg/kg), and RBGF (0.15–0.07 µg/kg) but an increase in AFB_2 (0.02–0.04 µg/kg) and AFG_1 (0.03–0.07 µg/kg) levels were recorded in RBGF during storage.

The amounts and relative proportions of individual AFs produced by toxigenic fungi are influenced by the strain, nutritional status of the substrate, temperature and relative humidity (Lin *et al.,* 1980). *Aspergillus flavus* can produce some AF within 24 h and a biologically

significant quantity in a few days when conditions are optimal (Achar and Sanchez, 2006). In a study by Pildain *et al.* (2004), two strains of *A. flavus* were identified with S- strains producing high amount of only B aflatoxin or both B and G aflatoxins while L-strains produced low amounts of only B aflatoxins. The detection of both B and G aflatoxin derivatives implies that Bambara groundnut flour may support the growth and AF production by *A. flavus* S-strain.

The higher concentration of AFB_1 noted in all the samples during storage shows that *A. flavus* produces more of AFB_1 than other AFs in Bambara groundnut. Aflatoxin B_1 is the most toxic of all the AFs and has been classified as a Group 1 human carcinogen (IARC, 1993). Maximum tolerable limit of 2 µg/kg has been set for AFB_1 in foods (European Commission, 2006). The initial concentration of AFB_1 in BGF produced from healthy Bambara groundnut seeds used in this study was 3.37 µg/kg, which exceeded the recommended tolerable EU limit. Gamma irradiation and further processing reduced the AFB_1 concentrations in BGF (2.86 µg/kg), FBGF (2.71 µg/kg) and RBGF (0.36 µg/kg). In a study by Seonyeong *et al.* (2015), AFB_1 was produced at much higher concentration than AFB_2 by *A. flavus* in rough rice, brown rice and white rice during storage. Similar findings have earlier been reported by Bircan *et al.* (2008). The detection of AFG_1 at concentrations higher than those detected for AFG_2 and AFB_2 may have been influenced by the storage temperature (25±2 °C). In a study by Lin *et al.* (1980), it was found that lower temperature of 25 °C favoured AFG_1 production and accumulation cultures of *A. parasiticus* were cycled between 25 and 15 °C or held at a constant temperature of 25 °C.

Several reports have documented variations in AF production and accumulation by *A. flavus* in different food matrices (Bircan *et al.*, 2008; Seonyeong *et al.*, 2015; Olagunju *et al.*, 2018a). These variations may be attributed to the decomposition of AFB_1 to form other metabolites or related to the physiological changes that occur during the growth cycle of *A. flavus* as has been suggested by some researchers. Further studies into the biosynthetic pathways and conditions that favour AF production by *A. flavus* are necessary to clarify the complexity in AF production by *A. flavus* (Bircan *et al.*, 2008).

Table 6.2 Concentration of aflatoxin B_1 (µg/kg) in processed Bambara groundnut flour stored at 25±2 °C and 75±2% for 10 weeks

Period (weeks)	BGF	FBGF	RBGF
0	A 2.86^{a} (0.01)	B 2.71^{a} (0.02)	C 0.36^{e} (0.00)
1	C 0.87^{f} (0.01)	A 2.14^{b} (0.01)	B 0.89^{b} (0.01)
2	C 0.79^{h} (0.00)	A 1.82^{e} (0.01)	B 0.84^{c} (0.01)
3	C 0.81^{g} (0.01)	A 1.95^{c} (0.04)	B 0.92^{a} (0.04)
4	B 1.26^{b} (0.01)	A 1.50^{g} (0.01)	C 0.58^{d} (0.02)
5	B 0.95^{e} (0.03)	A 1.53^{f} (0.01)	C 0.31^{f} (0.01)
6	B 1.11^{c} (0.01)	A 1.92^{d} (0.01)	C 0.30^{fg} (0.01)
7	A 1.01^{d} (0.01)	C 0.71^{h} (0.03)	C 0.20^{i} (0.01)
8	C 0.11^{i} (0.04)	A 0.70^{h} (0.02)	B 0.36^{e} (0.01)
9	C 0.06^{k} (0.01)	A 0.70^{h} (0.01)	B 0.29^{g} (0.02)
10	C 0.24^{i} (0.01)	A 0.70^{h} (0.01)	B 0.26^{h} (0.02)

Values represent mean (standard deviation) of duplicate determinations. Means followed by the same superscript within the column are not significantly different ($p \geq 0.05$). Means preceded by the same uppercase letter along the row are not significantly different ($p \geq 0.05$). Limits of detection, and quantification are 0.01 and 0.05 µg/kg, respectively. ND, not detected. BGF-Bambara groundnut flour, FBGF-Fermented Bambara groundnut flour, RBGF-Roasted Bambara groundnut flour.

Table 6.3 Concentration of aflatoxin B_2 (µg/kg) in processed Bambara groundnut flour stored at 25±2 °C and 75±2% for 10 weeks

Period (weeks)	BGF	FBGF	RBGF
0	A 0.14^{a} (0.04)	A 0.14^{a} (0.02)	B 0.02^{d} (0.01)
1	B 0.07^{b} (0.01)	A 0.13^{ab} (0.01)	B 0.08^{a} (0.01)
2	C 0.06^{c} (0.01)	A 0.12^{bc} (0.01)	B 0.08^{a} (0.02)
3	B 0.07^{b} (0.01)	A 0.13^{ab} (0.01)	B 0.08^{a} (0.01)
4	ND	A 0.11^{c} (0.00)	B 0.06^{b} (0.04)
5	A 0.07^{b} (0.01)	C 0.04^{e} (0.00)	BC 0.05^{bc} (0.01)
6	B 0.06^{c} (0.01)	A 0.14^{a} (0.01)	B 0.05^{bc} (0.01)
7	A 0.08^{b} (0.02)	A 0.07^{d} (0.01)	B 0.04^{c} (0.00)
8	ND	A 0.07^{d} (0.03)	B 0.05^{bc} (0.01)
9	BC 0.06^{c} (0.01)	A 0.07^{d} (0.01)	B 0.05^{bc} (0.02)
10	B 0.05^{c} (0.01)	A 0.07^{d} (0.03)	B 0.04^{c} (0.02)

Values represent mean (standard deviation) of duplicate determinations. Means followed by the same superscript within the column are not significantly different ($p \geq 0.05$). Means preceded by the same uppercase letter along the row are not significantly different ($p \geq 0.05$). Limits of detection, and quantification are 0.01 and 0.05 µg/kg, respectively. ND, not detected. BGF-Bambara groundnut flour, FBGF-Fermented Bambara groundnut flour, RBGF-Roasted Bambara groundnut flour.

Table 6.4 Concentration of aflatoxin G_1 (µg/kg) in processed Bambara groundnut flour stored at 25±2 °C and 75±2% for 10 weeks

Period (weeks)	BGF	FBGF	RBGF
0	A 1.38^{d} (0.01)	B 1.18^{a} (0.00)	C 0.15^{g} (0.01)
1	A 1.46^{b} (0.01)	B 0.98^{b} (0.00)	C 0.53^{b} (0.04)
2	A 0.99^{g} (0.01)	B 0.82^{d} (0.01)	C 0.50^{c} (0.01)
3	A 1.39^{cd} (0.03)	B 0.82^{d} (0.01)	C 0.55^{a} (0.03)
4	A 1.40^{c} (0.02)	B 0.71^{e} (0.04)	C 0.43^{d} (0.01)
5	A 1.53^{a} (0.01)	B 0.82^{d} (0.01)	C 0.27^{f} (0.00)
6	A 1.36^{e} (0.00)	B 0.87^{c} (0.03)	C 0.29^{e} (0.01)
7	A 0.93^{h} (0.01)	B 0.11^{f} (0.01)	C 0.07^{i} (0.02)
8	A 0.17^{i} (0.02)	B 0.10^{f} (0.02)	B 0.09^{h} (0.01)
9	A 1.16^{f} (0.01)	B 0.10^{f} (0.01)	ND
10	A 0.11^{j} (0.01)	A 0.10^{f} (0.01)	B 0.07^{i} (0.01)

Values represent mean (standard deviation) of duplicate determinations. Means followed by the same superscript within the column are not significantly different ($p \geq 0.05$). Means preceded by the same uppercase letter along the row are not significantly different ($p \geq 0.05$). Limits of detection, and quantification are 0.01 and 0.05 µg/kg, respectively. ND, not detected. BGF-Bambara groundnut flour, FBGF-Fermented Bambara groundnut flour, RBGF-Roasted Bambara groundnut flour.

Table 6.5 Concentration of aflatoxin G_2 (µg/kg) in processed Bambara groundnut flour stored at 25±2 °C and 75±2% for 10 weeks

Period (weeks)	BGF	FBGF	RBGF
0	A 0.14^{ab} (0.01)	A 0.13^{c} (0.00)	B 0.03^{f} (0.01)
1	A 0.15^{a} (0.01)	A 0.15^{b} (0.02)	A 0.14^{a} (0.01)
2	C 0.11^{de} (0.02)	AB 0.13^{c} (0.02)	BC 0.12^{b} (0.02)
3	B 0.10^{e} (0.01)	A 0.19^{a} (0.01)	B 0.11^{bc} (0.02)
4	A 0.15^{a} (0.00)	B 0.12^{cd} (0.01)	B 0.12^{b} (0.02)
5	A 0.13^{bc} (0.01)	A 0.13^{c} (0.00)	B 0.09^{d} (0.01)
6	B 0.13^{bc} (0.03)	A 0.16^{b} (0.01)	C 0.11^{bc} (0.01)
7	C 0.05^{f} (0.01)	A 0.11^{de} (0.03)	B 0.07^{e} (0.03)
8	B 0.05^{f} (0.01)	A 0.11^{de} (0.01)	A 0.12^{b} (0.01)
9	A 0.12^{cd} (0.01)	B 0.10^{e} (0.01)	B 0.10^{cd} (0.01)
10	B 0.06^{f} (0.00)	A 0.10^{e} (0.01)	B 0.06^{e} (0.00)

Values represent mean (standard deviation) of duplicate determinations. Means followed by the same superscript within the column are not significantly different ($p \geq 0.05$). Means preceded by the same uppercase letter along the row are not significantly different ($p \geq 0.05$). Limits of detection, and quantification are 0.01 and 0.05 µg/kg, respectively. ND, not detected. BGF-Bambara groundnut flour, FBGF-Fermented Bambara groundnut flour, RBGF-Roasted Bambara groundnut flour.

6.3.3 Effect of change in water activity on *Aspergillus flavus* growth in processed Bambara groundnut flours during storage

Changes in water activity on *A. flavus* growth in processed and inoculated BGF samples during storage are presented in Figures 6.1–6.3. Significant variations were observed in the population of *A. flavus* in all the flour samples during storage. Water activity values increased significantly ($p \leq 0.05$) over the storage period in FBGF (0.52–0.85), BGF (0.47–0.76), and RBGF (0.34–0.80). At low water activity levels (≤0.65), significant population of

A. flavus was still recorded in BGF and RBGF samples. However, despite the increase in water activity, *A. flavus* could not survive in FBGF after 7 weeks. The growth of an organism in an environment with reduced water activity is governed by factors such as physico-chemical properties of the environment and the species of organism involved (Troller, 1987:102). Survivor curves describe the effect of varying parameters such as processing on viable microbial populations. Microbial deaths are depicted by first-order chemical inactivation, generally resulting in linear plots. However, exceptions leading to "shoulder" and "tailing" effects may result (Lenovich, 1987:120). The non-linearity of survivor curves of microbes may be influenced by cell clumps or chains, cell injury, and physiological age (Lenovich, 1987:120).

The increase in water activity recorded in all the flour samples during storage can be attributed to active metabolism of *A. flavus* and partly due to absorption of moisture from the ambience. The decrease in population and eventual death of *A. flavus* in FBGF observed during storage may be related to the acidic environment of FBGF. The pH and type of acid contribute to the inhibition of microbial growth in food systems (Troller, 1987:107). At any given water activity level, survival rate of microorganisms decreases with a decrease in pH (Lenovich, 1987:127). The continued growth and activity of *A. flavus* at low water activity levels imply the ability of the organism to adapt favourably to the prevailing conditions. This further affirms the adaptability, continued growth and survival of the pathogen in BGF when water activity requirements are not optimal. However, a decrease in pH significantly ($p \leq 0.05$) decreased growth of the pathogen.

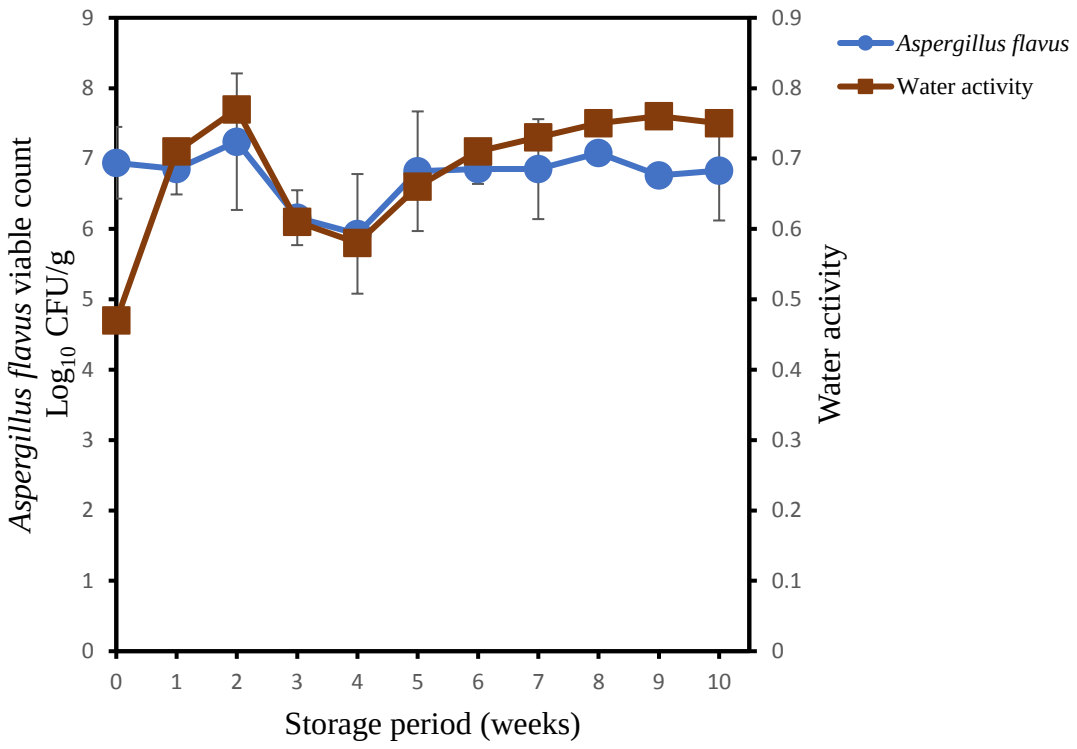

Figure 6.1 *Aspergillus flavus* viable count and water activity in Bambara groundnut flour stored at 25±2 °C and 75±2% relative humidity for 10 weeks.

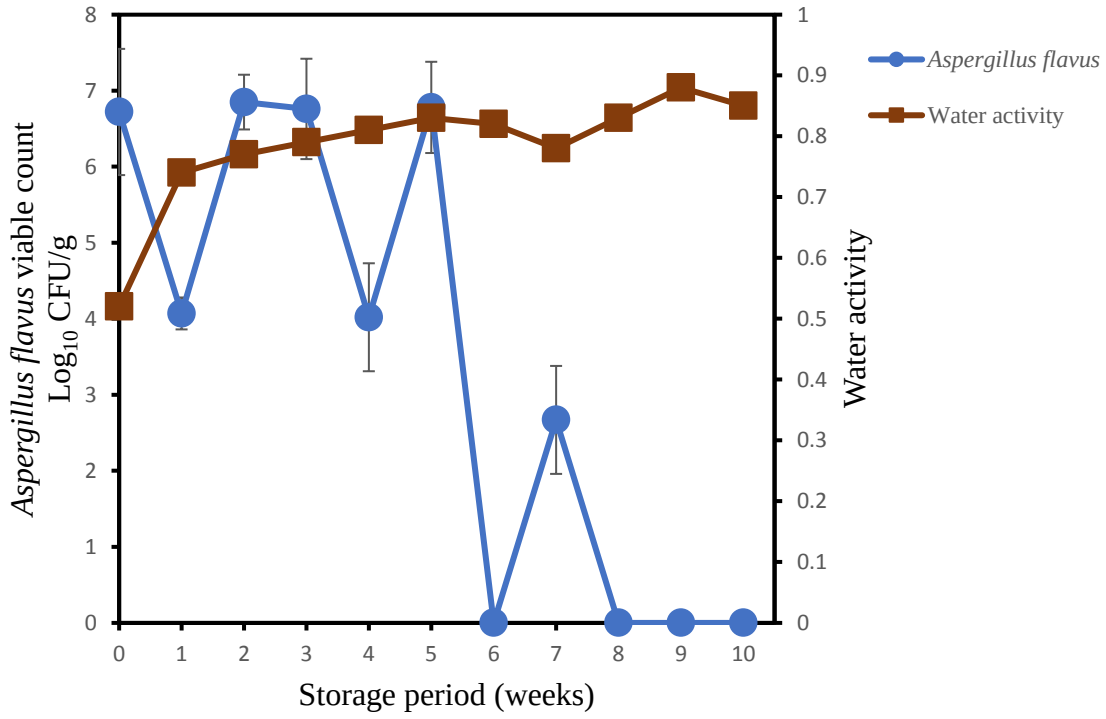

Figure 6.2 *Aspergillus flavus* viable count and water activity in Fermented Bambara groundnut flour stored at 25±2 °C and 75±2% relative humidity for 10 weeks.

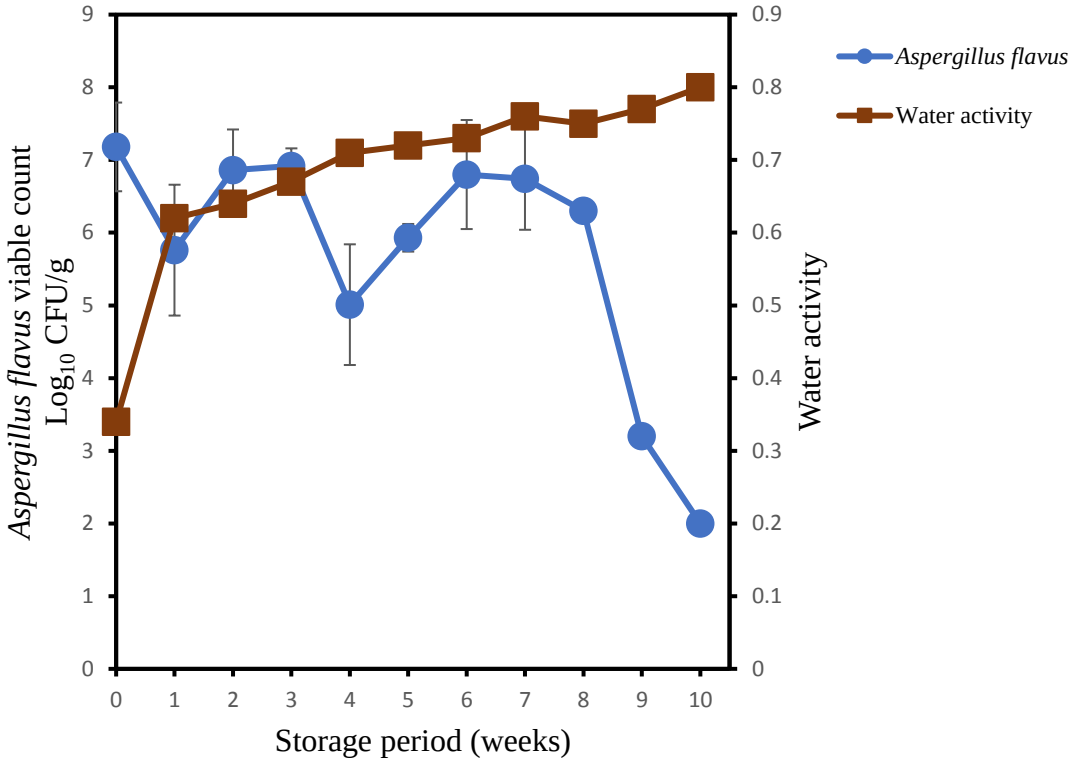

Figure 6.3 *Aspergillus flavus* viable count and water activity in Roasted Bambara groundnut flour stored at 25±2 °C and 75±2% relative humidity for 10 weeks.

6.3.4 Effect of compositing maize and Bambara groundnut on growth and aflatoxin production of *Aspergillus flavus* during storage

The growth of *A. flavus* in MBCF and MZF over the storage period are presented in Figures 6.4 and 6.5, respectively. The population of *A. flavus* in the flour samples over the 10-week storage period decreased significantly ($p \leq 0.05$) in MBCF from 6.90 to 6.72 Log_{10} CFU/g but increased significantly ($p \leq 0.05$) in MZF from 6.91 to7.02 Log_{10} CFU/g. The water activity values in the samples significantly ($p \leq 0.05$) increased in MBCF from 0.43 to 0.96 and in MZF from 0.43 to 0.85 during storage.

The production of aflatoxin B and G derivatives by *A. flavus* in the samples are shown in Tables 6.7 and 6.8. The ability of *A. flavus* to produce AFs was markedly affected by compositing the flour samples. AFB_1 concentration in the flour samples significantly ($p \leq 0.05$) increased in MBCF from 1.17 µg/kg at week 0 to 2.05 µg/kg after storage for 10 weeks. A relatively lower concentration was detected in MZF over the storage period, with the highest concentration of 0.62 µg/kg detected after storage for 4 weeks. During the storage period, the ability of *A. flavus* to produce AFB_2, AFG_1, and AFG_2 was supported better by MBCF than by MZF. Fungal presence in food commodities does not always correlate with

toxin production as the fungus may be present but the associated toxins may not be produced (Sulyok *et al.*, 2010). Also, the fungus may lose viability after toxin production. The higher population of *A flavus* in MZF did not yield any significant ($p \leq 0.05$) AFB_1 production while at a relatively lower population in MBCF, significant ($p \leq 0.05$) production of AFB_1 was noted. Results of this experiment also show that the inclusion of BGF (30%) significantly ($p \leq 0.05$) increased the production of both aflatoxin B and G derivatives by *A. flavus* during storage.

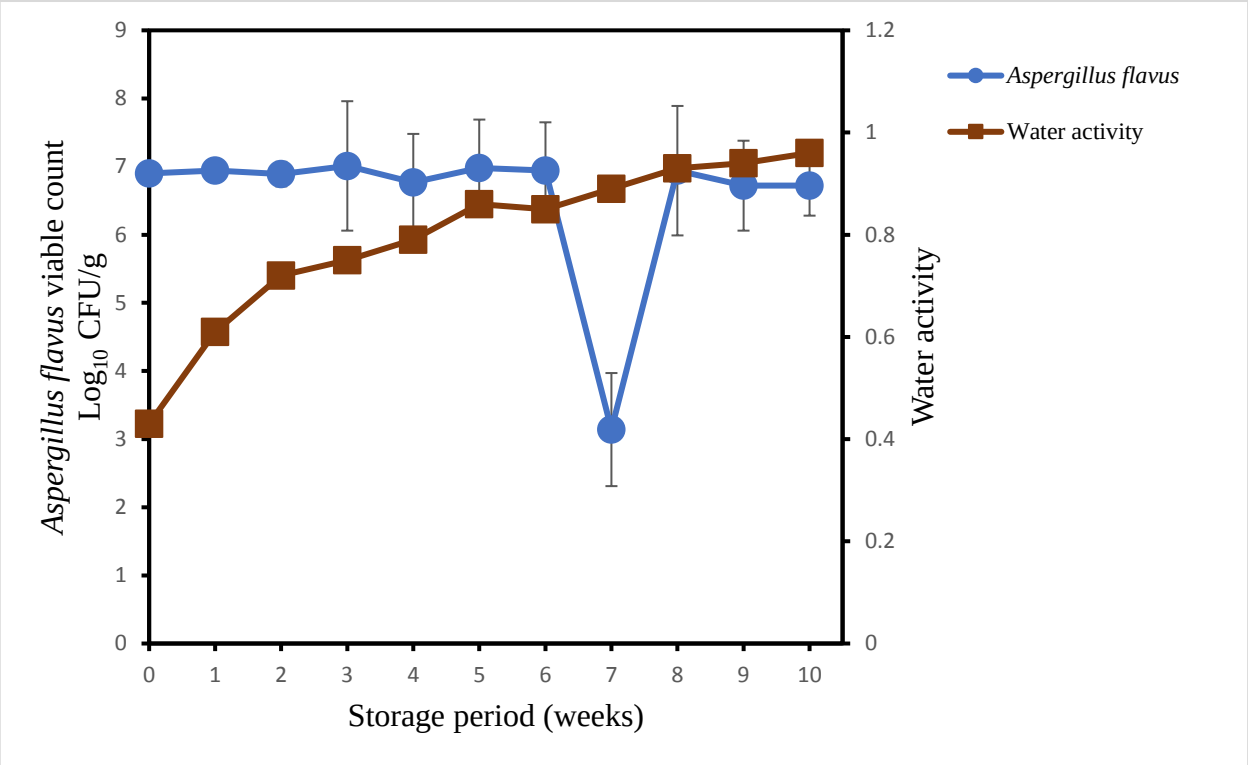

Figure 6.4 *Aspergillus flavus* viable count and water activity in maize-bambara groundnut composite flour stored at 25±2 °C and 75±2% relative humidity for 10 weeks.

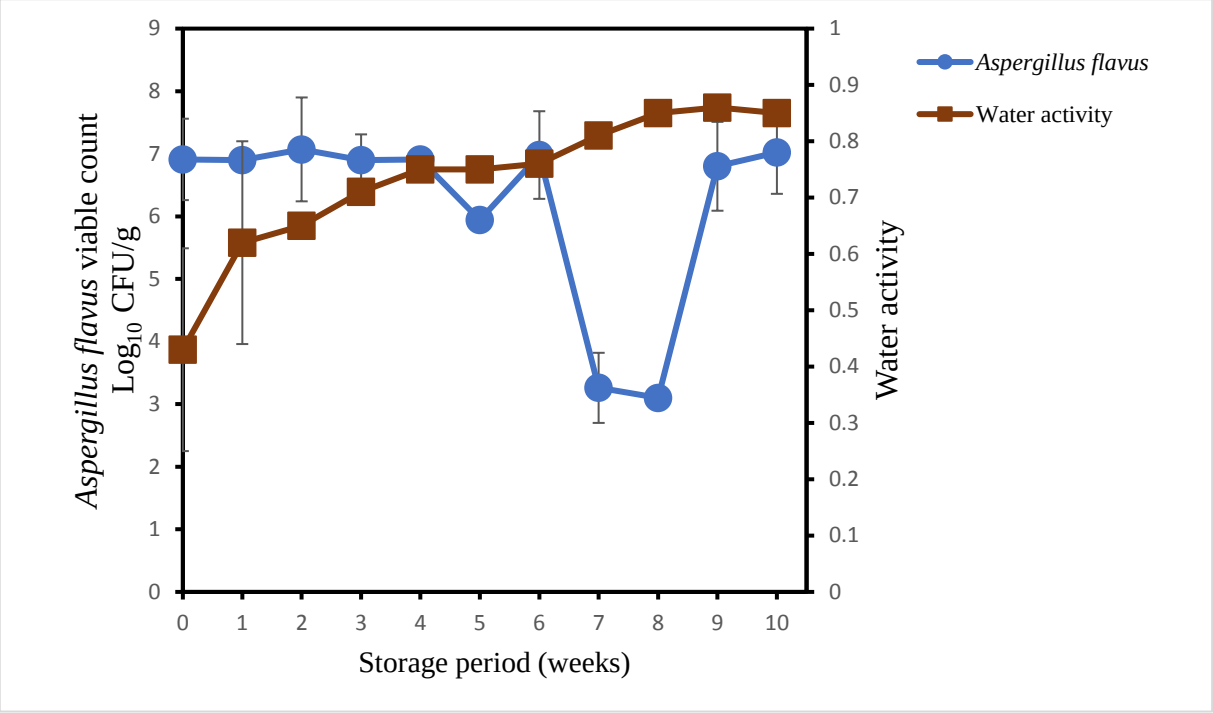

Figure 6.5 *Aspergillus flavus* viable count and water activity in maize flour stored at 25±2 °C and 75±2% relative humidity for 10 weeks.

Table 6.6 Concentration of aflatoxin B_1 and B_2 (µg/kg) in maize-bambara composite flour stored at 25±2 °C and 75±2% for 10 weeks

Period (weeks)	AFB_1		AFB_2	
	MBCF	MZF	MBCF	MZF
0	A 1.17^{g} (0.01)	B 0.27^{g} (0.03)	A 0.08^{d} (0.01)	B 0.05^{ab} (0.01)
1	A 1.24^{f} (0.01)	B 0.30^{f} (0.01)	A 0.07^{de} (0.01)	B 0.04^{b} (0.01)
2	A 1.45^{e} (0.01)	B 0.21^{j} (0.01)	A 0.06^{ef} (0.03)	B 0.04^{b} (0.01)
3	A 0.39^{k} (0.04)	B 0.34^{e} (0.00)	ND	0.04^{b} (0.01)
4	B 0.44^{j} (0.01)	A 0.62^{a} (0.01)	A 0.05^{f} (0.01)	A 0.04^{b} (0.00)
5	A 0.63^{i} (0.01)	B 0.47^{b} (0.02)	A 0.06^{ef} (0.01)	A 0.06^{a} (0.01)
6	A 0.80^{h} (0.00)	B 0.22^{ij} (0.02)	0.05^{f} (0.00)	ND
7	A 2.49^{b} (0.01)	B 0.40^{c} (0.02)	A 0.03^{g} (0.01)	A 0.04^{b} (0.01)
8	A 2.73^{a} (0.02)	B 0.36^{d} (0.02)	A 3.11^{a} (0.01)	B 0.04^{b} (0.04)
9	A 2.02^{d} (0.01)	B 0.25^{h} (0.01)	A 0.16^{c} (0.01)	B 0.04^{b} (0.01)
10	A 2.05^{c} (0.01)	B 0.23^{i} (0.01)	0.18^{b} (0.02)	ND

Values represent mean (standard deviation) of duplicate determinations. Means followed by the same superscript within the column are not significantly different ($p \geq 0.05$). Means preceded by the same uppercase letter along the row are not significantly different ($p \geq 0.05$). Limits of detection, and quantification are 0.01 and 0.05 µg/kg, respectively. ND, not detected. MBCF-Maize-bambara composite flour, MZF-Maize flour.

Table 6.7 Concentration of aflatoxin G_1 and G_2 (µg/kg) in maize-bambara composite flour stored at 25±2 °C and 75±2% for 10 weeks

Period (weeks)	AFG_1		AFG_2	
	MBCF	MZF	MBCF	MZF
0	0.54^{a} (0.04)	ND	0.10^{d} (0.01)	ND
1	0.47^{b} (0.01)	ND	A 0.10^{d} (0.01)	B 0.07^{cd} (0.01)
2	0.53^{a} (0.01)	ND	A 0.14^{b} (0.01)	B 0.06^{de} (0.02)
3	A 0.41^{c} (0.01)	B 0.06^{a} (0.01)	A 0.07^{ef} (0.01)	A 0.07^{cd} (0.01)
4	A 0.29^{e} (0.03)	B 0.06^{a} (0.01)	A 0.06^{fg} (0.01)	A 0.06^{de} (0.01)
5	0.22^{f} (0.01)	ND	A 0.12^{c} (0.01)	B 0.10^{a} (0.01)
6	0.36^{d} (0.02)	ND	A 0.08^{e} (0.01)	A 0.08^{bc} (0.03)
7	0.21^{f} (0.01)	ND	A 0.05^{g} (0.01)	A 0.05^{e} (0.01)
8	0.07^{g} (0.00)	ND	A 0.20^{a} (0.01)	B 0.09^{ab} (0.01)
9	ND	ND	0.07^{ef} (0.01)	ND
10	ND	ND	A 0.05^{g} (0.01)	A 0.05^{e} (0.02)

Values represent mean (standard deviation) of duplicate determinations. Means followed by the same superscript within the column are not significantly different ($p \geq 0.05$). Means preceded by the same uppercase letter along the row are not significantly different ($p \geq 0.05$). Limits of detection, and quantification are 0.01 and 0.05 µg/kg, respectively. ND, not detected. MBCF-Maize-bambara composite flour, MZF-Maize flour.

6.4 Conclusion

The growth of *A. flavus* and AF production in BGF during storage was significantly ($p \leq 0.05$) affected by the processing operations of milling, lactic acid bacteria fermentation and roasting. Growth of *A. flavus* was markedly influenced by fermentation; AF production was influenced by roasting. Higher amounts of AFB_1 and AFG_1 were produced by *A. flavus* in the flour samples under conditions of storage. Further studies to understand the growth kinetics and AF production pathways of *A. flavus* in BGF, and the development of resistance varieties of the seed are necessary as mitigatory measures.

CHAPTER SEVEN

Microscopic studies on *Aspergillus flavus* infection in Bambara groundnut (*Vigna subterranea* (L.) Verdc)

Abstract

Bambara groundnut (*Vigna subterranea* (L.) Verdc) is susceptible to fungal infection and aflatoxin (AF) contamination. *Aspergillus flavus* grows in the legume and produces AFs as secondary metabolites. However, there is a sparse information on the infection pattern of *A. flavus* in Bambara groundnut. Irradiated Bambara groundnut seeds were artificially inoculated with spore suspension of aflatoxigenic *A. flavus* (2 x 10^6 spores/mL) and stored at temperature of 25±2 °C and 75±2% relative humidity for 14 days. Samples were withdrawn at 24 h intervals for 4 days, and then at 7 and 14 days. Using scanning electron microscopy (SEM) and transmission electron microscopy (TEM), the mode of entry of *A. flavus*, and changes to the seed coat, storage cells and tissue structure due to fungal infection were studied. The seed coat of Bambara groundnut was rapidly colonized by *A. flavus* within 24 h of inoculation. The infection of internal tissues of the cotyledon was via the ruptured seed coat, resulting in a disruption of cellular architecture. Cell wall collapse, the presence of cavities in parenchymatous cells and ruptured storage cells resulted from *A. flavus* infection of the seed over storage period. Information from this study may assist in the development of effective post-harvest management practices to mitigate *A. flavus* infection in Bambara groundnut.

Key words: Bambara groundnut, *Aspergillus flavus*, SEM, TEM, colonization, infection

7.1 Introduction

Fungal infection is a major constraint in the utilization of many agricultural products. Contamination of food commodities by pathogenic fungi may impact seriously on the economy due to product recall and adversely affect international trade (Afsah-Hejri *et al.*, 2013). Agricultural products become susceptible to fungal infection while on the field and/or during storage (Ji *et al.*, 2016). Poor management practises further encourage the proliferation of pathogenic fungi in food commodities. Fungi belonging to *Aspergillus*, *Fusarium* and *Penicillium* genera have been isolated from many food commodities (Reddy *et al.*, 2011; Chilaka *et al.*, 2012; Yogendrarajah *et al.*, 2014; Egbuta *et al.*, 2015; Olagunju *et al.*, 2018b).

Aspergillus flavus is an opportunistic pathogen of plants, animals, and insects, causing storage rots in numerous crops, and produces AF that is highly regulated in almost all the countries with legislation on mycotoxins (Klich, 2007). Peanuts, corn and cotton seed are particularly susceptible to *A. flavus* infection, leading to rot in mature seed, ear, and boll respectively (Klich, 2007). Like peanut, Bambara groundnut is susceptible to *A. flavus* infection and equally supports AF contamination (Olagunju *et al.*, 2018a). *Aspergillus flavus* infection in peanut studied using SEM and TEM revealed the disruption on seed coat and intercellular structure of seeds and seedlings (Achar *et al.*, 2009). However, the infection pattern of *A. flavus* on Bambara groundnut has not been studied. The objective of this work was to study the mode of infection of *A. flavus* on Bambara groundnut, and the changes in the internal and external structures of the seed due to infection caused by this fungus.

7.2 Materials and methods

7.2.1 Materials

Bambara groundnut (cream variety) was obtained from Southern Africa and cleaned by hand-picking of all foreign materials and defective seeds.

7.2.2 Preparation and treatment of seeds

Whole healthy seeds (undehulled) weighing about 500 g were selected and packed in double-seal Tuff lock bags, and thereafter, subjected to gamma irradiation (20 kGy) to ensure the destruction of viable fungi or bacteria on the seed. The irradiated seeds were then used throughout the experiment.

7.2.3 *Aspergillus flavus* strains and preparation of inoculum

A 3-strain cocktail of *A. flavus* was prepared from the following strains, *i.e*, BG01, BG02 and ATCC 9346 as described in section 5.2.4 of Chapter Five. Using a hemocytometer, the cell suspension was adjusted at 2 x 10^6 spores/mL following the protocol reported in the same section. The spore suspension was stored at 4 °C for further use.

7.2.4 Artificial contamination and storage of seeds

The method described by Achar *et al.* (2009) with modifications, was used to artificially contaminate the sterile Bambara groundnuts. Under a laminar flow, whole irradiated Bambara nuts were immersed in *A. flavus* suspension for 2 min with continuous stirring using a sterile glass rod. Seeds were then removed from the suspension and placed (10 seeds per plate) in sterile petri plates with cover. Artificially contaminated seeds were stored in a sterile climatic oven at temperature of 25±2 °C and 75±2% relative humidity for 14 days to

monitor the colonization of the seed by *A. flavus*. Seeds withdrawn at 24-h intervals over the next 4 days, and at 7 and 14 days were studied using SEM and TEM.

7.2.5 Preparation of seeds for scanning electron microscopy

The changes due to *A. flavus* colonization in artificially contaminated seeds, and whole healthy non-contaminated seeds, which served as the control were studied. To view the seeds by SEM, each seed was cut into half and mounted on aluminium stubs. Gold sputtering of the seeds was carried out under high vacuum using a Quorum Q150R ES (Kent, UK) coater. Seeds were quoted twice with about 12 nm of gold and viewed at 5 kV using a ZEISS EVO LS15 SEM.

7.2.6 Fixation and preparation of seeds for transmission electron microscopy

To view the artificially contaminated and healthy seeds using TEM, a portion of the seed was cut into small pieces (2 mm x 2 mm). The sectioned seeds were then fixed in 2.5% glutaraldehyde (in 0.1 M phosphate buffer) overnight, followed by two washings (10 min each) in 0.1 M phosphate buffer. Using 0.5% aqueous osmium tetroxide, sections were post-fixed overnight and then washed twice (10 min each) in 0.1 M phosphate buffer. The specimens were then dehydrated through a graded acetone series [30% for 10 min (x 2), 50% for 10 min (x 2), 70% for 10 min (x 2), and 100% for 10 min (x 3)].

For the preparation of samples for TEM, acetone dehydration of the specimens was performed followed by infiltration with Resin [1:3 resin to acetone (overnight), 1:1 resin to acetone (overnight), 3:1 resin to acetone (overnight) and pure resin (overnight) (x 2)]. Following infiltration, polymerization was carried out whereby samples were imbedded in fresh 100% resin for 8 h at 70 °C. Resin blocks were then sectioned on a Leica UC7 ultra microtome set at wavelength of 100 nm. Sections were stained with Reynold's lead citrate and 2% uranyl acetate for 10 min each and then viewed on a JEOL 2100 HR TEM.

7.3 Results and discussion

The changes in the seed coat and intercellular structures of non-contaminated and artificially contaminated Bambara groundnut seeds, with *A. flavus* spores as observed with SEM and TEM are presented in Figures 7.1–7.3. Seed coat of healthy, uninoculated seed as seen under SEM is smooth, without pits, appendages, pores, hairs or wings (Figure 7.1A). Kola (2003) reported similar conditions of the seed coat of non-contaminated Bambara groundnut. Lersten (1981) reported variations in the testa topography of some seeds of *Leguminosae* (subfamily *Papilionoideae*) showing cuticular blisters, pits and inconspicuous mounds.

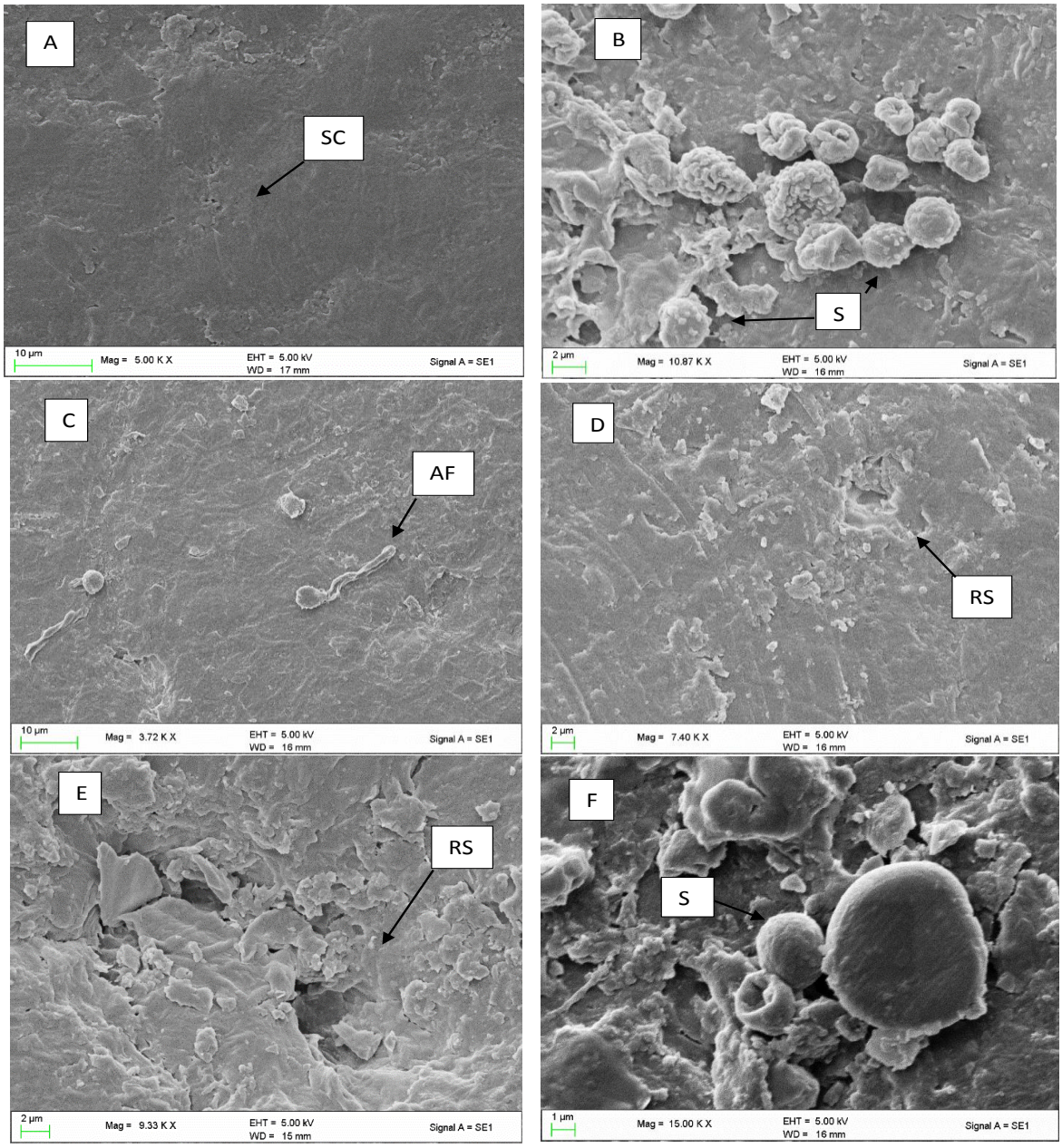

Figure 7.1 Scanning electron microscopy (SEM) micrographs of Bambara groundnut seed coat showing infection by *A. flavus*: (A) undamaged seed coat (SC) of healthy uninoculated seed (B) *A. flavus* spores (S) on seed coat of inoculated seed after 24 h, (C) *A. flavus* (AF) growing on seed coat of inoculated seed after 48 h, (D) – (E) ruptured seed coat (RS) of inoculated seed after 72 h, 7 days, (F) spore (S) entry through ruptured seed coat at 14 days.

Following artificial inoculation with *A. flavus* spores, Bambara groundnut seed coat was rapidly colonized within 24 h (Figure 7.1B). Micrographs obtained from SEM showed *A. flavus* growing on the seed coat after 48 h of inoculation (Figure 7.1C). Rupturing of the seed coat was observed up till 14 days of storage (Figures7.1D-F) and can be attributed to enzyme action of the germinating spores (Achar *et al.*, 2009). Figure 7.1F reveals the entry

of spore through the ruptured seed coat into the underlying tissues of the cotyledon. Further studies on the intercellular structure of the seed showed a healthy epidermis and intact parenchyma in healthy uninoculated seed (Figure 7.2A). Tightly packed parenchymatous cells with well-defined cell wall were observed after 48 h of inoculation (Figure 7.2B). However, cellular disintegration with fractured parenchyma and cell wall rupture was visible after 72 h of inoculation and storage (Figure 7.2C-D). Cell collapse and the appearance of cavities in the cellular structure was seen in inoculated seed after 7 days of storage (Figure 7.2E). SEM showed rupturing of the storage cells after 14 days of inoculation and storage (Figure7.2F).

The intercellular structure of a healthy, uninoculated Bambara groundnut seed studied with TEM showed abundant and well organized storage cells, unruptured cell wall and minimal intercellular spaces (Figure 7.3A). After 48 h of inoculation and incubation, changes in cell wall structure were observed (Figure 7.3B). After 7 days, cellular architecture was distorted in inoculated seed with ruptured storage cells, due to *A. flavus* infection (Figure 7.3C). Transmission electron microscopy sections revealed tissue disintegration in the Bambara groundnut seed after 14 days of inoculation (Figure 7.3D). The colonization of peanut (*Arachis hypogaea*) by *A. flavus* has been studied by Achar *et al.* (2009). The authors observed rapid colonization of the seed coat within 48 h of inoculation and establishment of fungal mycelia in the tissues of the cotyledon within 72 h. Partial or complete degradation of cell wall, evidence of amylolytic degradation of starch granules, and loss of protein matrix around the starch granules have been reported by Jackowiack *et al.* (2005) in spring wheat infected by *Fusarium culmorum*, due to the activities of the hydrolytic enzymes produced by the fungi growing in the infected kernels.

Fungi are heterotrophic in nature and obtain nutritional requirements through amylolytic degradation of complex organic molecules. Fungi penetrate the food matrice and produce hydrolytic enzymes, which break down the complex molecules (Bhat *et al.*, 2010). The cell wall, starch granules and storage proteins may be affected by the activity of fungal enzymes, leading to a deterioration in the quality of the grain and its suitability for processing applications (Jackowiack *et al.*, 2005). Cell wall degrading enzymes (cellulases, xylanases and pectinases) were produced during fungal infection and colonization of wheat spike tissues (Kang and Buchenauer, 2002).

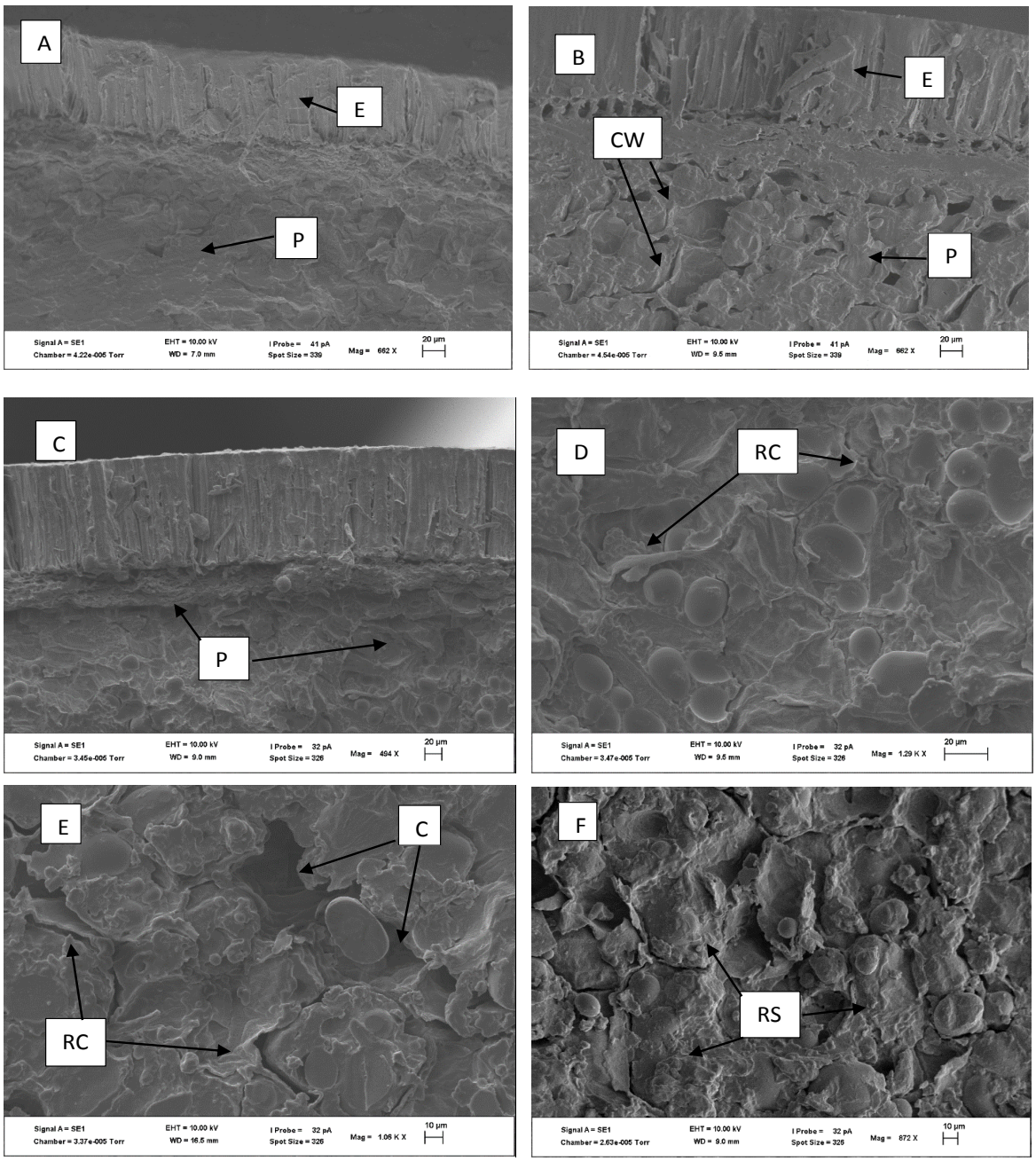

Figure 7.2 Scanning electron micrographs (SEM) of Bambara groundnut seed.(A) healthy uninoculated seed with undamaged epidermis (E) and parenchyma (P), (B) cell wall (CW) in parenchyma of infected seed after 48 h, (C) fractured parenchyma of infected seed after 72 h, (D) parenchyma of infected seed after 72 h showing ruptured cell wall (CW), (E) ruptured cell wall and cavities (C) in parenchyma of infected seed after 7 days, (F) ruptured storage cells (RS) of infected seed after 14 days.

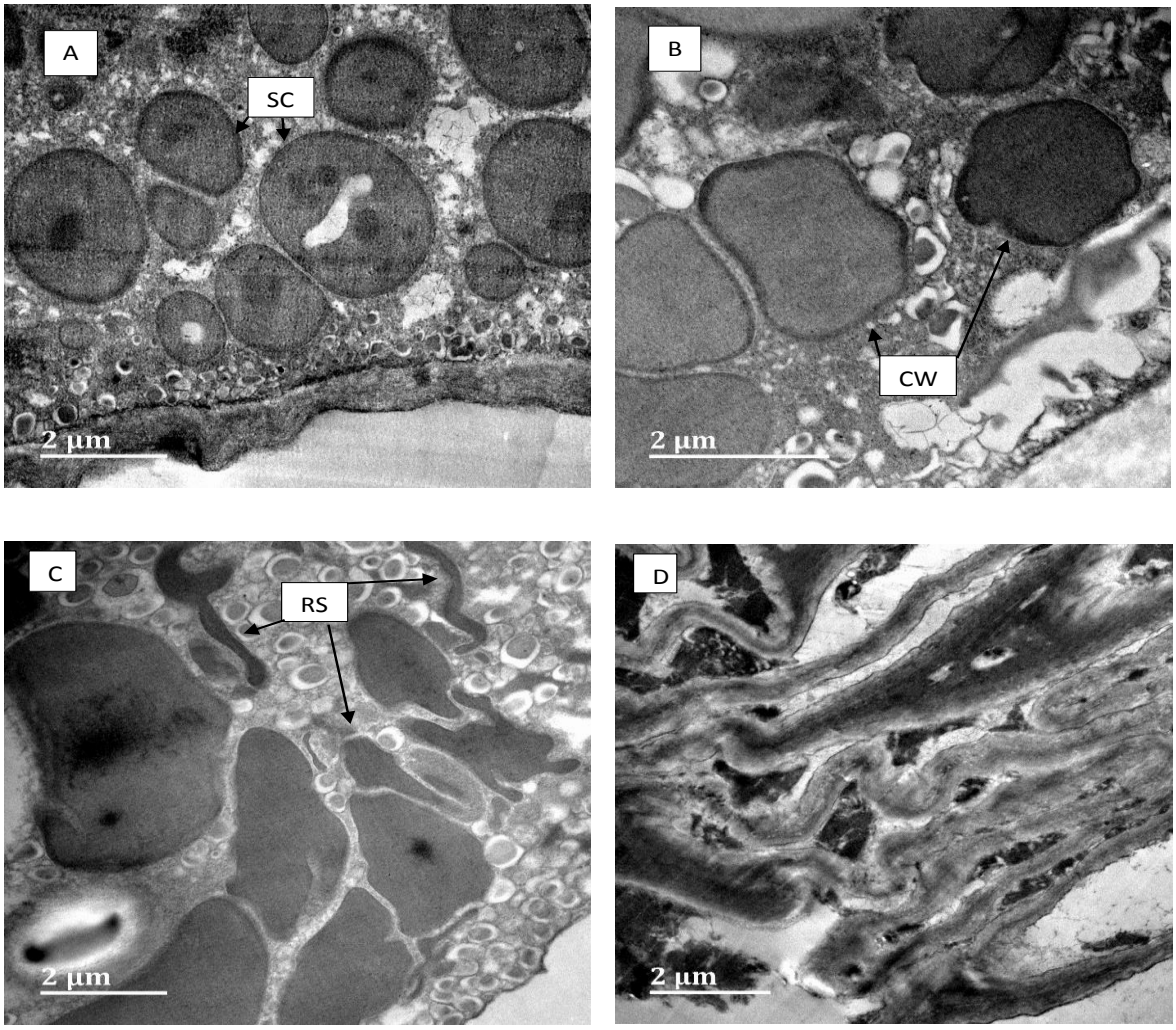

Figure 7.3 Transmission electron microscopy (TEM) sections of Bambara groundnut inoculated with *A. flavus*. (A) Uninoculated, healthy tissues with well-defined storage cells, (B) inoculated seed after 48 h showing signs of cell (CW) wall rupture, (C) ruptured storage cells (RS) of inoculated seed after 96 h, (D) tissue disintegration in inoculated seed after 7 days.

7.4 Conclusion

Aspergillus flavus is a pathogen of many agricultural products, including Bambara groundnut. Mycelium of the fungi become attached to the seed coat, through which it enters into the cotyledon and able to colonize the cotyledonal tissues, leading to breakdown in the cellular structure of the seed and tissue disintegration. Colonization and tissue disintegration occur within 7 days of infection. Mitigatory measures aimed at preventing fungal infection, which include the use of non-toxigenic strains of *A. flavus*, development of mold-resistant varieties of Bambara groundnut, good agronomic practices and educating farmers on proper seed handling are recommended.

CHAPTER EIGHT

8.1 General discussion

In this thesis, the behaviour of *Aspergillus flavus* in Bambara groundnut as affected by processing and during storage was studied. The first part of this section discusses fungal and mycotoxin contamination of Bambara groundnut (Chapters Three and Four) while the second part focuses on the behaviour of *A. flavus* in Bambara groundnut and maize composite flour during processing and storage (Chapters Five and Six). The third part discusses the colonization of Bambara groundnut by *A. flavus* (Chapter Seven). Chapter Eight presents a summary of the findings from this study, and recommendations to mitigate aflatoxin (AF) contamination in Bambara groundnut.

8.1.1 Fungal and mycotoxin contamination of Bambara groundnut

Bambara groundnut samples obtained from retail stores and open markets in Durban, South Africa and destined for human consumption were found to be contaminated by different fungal species. *Aspergillus flavus*, *A. niger*, *A. tamarii*, *Penicillium citrinum*, and *P. oxalicum* were isolated from the seeds. The pods of Bambara groundnut develop and mature in the soil or just above the ground with the soil serving as source of inoculum of pathogenic fungi (Murevanhema and Jideani, 2013; Guezlane-Tebibel *et al.*, 2013). Further analysis showed the contamination of Bambara groundnut by toxigenic strains of *A. flavus*. Although other fungal species were isolated from the seed, a multi-mycotoxin analysis showed the accumulation of only aflatoxin B and G derivatives. It has been noted how fungal infection does not necessarily correlate with production of attendant mycotoxin and vice versa (Sulyok *et al.*, 2010). S-strain of *A. flavus*, *A. parasiticus*, and other fungi have been reported to produce both aflatoxin derivatives (Pildain *et al.*, 2004; Matumba *et al.*, 2015b).

The concentration of AFB_1, the most common and potent of the AFs, detected in the samples under study exceeds the maximum tolerable limits permissible in groundnuts, other nuts, and baby foods for infants and young children according to European Commission (2006). The consumption of aflatoxin-contaminated foods, even at low concentrations or over a long period of time may have some effects on health (Marroquín-Cardona *et al.*, 2014). Previous studies have recovered AFs, especially AFB_1 from body fluids of malnourished children and cancer patients (Gong *et al.*, 2002; Tchana *et al.*, 2010). Traditional processing operations of dehulling, milling, roasting and spontaneous fermentation had varied effects on the natural fungal contaminants of Bambara groundnut and during storage. Although roasting resulted

in the degradation of AF, this heat treatment did not achieve complete destruction of infection in the internal tissues of the seed, leading to AF production and accumulation during storage.

8.1.2 The behaviour of *Aspergillus flavus* in Bambara groundnut and maize composite flour during processing and storage.

The growth of *A. flavus* and AF production by this fungal species in Bambara groundnut were markedly affected by lactic acid bacteria (LAB) fermentation. Complete elimination of *A. flavus* and significant reduction of AFB_1 concentration were achieved within 24 hours of inoculation. The effectiveness of LAB fermentation in reducing fungal contamination has been attributed to the production of anti-fungal compounds, organic acids and competitive inhibition effect (Lavermicocca *et al.*, 2000; Omemu, 2011; Kachouri *et al.*, 2014; Roger *et al.*, 2015). During storage, LAB fermentation also markedly affected the growth and survival of *A. flavus*. This can also be attributed to the inhibitory mechanisms of LAB. The population of *A. flavus* recovered from all the stored samples did not follow a linear progression characteristic of microbial survival plots, established by plotting the logarithm of survivors against time. Fluctuations in the growth of *A. flavus* and AF production may have been affected by several factors such as cell clumps or chains, cell injury, and physiological age of the culture (Lenovich, 1987:120). Further studies aimed at understanding the growth kinetics of *A. flavus* and its AF production pathways as affected by processing operations in Bambara groundnut may aid in proffering mitigatory measures against *A. flavus* infection.

The practice of compositing legumes with cereals to improve nutritional status of the composite flour has been adopted in many developing countries. The present study shows a marked effect of compositing Bambara groundnut with maize flour on aflatoxin-production potential of *A. flavus*. A significant increase in AF production and accumulation in Bambara-maize composite flour during storage was reported in this study, particularly in the case of AFB_1 and AFG_1, which are more toxic than the other types of aflatoxin.

8.1.3 Colonization of Bambara groundnut by *Aspergillus flavus*

Physical examination showed shrivelling of seed coat due to fungal infection. Scanning and transmission electron microscopy revealed the infection pattern of *A. flavus* on Bambara groundnut. Rapid colonization of seed coat by fungal spores and growth of *A. flavus* on seed coat were observed, with disruption in seed coat structure. *A. flavus* penetration occurred through the ruptured seed coat into the underlying cotyledonal cells. Cell wall collapse,

rupturing of storage cells and development of cavities in parenchymatous cells resulted from tissue invasion by *A. flavus*. Fungi being heterotrophic in nature, metabolize other complex organic molecules to meet nutritional requirements (Bhat *et al.*, 2010). Fungal penetration through the seed coat and secretion of hydrolytic enzymes required for degradation of storage cells resulted in the disruptions in seed coat and cellular structures of the seed. Cell wall degrading enzymes including, cellulases, xynalases and, pectinases are produced during fungal metabolism (Kang and Buchenauer, 2002). Transmission electron microscopy revealed similar cellular disruptions and tissue disintegration due to *A. flavus* infection.

Aflatoxin quantification using enzyme-linked immunosorbent assay was limited in detecting low levels of aflatoxin (< 2.5 ng/g) in the samples during incubation for 96 h. *Aspergillus flavus* has the ability to produce AF within 24 h under optimum conditions (Achar and Sanchez, 2006). The fungus was eliminated after roasting artificially inoculated Bambara groundnut at 140 °C for 20 min and was not detected in the flour during incubation for 96 h. In the flour obtained from similar processing of naturally contaminated Bambara groundnut, proliferation of *A. flavus* was recorded during storage. This disparity can be attributed to surviving spores in the internal tissues of the seed, which were not eliminated during roasting.

8.2 General conclusions and recommendations

Bambara groundnut is a susceptible substrate for *A. flavus* infection and production of B and G types of AF. Aflatoxin B_1 concentrations detected in some Bambara groundnut flour samples which ranged from 0.13 to 6.90 µg/kg, exceeds the maximum tolerable limit of 2 µg/kg for AFB_1 in groundnut and nuts, 0.1 µg/kg for baby foods for infants and young children, and total aflatoxin of 4 µg/kg. Processing methods, *i.e*., dehulling and milling, roasting, and LAB fermentation had varied effects on *A. flavus* growth and AF production. Compositing maize with Bambara groundnut increased AF accumulation during storage. *Aspergillus flavus* can thrive on Bambara groundnut and colonize the cotyledonal tissues, leading to breakdown in the cellular structure of the seed and tissue disintegration. Colonization and tissue disintegration occurred within 7 days of infection.

Bambara groundnut is a good source of plant protein and is especially important in developing countries where protein-energy malnutrition is still a challenge. Mitigatory measures are therefore necessary to prevent infection by *A. flavus*. These include the use of mold-resistant varieties of Bambara groundnut such as transgenic plants with anti-fungal or

anti-mycotoxin compounds. The use of non-toxigenic strains of *A. flavus* when applied to the seed or soil during crop planting can competitively inhibit growth of toxigenic strains. Cleaning and drying of seeds to low moisture content before storage, monitoring of temperature and moisture during storage, and educating smallholder farmers on the health hazards of mycotoxin contamination of food commodities and good agricultural practices such as crop rotation, avoiding overcrowding during planting, seed handling to minimize insect damage, avoiding planting during periods of high temperature and water stress etc., will further prevent mold growth. There is need for legislation of AF contamination in Bambara groundnut in Southern Africa and the establishment of surveillance systems to regularly monitor mycotoxin contamination in agricultural food commodities.

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