CHANGES IN FUNGAL COMMUNITY AND AFLATOXIN LEVELS DURING PEANUT BUTTER PRODUCTION AND STORAGE

By

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Declaration

I, **Hamiwe Abigail**, hereby declare that this dissertation is my original work. It has never been submitted at this University or any other University for the award of any academic qualification. All published work or material from other sources incorporated in this dissertation have been acknowledged and adequate reference thereby given.

Signature: …….. Date……..……

Certificate of Approval

This dissertation of Abigail Hamiwe has been approved as fulfilling the requirements for the award of Master of Science in Molecular Biology by The University of Zambia.

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Abstract

Most peanut butter brands on the Zambian market have been reported to contain high levels of aflatoxins (AFs). It is not clear whether these high levels are from the use of contaminated raw peanuts, proliferation during processing or storage. The changes in fungal community and AF levels during production and three months storage of peanut butter were monitored. The AF producing ability of isolated *Aspergillus* species was also assessed. The investigation was carried out on two peanut varieties: *Chalimbana* and *Kadononga*, both varieties obtained from a market and farm. Peanut butter was processed from sorted peanuts and stored at room temperature. Room temperature was recorded daily during storage. The raw peanuts were evaluated for moisture content, water activity, fungal and AF content, using established procedures. Changes in fungal and AF content of peanuts from various stages of the processing line including: roasting, blanching and grinding, as well as peanut butter samples under storage were assessed. The isolated fungi were identified by sequencing the ITS gene fragment and AF levels were assessed using lateral flow immunochromatography. The AF producing ability of isolated *Aspergillus* species from all peanut samples was also done. The following nine fungal genera were isolated from raw peanuts: *Cladosporium*, *Aspergillus*, *Fusarium*, *Penicillium*, *Epicoccum*, *Alternaria*, *Talaromyces*, *Curvularia* and *Xenocamarosporium*. A reduction in fungal diversity was observed during processing. Only three fungal genera were isolated from peanuts from the processing line: *Cladosporium*, *Penicillium* and *Talaromyces*. Fungal growth in peanut butter stored in a temperature range of 18.3 - 31.8 °C was minimal but changes in fungal diversity were observed during storage. The AF producing potential of four isolates of *Aspergillus* indicated that out of the four, three were atoxigenic and only one strain was toxigenic. Sorting of raw peanuts reduced AF levels by 38 - 92%. Further reduction was observed after processing and there was no significant change in AF levels during storage. The present study concludes that processing of peanuts into peanut butter reduces fungal diversity as well as AF levels and no significant changes occur in AF levels during three months of storage when peanut butter is stored in a temperature range of 18.3 - 31.8 °C. Therefore, high levels of AF in peanut butter are most likely due to the use of contaminated peanuts. Sorting is therefore recommended prior to processing.

Keywords: Peanut butter, storage, *Aspergillus*, aflatoxin, sorting

Dedications

This dissertation is dedicated to my sons Nachiloba and Nathan for giving me a reason to keep pushing on even when the odds were against me, you are my biggest motivators.

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Abbreviations and Acronyms

AF	Aflatoxin
AFB1	Aflatoxin B1
AFB2	Aflatoxin B2
AFG1	Aflatoxin G1
AFG2	Aflatoxin G2
AIDS	Acquired Immuno Deficiency Syndrome
AOAC	Association of Official Analytical Chemists
CAC	Codex Alimentarius Commission
DNA	Deoxyribonucleic Acid
ELISA	Enzyme-Linked Immunosorbent Assay
EU	European Union
FAO	Food and Agriculture Organization
HPLC	High Performance Liquid Chromatography
IARC	International Agency for Research on Cancer
ITS	Internal Transcribed Spacer
LSU	Large Sub-Unit
NIR	Near Infrared
PCR	Polymerase Chain Reaction
ppb	Parts per billion
R.H	Relative humidity
RT-PCR	Reverse transcriptase-Polymerase Chain Reaction
RTi-PCR	Real Time-Polymerase Chain Reaction
RNA	Ribonucleic Acid
SSU	Small Sub-Unit
TLC	Thin-Layer Chromatography
UV	Ultraviolet
Vis	Visible
WHO	World Health Organization
ZABS	Zambia Bureau of Standards

CHAPTER ONE: INTRODUCTION

1.1 Background

Peanut (*Arachis hypogaea* L.) also known as groundnut is a member of the *Fabaceae* family. It is a rich source of proteins, fats, carbohydrates, vitamins and minerals (Atasie *et al*., 2009; Chun, 2002). Due to its high nutritional value, it has several uses for human and animal consumption.

Peanuts are consumed raw, roasted, boiled or as a butter or paste (peanut butter). Peanut butter is produced by grinding dry roasted and blanched peanuts into a paste. Processing of peanuts into peanut butter has a number of benefits which include changing the eating quality of the raw peanuts and adding economic value to the raw peanuts. Peanut butter is widely used in porridge, on sandwiches and for thickening soups. In developing countries, peanut butter is also a major ingredient in therapeutic foods (Manary, 2006).

Peanuts are exclusively grown in the tropics and subtropics. In Zambia, peanuts are the second most grown crop after the staple food maize (Ross *et al*., 2012). One major constraint to the production and full exploitation of commercial benefits from peanuts and its products is the challenge of fungal and aflatoxin contamination. Contamination of peanuts can occur during production, storage, transportation and marketing (Nigam *et al.,* 2009).

Various fungi have been demonstrated to contaminate peanuts. These include: *Aspergillus*, *Alternaria*, *Cladosporium*, *Epicoccum*, *Eurotium*, *Fusarium*, *Penicillium*, *Rhizopus*, *Rhizoctonia*, *Rhodotorula* and *Sclerotium* (Gachomo *et al*., 2004; Hedayati *et al*., 2010; Embaby and Abdel-Galel, 2014). Some of these fungi produce mycotoxins, of which the most toxic are aflatoxins, fumonisins and ochratoxins, with aflatoxins being the most toxic to mammals (Reddy *et al*., 2010). Aflatoxins are a group of secondary metabolites produced by *Aspergillus* species, particularly, *A. flavus*, *A. parasiticus*, *A. tamorii*, *A. nominus*, *A. pseudotamarii*, *A. bombycis* and *A. ochraceoroseus* (Novas and Cabral, 2002; Cary *et al*., 2005; Varga *et al*., 2003; Frisvad *et al*., 2005; Njoroge *et al*., 2016b).

Aflatoxins occur in four main chemical types: aflatoxin B1 (AFB1), aflatoxin B2 (AFB2), aflatoxin G1 (AFG1) and aflatoxin G2 (AFG2), with AFB1 being the most toxic (Olaru *et al*., 2008).

Humans and animals are generally exposed to aflatoxins through their diet. Low level exposure to aflatoxins is associated with malnutrition (Okoth and Ohingo, 2004) and stunted growth in children (Castelino *et al*., 2015). Exposure to aflatoxins is also associated with increased risk of developing liver cancer, suppression of the immune system and increased rate of progression from human immunodeficiency virus infection to AIDS (Jiang *et al*., 2008; Jolly *et. al*., 2011). As a result of these health concerns, countries worldwide have set standards for the maximum amount of aflatoxin permissible in foods (William *et al*., 2004). The Codex Alimentarius Commission (CAC) has set maximum tolerance levels for total aflatoxins in peanuts at 10 parts per billion (ppb). The upper limit set by the European Union for total aflatoxin (AFB1+AFB2+AFG1+AFG2) in peanuts is 4 ppb and 2 ppb for AFB1 (Commission of the European Communities, 2006). Zambia complies with standards set by CAC hence the maximum acceptable limits for total aflatoxins is 10 ppb (Kachapulula *et al*., 2017). A number of studies have shown that majority of raw peanuts and peanut products on the Zambian market exceed the acceptable limits (Kachapulula *et al*., 2017; Njoroge *et al*., 2016a). This is a health concern and a hindrance to international trade.

Processing of peanut butter is known to reduce aflatoxins to levels acceptable by food safety regulatory agencies of many countries (Siwela *et al*., 2011). However, Njoroge *et al*., (2016a), in a three-year study (2012 - 2014), reported that most peanut butters on the Zambian market have high levels of aflatoxins with local brands having significantly higher levels than imported brands. The authors of this study suggested some ways of reducing aflatoxins including adherence to good manufacturing practices during crop production, monitoring of raw materials and finished products, as well as ensuring that suppliers of peanuts understand regulations pertaining to food standards and supply products that meet specifications. Ideally, if all this was followed, it is evident that the problem of aflatoxins would be tackled. However, it is not clear if these high levels that were found in the Zambian peanut butter samples were due to contaminated raw materials (peanuts) or produced during the production stages or subsequent storage

period (post production). The objectives of the present study included assessing changes in fungal community and aflatoxin contamination during processing and storage of peanut butter. The study also determined the aflatoxin producing ability of fungal isolates by screening them for presence of the seven aflatoxin biosynthetic genes-*aflR, aflS, aflD, aflM, aflO, aflP* and *aflQ*.

1.2 Justification of study

Numerous studies have been carried out on fungal and aflatoxin contamination in raw peanuts and peanut butter. However, there is limited work on changes in fungal community and aflatoxin levels during processing and storage of peanut butter. A number of studies have determined fungal and aflatoxin content in peanut butter. However, the peanut butter processing conditions were not always known as most samples tested in the studies were procured from retail shops. Additionally, the storage period at which the peanut butter was sampled may not have been recorded or known as well as the storage temperature. One study, (Baur, 1975) monitored aflatoxin levels in peanut butter stored at a constant temperature of 22.8 ℃ for a duration of two years. However, aflatoxin production occurs optimally at 24 ℃ (Joffe and Lisker, 1969) and peanut butters in most retail shops are not stored at constant ambient conditions. Therefore, the present study processed, packaged and stored the peanut butter under uncontrolled natural conditions, conditions under which most peanut butters in retail shops are stored.

To the best of my knowledge, there is currently no study that has monitored changes in fungal community of peanuts during processing stages or storage of peanut butter hence the present study was conceived to contribute to the understanding of the effects of peanut processing and storage on the quality of peanut butter.

The present study, also, assessed the toxigenicity or aflatoxin producing ability of *Aspergillus* species isolated from selected peanut varieties commonly grown in Zambia by screening the isolates for the presence or absence of seven aflatoxin biosynthetic genes. Numerous studies have determined toxigenicity in *Aspergillus* species, especially in *A. flavus* and *A. parasiticus* the two main aflatoxin producers. Information on toxigenicity in populations of *A. flavus* however varies considerably with strain,

substrate and geographic origin hence the attempt to determine toxigenicity of species in Zambia. There is a general lack of information on toxigenicity in *Aspergillus* species in Zambia hence the present study will broaden the knowledge of toxigenic and non-toxigenic *Aspergillus* species in select peanut varieties grown in Zambia.

1.3 Problem statement

Njoroge *et al.,* (2016a) in a three year study (2012 - 2014), have shown that some of the peanut butter brands on the Zambian market have high levels of aflatoxins, with local brands having significantly higher levels than imported ones. Results from the above study show that the problem of aflatoxin contamination in Zambian peanut butter was significant posing a considerable health risk to consumers. To contribute to the search for solutions to the problem of aflatoxin contamination, the present study monitored changes in fungal community and levels of aflatoxin in peanuts during peanut butter production and storage in attempts to determine if the high levels of aflatoxins in peanut butter could be attributed to the use of contaminated raw materials (peanuts) or accumulation of aflatoxins during the production stages or subsequent storage period (post production).

1.4 Aim

The aim of this study was to assess the changes in fungal community and aflatoxin levels during processing and storage of peanut butter. The study also assessed the aflatoxin producing ability of *Aspergillus* isolates that are prevalent in peanuts.

1.4.1 Specific objectives

The specific objectives of the study were to:

1. Isolate and determine the fungal species and aflatoxins present in samples of selected varieties of post-harvest peanuts.
2. Isolate and determine the fungal species and aflatoxins present at select stages of peanut butter making process
3. Monitor changes in fungal community and aflatoxin levels in peanut butter during a shelf life period of three months.

4. Determine the aflatoxin producing ability of the *Aspergillus* isolates extracted from the samples by screening the isolates for presence of seven genes usually attributed to aflatoxin production.

1.5 Research questions

1. What post-harvest fungal species are predominant in selected peanut varieties in Zambia?
2. What are the fungal species and aflatoxin levels present at select stages of peanut butter making process?
3. Are there any changes in fungal and aflatoxin levels during a storage period of three months?
4. Do the isolated *Aspergillus* species possess the ability to produce aflatoxins?

1.6 Significance of the study

The results generated from this study will be of use to peanut butter manufacturers and retailers to implement control measures in aflatoxin management. The knowledge will also be useful to the Ministry of Agriculture and the Ministry of Health who are all concerned with high levels of fungal and aflatoxin contamination in peanuts and its products. Consumers too, will also benefit from the results. The insights gained will also contribute to academic literature for use as reference material by academic institutions such as The University of Zambia.

1.7 Scope of study

The two main parameters analysed in this study were the fungal community and aflatoxin levels of raw peanuts and peanut butter produced from two varieties and two sets of raw peanuts. Two varieties of peanuts namely *Chalimbana* and *Kadononga* commonly grown in Zambia were procured from a farm in Eastern province and two other samples of the same varieties were purchased from an open market in Lusaka province of Zambia. Four sets of peanut butter were produced from the four samples and stored at room temperature for a period of three months. The peanut butter was stored at room temperature in order to mimic the storage conditions in retail shops and most households. The storage period was from 17th May to 15th September, 2017.

1.8 Definitions of terms

For the purpose of this study the following terms have been defined as follows;

1. **Processing**: The process of transforming raw materials into finished products.
2. **Blanching**: The mechanical removal of the coat or skin of peanut kernels.
3. **Storage**: The keeping of materials or samples in a place under controlled environmental conditions for future use.
4. **Water activity**: The water activity (a_w) of a particul$_a$r food is the ratio between the vapour pressure of the food itself, when in a completely undisturbed balance with the surrounding air media and the vapour pressure of distilled water under identical conditions.
5. **Toxigenicity**: The degree to which an organism is able to produce toxins or poisons**.**
6. **Gene**: A segment of DNA that codes for RNA and proteins.
7. **Biosynthesis**: A multi-step, enzyme-catalysed process where substrates are converted into more complex products in living organisms.
8. **Aflatoxins**: Poisonous carcinogenic secondary metabolites produced by certain fungi.

CHAPTER TWO: LITERATURE REVIEW

2.1 Introduction

This section gives detailed information on the following: fungal and aflatoxin contamination in raw peanuts and peanut butter, health concerns associated with aflatoxins, the use of Polymerase Chain Reaction (PCR) in determining the aflatoxin producing ability of *Aspergillus* species, effects of sorting, roasting, blanching and grinding processes on aflatoxin contamination, as well as the effects of storage on aflatoxin contamination in peanut butter.

2.2 Fungi associated with raw peanuts and peanut butter

Various fungi have been found to contaminate raw peanuts and peanut butter. Contamination of raw peanuts can happen in the field during production (pre-harvest) or during storage, transportation and marketing (post-harvest) (Nigam *et al.,* 2009). Field fungi that invade pre-harvest crops in the field include species belonging to the genera *Alternaria*, *Fusarium*, *Cladosporium*, *Aspergillus*, *Penicillium*, *Curvulari*, *Diplodia* and *Gibberella.* Fungal species that typically contact peanuts in storage include members of the genera *Aspergillus*, *Penicillium* and *Rhizopus.* Peanut butter, a product made from roasted peanuts, has been shown to be contaminated by some of these field and storage fungi (Boli *et al*., 2013; Mupunga *et al*., 2014).

Some of the fungi produce secondary metabolites called mycotoxins which are toxic to humans and animals when ingested. The mycotoxins of concern in this study are the aflatoxins produced by *A. flavus*, *A. parasiticus*, *A. tamorii*, *A. nominus*, *A. pseudotamarii*, *A. bombycis* and *A. ochraceoroseus* (Novas and Cabral, 2002; Cary, *et al*., 2005; Varga *et al*., 2003; Frisvad *et al*., 2005), with the first two being the most frequently encountered in agricultural products (Novas *et al*., 2002; Luo, 2014). The production of aflatoxins occurs optimally at a temperature of 25 °C and water activity of ≥ 0.99 (Ribeiro *et al*., 2006). The growing conditions of the two major aflatoxin producing *Aspergillus* species are tabulated in Table 2-1.

Table 2-1: Growing conditions of the most frequently encountered aflatoxin producing *Aspergillus* species.

Fungi	Temperature	Water activity	References
A. flavus	25-30 °C	0.83-0.99	Ribeiro *et al*., 2006
A. parasiticus	25 °C	0.95	Peromingo *et al*., 2016

Fungal species can be isolated from raw peanuts using various methods such as direct inoculation, pour plate, spread plate, agar dilution and blotter test, among others. Identification of the isolates can be achieved using morphological or molecular methods. Morphological identification of fungi makes use of culture appearance and microscopic appearance for positive identification. This type of identification is relatively cheaper but time consuming and not always precise, as demonstrated by Gallo *et al.*, (2012) who used molecular means to re-classify 67 strains of *Aspergillus* previously identified on morphological basis. Their results revealed that four isolates which had been previously described as *A. parasiticus* by Giorni *et al*., (2007) were in fact *A. flavus*. However, genetic similarity between species of certain organisms as well as a high degree of intraspecific variability can prevent a clear differentiation of various species using molecular methods (Gallo *et al.*, 2012).

2.3 Identification of fungal isolates using morphological methods

Identification of fungal isolates using morphological methods uses spore producing structures formed from asexual or sexual reproduction, as a means of identifying fungal species (Hyde *et al*., 2010). This method of identification has its drawbacks such as limited number of morphological characters that can be used for identification. In addition, these morphological characters can lead to false identification due to hybridization, cryptic speciation and convergent evolution (Lücking *et al*., 2014; Foltz *et al*., 2013). As a result of these draw backs, morphological identification often fails to differentiate fungi at specie level thus leading to false identification (Gallo *et al*., 2012). Moreover, some species may not grow or produce reproductive structures in culture and as such may be missed by traditional sampling methods, even though they could be important members of the fungal community (Schmit and Lodge, 2004).

A number of researchers have used morphological methods to identify fungi isolated from raw peanuts and peanut butter. Embaby and Abdel-Galel, (2014), following established procedures isolated and identified *Fusarium*, *Aspergillus*, *Rhizopus*,

Epicoccum and *Penicillium* fungal species from peanut kernels and pods sampled from five different provinces in Egypt. Similarly, following established procedures, Gachomo *et al.*, (2004) isolated and identified various species of members of the *Rhizopus, Fusarium, Aspergillus, Penicillium, Eurotium, Sclerotium* and *Rhizoctonia* groups from fresh peanuts sampled from selected informal markets in Kenya.

Fungi have also been isolated from peanut butter as reported for instance by Boli *et al*., (2013), following established procedures. The researchers isolated and identified fungal species from peanut butter sampled from retail markets in Ivory Coast: *Mucor, Alternaria, Helmintosporium, Geotrichum, Fusarium, Cladosporium, Penicillium* and *Aspergillus*. In a related study, Mupunga *et al.*, (2014), isolated and identified *A. flavus* and *A. parasiticus* from peanut butter sampled from retail shops and the informal market of Bulawayo, Zimbabwe.

2.4 Identification of fungal isolates using molecular methods

Several innovations have been developed for the purpose of identifying and classifying fungi at molecular level to higher orders using deoxyribonucleic acid (DNA) or ribonucleic acid (RNA) obtained directly from environmental sources such as soil, water, air and tissues of other organisms or from cultured fungal isolates (Hibbett *et al.*, 2011; Lindahl *et al*., 2013). These include the use of barcoding technology based PCR-amplification methods for both coding and non-coding DNA sequences (Raja *et al.*, 2017). Among the non-coding sequences typically used for fungal identification is the internal transcribed spacer (ITS) region of the ribosomal DNA (rDNA) gene complex. Also found within this complex is the large subunit (LSU-28S) and small subunit (SSU-18S) (Raja *et al*., 2017), Figure 2-1. The ITS region of fungi which comprises of ITS1, 5.8S, ITS2 is typically 450-800 bp in size (Raja *et al*., 2017). Certain regions of the LSU, SSU and ITS are used as gene markers. The choice of which marker to use depends on the level of classification required. The three regions have different rates of evolution, resulting in varying levels of genetic variation (Raja *et al.*, 2017). SSU evolves the slowest, thus producing the lowest amount of variation among taxa, while the ITS evolves the fastest and exhibits the highest variation (Raja *et al*., 2017; Bruns *et al*., 1991; Mitchell *et al*., 2006). Therefore, if fungi are to be identified at the family, order, class or phyla level, the SSU can be used (White *et al*., 1990), if the aim of a

study is to identify fungi at the family or genera level the LSU can be used (Vilgalys *et al.*, 1990). If identification needs to be made at the species level, then the ITS which is the fastest evolving portion of the rRNA cistron is used (White *et al.*, 1990). The universal primer pairs ITS 1 and ITS 4 designed by White *et al.*, (1990) have been the most commonly used in the identification of many fungi, while many other primer sequences within the rDNA gene complex have also been published and could be used for identification purposes (Raja *et al.*, 2017).

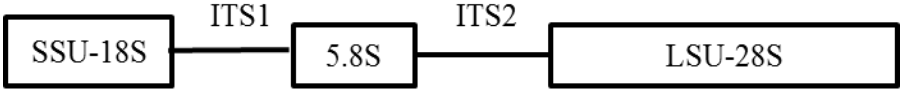

Figure 2-1: Ribosomal DNA (rDNA) gene complex (source:https://ars.els-cdn.com/content/image/1-s2.0-S0975947617301365figs5_lrg.jpg).

The ITS region has large barcode gaps and is easy to amplify and as such it was chosen as the official barcode for fungi by a consortium of mycologists (Hibbert *et al.*, 2016). Even though the ITS region and the DNA regions encompassing the small and large subunits have been instrumental in the work on fungal species identification and highly useful in generating information in databases on mycological identification, it has been demonstrated to have certain limitations. In some species that have no or narrow barcode gaps such as *Aspergillus, Trichoderma and Fusarium* (Raja *et al.*, 2017), the applicability of the ITS has been found to be limited. For this reason, protein-coding genes have also been identified as useful for species identification. Among the reasons these are useful is that in addition to containing exons, they contain introns, which have been hypothesised to evolve at a faster rate than the ITS region (Raja *et al.*, 2017). For purposes of higher taxonomic level identification, protein-coding sequences have also been proposed to have higher resolutions than genes that code for ribosomal RNA (Raja *et al.*, 2017). Examples of protein coding gene markers include: *β*-tubulin (*tub2/BenA*), translation elongation factor 1-α (*tef1*), RNA polymerase II largest (RPB1) and second largest subunits (RPB2) and mini-chromosome maintenance protein (MCM7), among others. Although these protein coding genes offer better resolution than ITS in certain genera, they are often difficult to amplify and sequence. This is because they occur as a single copy within the genome rather than as multiple copy tandem repeats as with the ribosomal genes.

A number of researchers have used ITS1-5.8S-ITS2 based primers to identify fungi in stored peanut butter. For example, Mahmoud (2015) used the ITS1-5.8S-ITS2 based primers FLA1 and FLA2 to ascertain the identity of 22 *A. flavus* isolates from raw peanuts collected from various markets in the Riyadh region of Saudi Arabia.

2.5 The use of PCR in determining the aflatoxin producing ability of *Aspergillus* species

The major species of *Aspergillus* that produce aflatoxins are *A. flavus*, *A. parasiticus*, *A. tamorii*, *A. nominus*, *A. pseudotamarii*, *A. bombycis* and *A. ochraceoroseus* (Novas and Cabral, 2002; Cary, *et al*., 2005; Varga *et al*., 2003; Frisvad *et al*., 2005). Gallo *et al.,* (2012), however, noted that presence of these *Aspergillus* species would not necessarily culminate into accumulation of aflatoxins as some species and strains of these *Aspergillus* species were observed to be atoxigenic.

Toxigenic and atoxigenic *Aspergillus* species can be differentiated by detecting the presence or expression of DNA and RNA of the seven aflatoxin biosynthesis genes or the lack of it (Mahmoud, 2015). The genes associated with aflatoxin biosynthesis include the regulatory genes *aflR* and *alfS*, and the structural genes *alfD, alfM, aflO, aflP* and *alfQ* (Gallo *et al*., 2012). Of the seven aflatoxin biosynthesis genes, *aflD* and *aflQ* are the best markers for differentiating toxigenic from atoxigenic isolates (Mahmoud, 2015).

Detection of presence of aflatoxin biosynthetic genes by fungi in selected food commodities can be done using PCR whereas the expression of the aflatoxin biosynthetic genes can be done using reverse-transcription PCR (RT-PCR) and real-time PCR (RTi-PCR). The detection of expression of genes is more precise than detecting the presence of genes as presence of a gene does not always result into expression of that gene as demonstrated by Gallo *et al.,* (2012) and Mahmoud *(*2015). Gallo *et al.,* (2012) used PCR to screen *Aspergillus* isolates for presence of the seven biosynthetic genes in order to determine their ability to produce AFB1 and they also determined the actual capability of the isolates to produce AFB1. They observed that atoxigenic isolates lacked the PCR products corresponding to three, four or all seven genes. However, three strains were not able to produce AFB1 although they possessed

all seven amplicons. Mahmoud (2015) on the other hand used PCR and RT-PCR to determine the aflatoxin producing ability of *A. flavus* isolates by screening for both the presence and expression of only four of the seven genes-*aflD, aflM, aflP* and *aflQ*. The PCR results showed that both toxigenic and atoxigenic *A. flavus* isolates harboured all the four genes whereas RT-PCR showed that gene presence doesn't correlate with aflatoxin production. Other authors have merely detected the actual production of aflatoxins as a means of differentiating toxigenic from atoxigenic strains. Kachapulula *et al*., (2017) conducted a study in which they determined the toxigenicity of *A. flavus* and *A. parasiticus* isolated from maize and groundnuts from 23 districts in Zambia by screening isolates for actual production of aflatoxin. Njoroge *et al.*, (2016b), also assessed toxigenicity of various *Aspergillus* species isolated from soils in Eastern province of Zambia by screening isolates for production of aflatoxins.

This study used PCR to determine the aflatoxin producing ability of *Aspergillus* species isolated from raw peanuts and peanut butter by screening them for the presence of the seven aflatoxin genes.

2.6 Aflatoxin contamination of raw peanuts

Aflatoxin contamination of raw peanuts is caused by infection of the crop by one or more of the *Aspergillus* aflatoxin producing species. Contamination of raw peanuts can occur during production, storage, transportation and marketing (Nigam *et al.,* 2009). Consumption of contaminated raw peanuts or peanut products made from contaminated raw peanuts has health implications. Lovelace and Salter, 1979 and Njapau *et al*., 1985 confirmed the presence of aflatoxins in Zambian meals with aflatoxin levels ranging from 1-50 ppb. Further studies confirmed the presence of aflatoxin metabolities in the urine of both children and adults (Lovelace *et al*., 1982; Dil 1986). Consumption of aflatoxins has health implications such as malnutrition (Lovelace *et al*., 1988; Okoth and Ohingo, 2004) and stunted growth in children (Castelino *et al*., 2015). Exposure to aflatoxins is also associated with increased risk of developing liver cancer, suppression of the immune system and increased rate of progression from human immunodeficiency virus infection to AIDS (Jiang *et al*., 2008; Jolly *et. al*., 2011). As a result of these health concerns, countries worldwide have set standards for the maximum amount of aflatoxin permissible in foods (William *et al*., 2004). The Codex Alimentarius Commission

(CAC) has set maximum tolerance levels for total aflatoxins in peanuts at 10 parts per billion (ppb). While, the upper limit set by the European Union for total aflatoxin (AFB1+AFB2+AFG1+AFG2) in peanuts is 4 ppb and 2 ppb for AFB1 (Commission of the European Communities, 2006).

The methods for aflatoxin detection and quantification in food products vary. These include the use of thin-layer chromatography (TLC), high-performance liquid chromatography (HPLC), mass spectroscopy, enzyme-linked immune-sorbent assay (ELISA), lateral flow devices (Immuno dip sticks) and electrochemical immunosensors, among others.

A number of researchers have used the above methods to detect or quantify aflatoxins in raw peanuts and peanut butter. For example, Bumbangi *et al.,* (2016) used HPLC to analyse aflatoxin contamination in raw peanuts from open markets and supermarkets from Lusaka district. They observed that 55.4% (51 out of 92) of the samples were contaminated with aflatoxin. The concentration levels of aflatoxins were generally low and ranged from 0.014 - 48.67 ppb. In a related study, Kachapulula *et al*., (2017) used lateral-flow immunochromatography to analyse aflatoxin contamination in 162 samples of raw peanuts sampled from markets and farm storages in 27 districts of three agro-ecological regions of Zambia. They observed that raw peanuts from two of the three regions had average aflatoxin levels higher than the permissible limits of 10 ppb. The high levels of aflatoxin contamination reported by Kachapulula *et al*., (2017) and Bumbangi *et al.,* (2016) are a health concern.

2.7 Interventions to tackle aflatoxin contamination

Aspergillus and aflatoxin contamination of raw peanuts often begins before harvest. Factors such as genotype (Mehan *et al*., 1986), soil type (Mehan *et al*., 1991), drought (Holbrook *et al*., 2000), insect activity (Singsit *et al*., 1997) and harvest conditions promote aflatoxin contamination before harvest. Pre-harvest intervention include: inoculating the fields with nonaflatoxigenic strains (Dorner *et al*., 1992), insect control and timely harvest. Post-harvest interventions include: rapid and adequate drying after harvest (Awuah and Ellis, 2002) and sorting of peanuts in shell before storage and again on kernels before processing into other food products (Meghan *et al*., 2012).

Sorting is a post-harvest intervention method that seeks to eliminate agricultural products with sub-standard quality (Udomkun *et al*., 2017). Sorting of grains can be achieved based on differentiation of physical properties such as colour, size, shape and density, as well as visible identification of fungal growth in affected grains (Udomkun *et al*., 2017). Methods for sorting include physical and computer-based image processing techniques. Physical methods include sorting by hand, floating and density separation, and electronic colour sorting (Udomkun *et al*., 2017). These physical methods are cost effective but often laborious and impractical for large-scale screening of fungal and toxin contamination. Computer-based image processing methods make use of Vis, UV and NIR to detect contaminated grains (Udomkun *et al*., 2017). These methods are more costly but are less laborious and therefore, more applicable for large-scale screening of fungal and toxin contamination (Udomkun *et al*., 2017). A number of researchers have demonstrated the effectiveness of sorting in reducing aflatoxin contamination in foods. Galvez *et al*., (2003) used the manual hand sorting method to sort in shell peanuts. They observed a 95 - 100% reduction in aflatoxins contamination. Sorting has also been shown to reduce aflatoxins in other foods. Shakerardekani *et al*., (2012) used the colour sorting method to sort pistachio nuts. They observed a > 95% reduction in aflatoxin contamination. In another study, Özlüoymak (2014) used an image based sorting method coupled with a colour detection system to sort figs. He observed a 98% reduction in aflatoxin contamination. The present study used the hand sorting method for sorting peanut kernels prior to peanut butter production due to its cost advantage.

2.8 Changes in fungal community and aflatoxin contamination during stages of peanut butter making process; roasting, blanching and grinding

Roasting, blanching and grinding of peanuts have been shown to reduce levels of aflatoxin contamination (Siwela *et al.,* 2011). Siwela *et al.,* (2011) used HPLC to quantify aflatoxin levels during processing of peanuts into peanut butter. They observed a 51% reduction in aflatoxins after roasting, 27% after blanching followed by a further 11% after grinding to make peanut butter. This translated into a cumulative total reduction of 89% of aflatoxin concentration during the production process of peanut butter. In a related study, Ogunswanwo *et al.*, (2004) observed an 81.7% reduction in aflatoxins after roasting peanuts at 150 °C for 30 minutes. However, there are limited

studies that have monitored changes in fungal community during processing of peanuts into peanut butter.

2.9 Changes in fungal community and aflatoxin contamination in peanut butter during storage

Most peanut butter brands on the Zambian market have been reported to have high levels of aflatoxins approximated to be above 10 ppb (Njoroge *et al*., 2016a). Njoroge *et al.,* (2016a) used ELISA to analyse and compare AFB1 levels in imported brands of peanut butter with those in local brands on the Zambian market from 2012 - 2014. They observed that local brands had significantly higher levels of aflatoxin than imported brands. During the 2012, sampling period, they observed average aflatoxin concentration of 10 ppb and 24 ppb in imported brands and local brands, respectively. In 2013, they observed aflatoxin concentration averages of 55 ppb and 130 ppb in imported brands and local brands, respectively. In 2014, the observed aflatoxins concentrations in the samples ranged from the mean of 6 ppb in imported brands to 35 ppb in local brands.

In a related study, Mupunga *et al*., (2014) also used HPLC to analyse aflatoxin contamination in peanut butter purchased from retail shops and the informal market of Bulawayo. They observed that 91% (10 out of 11) of the samples were contaminated with aflatoxins. The concentration levels of aflatoxins ranged from 6.1 - 247 ppb and an average of 75.66 ppb. Chen *et al.,* (2013) also used HPLC to analyse various peanut products for aflatoxin contamination in Taiwan from 1997 - 2011. Aflatoxins were detected in 32.7% of samples with levels ranging from 0.2 - 513.4 ppb, with peanut butter having the highest aflatoxin contamination.

Two of the weaknesses in the studies that detected or quantified aflatoxin contamination in peanut butter are that the conditions under which peanut butter was processed were not always known as most samples used in the studies were procured from retail shops. The amount of time that the samples had spent on shelves from the time of manufacture as well as the various storage conditions to which they were exposed by the time of sampling may also not have been well-documented. This study was designed to account for the processing conditions and duration of storage.

There is generally limited literature on changes in aflatoxin contamination during storage of peanut butter. Baur, (1975) monitored aflatoxin levels in peanut butter stored at a constant temperature of 22.8 ℃ for a duration of two years. The peanut butter samples were withdrawn from storage at 0, 6, 12, and 24 months and analysed in comparison to materials retained at 0 ℃. The author did not observe any statistically significant changes in aflatoxin contamination throughout the 2 years of study. Nevertheless, storage conditions in stores fluctuate during storage and aflatoxin production is at its optimum at 24 ℃ (Joffe and Lisker, 1969; Schindler *et al*., 1967).

To the best of our knowledge, there is currently no study that has monitored changes in fungal community during storage of peanut butter.

CHAPTER THREE: MATERIALS AND METHODS

3.1 Sampling and collection of peanuts

3.1.1 Material collection

Four (4) 50 kg samples each of two peanut varieties locally known as *Chalimbana* and *Kadononga* and commonly grown in various parts of Zambia were used in this study. One set comprising of a 50 kg sample of *Chalimbana* and another 50 kg sample of *Kadononga* were purchased from a farm in Eastern province (latitude:-13°00′S and longitude:-32°15′E). The second set of the same varieties and quantities were purchased from an open market in Lusaka province of Zambia (latitude:-15°25'S and longitude:-28°16'E). The four peanut samples were stored at room temperature until production of peanut butter and laboratory analysis.

3.1.2 Handling of raw material

For each of the four peanut samples, approximately, 3 kg of unsorted sub-sample was drawn from three different positions in a 50 kg sack (top, middle and bottom) to analyse for aflatoxin content. The remaining peanuts were manually sorted and cleaned to eliminate broken, defective, discoloured, visibly infected and dirty kernels. From the sorted peanuts, two, 3 kg sub-samples were similarly drawn. One of the 3 kg sorted sub-sample was used for analysis of moisture content and water activity, and the other 3 kg sorted sub-sample was used for the analysis of fungal and aflatoxin content. Aflatoxin content of the grade-outs (broken, defective, discoloured, visibly infected and dirty kernels) was also assessed.

3.2 Processing and storage of peanut butter

Each of the four sorted peanut samples was processed into peanut butter according to the method outlined by Tressler and Woodroof (1983) with slight modifications in the roasting time and temperature. For each of the four sorted peanut samples roasting was done in batches of 3 kg in a modified peanut roaster for 65 minutes at a temperature of 147 °C. Roasted peanuts were left to cool at room temperature after which they were blanched by hand and ground into a fine homogeneous paste using a peanut butter

processing machine (Hebei Iron-Lion, China). The processed peanut butters were packed in 150 g plastic bottles with airtight lids and labelled to differentiate the two varieties and their source. The peanut butter that was processed from *Chalimbana* peanut variety that were sourced from the market and farm were labelled or coded as CM and CF, respectively. While, the peanut butter that was processed from *Kadononga* peanut variety that were sourced from the market and farm were coded as KM and KF, respectively. The four sets of peanut butter: CM, CF, KM and KF were stored at room temperature on an open shelf in the Food Science and Technology laboratory at the University of Zambia for a period of three months. Figures 3-1 and 3-2 illustrate the processing stages.

Storage ambient temperature was monitored daily using a digital thermo-hygrometer (HDE, PA, USA).

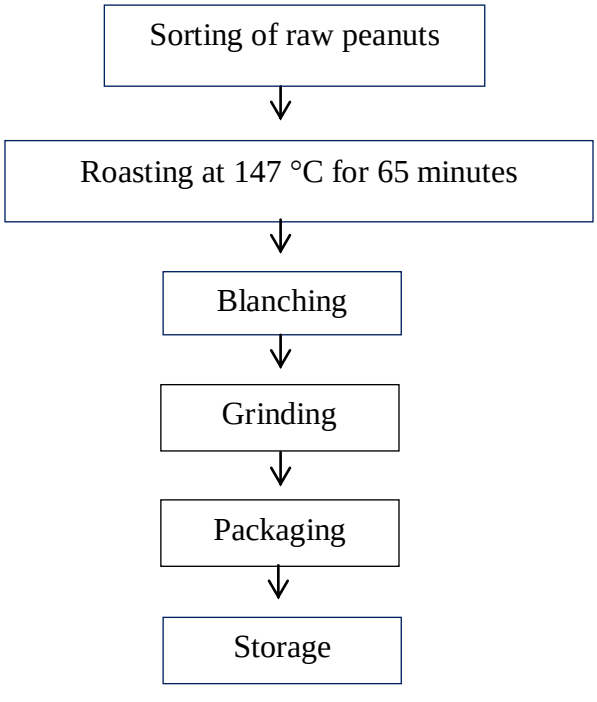

Figure 3-1: Flow chart of peanut butter production

Figure 3-2: Stages of peanut butter production. A-roasting, B-blanching, C-grinding and D-packaging.

3.3 Laboratory analyses of raw peanuts

3.3.1 Preparation of test samples

Laboratory sub-samples for each of the four peanut samples comprised of one 3 kg unsorted peanuts, two 3 kg sorted peanuts and grade-out peanuts. One of the sorted sub-samples was milled using a high speed kitchen blender (Kenwood, Japan) and sieved through a 0.5 mm sieve while the other sub-samples were milled but not sieved. The samples were stored in Ziploc bags prior to analysis. All analysis were conducted in triplicate unless otherwise stated.

3.3.2 Analysis of physical properties of sorted peanuts

3.3.2.1 Moisture content of sorted peanuts

The moisture content of the sorted and milled peanuts was determined using the oven drying method outlined by AOAC (AOAC, 1997). Three (3) grams of the milled and sieved peanuts were weighed in pre-weighed aluminium dishes. The weighed sample

was placed in a dry air oven (Memmert, Germany) and dried at 103 °C overnight. The samples were removed from the oven and cooled in a desiccator. After cooling the samples were weighed and the moisture content of the peanuts was computed from loss in mass using equation 1.

$$\text{Moisture content (\% weight basis)} = \frac{(M_0 - M_1)}{M_0} \times 100 \quad (1)$$

Where:

M_0 - initial weight, in grams of test portion

M_1 - final weight, in grams of dried test portion

3.3.2.2 Water activity of sorted peanuts

The water activity of the sorted and milled peanuts was determined using an AquaLab water activity meter (Decagon Devices, WA, USA) according to manufacturer's instructions. Briefly, the sample cup was half filled with milled and sieved peanuts. The sample cup was placed in the water activity meter and readings were taken in triplicates and recorded.

3.3.3 Analysis of fungal species in sorted peanuts

Fungal species in sorted and milled peanuts were isolated and identified using molecular techniques. Figure 3-3 illustrates the procedures and techniques used in identifying fungal isolates.

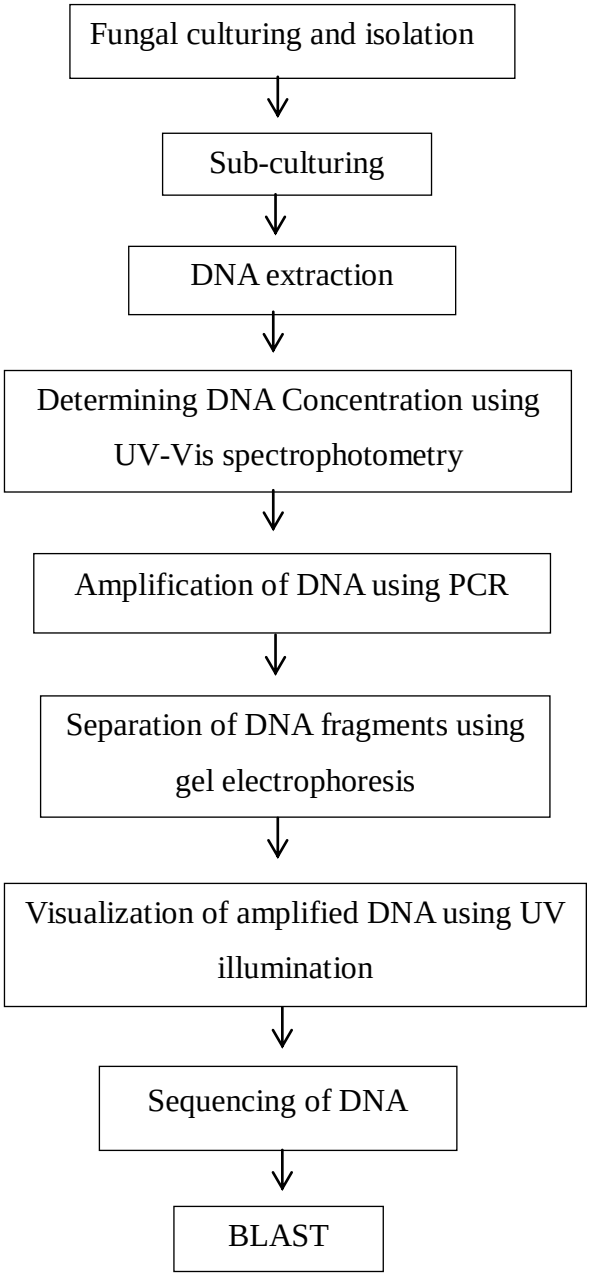

Figure 3-3: Flowchart of procedures and techniques used in identifying fungal isolates from raw sorted peanuts, peanut samples from the processing line and peanut butter during storage.

3.3.3.1 Fungal culturing and Isolation

3.3.3.1.1 Preparation of microbial reagents

Potato Dextrose Agar (PDA; HiMedia, India) medium was prepared by dissolving 39 g of PDA powder in 1000 ml of distilled water. The media was sterilised at 121 °C and 15 psi for 15 minutes using an autoclave then cooled in a water bath at 50 °C. Approximately, 20 ml of the medium was dispensed in disposable Petri dishes and allowed to set before refrigeration.

Yeast extract peptone (YEP; HiMedia, India) was prepared by dissolving 1 g granulated sugar, 2.5 g and 5 g in 1000 ml distilled water. Approximately, 5 ml of the YEP media was dispensed in test tubes and sterilised at 121 °C and 15 psi for 15 minutes in an autoclave. The solution was then cooled and refrigerated.

Buffered peptone water (Oxoid, Canada) was prepared by dissolving 20 g of buffered peptone water in 1000 ml distilled water. Approximately, 225 ml of the peptone water was dispensed in 250 ml conical flasks and sterilised at 121 °C and 15 pounds per square inch (psi) for 15 minutes using an autoclave. The solution was then cooled and refrigerated.

Ringer solution (Oxoid, Canada) was prepared by dissolving 1 tablet of ringer tablet in 500 ml of distilled water. Approximately, 9 ml of the ringer solution was dispensed in test tubes and sterilised at 121 °C and 15 psi for 15 minutes using an autoclave (Dixons, UK). The solution was then cooled and refrigerated.

Extraction buffer was prepared by dissolving 7.45 g potassium chloride in 10 ml of 1 M Tris-HCl (pH 8.0) and 2 ml 0.5 M EDTA (pH 8.0) and making up to 100 ml using distilled water. The solution was stored at room temperature.

3.3.3.1.2 Fungal culturing and isolation

Fungi were isolated from sorted raw peanuts using the spread plate culturing method outlined in the Oxoid manual (1998). Briefly, 25 g of the milled peanuts was incubated in 225 ml of buffered peptone water for 1 hour after which serial dilutions (10^{-1} to 10^{-6}) were made using Ringer solution. Approximately, 0.1 ml of aliquots of a series of

dilutions (10^{-1}, 10^{-3}, $10^{-5}$and 10^{-6}) of peanut samples was inoculated on PDA. Each dilution was run in duplicates and the samples were incubated for 5 days at 25 °C in an incubator (Boekel, PN, USA).

Sub-culturing was repeated two or three times using PDA media so as to obtain pure cultures (Figure 3-4). The pure cultures were preserved in a refrigerator till further analysis.

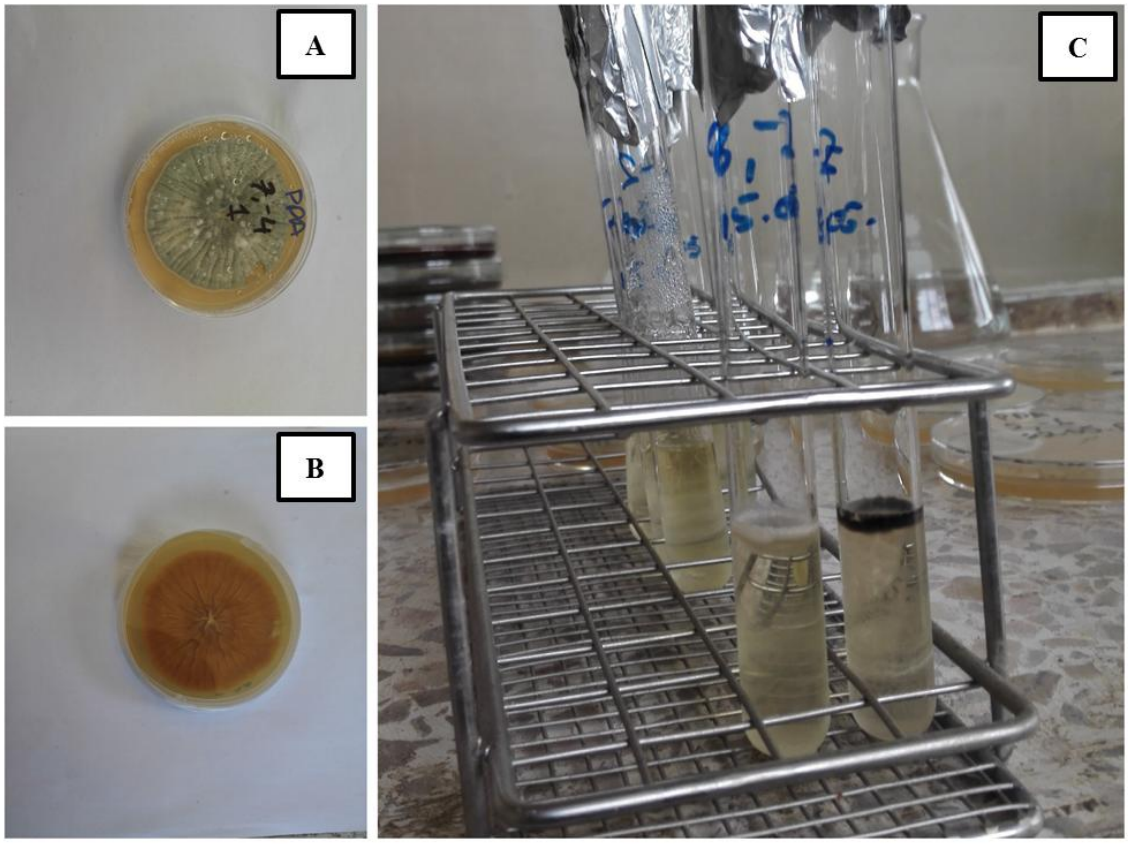

Figure 3-4: Fungal culturing and isolation. A-front side of a pure fungal colony on PDA media, B-reverse side of fungal colony on PDA media and C-sub-culturing in YEP media.

To obtain mycelia for DNA extraction, a loopful of a pure colony was transferred to sterile YEP medium and incubated at 25 °C for 48 hours in a water bath (Figure 3-4).

3.3.3.1.3 DNA extraction

Approximately, 20-70 mg of fresh fungal mycelium was collected using a heat sterilised wire loop and added to extraction buffer. The fungal mass was pulverized in extraction

buffer for 1-2 seconds using a plastic pestle and sand. To minimise cross contamination the plastic pestle was washed in distilled water then 70% ethanol in between samples. (Care was taken so as not to push pestle against the tube and also not to operate the pestle for more than 5 seconds.)

The cell lysate was centrifuged at 5,000 revolutions per minute (rpm) for 10 minutes using a biofuge microcentrifuge (Heraeus, Germany). The supernatant was decanted into a fresh microtube containing 0.3 ml of isopropanol and the remaining cell debris and lysate were discarded. The supernatant and isopropanol were mixed by inverting the tubes several times after which the tubes were centrifuged at 8,000 rpm for 10 minutes and the supernatant was discarded to leave a DNA pellet. The pellet was washed in chilled 70% ethanol by centrifuging at 5000 rpm for 5 minutes and the ethanol was discarded. The remainder of the ethanol was evaporated by incubating at 37 °C for 15 minutes in an incubator (Boekel, PN, USA). Approximately, 50 µl of distilled water was added to the tube and the DNA pellet was dissolved by vortexing at low speed.

The concentration of the extracted genomic DNA was determined using a UV-Vis nanodrop spectrophotometer (Thermo Fisher Scientific, MA, USA).

3.3.3.1.4 Amplification of DNA extracts using PCR

Molecular characterization of fungal isolates was performed according to the method outlined in the Qiagen DNeasy Plant DNA PCR handbook (2003), with slight modifications, using the primer pair ITS1 and ITS4 (Eurofins genomics, Netherlands). Briefly, a master mix for one DNA sample was prepared by mixing the following in an Eppendorf tube: 5 µl of 5×Green GoTaq buffer, 0.5 µl of 25 mM $MgCl_2$, 0.5 µl of 10 mMdNTP, 0.5 µl of forward primer, 0.5 µl of reverse primer, 0.1 µl GoTaq polymerase and 1 µl of DNA template per sample. The final volume of the master mix was adjusted to 25 µl by adding milli-Q water. The PCR amplification was carried out in a thermal cycler (Biorad, CA, USA) using the following amplification parameters: an initial heating to 98 °C for 1 min, 30 cycles of denaturation at 98 °C for 10 s, annealing at 50 °C for 30 s and extension at 72 °C for 60 s, followed by a final 5 min extension at 72 °C. *A. flavus* DNA (Eurofins genomics, Netherlands) was used as a positive control in the PCR assay and negative controls consisted of PCR reagents and 1 µl of milli-Q water.

3.3.3.1.5 Separation of amplified DNA using Gel electrophoresis

Amplified DNA products were separated by agarose DNA gel electrophoresis according to the method outlined in the Qiagen DNeasy Plant DNA PCR handbook (2003) with slight modification, using an electrophoresis equipment (Biorad, CA, USA). The electrophoresis gel was prepared by weighing 3 g of agarose in a glass beaker and adding to it, 150 ml 0.5 × TBE (Tris/Boric acid/EDTA) buffer to give a 2% solution. The mixture was heated in a microwave till the agarose was dissolved. Thereafter, the solution was cooled to 60 °C in a water bath. After cooling, 7.5 µL of GelRed was added to the solution and mixed thoroughly. The solution was then poured into a gel-casting tray and allowed to solidify.

Five (5) microliters of a 100-bp DNA gene ladder (Eurofins genomics, Netherlands) was mixed with 2 µl of the loading buffer and placed in the first well of the gel. Six (6) microliters of each of the PCR products, a positive control and a blank were mixed with 2 µl of the loading buffer prior to loading on the gel. A voltage of 120 Volts was applied to the gel for not more than 60 minutes to allow for DNA separation (Figure 3-5). PCR products were viewed using a UV illuminator (Vilberloumat, France). Successfully amplified PCR samples were shipped to the Netherlands for sequencing by Eurofins genomics. To identify the fungal isolates, sequence similarity searches were performed using BLAST algorithm at the NCBI (National Centre for Biotechnology Information) website (https://www.ncbi.nlm.nih.gov/).

The total number of fungal colonies isolated from all four raw sorted peanut samples was computed and the percentage occurrence of each genus was calculated.

3.3.4 Screening of *Aspergillus* isolates for aflatoxin biosynthetic genes

The DNA extracts which were used for identification of fungal isolates were used for detection of aflatoxin biosynthetic genes. To determine the aflatoxin producing ability of the fungal isolates, the isolates confirmed to be *Aspergillus* species were screened for the presence of seven aflatoxin biosynthetic genes (sequences of the PCR primers designed to amplify the seven aflatoxin biosynthetic genes are shown in Table 3-1**)** using the PCR and agarose gel DNA electrophoresis procedures used for the identification of fungi. An *A. flavus* DNA (Eurofins genomics, Netherlands) was used as a positive control in

the PCR assay and negative controls consisted of PCR reagents and 1 µl of MQ water. After conducting gel electrophoresis, the PCR products were viewed using a UV illuminator (Vilberloumat, France). The presence or absence of a PCR product was indicative of presence or absence of a particular gene, respectively.

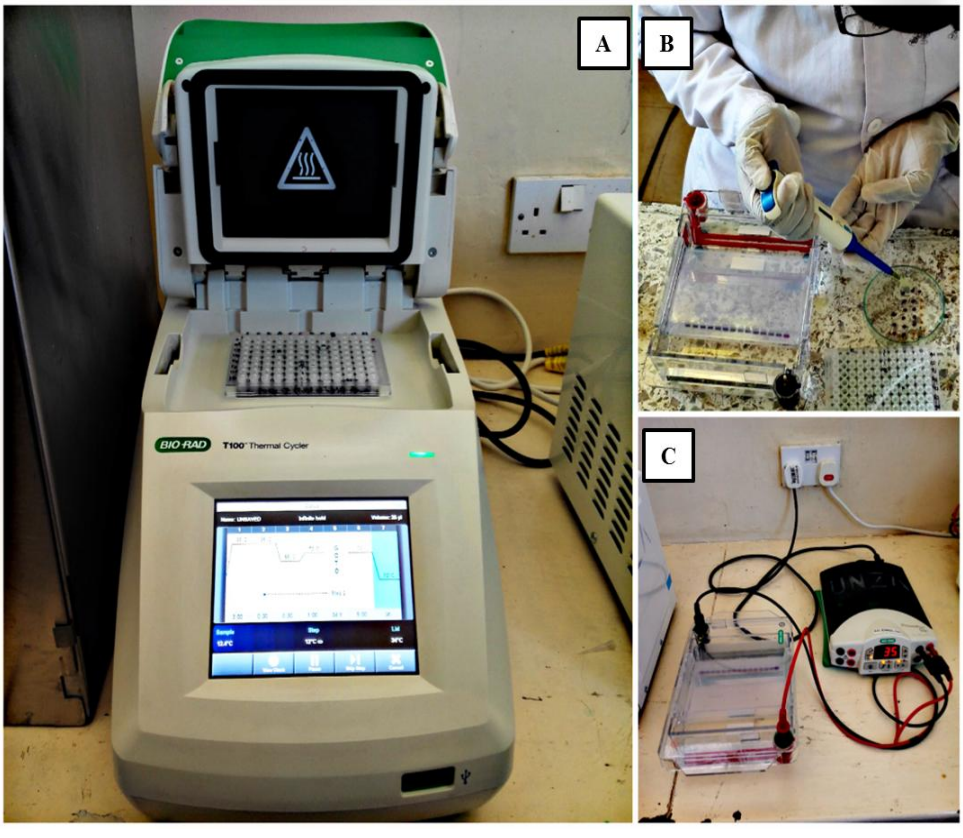

Figure 3-5: PCR and gel electrophoresis analysis of fungal DNA. PCR amplification of fungal DNA was conducted as shown, A. Amplified DNA was loaded in a horizontal agarose gel electrophoresis apparatus for band separation, B and C.

Table 3-1: Details of the PCR primers used for amplifying the seven aflatoxin biosynthetic genes

Primer code	Target gene	Primer sequences	PCR product size (bp)
AflR-1for	*AflR*	5'-AAGCTCCGGGATAGCTGTA-3'	1079
AflR-2rev		5'-AGGCCACTAAACCCGAGTA-3'	
AflS-1for	*AflS*	5'-TGAATCCGTACCCTTTGAGG-3'	684
AflS-2rev		5'-GGAATGGGATGGAGATGAGA-3'	
AflO-1for	*AflO*	5'-TCCAGAACAGACGATGTGG-3'	790
AflO-2rev		5'-CGTTGGCTAGAGTTTGAGG-3'	
aflM F	*AflM*	5'-GCCGCAGGCCGCGGAGAAAGTGT-3'	537
aflM R		5'-GGGGATATACTCCCGCGACACAGC-3'	
aflD F	*AflD*	5'-ACCGCTACGCCGGCACTCTCGGAC-3'	400
aflD R		5'-GTTGGCCGCCAGCTTCGACACTCG-3'	
aflP F	*AflP*	5'-GTGGACGGACCTAGTCCGACATCC-3'	797
aflP R		5'-GTCGGCGCCACGCACTGGGTTGGG-3'	
aflQ F	*AflQ*	5'-TTAAGGCAGCGAATACAAG-3'	719
aflQ R		5'-GACGCCCAAAGCCGAACACAAA-3'	

[1]Codes, target gene, primer sequences and sizes of all primers used; All primers were sourced from Eurofins genomics (Netherlands)

3.3.5 Quantification of total aflatoxin in raw peanuts

Total aflatoxins were quantified in the raw peanuts (sorted, unsorted and grade-outs) using a GIPSA approved lateral-flow immunochromatography assay (Reveal Q+ for Aflatoxin; Neogen Corporation, Lansing, MI, USA) according to manufacturer's instructions.

To extract aflatoxin, 30 ml of 65% ethanol was added to 10 g of milled sample and mixed thoroughly using a rotary shaker (Labinco, Netherlands) for 3 minutes. After shaking, the mixture was allowed to settle after which it was filtered using a Whatman No. 4 filter paper (Whatman, Maidstone, UK). Thereafter, 500 µl of diluent solution (Reveal Aflatoxin Q+) was pipetted into a sample dilution cup, to which 100 µl of filtrate was added. The mixture was mixed 4 - 5 times by drawing and releasing the mixture using a pipette. From the mixture, 100 µl was transferred into a new sample dilution cup. A test strip was placed in the dilution cup for 5 minutes after which it was placed in an mReader tablet and the reading was taken at the 6^{th} minute to determine the quantity of aflatoxin in the sample. The total aflatoxin contents of the samples were expressed in parts per billion (ppb).

3.4 Analysis of peanut samples from the processing line: roasted, blanched and ground peanut butter

3.4.1 Sampling plan and preparation of test samples

Three (3) kilogram each of peanuts was randomly drawn from the roasting and blanching stages. The samples were milled using a high speed kitchen blender (Kenwood, Japan) and stored in Ziploc bags prior to analysis.

Samples at the grinding stage comprised of three randomly selected bottles of freshly processed and packed peanut butter. The peanut butter was thoroughly mixed using a sterilised spatula before analysis.

3.4.2 Analysis of fungal species in roasted and blanched peanuts, and freshly ground peanut butter (day 0)

Isolation and identification of fungal species in roasted and blanched peanuts, and freshly ground peanuts was carried out as described in method **3.3.3**.

The total number of fungal colonies isolated at each stage of processing roasting, blanching and grinding for all the four peanut samples were computed.

3.4.3 Quantification of total aflatoxin in ground peanut butter

Total aflatoxins in peanut butter were quantified as described in method **3.3.5** with slight modification. Extraction of aflatoxins was achieved by adding 30 ml of 65% ethanol to the sample and mixing thoroughly. Mixing was done manually for 3 - 5 minutes because the rotary shaker did not achieve sufficient mixing due to the thick consistency of the peanut butter.

3.5 Analysis of peanut butter samples during a shelf life period of three months

3.5.1 Sampling plan and preparation of test samples

Three bottles of peanut butter were randomly selected every two weeks for analysis and discarded after use. Before analysis the peanut butter was homogenised using a sterilised spatula. All analysis were conducted in triplicates unless otherwise stated.

3.5.2 Water activity of stored peanut butter

The water activity of peanut butter was carried out as described in method **3.3.2.2**. Figure 3-6 illustrates the procedure.

Figure 3-6: Analysis of water activity in peanut butter

3.5.3 Analysis of fungal species in stored peanut butter

Isolation and identification of fungal species in peanut butter was carried out as described in method **3.3.3**.

The total number of fungal colonies isolated from all four peanut butter batches every two weeks was computed.

3.5.4 Screening of *Aspergillus* isolates for aflatoxin biosynthetic genes.

Isolated *Aspergillus* species were screened for aflatoxin biosynthetic genes as described in method **3.3.4.**

3.5.5 Quantification of total aflatoxin in stored peanut butter

Total aflatoxins in peanut butter were quantified as described in method **3.4.3**.

3.6 Statistical analysis

Statistical analyses were performed using GenStat 14th edition Statistical Software. The Student T-test was used to determine significant difference in physical properties of raw peanuts and peanut butter and to determine significant differences in levels of aflatoxin after processing raw peanuts into peanut butter. One-way analysis of variance (ANOVA) was used to detect significant differences in water activity and aflatoxin levels of peanut butter during storage. Fisher's Least Significance differences (LSD) was used to determine whether there were "true differences". A significance level of ($p < 0.05$) was used for the study. All graphs were generated using Microsoft Excel (2010).

CHAPTER FOUR: RESULTS AND DISCUSSION

4.1 Storage conditions of peanut butter

The four sets of peanut butter samples prepared in this study were stored at room temperature over a period of three months. The daily temperature during storage ranged from 18.3 - 31.8 °C, with a mean of 24 °C (Figure 4-1).

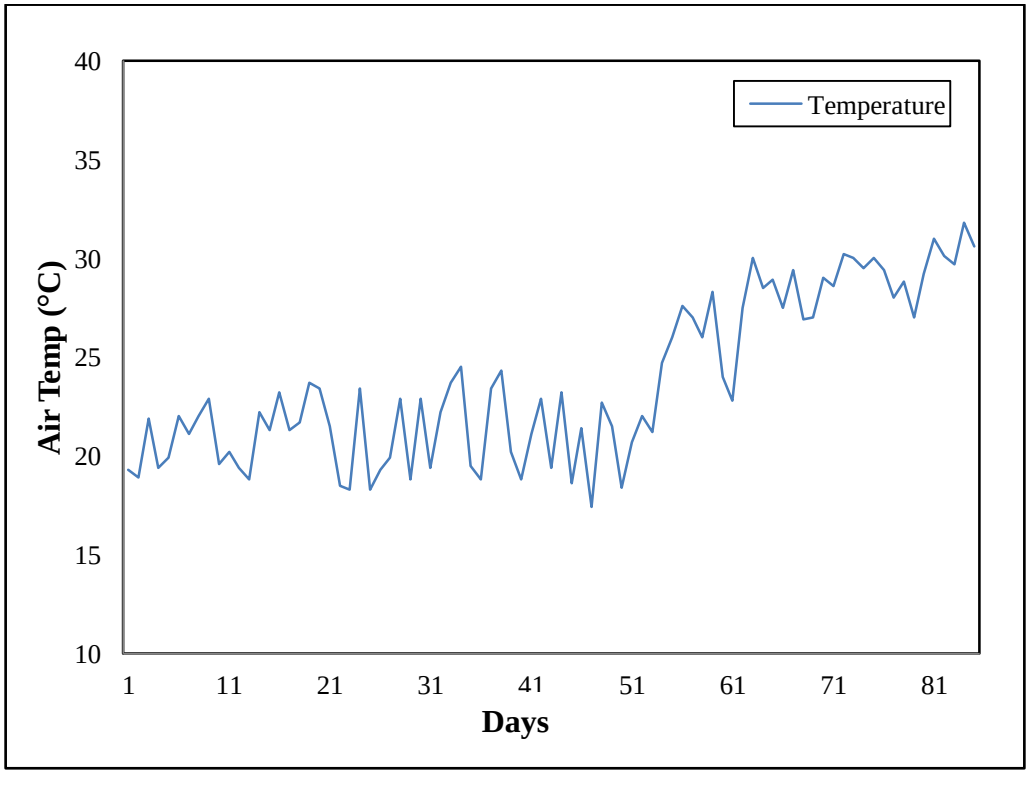

Figure 4-1: Variation of ambient temperature during storage of peanut butter samples made from *Chalimbana* and *Kadononga* in the Food Science and Technology laboratory, UNZA

4.2 Physical characteristics of raw peanuts, peanut butter from production (day 0) and stored peanut butter

4.2.1 The moisture content of raw peanuts

The moisture content of all the four raw peanut samples was analysed and found to be in the range of 3.24±0.01 - 4.06±0.47% (Figure 4-2). These values were lower than the values (6.0 – 8.9%) reported by Musa *et al*, (2010). There was no significant difference in the moisture content of *Chalimbana* peanut variety that were purchased from the farm

and market at ($p < 0.05$). Equally, there was no significant difference in the moisture content of *Kadononga* peanut variety that were purchased from the farm and market at ($p < 0.05$). There were no significant differences in the moisture content of the two peanut varieties *Kadononga* and *Chalimbana* that were purchased from the same source, the farm or market at ($p < 0.05$).

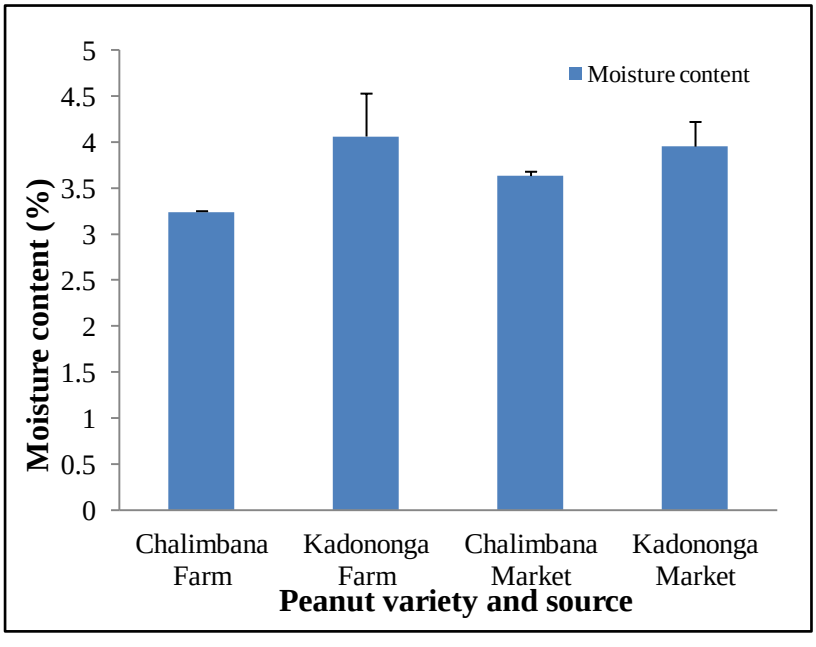

Figure 4-2: Moisture content of raw peanuts. n=3, values are not significantly different, ANOVA, ($p < 0.05$)

The moisture content of all the four raw peanut samples in the present study was below the > 10% moisture content threshold known to favour the growth of fungi in food (Ribeiro *et al.*, 2006). However, Boli *et al.*, (2013) isolated fungi from peanut butter with moisture content ranging from 1.23±0.20 - 4.17±0.25%.

4.2.2 Water activity of sorted raw peanuts

Water activity of raw sorted milled peanuts from each variety and location are shown (Table 4-1). The water activity of all the four raw sorted peanut samples ranged from 0.56±0.00 - 0.61±0.00. The water activity of all the four raw peanut samples was below 0.80, levels that do not fully support the growth of fungi as fungal growth in food is limited by water activity < 0.80 (Ribeiro *et al.*, 2006).

Table 4-1: Water activity of milled sorted raw peanuts, peanut butter from production (day 0) and peanut butter during storage

Source	**Farm**		**Market**	
Peanut variety	***Chalimbana***	***Kadononga***	***Chalimbana***	*Kadononga*
Raw peanuts	0.58±0.01	0.59±0.00	0.56±0.00a	0.61±00b
Peanut butter day 0	0.57±0.00	0.59±0.00	0.57±0.00a	0.66±0.00b
Peanut butter at week 2	0.40±0.00a	0.59±0.01b	0.41±0.01	0.41±0.00
Peanut butter at week 4	0.39±0.00a	0.69±0.00b	0.36±0.03	0.37±0.01
Peanut butter at week 6	0.45±0.02a	0.60±0.00b	0.42±0.00	0.42±0.03
Peanut butter at week 8	0.39±0.00a	0.41±0.00b	0.27±0.01a	0.31±0.00b
Peanut butter at week 10	0.59±0.00a	0.40±0.00b	0.33±0.00	0.33±0.01
Peanut butter at week 12	0.42±0.01a	0.59±0.01b	0.42±0.00	0.42±0.00

[1]Values are expressed as means ± standard deviation, n = 3. Different letters in rows indicate significant differences between water activity of *Chalimbana* and *Kadononga* peanut varieties from the same source, Student's t test, ($p < 0.05$)

There was no significant difference in the water activity of raw *Chalimbana* peanut varieties that were purchased from the market and farm ($p = 0.30$). Equally, there were no significant differences in the water activity of raw *Kadononga* peanut varieties that were purchased from the farm and market ($p = 0.15$). Therefore, the source of the peanuts didn't have an effect on water activity. However, there were significant differences in the water activity of the two peanut varieties *Chalimbana* and *Kadononga* that were purchased from the market ($p = 0.00$), with *Kadononga* peanuts having higher water activity. On the other hand, there was no significant difference in the water activity of the same peanut varieties that were purchased from the farm ($p = 0.18$).

4.2.3 Water activity of peanut butter from the production line and peanut butter during storage

The water activity of peanut butter withdrawn from the production line (day 0) ranged from 0.57±0.03 - 0.66±0.00 (Table 4-1). There was no significant difference in the water activity of peanut butter that was processed from *Chalimbana* peanut varieties that were purchased from the market and farm ($p < 0.05$). However, there were significant differences in the water activity of peanut butter that was processed from *Kadononga* peanuts that were purchased from the market and farm ($p < 0.05$), with the former having levels. The water activity of peanut butter that was processed from *Chalimbana* and *Kadononga* peanut varieties that were purchased from the market were also significantly different from each other ($p = 0.048$), with the later having higher levels.

Equally, there was a significant difference in the water activity of peanut butter that was processed from *Chalimbana* and *Kadononga* peanuts that were processed from the farm ($p = 0.02$), with the later having higher levels.

The water activity of the four sets of peanut butter during storage are shown (Table 4-1). In week 2, the water activity of the peanut butter ranged from 0.40±0.00 - 0.59±0.01. In week 4, the water activity ranged from 0.36±0.03 - 0.69±0.01. While, in week 6 the water activity ranged from 0.42±0.00 - 0.60±0.00. In week 8, the water activity ranged from 0.27±0.01 - 0.41±0.00. In week 10, the water activity ranged from 0.325±0.01 - 0.40±0.00 and in week 12 the range was from 0.42±0.0 - 0.59±0.01. There were significant changes in the water activity of all the four batches of peanut butter during the 12 weeks of storage at ($p < 0.05$). However, the water activity of all the four sets of peanut butter during the 12 weeks of storage were also below 0.80, levels that do not fully support the growth of fungi.

4.3 Fungal species in raw peanuts, peanuts along processing line: roasted, blanched and freshly processed peanut butter, and stored peanut butter

4.3.1 Fungal species in raw peanuts

Based on PCR results from rDNA region, all four sorted raw peanut samples had presence of fungal genera typically associated with peanuts. The distributions of the fungal species isolated from the four sorted raw peanut samples are shown (Table 4-2). Fungi that were isolated from all the four sorted raw peanut samples included 32 fungal colonies belonging to nine fungal genera which included *Cladosporium*, *Aspergillus*, *Fusarium*, *Penicillium*, *Epicoccum*, *Alternaria*, *Talaromyces*, *Curvularia* and *Xenocamarosporium*. The percentage occurrences of the isolated fungi showed that *Cladosporium* sp. was predominant (53%), *Aspergillus*, *Fusarium* and *Penicillium* showed percentage occurrence of (9%), *Epicoccum* (6%), while the rest of the fungi showed occurrence of just 3%. The following fungi were isolated from both *Chalimbana* and *Kadononga* peanuts: *Cladosporium*, *Aspergillus*, *Fusarium* and *Penicillium*. *Epicoccum*, *Alternaria* and *Xenocamarosporium* were only isolated from *Chalimbana peanuts*, whereas *Talaromyces* and *Curvularia* were isolated from *Kadononga* peanuts.

Table 4-2: Colonies of fungal species isolated from selected raw peanut varieties purchased from Lusaka and Eastern province of Zambia

Source	**Farm**		**Market**		
Peanut variety	***Chalimbana***	***Kadononga***	***Chalimbana***	***Kadononga***	**Total number of fungal colonies**
Alternaria longissima	-	-	1	-	1
Aspergillus flavus	-	1	-	-	1
Aspergillus multicolour	-	-	-	1	1
Aspergillus pseudodeflectus	-	-	1	-	1
Cladosporium cladosporioides	4	2	-	-	6
Cladosporium sphaerospermum	-	-	-	2	2
***Cladosporium* sp.**	2	2	3	2	9
***Curvularia* sp.**	-	1	-	-	1
Epicoccum nigram	-	-	1	-	1
***Epicoccum* sp.**	1	-	-	-	1
***Fusarium* sp.**	1	1	1	-	3
Penicillium tularense	2	-	-	-	2
***Penicillium* sp.**	-	1	-	-	1
Talaromyces pinophilus	-	-	-	1	1
Xenocamarosporium acaciae	-	-	1	-	1
Total number of fungal colonies	10	8	8	6	32

Other studies have also isolated and identified similar fungal species in other varieties of peanuts hence the variety of peanuts may not be a determining factor in the type of fungal community. Embaby and Abdel-Galel (2014) isolated and identified the following fungi from raw peanut kernels: *Fusarium, Aspergillus, Rhizopus, Epicoccum* and *Penicillium*, with *Aspergillus* being the predominant species. In another study, Gachomo *et al.*, (2004) isolated and identified the following fungi from raw peanut kernels: *Rhizopus, Fusarium, Aspergillus and Penicillium*, with *Rhizopus* being the predominant specie. This variation in fungal species and frequency of occurrence in the different studies is expected because fungal contamination of peanuts can occur during production, storage, transportation and marketing (Nigam *et al.,* 2009) and these factors vary.

Fungi were able to grow on the raw peanut samples because peanuts have a high nutritional content useful to numerous fungi (Boli *et al*., 2013). However, the water activity (0.56±0.00 - 0.61±0.00) and moisture content (3.24±0.01 - 4.06±0.47%) of the four raw peanut samples were a limiting factor as they were below levels that fully support the growth of majority of fungi because majority of fungi grow best in foods that have a water activity and moisture content of ≥ 0.80 and > 10%, respectively (Ribeiro *et al*., 2006). However, Boli *et al*., (2013) isolated fungi in peanut butter with moisture levels ranging from 1.23±0.20 - 4.17±0.25%.

4.3.2 Fungal species in peanuts from processing line; roasted, blanched and freshly processed peanut putter

Fungal species were isolated from peanuts along the processing line. The identity of all the fungal isolates was confirmed based on partial sequences of the ITS1-5.8S-ITS2 regions using the primer pair ITS1 and ITS4. A total of 18 fungal isolates belonging to three fungal genera were isolated from all four samples at the roasting, blanching and grinding stages (Table 4-3). The three genera comprised of *Cladosporium*, *Penicillium* and *Talaromyces.* Of the 18 fungal isolates, nine where isolated from roasted peanuts, four from blanched peanuts and five from ground peanut butter. The following fungal species were isolated from the roasted peanuts: *Cladosporium* spp. and *Penicillium* spp.

Table 4-3: Colonies of fungal species isolated from peanut samples during the processing stages of peanut butters from *Chalimbana* and *Kadononga*

Source	**Farm**		**Market**		
Peanut variety	***Chalimbana***	***Kadononga***	***Chalimbana***	***Kadononga***	**Total number of fungal colonies**
Roasting	*Penicillium* sp. (1) *Cladosporium* sp. (2)	*Penicillium* sp. (1) *Cladosporium* sp. (2)	*Cladosporium* sp. (2)	*Cladosporium* sp. (1)	9
Blanching	*Cladosporium* sp. (1)	*Cladosporium* sp. (1)	*Cladosporium* sp. (1)	*Talaromyces pinophilus*(1)	4
Grinding	*Cladosporium* sp. (1)	*Cladosporium* sp. (1)	*Cladosporium* sp. (2)	*Cladosporium* sp. (1)	5

Cladosporium spp. and *Talaromyces pinophilus* were isolated from the blanched peanuts and only *Cladosporium* spp. were isolated from the freshly ground peanut butter. A total of 32 fungal isolates belonging to nine different genera were isolated from the raw peanuts before processing (Table 4-2). The difference in fungal species in raw peanuts and in peanuts from the roasting, blanching and grinding stages show that processing has a reductive effect on fungal diversity. This reduction can be attributed to roasting killing some of the fungi, an effect that was demonstrated by Darko (2016). As observed in this study, not all fungi are killed by roasting as some still grew after roasting. Nyirahakizimana *et al*., 2013 also isolated fungi from roasted peanuts, with roasted peanuts having less fungal contamination than raw peanuts. Blanching also had a reductive effect on fungal diversity and this finding also agrees with Darko (2016) who observed a reduction in fungal contamination after blanching. The reason for this reduction is that some fungi grow on the surface or skin of the peanut kernels so removal of this skin eliminates some fungi. Grinding on the other hand did not seem to have an effect on fungal community.

The present study has shown that processing of raw peanuts into peanut butter reduces fungal diversity but processing does not completely eliminate fungi as fungi were isolated from freshly processed peanut butter. However, good hygiene practices during processing are encouraged to help reduce fungal contamination.

4.3.3 Fungal species in peanut butter stored over a period of three months

During the storage period, fungal species were isolated from peanut butter every fortnight. The identity of all the fungal isolates was confirmed based on partial sequences of the ITS1-5.8S-ITS2 regions using the primer pair ITS1 and ITS4. Fungal growth during storage was very minimal with some sampling intervals showing no fungal growth. A total of five fungal colonies were isolated from all four sets of peanut butter on day 0 of storage (Table 4-4). Four fungal colonies were isolated from all four sets of peanut butter in week two of storage. In week four of storage, six fungal colonies were isolated from all the four sets of peanut butter. In week six of storage, seven fungal colonies were isolated from the four sets of peanut butter.

Table 4-4: Colonies of fungal species isolated from peanut butters processed from *Chalimbana* and *Kadononga* during a three-month storage period[a]

Source	**Farm**		**Market**		
Peanut variety	***Chalimbana***	***Kadononga***	***Chalimbana***	***Kadononga***	**Total**
Week 0[b]	*Cladosporium* sp. (1)	*Cladosporium* sp. (1)	*Cladosporium* sp. (1)	*Cladosporium* sp. (2)	5
Week 2	*Cladosporium* sp. (1)	*Cladosporium* sp. (1)	*Cladosporium* sp. (1)	*Cladosporium* sp. (1)	4
Week 4	*Penicillium tularense* (1)	*Cladosporium cladosporioides* (1) *Penicillium glabrum*[c] (1)	*Cladosporium* sp. (1) *Epicoccum nigram* (1)	*Cladosporium* sp. (1)	6
Week 6	*Penicillium sizovae*[c](1)	*Fusarium* (1) *Aspergillus flavus* (1) *Penicillium* sp. (1)	*Cladosporium* sp. (1) *Cladosporium oxysporium*[c](1)	*Cladosporium* sp. (1)	7
Week 8	-	*Cladosporium* sp. (1)	*Cladosporium* sp. (1)	*Cladosporium* sp. (1)	3
Week 10	*Cladosporium* sp. (1)	*Cladosporium* sp. (1)	*Cladosporium* sp. (1)	*Cladosporium* sp. (1)	4
Week 12	*Cladosporium* sp. (1)	-	*Cladosporium* sp. (1)	*Talaromyces pinophilus* (1)	3

[a]Samples were drawn every two weeks for analyses; [b]Freshly ground peanut butter from day 0
[c]These fungal species were isolated from stored peanut butters but not from the sorted raw peanut

In week eight of storage, three fungal colonies were isolated from three of the four sets of peanut butter. *Chalimbana* peanut butter which was processed from *Chalimbana* peanuts that were purchased from the farm did not have any fungal growth. In week ten of storage, four fungal colonies were isolated from the four sets of peanut butter. In week twelve of storage, three fungal colonies were isolated from three of the four sets of peanut butter. There was no fungal growth in peanut butter that was processed from *Kadononga* peanut variety that was purchased from the farm in week 12 of storage.

The isolated fungi belonged to six fungal genera previously isolated from the sorted raw peanuts. These included: *Cladosporium*, *Aspergillus*, *Penicillium*, *Epicoccum*, *Talaromyces* and *Fusarium*. Of these fungal genera, *Cladosporium* and *Penicillium* were isolated from both *Chalimbana* and *Kadononga* peanut butters, while, *Epicoccum* was isolated from *Chalimbana* peanut butter alone. Whereas, *Aspergillus*, *Talaromyces* and *Fusarium* were isolated from *Kadononga* peanut butter. The respective species are shown in Table 4-4. Most of the isolated fungal species were isolated from the raw peanuts; however, some fungal species not originally present in the sorted raw peanuts were also isolated from the stored peanut butter. These included three fungal species: *Cladosporium oxysporium*, *Penicillium sizovae* and *Penicillium glabrum*. *Cladosporium oxysporium* and *Penicillium sizovae* were isolated from peanut butter made from *Chalimbana* peanut variety that was purchased from the market and farm, respectively, in week 6 of storage. While, *Penicillium glabrum* was isolated from peanut butter made from *Kadononga* peanuts that were purchased from the farm in week 4 of storage. The fungal species not originally present in the sorted raw peanuts may be post process contaminants or they may be fungi whose growth was suppressed by other microorganisms in the raw peanuts and only grew in the peanut butter after changes occurred in the fungal community. Of the fungi that grew in the four sets of peanut butter during storage, *Cladosporium* species were still the dominant species with only one isolate of *Aspergillus flavus* being isolated in peanut butter that was processed from *Kadononga* peanut variety that was purchased from the farm. The predominance of *Cladosporium* species can be attributed to its growing conditions being slightly lower than most fungal species. They grow in a temperature range of 15 - 30 °C (Ibtisam,

2013), with some species such as *Cladosporium cladiosoporioides* growing at even lower temperatures of -10 °C. *Cladosporium* species are widely distributed and have been isolated from soil, organic matter and air. Other researchers have also isolated fungi from peanut butter. Boli *et al*., (2013) isolated and identified the following fungi from peanut butter: *Mucor, Alternaria, Helmintosporium, Geotrichum, Fusarium, Cladosporium, Penicillium* and *Aspergillus.* Mupunga *et al.*, (2014) also isolated and identified *A. flavus* and *A. parasiticus* from peanut butter.

Factors that determine fungal growth in food include: nutritional content of the food, temperature, relative humidity, water activity and moisture content, among other factors. Fungal growth in the stored peanut butter can be attributed to the high nutritional content of peanut butter useful to numerous fungi (Boli *et al*, 2013) as well as favourable temperatures during storage. Temperatures during storage ranged from 18.3 - 31.8 °C, with a mean of 24 °C. Majority of fungi are mesophilic, therefore, they can grow at temperatures ranging from 10 - 35 °C (Sadiq *et al*., 2017) and most *Aspergillus* species grow best in the temperature range of 25 - 30 °C (Ribeiro *et al*., 2006 and Peromingo *et al*., 2016), therefore, temperatures during storage were mostly supportive of fungal growth. Fungal growth was, however, limited by low water activity. The water activity of the four peanut butter batches during the course of storage ranged from 0.27±0.01 - 0.67±0.00 (Table 4-1) which is below what is favourable for the growth of most fungi ≥ 0.80 (Ribeiro *et al*., 2006).

The results of the present study show a change in fungal community during storage but the fungal growth was generally minimal compared to growth in the raw peanuts.

4.4 Aflatoxin producing potential of *Aspergillus* isolates

For fungi to produce aflatoxins, they need to possess seven aflatoxin biosynthetic genes. These include the following genes: *aflQ, aflD, aflO, aflM, aflP, aflS* and *aflR*. The ability to produce aflatoxins was tested in the isolated *Aspergillus* species and demonstrated (Table 4-5). From the four *Aspergillus* species that were isolated in the present study *A. flavus*, *A. multicolour* and *A. pseudodeflectus* from the raw peanuts and another *A. flavus* from stored peanut butter, only one was found to be toxigenic.

Table 4-5: PCR amplification products of *Aspergillus species* isolated from raw peanuts and stored peanut butter[a].

Aspergillus isolate	*aflR*	*aflS*	*aflQ*	*aflM*	*aflD*	*aflP*	*aflO*	Aflatoxin producing Potential[b]	Toxigenicity
A. flavus[c]								+	Toxigenic
A. flavus[d]								–	Atoxigenic
A. multicolour[c]								–	Atoxigenic
A. pseudodeflectus[c]								–	Atoxigenic

[a]Presence (blue box) or absence (red box) of PCR products of the tested aflatoxin genes
[b](+/–): aflatoxin and no aflatoxin producing potential
[c]Fungi isolated from raw peanuts
[d] Fungi isolated from stored peanut butter

A correlation exists between aflatoxin production and possession of the seven aflatoxin biosynthetic genes (Gallo *et al*., 2012; Chang *et al*., 2005). Therefore, the *A. flavus* isolated from raw peanuts was toxigenic as it possessed all the seven aflatoxin biosynthetic genes while the *A. flavus* isolated from stored peanut was atoxigenic as it possessed only 6 genes with the exception of *alfM*. The other two *Aspergillus* species tested (*A. multicolour* and *A. pseudodeflectus*) lacked all the seven aflatoxin biosynthetic genes. This finding is in agreement with literature which states that aflatoxins are mainly produced by seven species of *Aspergillus*: *A. flavus*, *A. parasiticus*, *A. tamorii*, *A. nominus*, *A. pseudotamarii*, *A. bombycis* and *A. ochraceoroseus* (Novas and Cabral, 2002; Cary *et el*., 2005; Varga *et al*., 2003; Frisvad *et al*., 2005). Therefore, the species *A. multicolour* and *A. pseudodeflectus* are not aflatoxin producers. To the best of our

knowledge, this is the first report on the identification of *A. multicolour* and *A. pseudodeflectus* in Zambia.

Other authors (Kachapulula *et al*., 2017; Njoroge *et al*., 2016b) have isolated *Aspergillus* species in Zambia and determined the actual capability of the isolates to produce aflatoxin. Both toxigenic and atoxigenic strains belonging to *A. flavus, A. nominus* and *A. Parasiticus, A tamorri* were isolated. Non aflatoxin producers included: *A. niger, A. terreus,* and *A. oryzae.*

4.5 Aflatoxin levels in raw peanuts, peanut butter from production (day 0) and stored peanut butter

4.5.1 Aflatoxin levels in raw peanuts

Results of aflatoxin levels in the four unsorted peanut samples that were determined using a GIPSA approved lateral-flow immunochromatography assay are shown (Figure 4-3). Aflatoxin levels in unsorted raw peanuts which were sourced from the farm and market ranged from 2.50±0.36 - 6.20±3.34 ppb and 14.9±0.05 - 42.7±5.25 ppb, respectively. The levels of aflatoxin contamination in the unsorted raw peanuts fall within the ranges reported by Bumbangi *et al.,* (2016) who found that aflatoxin levels in raw peanuts from open markets and supermarkets from Lusaka district ranged from 0.01 - 48.67 ppb. Aflatoxin levels in unsorted peanut samples that were purchased from the market are of concern as they were above the maximum permissible limits set by CAC (10 ppb). There were significant differences between unsorted *Chalimbana* peanut variety which were sourced from the farm and market ($p = 0.01$). Whereas no significant differences were observed between *Kadononga* peanut variety that were sourced from the farm and market ($p = 0.07$). Aflatoxin levels in unsorted *Chalimbana* peanut varieties that were sourced from the market were significantly higher (42.7±5.25 ppb) than in unsorted *Kadononga* peanut variety from the same source (14.9±0.05 ppb) at ($p < 0.05$). Bumbangi *et al.,* (2016) also reported that *Chalimbana* peanut variety is more susceptible to aflatoxin contamination than *Kadononga* peanut variety. However, there was no significant difference in levels of aflatoxins in unsorted *Chalimbana* and *Kadononga* peanut varieties which were purchased from the same farm ($p = 0.26$). The levels of aflatoxin contamination in the unsorted *Chalimbana* and *Kadononga* peanuts

that were sourced from the same farm were 2.50±0.36 ppb and 6.20±3.33 ppb, respectively.

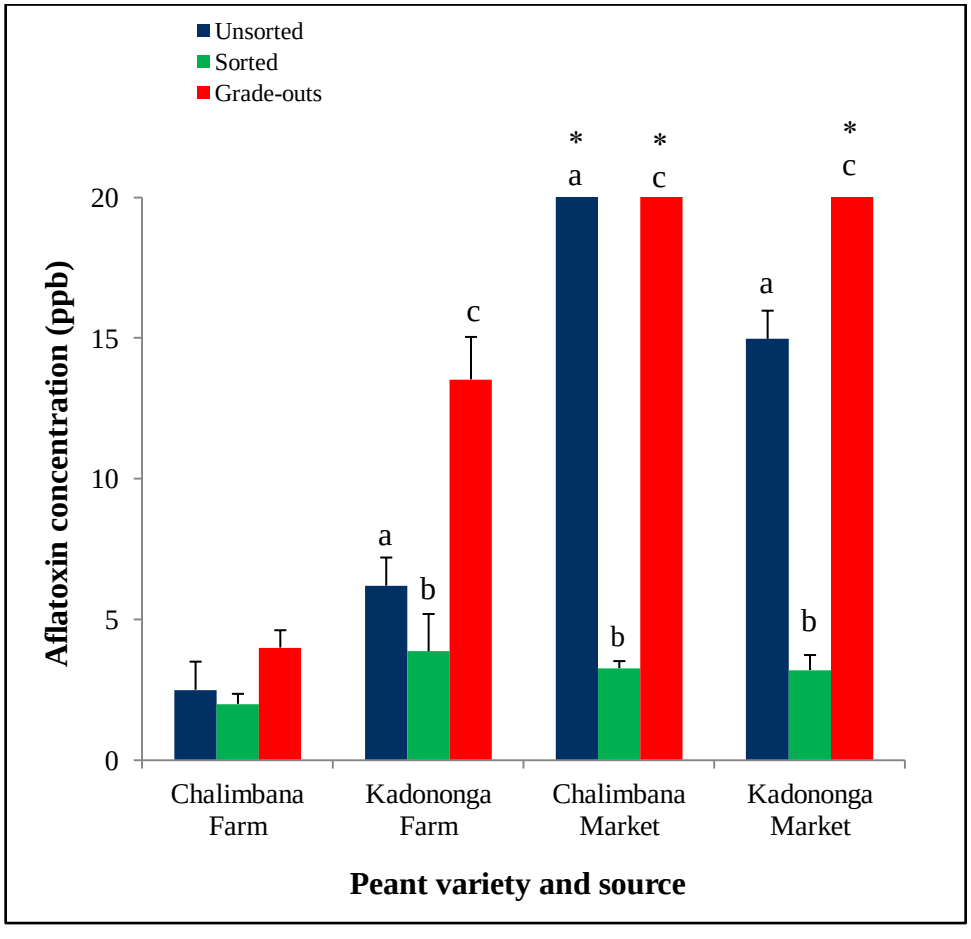

Figure 4-3: Effect of sorting on aflatoxin contamination of raw peanuts. ∗Aflatoxin concentration exceeding 20 ppb. Different letters above vertical bars for each peanut sample indicate that aflatoxin concentrations in unsorted, sorted and grade-out peanuts are significantly different from each other, ANOVA, ($p < 0.05$).

The raw peanut samples were sorted before use in production of peanut butter. To determine whether sorting could reduce the quantity of aflatoxin in raw peanut samples, levels of aflatoxin in sorted peanuts were assessed and compared to aflatoxins in the unsorted samples (Figure 4-3). Three of the four peanut samples showed significant reduction in aflatoxins after sorting at ($p < 0.05$). Levels of aflatoxin in *Chalimbana* peanut variety that was purchased from the market reduced from 42.7±5.24 to 3.27±0.25 ppb (92%). Reductions in *Kadononga* peanut variety that were purchased from the market and farm were from 14.90±0.05 to 3.20±0.53ppb (79%) and 6.20±3.34 to

3.87±1.32 ppb (38%), respectively. However, there was no significant reduction in levels of aflatoxin after sorting in *Chalimbana* peanut variety that was purchased from the farm. Levels of aflatoxins reduced from 2.50±0.35 to 2.00±0.35 ppb. The insignificant reduction observed can be attributed to the *Chalimbana* peanut sample having very few mouldy, discoloured, disfigured or broken kernels.

The reduction in levels of aflatoxin after sorting ranged from 38 - 92% thus reducing the total levels of aflatoxins in all four samples to less than 5 ppb, levels acceptable by CAC. The effect of sorting on levels of aflatoxin in peanuts has also been reported by Galvez *et al*., (2003) who observed a 95 - 100% reduction in aflatoxins after manually sorting in shell peanuts. The effectiveness of sorting in reducing aflatoxin contamination has further been demonstrated by the high levels of aflatoxins in the grade-out peanuts. Three of the four grade-out peanut samples had significantly higher levels of aflatoxin than the unsorted and sorted peanut samples at ($p < 0.05$). The highest levels of aflatoxin recorded were in *Chalimbana* peanut variety that was purchased from the market (109±2.62 ppb). Therefore, sorting can be used as a tool to help tackle the problem of aflatoxin contamination in raw peanuts. However, sorting of raw peanuts often reduces the quantity of peanuts and in turn reduces income from the peanuts, therefore, it is likely that few farmers, retailers and processors carry it out thoroughly hence the need to come up with means of reducing income loss. One suggestion is the extraction of edible oil from contaminated grade-outs. Schwartzbord *et al*., (2015) observed that oil made from contaminated peanuts contained 99.5% less aflatoxins than the contaminated peanuts. Therefore extracting oil from grade-outs can serve as a means of both disposing the grade-outs and at the same time it can encourage sorting of raw peanuts as grade-out peanuts can still be a source of income.

4.5.2 Aflatoxin levels in peanut butter from production (day 0)

Figure 4-4 illustrates changes in levels of aflatoxin in sorted raw peanuts and peanut butter withdrawn at commencement of production or day 0.

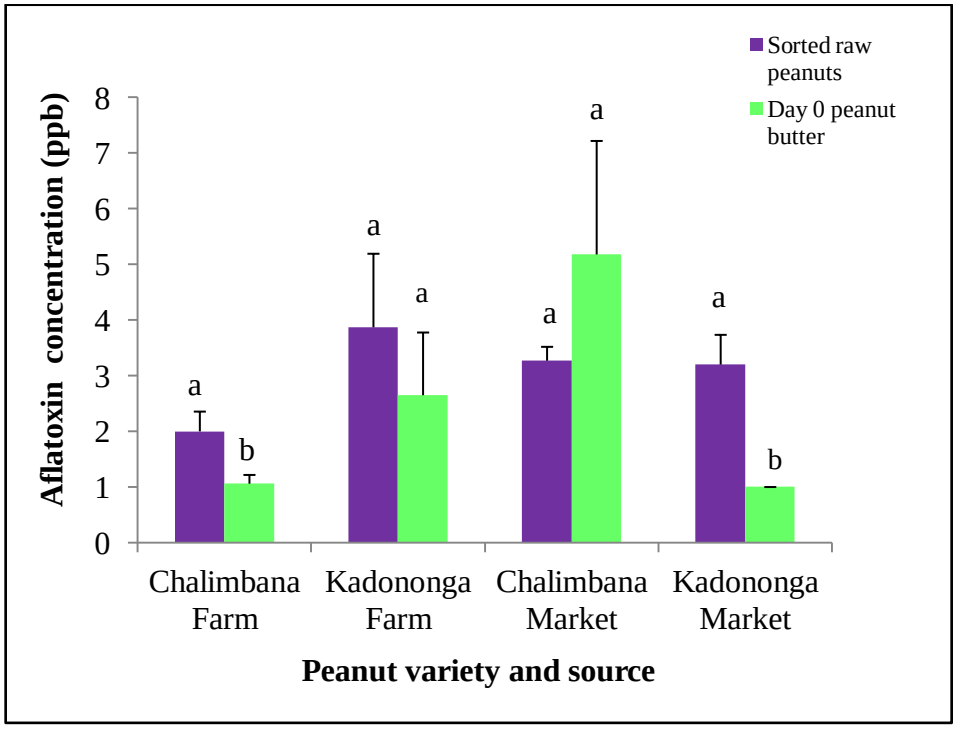

Figure 4-4: Mean aflatoxin concentration in raw peanuts and peanut butter withdrawn from samples at the start of peanut butter production. n=3, different letters above error bars for each aflatoxin indicate that concentrations are significantly different from each other, student t-test ($p < 0.05$)

The three stages of peanut butter production: roasting at 147 °C, blanching and grinding collectively, reduced levels of aflatoxin in two of the four samples *Chalimbana* peanut variety that was sourced from the farm and *Kadononga* peanut variety that was purchased from the market but no significant reductions were observed in *Chalimbana* and *Kadononga* peanut varieties that were purchased from the market and farm, respectively. Aflatoxin levels in *Chalimbana* peanut variety that was purchased from the farm reduced from 2.00±0.36 to 1.06±0.16 ppb whereas the reduction in Kadononga peanuts which were purchased from the market was from 3.20±0.53 to 1.00±0.00 ppb. Aflatoxin levels of peanut butter withdrawn from production or day 0 ranged from 1.06±0.06 - 5.18±2.03 ppb. These aflatoxin levels fall within the range of those reported by Elshafie *et al.*, 2010 (0 - 6 ppb) in laboratory produced peanut butter of another

variety that was sorted prior to processing. Siwela *et al*., (2011) also observed that processing of raw peanuts into peanut butter reduces aflatoxin contamination. They observed an 89% cumulative total reduction during the production process of peanut butter with roasting temperatures of 160 °C. The 47% and 69% reduction in aflatoxin levels observed after processing *Chalimbana* peanut variety that was purchased from the farm and *Kadononga* peanut variety that was purchased from the market, respectively, can mainly be attributed to the effect that roasting and blanching have on aflatoxins (Siwela *et al*., 2011). The temperature required to destroy aflatoxin is in the range of 237 - 289 °C (O'Neil *et al*., 2001) and the roasting temperature used in the preparation of the peanut butter in the present study was 147 °C. This temperature was not sufficient to completely destroy the aflatoxins but was enough to cause changes in the chemical structure of the aflatoxins (Siwela *et al*., 2011) possibly rendering them undetectable by the method used hence the reduction in number. Blanching also contributed to the reduction in aflatoxins as some aflatoxins are found on the skin of the peanuts (Siwela *et al*., 2011).

The three stages of peanut butter processing: roasting, blanching and grinding individually reduce levels of aflatoxins (Siwela *et al*., 2011). Therefore, the increase in levels of aflatoxins observed after processing *Chalimbana* peanuts which were sourced from the farm (Figure 4-4) is unusual. The increase in aflatoxins after processing can be attributed to the size of the sorted sub-sample. Only 3 kg of peanuts was drawn from a 50 kg bag for analysis of aflatoxins of which the rest was processed into peanut butter. This may have brought up some bias as one peanut kernel can be more contaminated that the other. It is very likely that aflatoxin levels in the raw peanuts were higher than those recorded..

4.5.3 Aflatoxin levels in peanut butter a shelf life of 90 days or three months of storage

In this study, during the storage period of the four sets of peanut butter, the ambient temperature ranged from 18.3 - 31.8 °C, with a mean of 24 °C. Figure 4-5 illustrates changes in levels of aflatoxin during storage. There were no significant changes in levels of aflatoxin in all the four sets of peanut butter during three months of storage at ($p < 0.05$). Levels of aflatoxin in peanut butter made from *Chalimbana* peanut variety that

were purchased from the farm and market ranged from 1.08±0.11 - 1.11±0.11 ppb and 4.99±0.63 - 6.80±0.46 ppb, respectively. While aflatoxin levels in peanut butter made from *Kadononga* peanut variety that were purchased from the farm and market ranged from 1.46±0.08 - 2.71±0.83 ppb and 1.00±0.00 - 1.34±0.17 ppb, respectively. The lack of change in aflatoxin levels during storage can be attributed to the absence of toxigenic *Aspergillus* species in the peanut butter. The findings of the present study corroborate data by Baur (1975) who monitored levels of aflatoxins in highly contaminated roasted peanut butter and raw peanut butter stored at 22.8 °C for a period of two years.

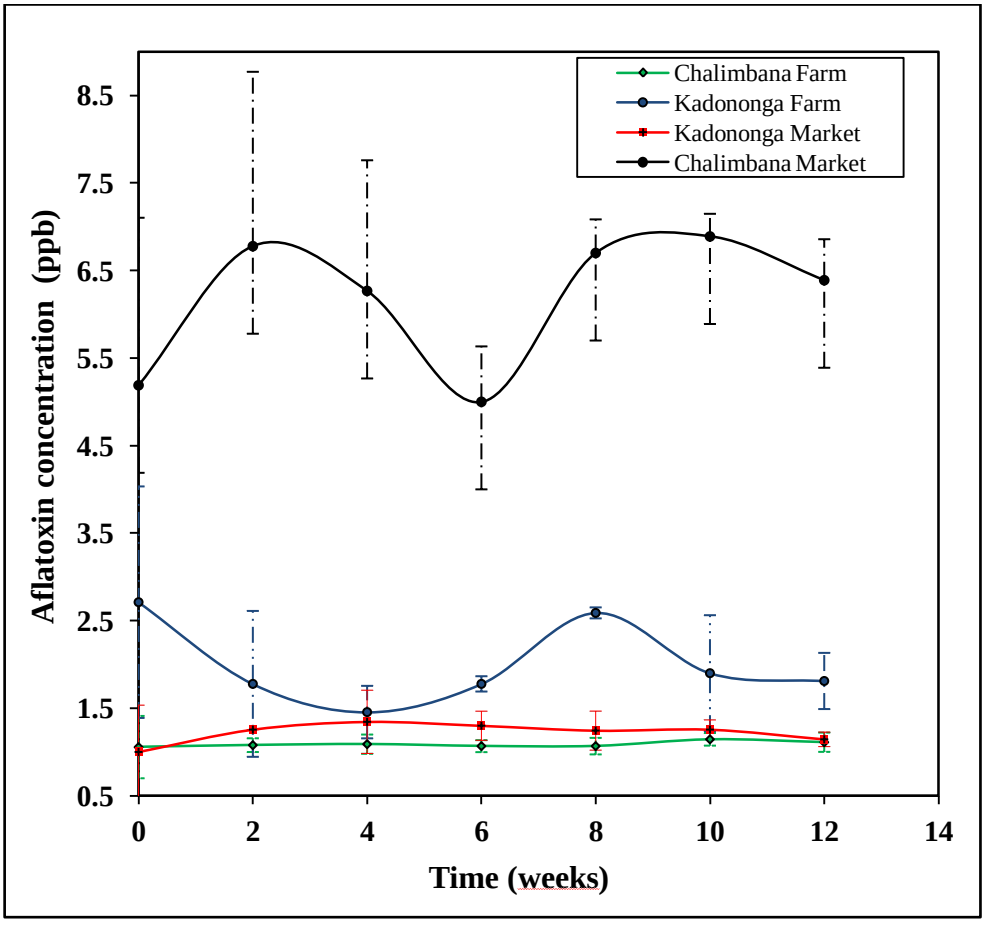

Figure 4-5: Mean aflatoxin concentrations in peanut butter during storage n=3. Values are not significantly different, ANOVA, ($p < 0.05$).

CHAPTER FIVE: CONCLUSION AND RECOMMENDATIONS

Peanut butter has been reported to have high levels of aflatoxin (Njoroge *et al.,* 2016; Mupunga *et al*., 2014; Chen *et al*., 2013). It is not clear whether these high levels are from the use of contaminated raw peanuts, proliferation during the processing technology or during storage. The present study aimed at assessing changes in fungal community and aflatoxin levels during production and storage of peanut butter, as well as assessing the aflatoxin producing ability of the isolated *Aspergillus* species. These aims were made to answer the question of the source of the high levels of aflatoxin in peanut butter, with specific interest being high levels of aflatoxins in Zambian local brands of peanut butter.

The present study has demonstrated that the three stages of peanut butter production: roasting, blanching and grinding reduce fungal diversity but do not completely eliminate fungi from peanut butter as fungal species were isolated from freshly produced peanut butter. However, enumeration of fungi at different stages of processing is recommended in order to determine the actual percentage reduction. Fungal growth in peanut butter stored at temperatures ranging from 18.3 - 31.8 °C for a period of three months was very minimal but there were some changes in fungal diversity during storage. It is however, recommended that other than measuring the external temperature under which the peanut butter was stored that the actual temperature inside the peanut butter be monitored too.

The three stages of peanut butter production also significantly reduce levels of aflatoxin in peanut butter and no significant changes occur in the peanut butter during three months of storage. Examination of the *Aspergillus* species from raw peanuts and peanut butter under storage revealed that only one strain was toxigenic and this strain was only present in the raw peanuts but not in the processed peanut butter.

In this study, sorting of peanuts prior to processing was shown to significantly reduce levels of aflatoxin contamination. Therefore, high levels of aflatoxin in peanut are most likely from the use of contaminated raw peanuts than from proliferation during

processing or storage. Sorting of peanuts prior to peanut butter production is, therefore, highly advised in addition to good hygiene practices during processing and packaging.

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APPENDICES

Appendix A: Statistical analysis results

1. Difference in moisture content of the four raw peanuts samples (Student T-test):

    a) Differences in Moisture content of *Chalimbana* peanuts from market and farm
    Test statistic t = 0.23 on 4 (degree of freedom) d.f.
    Probability = 0.832
    b) Differences in Moisture content of *Kadononga* peanuts from market and farm
    Test Test statistic t = 1.28 on 4 d.f.
    Probability = 0.269
    c) Differences in Moisture content of *Kadononga* and *Chalimbana* peanuts from the farm
    Test statistic t = 0.23 on 4 d.f.
    Probability = 0.831
    d) Differences in Moisture content of *Kadononga* and *Chalimbana* peanuts from the market
    Test statistic t = -0.36 on 4 d.f.
    Probability = 0.740

2. Difference in water activity of the four raw peanuts samples (Student T-test):

    a) Difference in water activity of raw *Chalimbana* and *Kadononga* sourced from the marketTest statistic t = -52.92 on 4 d.f.
    Probability < 0.001
    b) Differences in water activity of *Chalimbana* and *Kadononga* sourced from the farm
    Test statistic t = -1.64 on 4 d.f.
    Probability = 0.176
    c) Difference in water activity of *Chalimbana* peanut sourced from farm and market
    Test statistic t = -1.38 on approximately 2.01 d.f.
    Probability = 0.301
    d) Difference in water activity of *Kadononga* peanuts that were sourced from the farm and market
    Test statistic t = 2.23 on approximately 2.05 d.f.
    Probability = 0.152

3. Water activity of peanut butter samples during storage (Analysis of variance):

a) *Chalimbana* peanut variety from market

Variate: water activity

Source	*df*	*SS*	*MS*	*F*	*Pr*
Week	**6**	**0.1673105**	**0.0278851**	**66.31**	**<.001**
Residual	**14**	**0.0058873**	**0.0004205**		
Total	**20**	**0.1731978**			

b) *Kadononga* peanut variety from a market

Variate: water activity

Source	*df*	*SS*	*MS*	*F*	*Pr*
Week	**6**	**0.2436365**	**0.0406061**	**165.91**	**<.001**
Residual	**14**	**0.0034265**	**0.0002447**		
Total	**20**	**0.2470630**			

c) *Chalimbana* peanut variety from a farm

Variate: water activity

Source	*df*	*SS*	*MS*	*F*	*Pr*
Week	**6**	**0.08110473**	**0.01351746**	**184.6**	**<.001**
Residual	**14**	**0.00102519**	**0.00007323**		
Total	**20**	**0.08212992**			

d) *Kadononga* peanut variety from a farm

Variate: water activity

Source	*df*	*SS*	*MS*	*F*	*Pr*
Week	**6**	**0.08110473**	**0.01351746**	**184.60**	**<.001**
Residual	**14**	**0.00102519**	**0.00007323**		
Total	**20**	**0.08212992**			

4. Difference in aflatoxin levels of *Chalimbana* and *Kadononga* raw peanut varieties that were sourced from the farm (student's T-test):
   Test statistic t = -1.56 on approximately 2.05 d.f.
   Probability = 0.256

5. Difference between aflatoxin levels in *Chalimbana* and *Kadononga* raw peanut varieties that were sourced from the market (student's T-test):
   Test statistic t = 7.46 on approximately 2.00 d.f.
   Probability = 0.017

6. Differences in aflatoxin levels of *Chalimbana* raw peanuts from the market and farm:
   Test statistic t = 7.46 on approximately 2.00 d.f.
   Probability = 0.017
7. Differences in aflatoxin levels of *Kadononga* raw peanuts from the farm and market (Student's T-test):
   Test statistic t = 3.72 on approximately 2.00 d.f.
   Probability = 0.065

8. Effect of sorting on aflatoxin levels (Analysis of Variance results):

    a) *Chalimbana* peanut variety from a market

Variate: aflatoxin

Source	***df***	***SS***	***MS***	***F***	***Pr***
Sorting	**2**	**17246.94**	**8623.47**	**499.81**	**<.001**
Residual	**6**	**103.52**	**17.25**		
Total	**8**	**17350.46**			

    b) *Kadononga* peanut variety from a market

Variate: aflatoxin

Source	***df***	***SS***	***MS***	***F***	***Pr***
Sorting	**2**	**152.667**	**76.333**	**10.08**	**0.012**
Residual	**6**	**45.433**	**7.572**		
Total	**8**	**198.100**			

c) *Chalimbana* peanut variety from a farm

Variate: aflatoxin

Source	*df*	*SS*	*MS*	*F*	*Pr*
Sorting	**2**	**0.5000**	**0.2500**	**0.79**	**0.496**
Residual	**6**	**1.9000**	**0.3167**		
Total	**8**	**2.4000**			

d) Kadononga peanut variety from a farm

Variate: aflatoxin

Source	*df*	*SS*	*MS*	*F*	*Pr*
Sorting	**2**	**152.667**	**76.333**	**10.08**	**0.012**
Residual	**6**	**45.433**	**7.572**		
Total	**8**	**198.100**			

11. Effect of processing on aflatoxin levels (Student T-test):

a) *Chalimbana* peanut variety from a market

Test statistic t = 1.41 on approximately 2.07 d.f.
Probability = 0.291

b) *Kadononga* peanut variety from a market

Test statistic t = -5.81 on 4 d.f.
Probability = 0.004

c) Chalimbana peanut variety from a farm

Test statistic t = -3.67 on 4 d.f.
Probability = 0.021

d) *Kadononga* peanut variety from a farm

Test statistic t = -1.05 on 4 d.f.
Probability = 0.354

9. Changes in aflatoxin levels in peanut butter during storage (Analysis of variance):
    a) *Chalimbana* peanut variety from a market

Variate: aflatoxin

Source	*df*	*SS*	*MS*	*F*	*Pr*
Weeks	**6**	**10.664**	**1.777**	**0.78**	**0.602**
Residual	**14**	**32.094**	**2.292**		
Total	**20**	**42.759**			

    b) Kadononga peanut variety from a market

Variate: aflatoxin

Source	*df*	*SS*	*MS*	*F*	*Pr*
Weeks	**6**	**3.9165**	**0.6528**	**2.06**	**0.124**
Residual	**14**	**4.4302**	**0.3164**		
Total	**20**	**8.3467**			

    c) *Chalimbana* peanut variety from a farm

Variate: aflatoxin

Source	*df*	*SS*	*MS*	*F*	*Pr*
Weeks	**6**	**0.01819**	**0.00303**	**0.24**	**0.955**
Residual	**14**	**0.17578**	**0.01256**		
Total	**20**	**0.19397**			

    d) *Kadononga* peanut variety from a farm

Variate: aflatoxin

Source	*df*	*SS*	*MS*	*F*	*Pr*
Weeks	**6**	**433586**	**72264**	**1.00**	**0.463**
Residual	**14**	**1011446**	**72246**		
Total	**20**	**1445032**			