# ISOLATION AND CHARACTERIZATION OF *LACTOBACILLUS* AND *WEISSELLA* STRAINS FROM *KIMERE* TOWARDS DEVELOPMENT OF A NOVEL PROBIOTIC YOGHURT FOR AFLATOXIN $B_1$ CONTROL


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## DECLARATION

This thesis is my original work and has not been presented in any other institution for a degree award or other qualification.

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## DEDICATION

Dedicated to my late mother. Support from my family, my wife and children and particularly their understanding while I was away from them gave me the will to achieve.

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There was timely response of the sample transporter, the World Courier. The cooperation by the elders within Mbeere community where Kimere samples were collected warrant mention.

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## ABSTRACT

In Kenya, maize is an important staple food. Unfortunately, household maize is generally spoilt by fungi whose mycotoxin metabolites have been found to cause adverse effect to animal and human health. The study had four objectives: i) to determine aflatoxin levels in maize and maize flours in three different selected regions in Kenya. ii) To isolate and characterize *Lactobacillus* and *Weissella* strains in *Kimere* and determine their individual strain capacity to sequester aflatoxins. iii) To assess growth of the isolated *Lactobacillus* and *Weissella* strains in milk so as to develop a novel yoghurt using the highest *Lactobacillus* and *Weissella* aflatoxins binding strains. iv) To determine the capacity to lower aflatoxin B1 (AFB1) metabolite (aflatoxin M1) in urine of children from Eastern Kenya by *Lactobacillus* and *Weissella* isolated strains. To achieve the objectives, 75 maize grains and 27 flour samples were collected from three parts of Kenya and analysed for aflatoxins by Enzyme linked immunosorbent assay (ELISA) plus spectrophotometric technique. Basic and advanced molecular microbiology techniques were employed to obtain *Lactobacillus* and *Weissella* isolates from *Kimere.* These included growing the *Lactobacillus* and *Weissella* in a selective media followed by Gram staining. To identify the species of the isolated *Lactobacillus* and *Weissella*, Polymerase Chain Reaction (PCR) was used to amplify the DNA extracts. DNA blocks were sequenced and identified using the Blast software process. A novel yoghurt was developed using an isolate of *Lactobacillus rhamnosus* and *Weissella*, along with starter culture *Streptococcus thermophilus*. The capacity of the isolates to sequester or bind AFB1, *in vitro* and *in vivo* was determined using ELISA. Where applicable High Performance Liquid Chromatography (HPLC) as well as Spectrophotometric techniques were used. Sequestration of AFB1 *in vivo* was examined indirectly by measuring aflatoxin M1 (AFM1) in urine of school children in Eastern Kenya whose diet was mainly maize or maize-based. Samples of urine from children were analyzed by ELISA, and Liquid chromatography-mass spectrophotometry combination (LC-MS). The results for objective one showed a significant difference between the AFB1 levels in Maize grains ($P<0.05$) from different regions. Maize samples from Eastern Kenya region had the highest contamination. Maize samples from Nairobi had the lowest concentration at 6.02±0.31145 ppb. There was no significant difference in the total aflatoxins in Maize flour samples from all regions ($P>0.05$). Moreover there was significant difference in AFB1 concentrations between stores for example from Western and Eastern regions ($P<0.05$). Total aflatoxin levels in maize flours were slightly above international standard of 5ppb but lower than the Kenyan standard which is 10 ppb. The results for objective two indicated that out of 300 isolates from *Kimere* that were analysed for capacity to lower the risk of aflatoxin exposure *in vivo* or *in vitro, Weissella cibaria* NN20 had highest but statistically insignificant survival in low acidic condition than probiotic *Lactobacillus rhamnosus* GR-1 and *Escherichia coli* GR12 ($P>0.05$). *Weissella cibaria* NN20 bound 43.7±2.3 % of total available AFB1 in modulated media. Objective three and four had results showing that lactobacillus isolated from *Kimere* had capacity to ferment milk same as yoghurt and be able to reduce aflatoxin M1 in urine *in vivo* from 6.3ppb to 2.6ppb. The results confirmed the hypothesis that consumers were at a risk of aflatoxin exposure and that they were indeed consuming the AFB1 contaminated maize and maize products. In conclusion, the findings confirmed relatively high concentrations of Aflatoxins in maize and maize flours despite government efforts to prevent this. These results call for further intervention at table level. The *Lactobacillus* and *Weissella* isolates reduced AFB1 both *in vitro* and *in vivo*, suggesting that probiotic yoghurt has the potential to prevent aflatoxicosis among consumers of maize and maize based diets.

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## LIST OF ABREVIATIONS

| | |
|---|---|
| AFB1 | Aflatoxin B1 |
| AFM1 | Aflatoxin M1 |
| ANOVA | Analysis of Variance |
| DNA | Deoxyribonucleic Acid |
| E. Coli | Escherichia Coli |
| ELISA | Enzyme linked immunosorbent assay |
| HAD | Helica Dependent Amplification |
| HCC | Hepatocellular Carcinoma |
| HCl | Hydrogen Chloride |
| HPLC | High Performance Liquid Chromatography |
| KCl | Potassium Chloride |
| LAB | Lactic Acid Bacteria |
| LC-MS | Liquid Chromatography Mass Spectrophotometry |
| LHRI | Lawson Health Research Institute |
| LSD | Least Significant difference |
| MRS | De Man, Rogosa and Sharpe agar |
| PBS | Phosphate Buffer Solution |
| PCR | Polymer Chain Reaction |
| ppb | part per billion |
| ppm | part per million |
| SSA | Sub-Sahara Africa |
| TUK | Technical University of Kenya |

| | |
|---|---|
| WHO | World Health Organization |

# CHAPTER ONE

# INTRODUCTION

## 1.1 Background

*Kimere* is a wet millet dough made from pearl millet (*Pennisetum glaneum*) that is spontaneously fermented. It Contains LAB including *Lactobacillus* and *Weissella* (Njeru 2009). *Weissella* are closely related to *Lactobacillus* and have been wrongly identified as *Lactobacillus* and sometime *Leuconostoc* (Abriouel *et al.,* 2015). *Lactobacillus* have been used as probiotics which are microorganisms that provides health benefits to the host (Njeru 2009).

On the other hand aflatoxins are mycotoxins that belong to fungal metabolites (Jimenez-Garcia *et al.,* 2018). They are rampant, invisible in the environment with severe effect in food security and safety especially within Sub Saharan Africa (SSA) societies (International Agency for Research on Cancer, New report 2016) (Figure 1.1). They can be classified as AFB1, AFB2, AFG1, AFG2 with AFB1 being the most Lethal (Jimenez-Garcia *et al.,* 2018). This is due to their toxicological capacity. The most affected food includes maize, rice, peanut and cassava (Williams *et al.,* 2010). In Kenya, maize is mainly the target crop but Sorghum, Groundnuts and Cotton are also at risk (Guchi, 2015). Crop produce infected by *Aspergillus* fungus has some black coloration, is bitter and has an offensive smell (Rawat, 2015). After infection, optimum environmental conditions are necessary for the *Aspergillus* to produce metabolites - the aflatoxins. *Aspergillus* can attack crops while in the field or after harvest in favorable conditions such as drought, temperature range of 25-32$^{o}$C, high humidity and moisture content above 13% in the harvested grains (Pitt *et al.,* 2013).

As they are odorless, tasteless and colorless, aflatoxins are difficult to detect with basic sensory organs (Negash, 2018). For this reason, they pose a challenge to food handlers, consumers and regulators in a bid to control initiation or to eradicate (Negash, 2018). The most rampant mycotoxins of concern to human health and livestock include aflatoxins, ochratoxin A, patulin, fumonisins, zearalenone and nivalenol/deoxynivalenol (WHO, 2018).

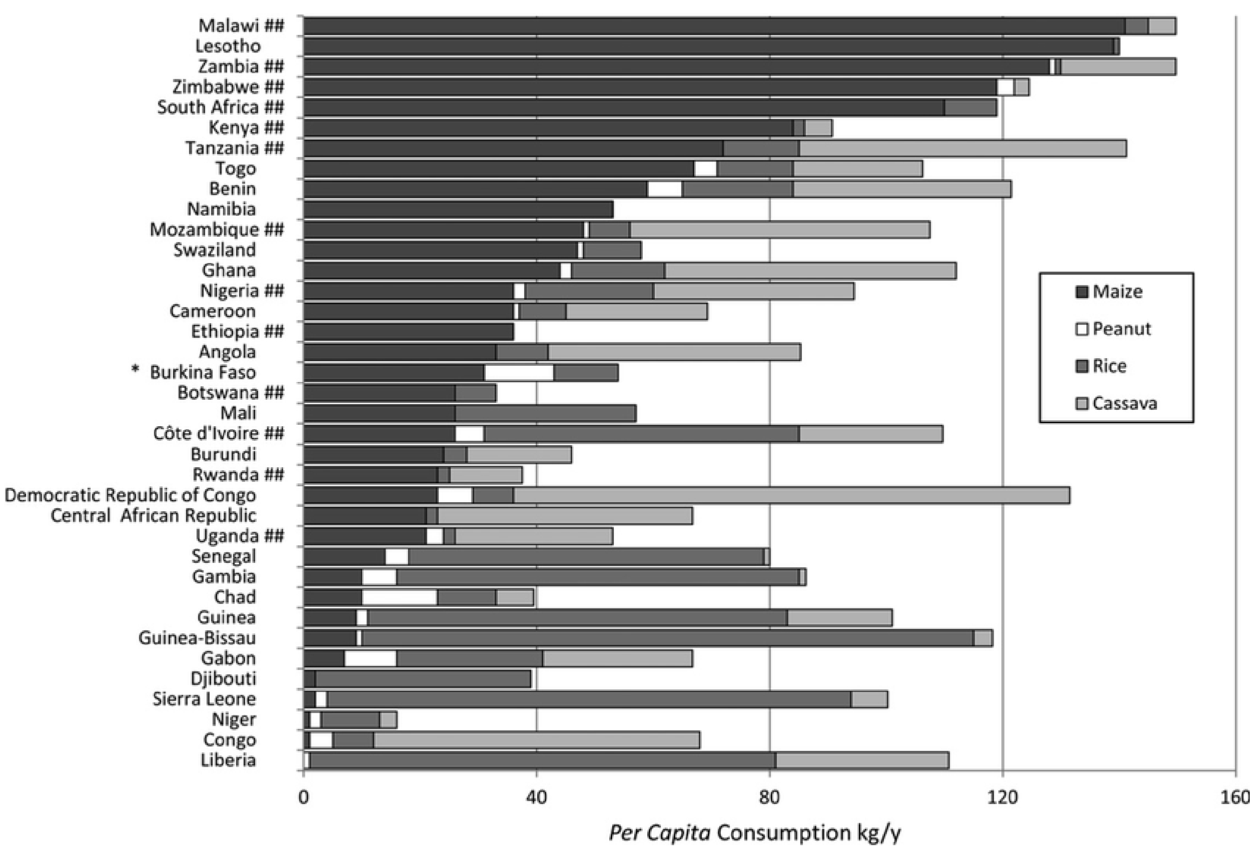



**Figure 1.1. Per capita consumption of four mycotoxin-prone foods in sub-Saharan African countries in 1993.**

*Denotes median country for maize consumption.
## Denotes 2004 President's Emergency Plan for AIDS Relief country.
(William *et al.,* 2010)

Aflatoxins, often referred to as bisfuranocoumarins are the most poisonous mycotoxins produced by *Aspergillus flavus* and *Aspergillus parasiticus* molds that grow in soil, decaying vegetation, hay, and grains (Tao *et al.,* 2018; Pizzolitto *et al.,* 2013). Aflatotoxins, fumonisins & ochratoxin A have been found rampant across sub-Saharan Africa (Darwish *et al.,* 2014). Their effects include health risk to human, economical loss and low efficacy of animal husbandry (Rocha *et al.,* 2014). Adverse effects of mycotoxins to humans include mutagenic, carcinogenic, teratogenic, hepatotoxic, and immunosuppressive properties (Figure 1.2) (Bbosa *et al.,* 2013). These effects are referred to as aflatoxicosis (William *et al.,* 2004) with aflatoxins G1, G2, M1 and M2 the most common derivatives.

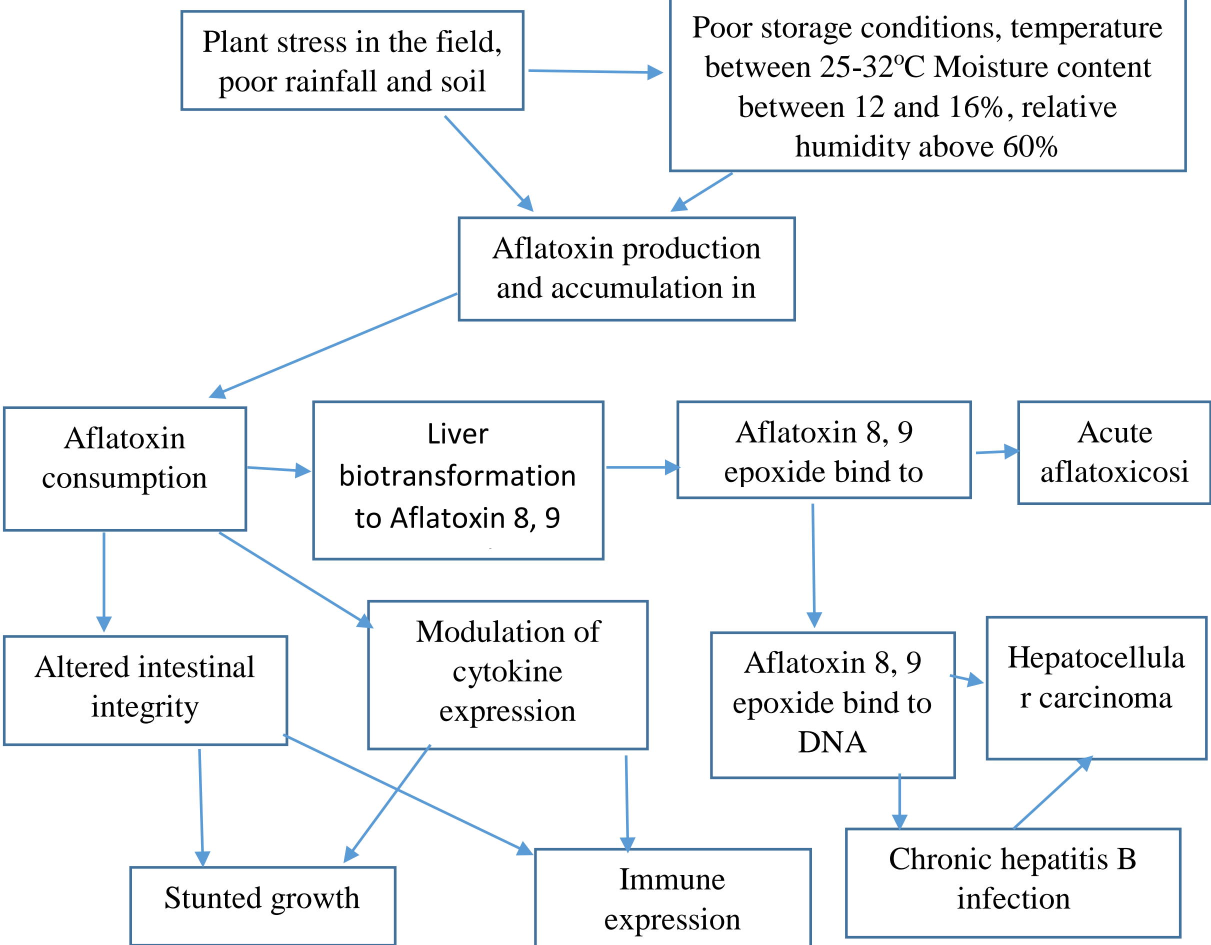



**Figure 1. 2. Aflatoxin disease pathways in humans**

(Bbosa *et al.,* 2012)

In Kenya, aflatoxin B1 is the most prevalent and commonly found in maize and maize products as well as peanuts (Williams et al., 2010). Studies have shown that there is high level of aflatoxins consumption in Kenya (Kang'ethe *et al.,* 2017; Mutegi *et al.,* 2018; Stepman, 2018). The allowable limit in Kenya is 10 ppb against 5ppb global standards (Sirma *et al.,* 2018). The point of reference for this study was Kenya standards where total allowable aflatoxins is 10ppb while total allowable AFB1 is 5ppb. After *Aspergillus fumigatus*, the second leading cause of invasive aspergillosis and cause of superficial infection is *Aspergillus flavus* (Murreil, 2017; Peraica, 2016).

Acute aflatoxicosis manifests either in humans or in animals with acute vomiting, abdominal pain, swelling of the lungs, convulsions, coma and death from the swelling of the brain, the liver, the kidneys and the heart (Peraica, 2016). Pathological findings such as acute necrosis and bile duct hyperplasia are seen in domestic and experimental animals exposed to aflatoxins. People of rural origin with maize as their staple food are presented with jaundice, brief febrile episode, vomiting, and anorexia (Murreil, 2017). In the liver, aflatoxin B1 is oxidized to various compounds which are then excreted in urine and blood. Those found in urine are aflatoxin M1, aflatoxin Q1, aflatoxin mercapturic acid, aflatoxin N7 guanine and aflatoxin glucuronide acid. The aflatoxin albumin adduct is found in the peripheral blood (Wacoo *et al.,* 2014).

In Kenya, the Eastern region has struggled with aflatoxicosis for a long time. In 2004 for example, 125 humans and countless wildlife died after consumption of contaminated maize and maize products (Lewis *et al.,* 2005). Indeed, in 2009, a study of 830 animal feeds and 613 milk samples from four Kenyan urban centers showed 86% of the former were positive for AFB1 and 67% of these exceeded the national standard limit level of 10 ppb in maize flour (Kang'ethe *et al*., 2009).

Before maize was a staple food in Eastern Kenya, *Kimere* was traditionally the food for many households (Njeru, 2009). This product was made by wet milling of pearl millet followed by fermentation which was primarily spontaneous, that is, without specific microbiological cultures. Unfortunately, the product was often made in unhygienic conditions and was prone to contamination. This was part of the reasons for its decline in consumption among the communities in Eastern Kenya (Njeru, 2009). Studies have shown that spontaneous fermentation is due to several type of microorganisms that the products picks from environment (Marco *et al.,* 2017). *Lactobacillus* species for example have been characterized from these spontaneously fermented products (Nduko *et al.,* 2017). *Weissella* species are closely related to *Lactobacillus* in terms of phenotypic and genotypic characteristics (Kamboj *et al.,* 2015). Indeed all selective media meant for isolation of *Lactobacillus* also favors growth of *Weissella* species. Furthermore, *Weissella* species are often misidentified by traditional and commercial phenotypic identification methods as *Lactobacillus spp.* or *Lactobacillus*-like organisms (Komboj *et al.,* 2015). The fermentation behavior of *Lactobacillus* and *Weissella* depend on specific strain, fermentation conditions and substrates (Le & Young, 2018).

## 1.2 Conceptual Framework

In the present study, maize and maize flour were the major source of aflatoxin B1; millet as the raw material for *Kimere*; and *Kimere* as source of the *Lactobacillus* or *Weissella*. In the event of lowered absorption of aflatoxin B1, the secreted aflatoxins volumes in the urine are affected considerably (Figure 1.3)

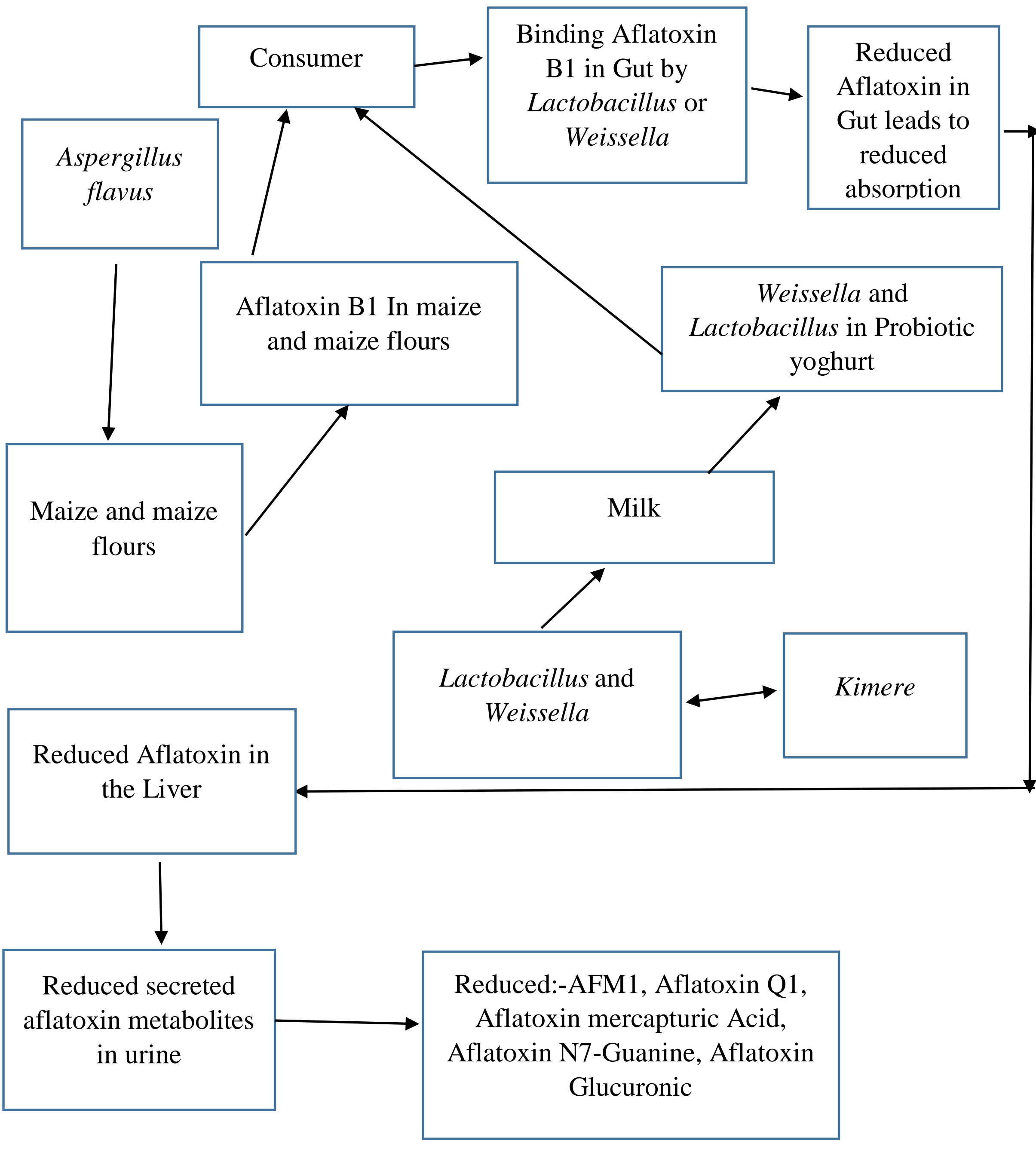



**Figure 1.3. Conceptual framework of the study**

### 1.3 Statement of the problem

Kenya has climatic conditions that are favorable for mold to thrive naturally and produce aflatoxins (Okoth & Kola, 2012). Moreover, maize, the preferred substrate of *Aspergillus flavus* (Tournas *et al.,* 2018), is the major staple food in Kenya. Consequently, aflatoxicosis continues to be reported in Kenya against the various control measures put in place by the Government agencies. Interestingly, some regions seem to be more prone than others. The explanation for the differences being due to exposure levels within the regions is debatable. Eating habits in Kenya are changing. As communities adopt westernized lifestyles, consumption of indigenous food is declining (Njeru *et al.,* 2010, Franz *et al.,* 2014). This coincides with an increase in health-related problems like aflatoxicosis (Baraca, 2019). The acceptable upper limit of aflatoxin levels in Kenya is 10 ppb against International standards at 5ppb (International Food Policy Research Institute, 2011). Methods of preventing aflatoxicosis in Kenya focus on agronomic practices (Ochieng *et al.,* 2013). For example, contaminated maize with levels above 10 ppb has previously been incinerated (Hoffman *et al.,* 2013). However, this affected food security and price instability. In some cases agronomic practices have lowered aflatoxins load in food (Kang'ethe *et al.,* 2009) but consumption of low aflatoxin doses still increases the risk of cancer (Wacoo *et al.,* 2013; *Bbosa et al.,* 2012). Currently, there are no known methods to eradicate aflatoxins in the cereal value chain (Negash *et al.,* 2018). Scientist continue to search for interventions that can act as antidotes of aflatoxicosis. *Kimere* has been reported to have *Lactobacillus* with some healthy benefits (Njeru, 2009). However, it is prepared in an unhygienic conditions which has minimized its consumption. There are no reports of *Lactobacillus* isolated from *Kimere* with the intent to prevent aflatoxicosis even though Njeru (2009) reported that mainly *Lactobacillus fermentum* has some healthy benefits.

It is now recognized that the efficacy of probiotics is strain specific. Until the present study, there were no studies carried out with *Lactobacillus* in prevention of aflatoxicosis *in vivo*.

## 1.4 Justification

Maize is the number one staple food in Kenya. It is logical to suspect that much of the aflatoxins consumed by people come from these products. Due to variations in the reported aflatoxicosis cases, this study was designed to confirm the contamination levels and compare these levels with the national and internationally maximum permitted standards. Further, *Kimere* has been reported to have *Lactobacillus* of health benefits but these *Lactobacillus* isolates were not tried specifically for aflatoxin control. Furthermore, the reviewed studies did not mention or find *Weissella,* thus it is assumed the species were not present or identified. In the present study, *Lactobacillus* and *Weissella* strains were viewed as candidate probiotics due to their presence in *Kimere* and as reported in previous studies (Njeru, 2009). *Lactobacillus* and *Weissella* are Gram-positive, facultative anaerobic or microaerophilic, rod-shaped, non-spore-forming bacteria (Huang *et al.,* 2018). They are part of the Lactic Acid Bacteria group (Ayeni *et al.,* 2011). In humans, *Lactobacillus* constitute a component of the microbiota of the oral cavity, intestine and vagina (Petrova *et al*., 2015; Gao *et al.,* 2018). In general aflatoxin levels in Kenya are high, and currently the only mitigation is at agronomy level even as cases of aflatoxicosis continues to increase. Moreover control at table level is largely not studied. In addition, use of *Weissella* or even *Lactobacillus* to control aflatoxicosis has not be studied. The ability of these strains to ferment milk and bind aflatoxin *in vivo* was not known.

## 1.5 General Objective

To isolate and characterize *Lactobacillus* and *Weissella* strains from *Kimere* toward development of a novel probiotic yoghurt for aflatoxin $B_1$ control

## 1.6 Specific Objectives

1. To determine aflatoxin levels in maize and maize flours in Western, Nairobi and Eastern Kenya.
2. To isolate and characterize *Lactobacillus* and *Weissella* strains in *Kimere* and determine their individual strain capacity to sequester aflatoxins
3. To assess growth of the isolated *Lactobacillus* and *Weissella* strains in milk so as to develop a novel yoghurt using the highest *Lactobacillus* and *Weissella* aflatoxins binding strain.
4. To determine the capacity to lower AFB1 metabolite (aflatoxin M1) in urine of children from Eastern Kenya by *Lactobacillus* and *Weissella*

## 1.7 Hypothesis

This study was guided by the following hypothesis;

1. Maize and maize flour samples from Western, Nairobi and Eastern Kenya have fungal toxin levels above the maximum permitted in Kenya and international standards.
2. *Kimere* contains *Lactobacillus* or *Weissella* and these microorganisms can bind aflatoxins rendering them unavailable to be absorbed in the body.
3. *Lactobacillus* and *Weissella* isolates from *Kimere* can ferment milk to make product with characteristics the same as yoghurt.
4. Addition to probiotic yoghurt of the highest binding strain of AFB1, identified and isolated from *Kimere* would lower the aflatoxin absorption in human gut.

# CHAPTER TWO

# LITERATURE REVIEW

## 2.1 Introduction

### 2.1.1 Aflatoxins

For many years *Aspergillus* have been known to grow in crops and food products like cereals, nuts, spices, dried fruits, apples and coffee beans, often under warm and humid conditions (Pitt *et al.,* 2013; Guchi, 2015). In the favorable conditions *Aspergillus* utilize the stored nutrients in food stuffs subsequently producing mycotoxins that are secondary metabolites with negative economic importance to growth and development (Figure 2.1).

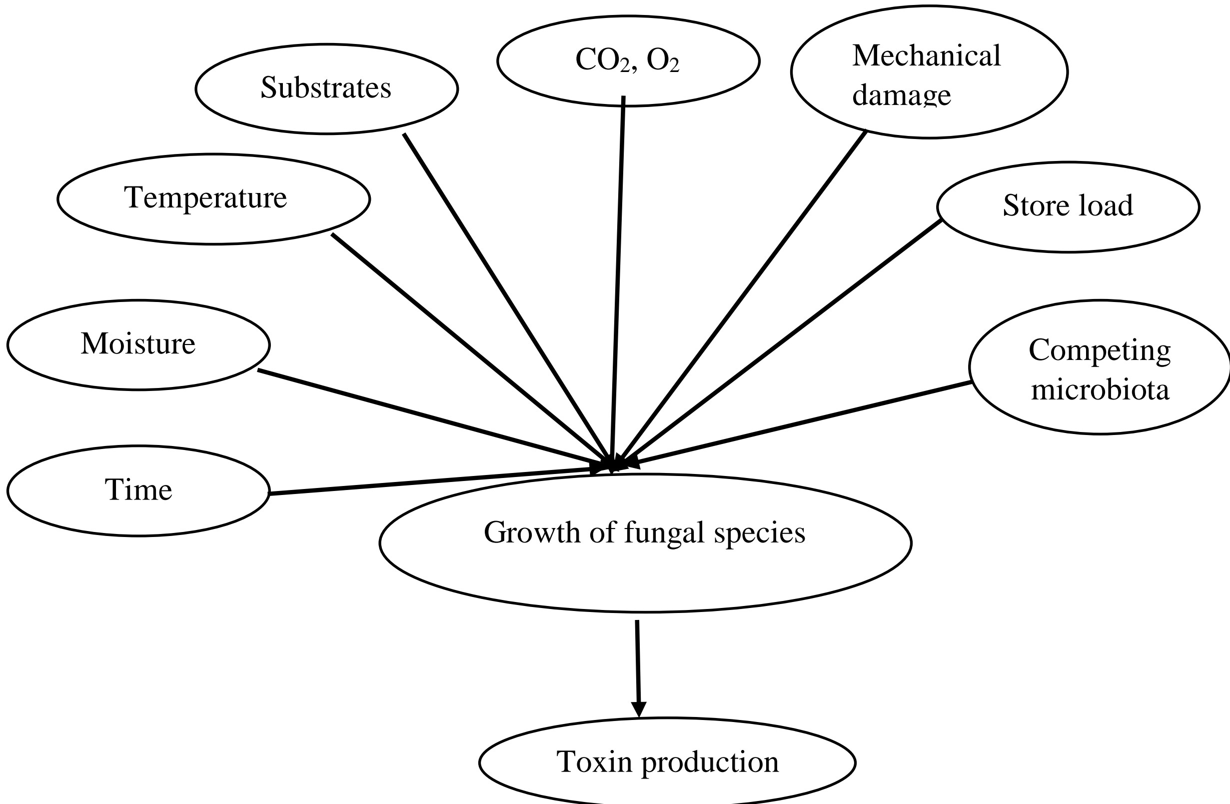



**Figure 2. 1. Ecological parameters that influence *Aspergillus* growth and toxin production.**

(Kalinina *et al.,* 2017)

The effect of food-borne mycotoxins is acute with symptoms of severe illness appearing quickly after consumption (WHO, 2018). Mycotoxins may also have long-term effects on health, including the induction of cancers and immune deficiency (Liew & Redzwon, 2018). Of the several hundred mycotoxins identified so far, about a dozen have gained the most attention due to their severe effects on human health and their occurrences in food (Table 2.1).

**Table 2.1 Most occurring mycotoxins and their effect in human health**

| Mycotoxins | **Chemical structure** | **Productivity loss** | **Immuno toxicity** | **Frequently related clinical signals** | **Main affected organs or system** |
|---|---|---|---|---|---|
| Aflatoxins | | Very high | Very high | Hepatitis, poor response to vaccination, unspecific infections, Increased susceptibility to diseases. | Liver, kidney, immune system |
| Zearalenone | | Very high | Slight | Hyperestrogenism, reproductive disorders | Reproductive tract, mainly females |
| Deoxynivalenol also called Vomitoxin | | Very high | High | Feed refusal, Vomiting | Central nervous system, Gut, epithelium, liver, immune system |
| T-2 toxin | | Very high | Very high | Oral and epithelial lesions, loss of appetite | ,Gut, epithelium, liver, immune system |
| Ochratoxin | | Very high | Very high | Nefritis( Kidney damage-enlarged Kidney) Hepatitis | Kidney, Liver, Immune system |
| Fumonisms | | Very high | Slight | Porcine pulmonary edema (PPE), equine Leukoencephalomalacia (ELEM) | Lung and heart, Central nervous system, liver, immune system |

(Andrade & Caldas, 2015; Bennett & Klich 2003)

Aflatoxins are considered the most poisonous mycotoxins (Alshannaq & Yu, 2017). They are produced by molds *Aspergillus flavus* and *Aspergillus parasiticus*, which grow in soil, decaying vegetation, hay, and grains (Andrade & Caldas, 2015). Crops that are frequently affected by *Aspergillus spp*. in sub-Saharan Africa have been well documented. These include cereals (corn, sorghum, wheat and rice), oilseeds (soybean, peanuts, sunflowers and cotton seeds), spices (chili

peppers, black pepper, coriander, turmeric and ginger) and tree nuts (pistachios, almonds, walnuts, coconut and Brazil nuts) (Kumar *et al.,* 2017). While AfB1, B2, G1 and G2 are primarily found in food crops, AFM1 is considered an aflatoxin metabolite that is found mainly in urine and other body secretions (Andrade & Caldas, 2015). Aflatoxin M1 can also be found in the milk of humans and animals that consume food contaminated by aflatoxins (Kang'ethe & Lang'a, 2009). Large doses of aflatoxins can lead to acute aflatoxicosis and can be life threatening, usually through damage to the liver (Bezera *et al.,* 2014). Aflatoxins have also been shown to be genotoxic; meaning they can damage DNA and cause cancer in animal species (Hamid *et al.,* 2013), as well as liver cancer in humans (Hamid *et al.,* 2013).

To minimize the risk of mycotoxin contamination, experts advise consumers to inspect whole grains for evidence of mold, and discard any that look moldy, discolored, or shriveled; avoid damage to grains before and during drying as these are more prone to invasion of molds and therefore mycotoxin contamination; buy grains and nuts as fresh as possible; make sure that foods are stored properly – kept free of insects, dry, and not too warm; and lastly not to keep foods for extended periods of time before consumption (Logrieco *et al.,* 2018). These precautions are difficult to observe, thus consumers have for a long time inadvertently consumed aflatoxins and suffered from the consequences.

### 2.1.2 Probiotics

Probiotics are live microorganisms which, when administered in adequate amounts, confer a health benefit on the host (FAO/WHO, 2001). Many health benefits have been reported and tend to be strain specific (Dolan *et al.,* 2017). In sub-Saharan Africa fermented foods are popular and some

contain species of bacteria that are often used as probiotic (Franz *et al.,* 2014). Notably fermented foods are not probiotic per se. Some researchers have suggested that strains work best when applied to niches from which they were isolated but this is not the case. The intent of a probiotic is to perform functions that promote health, not to colonize the host, therefore the strain origin is not critical (Oldak *et al.,* 2017).

Majority of probiotic strains are Gram-positive bacteria, often associated with fermentation (Behnsen *et al*., 2013). Gram-positive bacteria have a thick mesh-like cell wall made of peptidoglycan (50–90% of cell envelope), and as a result are stained purple by crystal violet (Amils, 2011). These organisms are rich in teichoic acids and some have lipids that form lipoteichoic acids, which serve as chelating agents, and aids in adherence to surfaces (Figure 2.2) (Monachese *et al*., 2012).

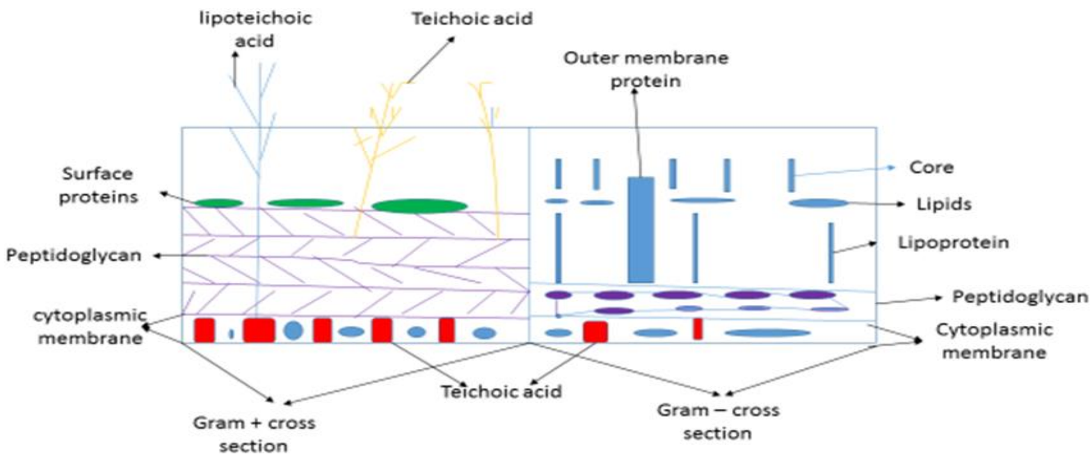



**Figure 2.2. A diagram illustrating the cell wall of Gram-positive and Gram- negative bacteria.**

(Microbeonline, 2013)

A case has been made to reclassify some species of *Lactobacillus* (Salvetti *et al.,* 2018). But until some are re-named, this thesis will retain their current nomenclature. According to metabolism, *Lactobacillus* species can be divided into three groups: Obligately homofermentative (group I) e.g. *Lactobacillus acidophilus, Lactobacillus delbrueckii, Lactobacillus helveticus* and *Lactobacillus salivarius;* Facultatively heterofermentative (group II) e.g. *Lactobacillus casei, Lactobacillus curvatus, Lactobacillus plantarum, Lactobacillus sakei* and obligatively heterofermentative (group III) e.g. *Lactobacillus brevis, Lactobacillus buchneri, Lactobacillus fermentum* and *Lactobacillus reuteri* (Salvetti *et al.,* 2012).

Some *Lactobacillus* species are used as starter cultures in industry for controlled fermentation in the production of yoghurt, cheese, sauerkraut, pickles, beer, cider, kimchi, cocoa, kefir, and other fermented foods, as well as animal feeds. Studies attest that the antibacterial and antifungal activity of *Lactobacillus* species rely on production of acids as well as some bacteriocins and low molecular weight compounds (Inglin, 2015).

*Weissella* is closely related to *Lactobacillus delbrueckii* subsp. *lactis* as shown in the phylogenetic tree in Figure 2.3. *Lactobacillus delbrueckii* subsp. *bulgaricus* has been used for a long time as a yoghurt starter culture (Rizzello & De Angelis, 2011).

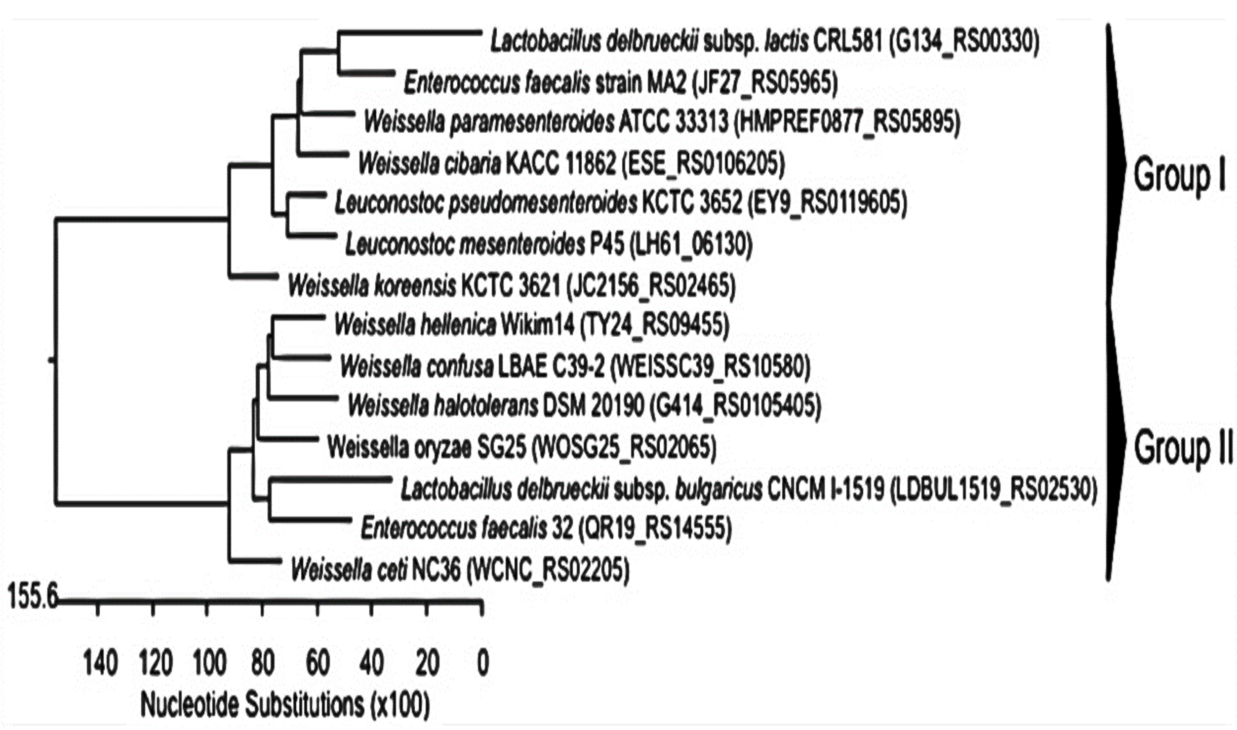



**Figure 2. 3. Phylogenetic relationship of *Weissella* species and *Lactobacillus delbrueckii.* Adopted from** (Abriouel *et al.,* 2015)

### 2.1.3 *Kimere*

*Kimere* is made from pearl millet (*Pennisetum glaneum*), a drought-tolerant crop widely grown all over the world including Kenya (Appendices 1 & 2). As with many cereals, the history of pearl millet is difficult to track (Manning *et al.,* 2011) but it seems to have originated in Africa or China. It is usually cultivated in climates with high temperatures, low precipitation, short growing seasons and acidic infertile soils with poor water holding capacity (Clotault *et al.,* 2012)

Further Clotault *et al.*2012 reports that among the several existing millet genera and cultivars, pearl millet is rich in protein. Like many cereals, pearl millet contain phytates and tannins that act as anti-nutrients which reduce bioavailability of other nutrients including iron and zinc (Gupta *et al.,* 2015). This makes their whole products nutritionally poor, and therefore further processing is

required to increase specific nutrients availability in the gastrointestinal tract. Pearl millet has lower endosperm to germ ratio compared to sorghum, and maize (Rosentrater 2018). Biochemical and chemical compositional changes occur during fermentation of pearl millet. Pearl millet contains gluco-sylvitexin, glycosylorienthin and vitexin, flavonoid compounds responsible for the yellow green discoloration of millet paste in the presence of base. The compounds are present in millet at a ratio of 29:11:4 depending on millet cultivar (Boncompagni *et al.,* 2018). The whole millet grain contains 87 to 259mg/100g C-glycosylflavones expressed as glycosylovitexin equivalents (Boncompagni *et al.,* 2018). The pearl millet moisture content is 7.4% lower than maize that of maize at 12.8% under normal conditions due to surface area to volume ratio. The low moisture content in pearl millet can be attributed to other properties such as, dimension, surface area to volume ratio and shape factor as indicated by the table of composition of selected cereals (Table 2.2). It is important to note that fungi of species that produce mycotoxins are largely influenced by cereal nutrients composition among other intrinsic and extrinsic factors.

Millets of various species have been suggested to act as functional foods and nutraceuticals as they provide dietary fibers, proteins, energy, minerals, vitamins, and antioxidants required for human health. Potential health benefits such as preventing cancer and cardiovascular diseases, reducing tumor incidence, lowering blood pressure, cholesterol and risk of heart disease, lowering the rate of fat absorption, delaying gastric emptying, and supplying gastrointestinal bulk have been reported for millets (Gupta *et al*., 2015)

**Table 2. 2. Nutrient composition of selected cereals**

| Food | Protein (g) | Fat (g) | Ash (g) | fiber (g) | Carhohy drate (g) | (kcal) | Ca (mg) | Fe (mg) | Thiamin (mg) | Riboflavin (mg) | Niacin (mg) |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Rice (brown) | 7.9 | 2.7 | 1.3 | 1.0 | 76.0 | 362 | 33 | 1.8 | 0.41 | 0.04 | 4.3 |
| Wheat | 11.6 | 2.0 | 1.6 | 2.0 | 71.0 | 348 | 30 | 3.5 | 0.41 | 0.10 | 5.1 |
| Maize | 9.2 | 4.6 | 1.2 | 2.8 | 73.0 | 358 | 26 | 2.7 | 0.38 | 0.20 | 3.6 |
| Sorghum | 10.4 | 3.1 | 1.6 | 2.0 | 70.7 | 329 | 25 | 5.4 | 0.38 | 0.15 | 4.3 |
| Pearl millet | 11.8 | 4.8 | 2.2 | 2.3 | 67.0 | 363 | 42 | 11.0 | 0.38 | 0.21 | 2.8 |
| Finger millet | 7.7 | 1.5 | 2.6 | 3.6 | 72.6 | 336 | 350 | 3.9 | 0.42 | 0.19 | 1.1 |
| Foxtail millet | 11.2 | 4.0 | 3.3 | 6.7 | 63.2 | 351 | 31 | 2.8 | 0.59 | 0.11 | 3.2 |
| Common millet | 12.5 | 3.5 | 3.1 | 5.2 | 63.8 | 364 | 8 | 2.9 | 0.41 | 0.28 | 4.5 |
| Little millet | 9.7 | 5.2 | 5.4 | 7.6 | 60.9 | 329 | 17 | 9.3 | 0.30 | 0.09 | 3.2 |
| Barnyard millet | 11.0 | 3.9 | 4.5 | 13.6 | 55.0 | 300 | 22 | 18.6 | 0.33 | 0.10 | 4.2 |
| Kodo millet | 9.8 | 3.6 | 3.3 | 5.2 | 66.6 | 353 | 35 | 1.7 | 0.15 | 0.09 | - |

**(Sidhu *et al.,* 2007; FAO/Government of Kenya, 2018).**

All values except protein are expressed on a dry weight basis.

Pearl millet is normally wet milled to make *Kimere* dough which spontaneously ferments with time. The preparation process is unhygienic because it involves a lot of hand contact. In addition, *Kimere* requires minimal cooking and consumption starts right after milling when *Kimere* is fresh. Some researchers have claimed that *Kimere* can boost libido perhaps due to being rich in calcium and iron (Ifesan & Kolawole, 2018). This needs further verification because the study did not provide data to support the relationship between *Kimere* being rich in the above mentioned nutrients and their boost to libido. The degradation of phytates during fermentation is a positive attribute (Pozrl *et al*., 2009) because fermented *Kimere* makes the protein bioavailable in the body.

This notwithstanding, the hygienic part of its production still makes fermented *Kimere* consumption unpopular within Eastern part of Kenya communities (Nduko *et al.,* 2017)).

A previous study of *Kimere*, suggested it has the potential of having health benefits (Njeru *et al.,* 2010), potentially due to its high abundance of *Lactobacillus.* As *Kimere* is spontaneously fermented, variations of microbial communities are expected from sample to sample and region to region (Franz *et al.,* 2014).

### 2.1.4 Aflatoxins binding trial *In Vivo* and *In Vitro*

Several methods of controlling aflatoxins absorption have been studied both *in vitro* and *in vivo.* The former normally utilize simulated body environment in laboratory while *in vivo* studies use body fluids as culture media (Gallab, 2013). Both *in vitro* and *in vivo* studies have challenges, for example *in vitro* experiments are performed in abnormal functions while *in vivo* provide drawback in differences in biokinetics and lack of extrapolations with small sample sizes. *In vivo* studies include use of humic acid as a binder in broiler chickens (Sabater & Vilar, 2007). One study by Diaz *et al.,* (2004) reported that dietary agents in cattle feeds were capable of reducing aflatoxins M1 metabolites in the cow's milk. Baere *et al.,* (2018) found a possible interaction of veterinary drugs binding to mycotoxin. This study review did not find research reports of studies that have used *Lactobacillus* and *Weissella* supplementation of *Kimere* in human trials.

# CHAPTER THREE

# DETERMINATION OF AFLATOXIN LEVELS IN MAIZE AND MAIZE FLOURS IN WESTERN, NAIROBI AND EASTERN KENYA

## 3.1 Introduction

Since the 1960s, deaths of animals and humans due to consumption of fungal intoxicated maize have been documented (Xu *et al,* 2018). In most cases the fungus attack maize and peanuts due to their available starch and relatively higher moisture content compared to other cereals (Edoh *et al.,* 2018). In Kenya, aflatoxicosis is mostly due to maize consumption. Various intervention methods ranging from agronomic to drastic incineration of grains have been employed. It is doubtful that these methods have been effective to eradicate aflatoxicosis, as evidenced by periodically reported cases of aflatoxicosis (Table 3.1).

Maize (*Zea mays*) or corn has been reported as among the best substrates that favors production of aflatoxin (Liu *et. al.,* 2016). It is a cereal crop that is produced annually more than any other grain and is grown widely throughout the world in a range of agro-ecological environments. Introduced into Africa in the 1500s, maize has become the most important cereal crop and a staple food for more than 1.2 billion people in sub-Saharan Africa (SSA) and Latin America (Nuss *et. al.,* 2010). In industrialized countries, maize is largely used as livestock feed and as a raw material for industrial products, but aflatoxicosis is still reported in both human and animals in these zones.

About 50 maize species exist, consisting of different colors, textures and grain shapes and sizes. In Eastern Kenya, the maize that is commonly consumed is white in color. Ninety percent of white maize is consumed in Africa and Central America. It is worthy to note that these regions lie within

the tropics with climatic conditions favorable for fungal toxins manifestation (Velásquez *et. al.,* 2018) (Figure 1.2).

Eastern and Southern Africans consume 85% of the maize produced (Ekpa *et al.,* 2018). About 5% is used as animal feeds while 10% is used in industrial processes such as wet milling for dextrose production (Ekpa *et al.,* 2019). The maize is processed and prepared in various forms, depending on the country. Ground maize is prepared into porridge, dough (ugali) in Eastern, Western and Southern Africa (Ekpa *et al.,* 2018) Ground maize is also fried or baked in many countries. In all parts of Africa, green (fresh) maize is boiled or roasted on its cob and served as a snack. Popcorn is also a popular snack. In Kenya, green or dried maize is also boiled together with beans, otherwise referred to as *githeri* in Kikuyu language. As aflatoxins are heat stable compounds, drying, boiling or frying the maize and maize products does not help to minimize concentrations (Karlovsky *et al.,* 2016).

Maize does not tolerate drought well and the grain can rot, be infested by mold and contaminated during storage in tropical climates (Suleiman *et al.,* 2013). Lack of sunshine and nitrogen can reduce the production potential of the crop. The grains are rich in vitamins A, C and E, carbohydrates, and essential minerals, and contain 9% protein as reported by Morris and Mouhidin (2021). They are also rich in dietary fiber and calories which are good source of nutrients for fungi (Mckevith, 2004).

### 3.1.1 Maize as a Mold Habitat

White maize is the most widely consumed in Kenya. Previously this has been associated with source of aflatoxins (Table 3.1).

**Table 3.1 Aflatoxicosis data in Kenya since 1960**

| Year | Those affected | Numbers | Sources of toxin | Observed complication/effect |
|---|---|---|---|---|
| 1960 | Duckling | 16000 | Aflatoxin contaminated ground nut | Death |
| 1977 | Dogs poultry | Not specified | Aflatoxin in maize | Death |
| 1981 | Human | 12 | Contaminated maize | Death |
| 1984/85 | Poultry | Not specified | Contaminated maize | Death |
| 1988 | Human | 3 | Contaminated maize | Death and acute effect |
| 2001 | Human | 29 | Contaminated maize | 16 people dead |
| 2002 | Poultry dogs | Unknown | Contaminated maize | Death |
| 2003 | Humans | 6 | Contaminated maize | 6 people dead |
| 2004 | Humans | 331 | Contaminated maize | Acute poisoning and 125 people reported dead |
| 2005 | Humans | 75 | Contaminated maize | Acute poisoning and 32 people reported dead |
| 2006 | Humans | 20 | Contaminated maize | Acute poisoning and 10 people reported dead |
| 2007 | Humans | 4 | Contaminated maize | 2 people reported dead |
| 2008 | Humans | 5 | Contaminated maize | Acute poisoning and 2 people reported dead |
| 2010 | Humans | Unknown | Contaminated maize | Price melt down and grain trade break down unconfirmed dog cases |
| 2015 | Human | Unknown | Contaminated maize | Price melt down |
| 2017 | Human | Unknown | Contaminated beer | - |

(Eduardo *et al.,* 2005; Wagacha *et al.,* 2008; Mutegi *et al.,* 2018)

Aflatoxins can be produced by mold in maize either in the field or during storage as shown in figure 3.1. In the field the source of mold can be soil or plant residue. The sporulating mycelia introduce mold in the crops at pathogenic stage either by winds and insect or soil contacts as shown in the figure 3.1.

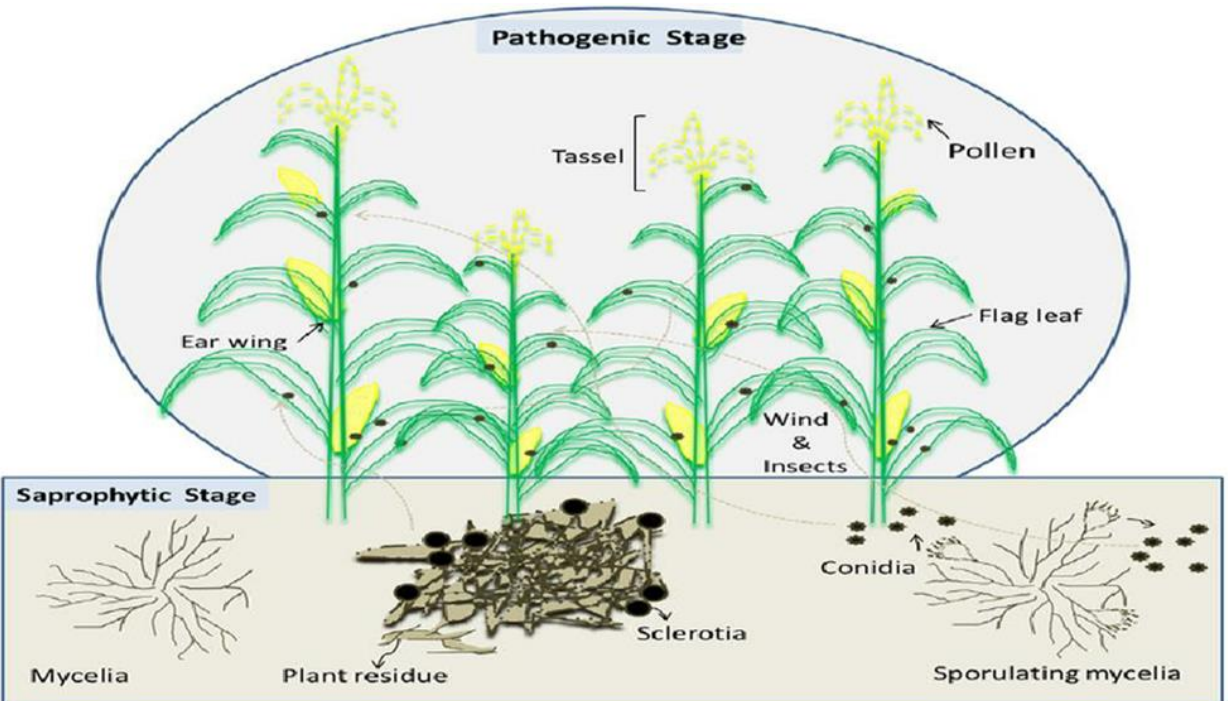



**Figure 3.1. Life cycle of *Aspergillus flavus***

(Lakkireddy *et al.,* 2014)

The main component of maize is starch, which is produced by dry and wet milling. Its functionality varies with the starch structure and composition as well as genotypes and cultural practices (Table 3.2) (Serna-Saldivar & Carrilo, 2019).

**Table 3 .2. Proximate Composition of Maize**

| Maize type | Moisture | Ash | Protein | Crude fibre | Ether extract | Carbohydrate |
|---|---|---|---|---|---|---|
| Salpor | 12.2 | 1.2 | 5.8 | 0.8 | 4.1 | 75.9 |
| Crystalline | 10.5 | 1.7 | 10.3 | 2.2 | 5.0 | 70.3 |
| Floury | 9.6 | 1.7 | 10.7 | 2.2 | 5.4 | 70.4 |
| Starch | 11.2 | 2.9 | 9.1 | 1.8 | 2 2 | 72 8 |
| Sweet | 9 5 | 1 5 | 12.9 | 2.9 | 3.9 | 69.3 |
| Pop | 10.4 | 1.7 | 13.7 | 2.5 | 5.7 | 66.0 |
| Black | 12.3 | 1.2 | 5.2 | 1.0 | 4.4 | 75.9 |

(Sidhu *et al.,* 2007)

Saldivar and Carrilo (2019) state that the average size of maize starch granules range between 1 and 7 μm and 15 and 20 μm, for small- and large-sized granules, respectively. Maize starch exhibit a typical A-type pattern, in which double helices comprising the crystallites are densely packed (Sidhu *et al.,* 2007). Sugary maize starch has lower crystallinity, while waxy maize starch has greater crystallinity as compared to normal maize starch (Wang *et al.,* 2017). The sugary maize starch has lower gelatinization temperature and enthalpy. The maize starch with long-branch chain length amylopectin and higher crystallinity has higher gelatinization temperature and enthalpy (Serna-Saldivar & Carrilo, 2019). Maize also contains various bioactive constituents, such as carotenoids, anthocyanins, and phenolic compounds, which vary with maize type (Lopez-Martinez and Garcia 2015). Studies have suggested that maize has higher anti-oxidant capacity compared to wheat, oat, and rice (Goufo *et al.,* 2014). On the other hand, rice has higher levels of vital

antioxidants that includes phenolic acids, flavonoids, anthocyanins, pro anthocyanidins, tocopherols, tocotrienols, γ-oryzanol, and phytic acid compared to other cereals (Goufo *et al*., 2014). As stated earlier in this study, intrinsic factors such as cereal nutrients greatly influence the fungal infestation.

The expansion of maize cultivation to more marginal lands and climates has been accompanied by increased risk of contamination with mycotoxins such as aflatoxins and fumonisins. Furthermore, conditions that stress plants make them more susceptible to fungal infection and render them more likely to be contaminated by one or more mycotoxins (Leslie *et al.,* 2014). In that case, maize in Eastern Kenya is more likely to be contaminated compared to Nairobi or Western Kenya (Hoffmann *et al.,* 2013).

### 3.1.2 Aflatoxin B1

Aflatoxin B1 (AFB1) is classified as a biological toxin. Biological toxins are toxic substances produced by microorganisms, animals, and plants (Drott *et al.,* 2019). AFB1 is activated to AFB1 exo-8, 0-epoxide primarily by cytochrome P450 (P450) enzymes, particularly P450 3A4. However, P450 3A4 and other P450s also oxidize AFB1 to less dangerous products (Mehrzad *et al.,* 2014). It has the capability of causing harmful effects when inhaled, ingested, injected or absorbed. The health effects of exposure can vary greatly depending on the toxin, the amount, and the route of exposure, ranging from minor (skin or eye irritation, headache, nausea) to severe (respiratory distress, muscle weakness, seizures, death) (Bezzerra *et al.,* 2011).

Secondary metabolites are metabolites by microorganisms that have no economic value to their growth and development. AFB1 is a secondary metabolite produced by *Aspergillus flavus* and *Aspergillus parasiticus* when environmental factors are favorable (Xing *et al.,* 2017). The spores of the fungi germinate by attaching their mycelium to food substrate followed by secreting enzymes that break down nutrients into simpler compounds able to be metabolized by the fungus. The aflatoxin B1 gives the fungi a competitive edge against other microorganisms (Taheur *et al.,* 2019).

Studies have been carried out to find ways of containing aflatoxin contamination along food value chain. The majority of these studies have been at agronomy level (Figure 3.2).

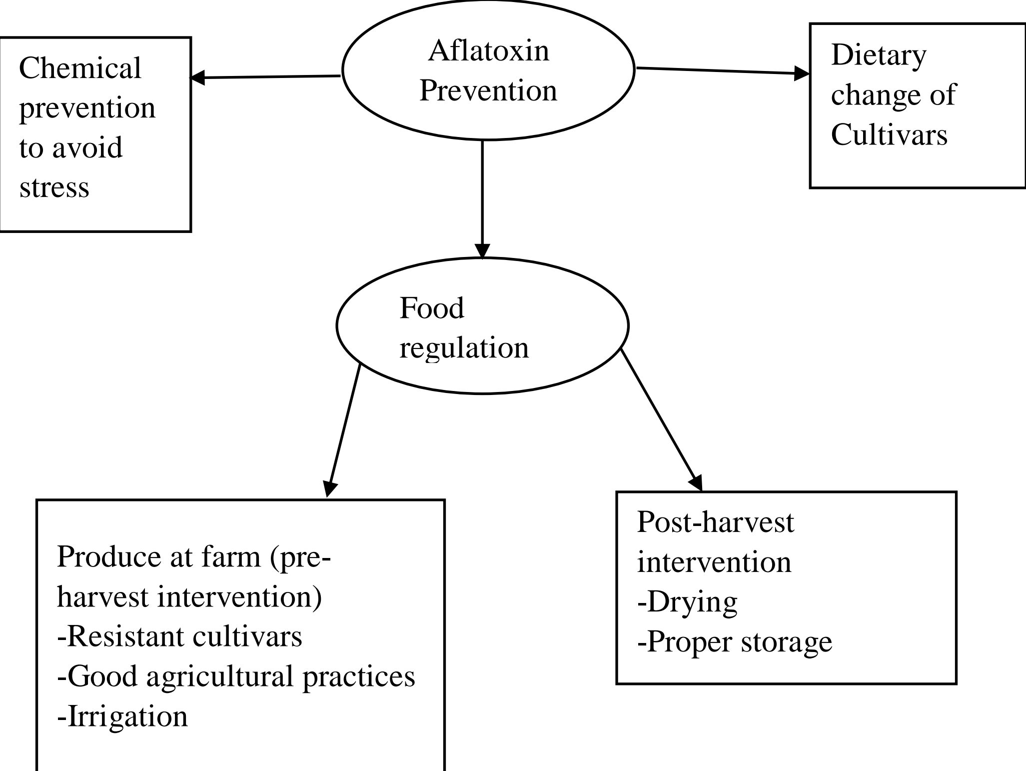



**Figure 3.2. Various Levels of Aflatoxicosis Intervention.**

(Goncalves *et al.,* 2019)

Results from previous studies have shown that it is difficult if not impossible to eradicate AFB1 in cereals once produced (Goncalves *et al.,* 2019). Thus, consumers are left vulnerable to exposure because they do not burn contaminated cereals. This has caused problems of food insecurity (Mahato *et. al.,* 2019). Once ingested, AFB1 is adsorbed into the blood stream from the gastrointestinal tract. In the liver, it is oxidized to various compounds as shown in figure 3.3.

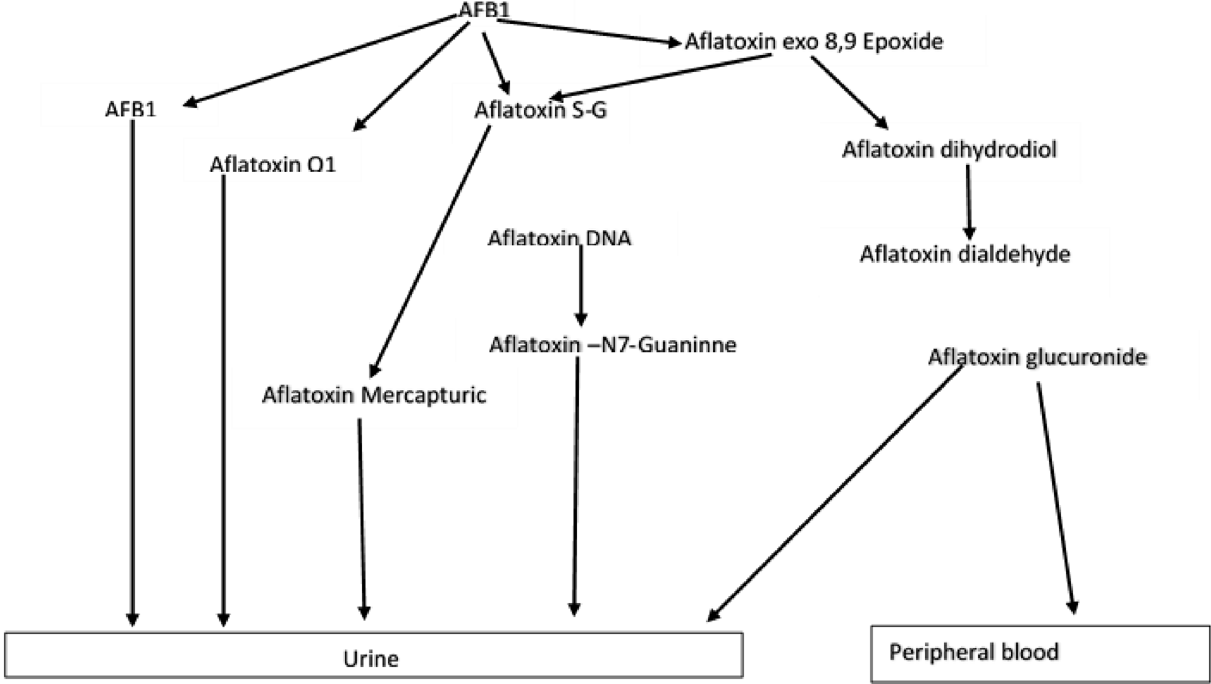



**Figure 3.3. AFB1 end products after oxidation in the liver.**

(Smith et al., 2017)

### 3.1.2 Aflatoxicosis in the selected Regions

Aflatoxin contamination in maize and maize products is problem all over Kenya especially in maize. As a result, aflatoxins issues in Kenya have received a great deal of attention from the academia, policy makers, farmers, processors, the international community and food aid agencies, among others (Mutegi *et al.,* 2018). In his review, Mutegi provides an insight about aflatoxins preference in selected regions in Kenya. Notably Nairobi, Eastern, Western and Nyanza are among the regions with the highest reported cases of aflatoxicosis (Kang'ethe *et al.,* 2017).

## 3.2 MATERIALS AND METHODS

### 3.2.1 Sampling

To establish the levels of aflatoxins contamination in the maize, samples from most consumed brands of flours and grains were targeted. In that case, 27 maize flour samples of three different brands coded X, Y, Z, were sampled from leading supermarkets stores within Nairobi, Eastern and Western regions of Kenya. The samples were in 2 kilograms packages as is done in Kenya during processing and packaging. From each region 9 samples were collected. Thus 3 samples of every brand were sampled from each region. The three regions that were selected were assumed to be sufficient enough to give a clear picture of the fungal toxin consumption. In that respect, Eastern region was selected due to rampant reported cases of aflatoxicosis as reported earlier in this thesis. Nairobi was selected for its high quantity in terms of maize consumption, while Western Kenya was selected as the number one maize producer. At the same time, 75 different samples of maize grains from 5 major outlets in every region were collected. To arrive at the sample size, generally accepted universal Slovin's formula was used. This formula takes into consideration the total population and the marginal error (0.05) in the following equation: - n= N/1+2Ne where n=sample size, N=population gotten by considering various storage points at each store and e is the marginal error in the current study that is 0.05. The specific identity of the stores were concealed to avoid legal implication. Nevertheless samples from Western were collected from stores within Bungoma town because during sampling the harvesting had recently taken place in that region. In Nairobi samples were collected from Kariobangi, Umoja, Kawangware, Kangemi and Kasarani. Each contributing one store. In Eastern the stores were from Embu, Kitui, Meru and Mwingi. At the same time mean temperature and humidity from the store records were noted for comparison

during sampling. The samples were transported immediately in cool boxes to an ISO 17025 accredited laboratory in Nairobi and stored at -20$^{o}$C until analysis.

### 3.2.2 Materials

An Enzyme Linked Immunosorbent Assay (ELISA) kit specific for AFB1 (Helica Biosystems Inc. cat log no 941BAFL01B1-96) was used. Laboratory apparatus included beakers, conical flasks, funnels, filter paper (Whatman No. 1) and 70% analytical grade methanol solution.

### 3.2.3 Methods

Method of Leszczynka *et al.,* (2001) was used to analyze aflatoxin levels in maize and maize flours Twenty grams for every sample (flour and maize) weighed into clean disinfected beakers and labelled. Thereafter the samples were ground to the texture of fine coffee for optimum extraction. To extract aflatoxins from the samples, 70% methanol solution was first prepared by mixing 70 parts of concentrated methanol (analytical grade) with 30 parts of distilled water. The ground samples was added to the beaker containing 100ml extraction solution of the70% methanol solution and kept for 15minutes. (Ratio of sample to extraction solvent was 1:5) (Figure 3.4). The mixture was stirred and then filtered into clean conical flask using Whatman filter paper No.1. The residue on the filter paper was discarded and the filtrate preserved in the beaker for analysis.

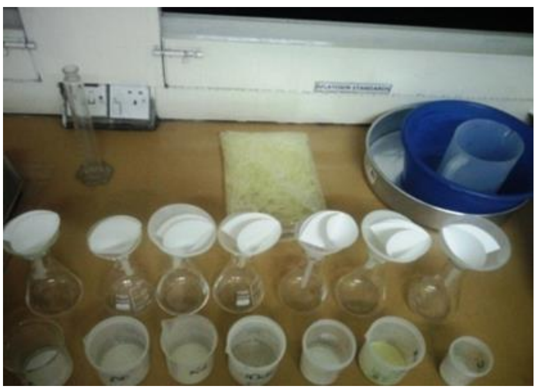


**Figure 3.4. Extraction of samples for aflatoxin analysis.**

To test for total Aflatoxins in the filtrate, 100 µls of conjugate was introduced to the colored premixing micro wells using a micropipette. Aliquots of 100 µl for every filtrate were added and mixed by priming with the micropipette. A standard sample of 20 ppb conc. Of aflatoxin was introduced as a control. One hundred micro liters of the sample plus conjugate mixture was then transferred to antibody-coated micro wells and incubated for 15 minutes. A specific conjugate mixture was used thus eliminating the need for pre-washing well with phosphate buffer saline (PBS). The PBS cleaned the unbound proteins but also reduced sensitivity at the enzyme reaction site. After incubation, the contents of the micro wells were discarded, and the micro wells washed 5 times with distilled water to remove the non-toxin reactants. Then, water was drained and 100 µl of the substrate solution added into each of the micro wells and incubated for 5 minutes. The reaction in this process resulted in a change from clear to blue coloration, whose intensity indicated the aflatoxin content.

To stop the reaction from proceeding, an acidic stop solution was added, which resulted in color change from blue to yellow, with varying intensities depending on the aflatoxin concentration. The resultant solutions in the micro wells were placed into a microtiter plate reader (Neogen model) where the optical density of each micro well was read using a 450nm filter, which gave the amount of total aflatoxin present in each sample quantitatively. Data was analyzed by calculating the means, standard deviations, and ANOVA using SPSS version 21. Graphs were plotted using sigma plot 14.0.

## 3.3 RESULTS

### 3.3.1 Aflatoxins in Maize Flours.

The mean temperature in Nairobi, Western and Eastern were 20, 22 and 25$^{o}$C respectively. While mean relative humidity for respective regions were 69%, 65% 63% respectively. This data is reported as was taken from respective stores during sampling.

After the stop solution during the ELISA technique, the intensity of color change from blue to yellow indicated the concentration of aflatoxins in the sample. Thus increase in intensity shows increase in aflatoxins in the samples as shown in figure 3.5.

**Figure 3.5 Aflatoxins end products by ELISA method**

There was significance difference between the brands among the three regions ($P<0.05$) as shown by the results in Figure 3.6 and ANOVA table in Appendix 1. Each brand had similar aflatoxin levels among the three areas $p>0.05$.

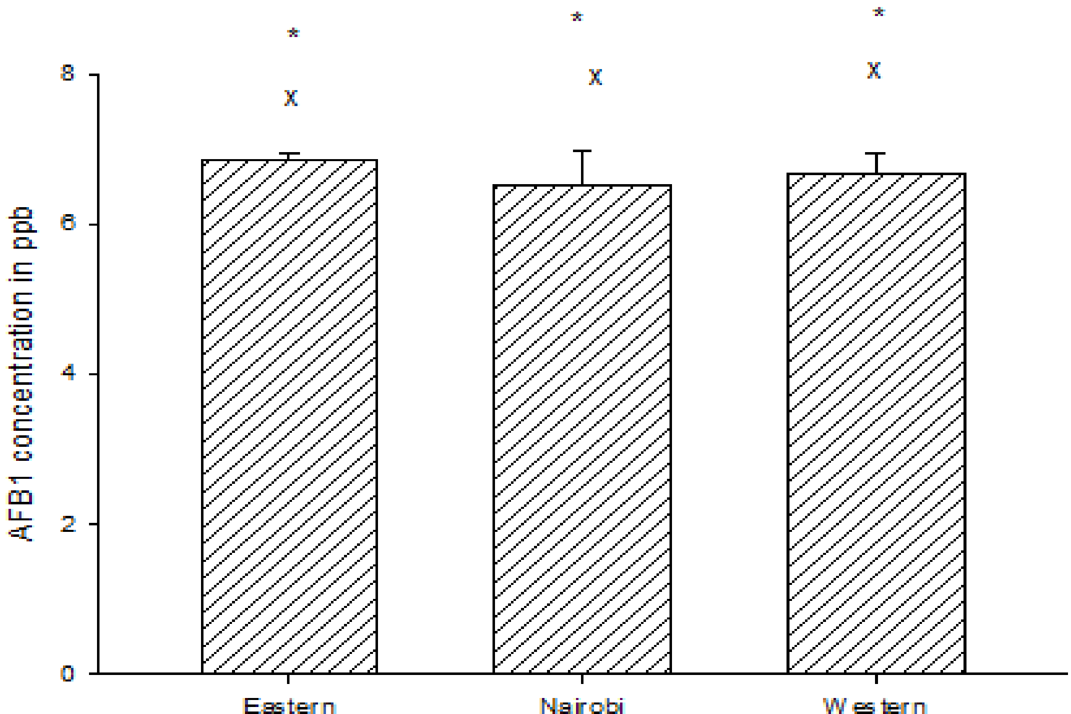



**Figure 3.6. Comparison of total aflatoxins level of brand X, Y, Z in Nairobi, Western and Eastern Regions**

X-no significant difference in AFB1 concentration between regions.

*Mean concentration above Kenya standards

With respect to the three stores in respective regions, Nairobi stores had similar concentrations among the brands (P>0.05). In Eastern region, the concentrations of total aflatoxins in the flour samples were different between the stores as confirmed by post hoc analysis in ANOVA tables of appendix 3, 4 and 5. The concentration differences of aflatoxins in the samples from the three different brands were not significantly different (Figure 3.7)

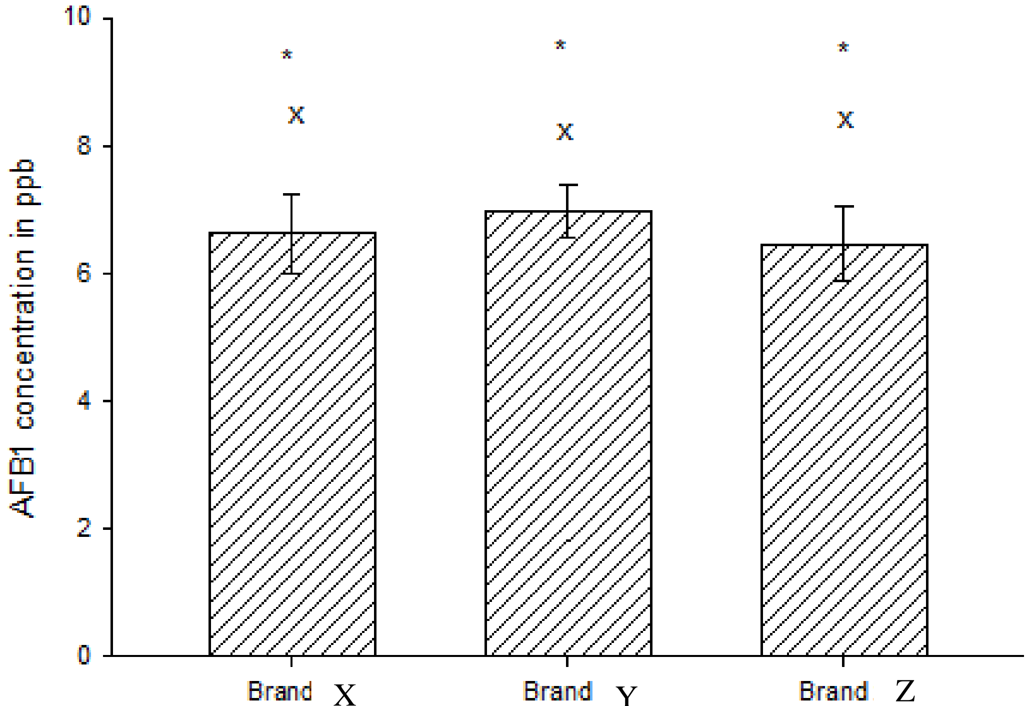



**Figure 3.7. Aflatoxins concentrations in samples of three Brands**

X- Concentration not significantly different at 95 % confidence interval

*-mean concentrations higher than Kenya standards

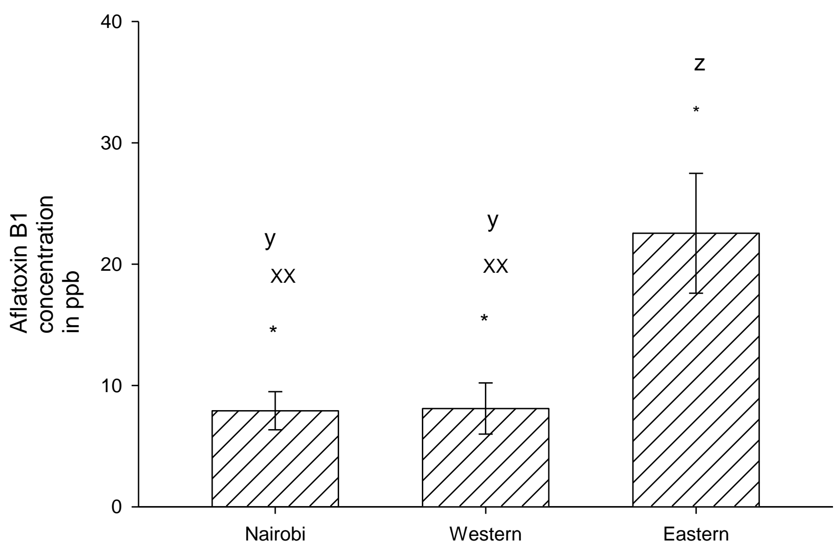
In Western Kenya, the concentrations of aflatoxins in brand x, from store 1 was different from aflatoxin level in store 2, $p < 0.05$, however the level was not significantly different between store 1 and store 3, $p > 0.05$. There was difference in aflatoxin concentration in brand X between store 2 and store 3, $p < 0.05$. For brand Y and Z, aflatoxin levels were significantly different between any two of the three stores in Western, $p < 0.05$ as shown in had hoc analysis for ANOVA analysis in appendix 3.

### 3.3.2 Aflatoxins in Maize Grains Samples

Aflatoxins concentrations in maize samples exceeded Kenya and International standards of 5ppb and 10ppb. The total aflatoxins levels in maize grains were highest in Eastern province and lowest in Nairobi. There were significant differences $p<0.05$ in samples between regions and some stores.

In Nairobi for example in store 1, aflatoxin concentration was not different from store 2 ($P>0.5$), store 1 was not different from store 3 ($P>0.5$), store 1 was not different from store 5 ($P>0.5$), ($P>0.5$), store 3 is was not different from store 5 ($P>0.5$) and store 4 different from store 2 ($P<0.05$) but not different from store 1, 3 and 5 ($P>0.05$).

The highest concentration was 28.19± 1.36 ppb in store 5 from Eastern Kenya. The lowest aflatoxin mean concentration was 5.5 ± 0.43 in store 3 from Western Kenya. Concentration range in Eastern was 10.01ppb with lowest and highest at 18.18 ± 0. 57 ppb and 28.19 ± 1.36ppb respectively. Western Kenya had a range of 5.50ppb with lowest and highest at 5.50 ±0.43 ppb and 11.30 ± 0.91 ppb respectively. Nairobi recorded the lowest range of 3.98 ppb with lowest and highest at 6.02 ±0.31 and 10.1 ±0.73 ppb respectively. These results are shown in Figure 3.8 and in appendices 3, 4, 5, 6 & 7.




* Significantly different compared to Eastern
xx- Nairobi and western not significant

y- Concentrations higher than Kenya standard 5ppb
z-concentration higher (twice) than international standard 10ppb

## 3.4 DISCUSSION

The results show considerable concentrations of aflatoxins were being ingested by consumers of maize grains and maize flour in regions of Kenya. The detected aflatoxins concentrations in maize flours are within Kenyan acceptable limit of 10ppb but the levels are above international standards of 5 ppb. This is well depicted by results which ranged between 6ppb to 8ppb in maize flours. These levels may not trigger acute aflatoxicosis, but continuous exposure may induce health problems like hepatocellular carcinoma, teratogenic effects as well as stunted growth (Barca, 2019). The results show that flours processed and sold in different three major outlets within the three regions were similar in terms of aflatoxin concentrations. While this indicates that manufacturers are keen on good processing methods e.g. quality assurance, it is unacceptable since aflatoxins are not eradicated.

The results show that some stores had maize flour samples with particularly high concentrations. This could have been contributed by different storage conditions as well as the duration of storage. Those with high concentrations were likely stored in conditions that favor mold growth and production of the bisfuranocoumarins. Higher aflatoxin contamination was detected in maize grains from Eastern Kenya than the other regions. This corroborated the previous findings of studies in that region (Mutiga *et al.,* 2017; Mahuku *et al.,* 2019). It is disconcerting that despite several measures that are in place to prevent the problem, excessive levels are clearly still being observed. The high concentration in Eastern Kenya could be due to variations in water stress and the nature of soils. Different methods of sorting, cleaning, bram removal and use of chemical and biological agents have attempted to lower levels of aflatoxins in grains (Isilay *et al.,* 2017). This

has not been universally achieved, likely explaining why differences were noted between Nairobi and Western Kenya

Within the three regions, some stores had maize grain samples with notably high concentrations of aflatoxins. It is important to note that all the samples from three regions that were analysed had concentrations above 5 ppb even though the range was notably high. Nairobi which is relatively more humid than Eastern Kenya had lower levels of aflatoxin concentration. Eastern Kenya experiences low rainfall in comparison to the other two region and it has wide variation of soil type ranging from loam to clay. It can therefore be argued that the only unique variation of experiences among the three regions were water stress and the composition of soils as well as daily and annual temperatures during sampling. These climatic differences affect residual grain moisture content and density of the aflatoxigenic fungi (Obonyo *et al.,* 2018). While anthropogenic causes such as delayed harvesting, poor drying and storage conditions (Kumar *et al.* 2017) may have affected the variations as observed in maize grains, these factors may not have been of major effect to maize flours because sorting, cleaning and bram removal is practised during flour processing.

## 3.5 CONCLUSION

This studies confirmed that consumers are at risk of aflatoxicosis caused by the major staple food in Kenya. Presently, maize millers in Kenya are required to test for aflatoxin in incoming consignments prior to milling. Most millers treat this process step as a Critical Control Point (CCP). Nevertheless, there are still many families who do not buy maize flours from the retail markets. They harvest maize from their farms or buy from their neighbors and mill it using hammer mills. This trend is common in the rural set up. In addition, some families occasionally dispatch packages of milled maize to their loved ones in the cities. This maize will not have undergone any quality assurance test and its aflatoxin concentrations would not be tested. This implies that regardless of the efforts put forth by big commercial maize millers to control aflatoxin, the oblivious consumers may still be consuming maize laden with high concentrations of aflatoxin in *ugali*, *githeri* and porridge. There is clearly an urgent need to establish further measures to protect consumers.

# CHAPTER FOUR

# ISOLATION AND CHARACTERIZATION OF *LACTOBACILLUS* AND*WEISSELLA* STRAINS IN KIMERE, DETERMINATION OF THEIR CAPACITY TO SEQUESTER AFLATOXINS AND DEVELOPMENT OF PROBIOTIC YOGHURT

## 4.1 Introduction

Fermented foods have long been part of the human diet. Many of these foods contain lactobacilli, organisms that have come under intense study due to the application of some of their strains as probiotics (Huang *et al.,* 2018). Many have been granted "generally recognized as safe" (GRAS) status by the Food and Drug Administration of the USA and can be incorporated into food and consumer products. In the past decade lactobacilli have been investigated for their potential as detoxification tools (Dolan *et al.,* 2017). Research has focused on a variety of environmental pollutants including AFB1 and heavy metals such as lead, cadmium and arsenic (Bisanz *et al*., 2014). The detoxification is suggested to be dependent of factors including pH and the strain of probiotics (Markowiak & Slizewska, 2017)

*Kimere* falls under category of spontaneously fermented foods. A fermented dough product made from Pearl Millet, *Kimere* is commonly consumed in Eastern Kenya. The consumption starts after wet milling and continues until after fermentation. Preparation of *Kimere* is simple and thus it requires rudimentary equipment with unskilled labor (Figure 4.1). Fermentation of *Kimere* is spontaneous due to the plethora of microbes that it picks up from the environments (Njeru, 2009).

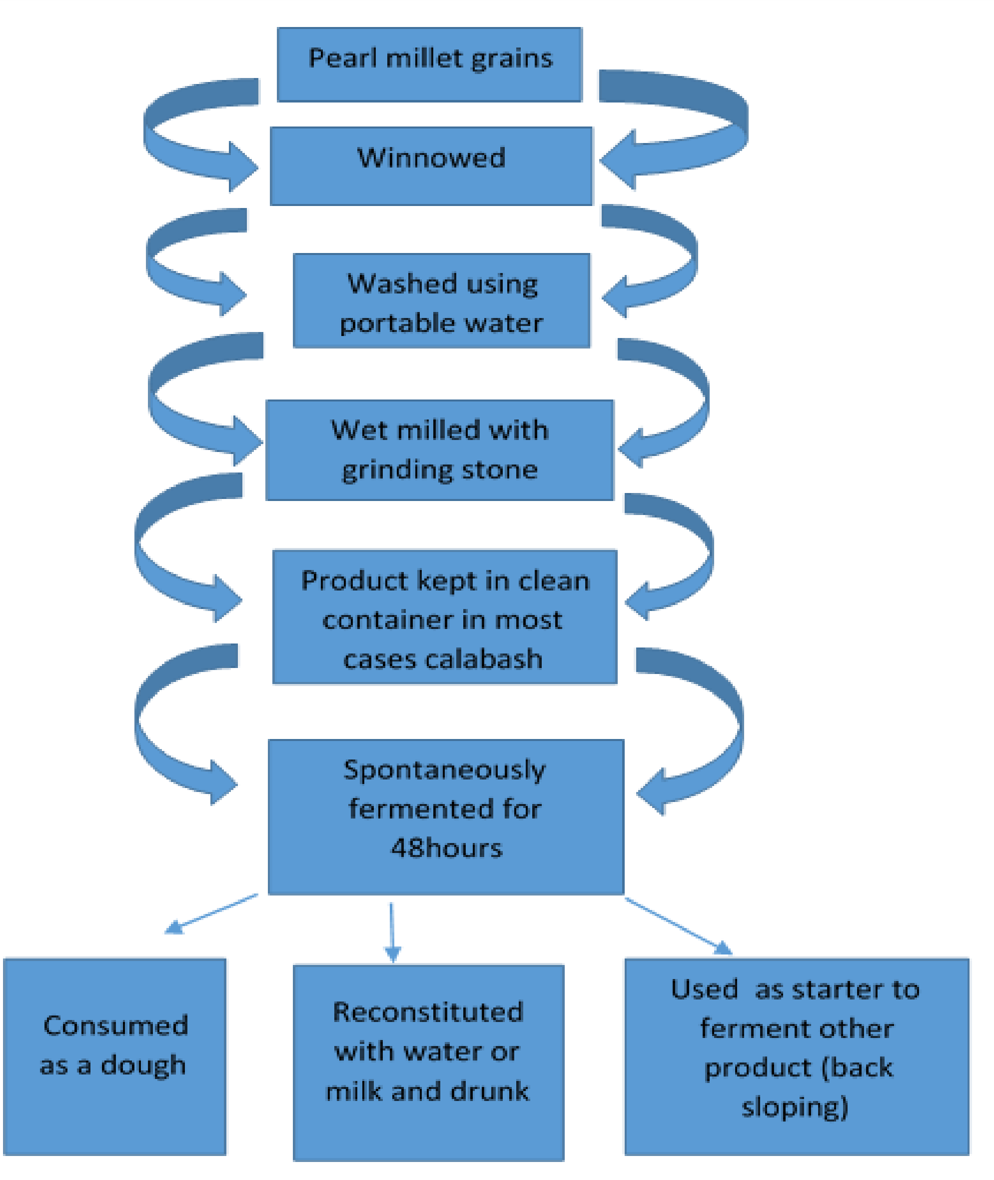



**Figure 4.1. Flow diagram for production of *Kimere*.**

### 4.1.1 Isolation of *Lactobacillus* and *Weissella*

Several guidelines have been published for the extraction of *Lactobacillus* from crude samples to pure isolates. They include initial homogenization of material, media resuspension, use of selective media as in extraction of *Weissella* species (Assamoi *et al.,* 2016). In dairy products, for example,

cells need be separated from the particles for ease of enumeration (DF 1997). The most commonly used probiotics are represented by *Lactobacilli,* bifidobacteria and non-pathogenic yeasts.

### 4.1.2 Selective Media

Several elective and selective media have been developed for the isolation and counting of *Lactobacillus* strains and for the differential counting of mixed populations of lactic acid bacteria. Oxygen tolerance, nutritional requirements, antibiotic susceptibility, and colony morphology and color are used to differentiate strains in these methods (Fusco *et al.,* 2015). To date, specific media have been developed for enumeration of *Lactobacillus*. However, these media support other bacteria such as *Lactococcus, Enterococcus, Leuconostoc, Weissella, Bifidobacterium* and *Pediococcus* thus no medium has yet been described on which only lactobacilli grow solely (Assamoi *et al.,* 2016). Routinely MRS (de Man and Rogosa) media has been used in isolation and enumeration of *Lactobacillus* (Ashraf & Shah, 2011)

## 4.2 MATERIALS AND METHODS

### 4.2.1 *Kimere* Sampling

Using Slovin's formula to calculate sample size, 100 grams of 30 *Kimere* samples that had spontaneously fermented for 24 hours were collected at random from 30 homesteads in Eastern Kenya. The samples were dispersed in sterile screw caps tubes, rapidly cooled using dry ice parks. Immediately they were transported in cool boxes, and stored at -20$^{o}$C in the Food laboratory of University of Nairobi until microbiological analysis.

### 4.2.2 Isolation of Lactic Acid Bacteria (LAB)

Standard methods to isolate LAB were used (Mwangi *et al*., 2016). Thus, 10 serial diluents of 10ml per sample were prepared aseptically using PBS autoclaved and cooled to room temperature resulting to dilutions up to $10^{-10}$. Thereafter, MRS (Hi media laboratories Pvt Ltd) agar was used aiming to isolate *Lactobacillus* for further tests. The agar was prepared according to manufacturer specification and aseptically dispensed in disposable sterile petri dishes, allowed to cool and later stored inverted until use. One milliliter of serial dilutions of $10^{-6}$ to $10^{-10}$ was inoculated for every sample in the petri dishes in triplicates and inoculants spread on the agar using a sterile spread plate glass rod. The petri dishes were then incubated for 24 hours at 37$^{o}$C. Later the colonies were grouped according to their shine, shape, size and gross. Further, Gram stain, spore stain, and microscopy tests were performed. Colonies which were Gram positive, rod shaped cells and with red brown color after spore stain were selected for further testing (Figure 4.2). The colonies were then streaked on the MRS agar incubated at 37$^{o}$C for 24 hours three times for purity and shipped in a dry ice packs to Lawson Health Research Institute (LHRI) in London, Ontario for further

analysis. At the LHRI, the cells were stored in analytical grade sucrose solutions in a freezer at -80$^{o}$C until further analysis.

Many fastidious organism removed lactic acid bacteria favored

Kimere

Serial dilutions

MRS agar 6.2

Anaerobic

Obligates anaerobes removed

Streak and sub cultures

Pure cultures

Streak onto MRS agar pH 4.3

*Cyanobacterium* and other bacteria that cannot tolerate low pH are removed, some species of *Lactobacillus* removed

Single colonies tested

Gram stain

Pink

Positive

Microscopic morphology

Coccoid vibrio

Rod shaped

Endospore test

Positive (green)

Not *Lactobacillus*

Negative (red)

Motility test

Motile

Non motile

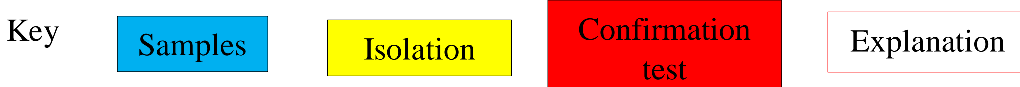
Catalase test

Positive (bubbling)

Negative (no bubbling)

*Lactobacillus*

**Figure 4.2. *Lactobacillus* or *Weissella* isolation flow chart.**
(Assamoi *et al.*, 2016; Mwangi *et al.*, 2016; Fusco *et al.*, 2015; Ashraf &Shah 2011)



### 4.2.3 Preparation of PBS

A standard method for preparing PBS was used. One liter stock of 1xPBS was made by dissolving 8g sodium chloride (NaCl), 0.2 g of potassium chloride (KCl), 1.44g of sodium hydrogen phosphate ($Na_2HP0_4$), 0.24g of potassium hydrogen phosphate ($KH_2P0_4$) all analytical grade in a 800ml distilled water. Finally, the volume was adjusted to one liter and pH adjusted to 7.4 using hydrochloric acid (HCl) acid.

### 4.2.4 Survival of Strains in AFB1 Solution

Eight ppm solution was prepared from a known pure AFB1 (Sigma ALdrich A6636-1mg) of a known concentration. Strains concentrations were standardized in PBS to a known Optic Density (OD) reading and which had AFB1 concentration of 8ppm (Lopez *et al.,* 2002). Then the strains were transferred into a 96 well plate, each strain in three wells per plate. The ODs were captured every 30min for a period of 18 hours.

### 4.2.5 Acid Tolerance Test

To test for survival of strains in a high-acidity environment, PBS solution had pH adjusted to pH2, pH3 and pH7 (neutral environment) using HCl. With strains' concentration standardized by Optical Densities and concentration adjusted following the standard formula C1 x V1=C2 x V2 where C1 is OD of known concentration of strain, V1 is the known volume of the strain, V2 is unknown final volume after adjusting and C2 is the desired standard. The bacterial strains solution at pH2, pH3, pH7 were transferred in a 96 micro wells plate, each strain in three wells per plate per pH. The ODs were captured after every 30 minutes for 18 hours using multi scan machine (thermal electron corporation USA) at 600nm.

### 4.2.6 PCR Amplification

After DNA extraction (Instagene), amplification was done on 10 ul, Instagene products, by PCR method, after preparing the master mix 60ul. Thus, the PCR would involve template DNA (from strains) melting and enzymatic replication of DNA primers (short DNA fragments) – containing sequences complimentary to the target region (in the study case 16s DNA region. During amplification DNA polymerase enhances the chain reaction by enabling assembly of new nucleotide by comparing the initial blocks (template DNA) and the DNA primers in heating and cooling steps referred to as thermal cycling. Thermal cycling steps are necessary first to physically separate the two strands in DNA double helix in a process called melting; and to use each strand as a template in DNA synthesis by the DNA polymerase to selectively amplify the target DNA. The selectivity of PCR is a function of specific primers that are complementary to the DNA region target for amplification. Thus, in this study HAD-1 (forward primer), CCATCTCATCCCTGCGTGTCTCCGACTCAGCWACGCGARGAACCTTACC) and HAD-2 reverse (CCACTACGCCTCCGCTTTCCTCTCTATGGGCAGTCGGTGATACRACACGAGCTGACGAC) primer was used (Janda & Abott, 2007).

### 4.2.7 Master Mix Preparation

Fifty microlitres of different reagents were added to 10 ul Instagene product to prepare 60 ul of master mix. Thus 20.4ul of distilled water ($dH_20$) was added first in a 100ul vials. This followed 8.4 ul of dinitrogen phosphates (dNTPs) of 1.25mM. Thereafter 5ul of 10x PCR buffer was added. 2.5 ul 0f Magnesium chloride (MgCl2) of 50mM concentration was added. 1.6 ul of HAD-1 and HAD-2 of 20uM strength were added, respectively. Finally, Taq (5U/Ml) according to

manufacturer specification, was added before the master mix was mixed thoroughly and inserted in PCR machine for thermal cycling.

### 4.2.8 PCR cycling conditions

Strains for DNA analysis were extracted using the QIAamp DNA stool mini kit (Qiagen). Strains were vortexed in 1 mL buffer ASL before removal of the strains and addition of 0.1 mm zirconia/silica beads (Biospec Products) with 2, 30 seconds rounds of bead beating at full speed with cooling on ice in between (Mini-Bead Beater; Biospec Products). The study used the forward primer (CCATCTCATCCCTGCGTGTCTCCGACTCAGCWACGCGARGAACCTTACC) and reverseprimer (CCACTACGCCTCCGCTTTCCTCTCTATGGGCAGTCGGTGATACRACACGAGCTGACGAC) for sequencing (Janda & Abott, 2007). Amplification was carried out in 42 mL with each primer present at a 10 mL (3.2 pMol/ml stock), 20 ml GoTaq hot start colorless master mix (Promega) and 2 mL extracted DNA. The PCR protocol was as follows: initial activation step at 95uC for 2 minutes and 25 cycles of 1 minute 95uC, 1 minute 55uC and 1 minute 72uC. PCR products were quantified by use of gel electrophoresis. Later at the London Regional Genomics Centre (LRGC, lrgc.ca, London, Ontario, Canada) samples were treated with an Ion OneTouch System (Life Technologies) and sequenced on an Ion Torrent Personal Genome Machine sequencer on a 316 chip (Life Technologies) (Bisanz *et. al* 2014).

### 4.2.9 Gel Electrophoresis

To establish quality and quantity of PCR products, gel electrophoresis was performed. To prepare the gel, 0.5 g of agarose was weighed into conical flask. 50ml of 0.5X TBE was added and swirled to mix according to standard protocol and safety. 0.5x TBE buffer was poured into the gel tank to submerge the gel to 2-5mm depth. This was the running buffer.

### 4.2.10 Preparation of the Samples for Gel Electrophoresis

Ten microliter of PCR products was transferred to a fresh microfuge tube which were well labelled so as to identify the lanes on the gel photograph. 2ul of loading buffer (bromophenol blue (Sigma B8026))-to give the color and density to the sample to make it easy to load into the well- was added into 10ul sample using a tip left into the microfuge so as to use it again in loading on the gel. As the dye are negatively charged in neutral buffer, they would move in the same direction as the DNA during electrophoresis making it possible to monitor the progress of the gel. The first well established after removing the comb from a well-set gel was loaded with the marker. Then other wells were loaded with the samples. The gel tank was closed, and the power switched on to run the gel. After the marker dye had run ¾ of the tank the gel was stopped and viewed at the UV light box in dark room.

### 4.2.11 PCR product analysis and Identifications

To analyze for species, a rapid approach to the 16s DNA based bacterial identification was used as per Janda & Abott (2007). This technique combines uracil DNA glycosylase (UDG), mediated base specific fragmentation of PCR product with matrix and assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF MS). 16s rDNA signature of strains were PCR

amplified. The complete amplification as well as extent and amplicons quantities and qualities were checked by gel electrophoresis. The PCR products were immobilized onto a streptavidin coated solid support to selectively generate either sense or antisense templates. Single stranded amplicons were subsequently treated with uracil – DNA- glycolase to generate T- specific basic sites and fragmented by alkaline treatment. The resulting fragments patterns were analyzed by MALDI-TOF MS. Mass signals of 16s rDNA fragments were compared with patterns calculated from published 16s rDNA sequences using blast software.

### 4.2.12 AFB1 Binding by the Strains in Yoghurt

To test for efficacy of strains to sequester AFB1 in yoghurt, fermented milk products were made from the reconstituted skim milk containing 12% nonfat dry matter (Isleten *et. al.,* 2006). Prepared skim milk was heated at 90°C for 5 min and then cooled to 42°C. Stock solution of AFB1(sigma ALdrich A6636-1mg) was collected from 5 μg/ml AFB1 in ben-zene/acetonitrile and the benzene/acetonitrile derived from the stock was evaporated by heating in a water bath at 80°C. AFB1 residue was dissolved in 2 ml methanol. A volume of 0.08 ml was transferred from the contaminated methanol to 20 ml of skim milk, resulting in milk containing 10 ng/ml AFB1 (Isleten *et. al*., 2006). After that, 20 ml milk was inoculated with 2% cells of isolated strains. *Streptococcus thermophilus* was added as a requirement of the yoghurt preparation protocol. But the same was done to our control thus any unforeseen effect of *Streptococcus thermophilus* to the binding statistically was controlled. The ratio of isolates to *Streptococcus thermophilus* was 1:1. Cell-free reconstituted milk contaminated with AFB1was used for positive control with only *Streptococcus thermophilus*, while fermented products made from uncontaminated reconstituted milk was used for negative control. All groups were incubated at 42°C for 4 h. Fermented milk products were

centrifuged at the end of incubation and unbound AFB1content in the supernatant was determined by High Performance Liquid Chromatography (HPLC). Each sample for the HPLC analysis was diluted 1:125 in PBS; 1.5ml of this solution was transferred into Eppendorf safe lock vials for aflatoxin binding analysis using high performance liquid chromatography technique (Wacoo *et al.,* 2014). The percentage of AFB1 bound to the cells was calculated as the difference between the total AFB1 the amount of free AFB1 in the resultant solution. Data from triplicate experiments were integrated and recorded using a Millennium chromatography manager Software 2010 (Waters, Milford, MA) as appropriate. In this study all assays were performed three times and both positive and negative controls were included. At the same time fermented milk products with uncontaminated milk for viscosity and pH analysis was prepared. In this case yoghurt prepared using *Lactobacillus delbreuckii* subsp. *bulgaricus* and *S. thermophilus* at the ratio of 1:1 was used as positive control and labelled GR 28.

**4.2.13 Probiotic Yoghurt Development.**

Different experiments were done to ascertain the best strain to prepare probiotic yoghurt. *Streptococcus thermophilus* enhances yoghurt gel initiation and gives it the characteristic yoghurt flavor. Propagation of the probiotic strains was done in MRS broth before harvesting after 24 hours incubation at 37$^{o}$C anaerobically. *Streptococcus thermophilus* was grown in de Mane 17 (M17) broth and incubated accordingly. To prepare the yoghurt, milk was boiled to 80$^{o}$C for 30minutes, followed by cooling to 38$^{o}$C (close to body temperature). Thereafter the starter cultures were inoculated at a ratio of 1:1 for *streptococcus thermophiles* and selected strains respectively. Incubation was done for 4 after when gelled yoghurt was agitated and stored at refrigeration until further analysis. Finally pH and viscosity were tested using the final probiotic yoghurt.

## 4.3 RESULTS

### 4.3.1 Isolation

Three hundred Gram-positive rod LAB isolates from *Kimere* were identified for further screening. Out of the three hundred strains, 16 strains were identified based on ability to survive in high acidity (pH2 to pH3), catalase test negative, non-motile and endospore test negative. (Figure 4.3, Figure 4.4). Figure 4.3 shows a photograph of a gram stained *Weissella cibaria* NN20 cells. The cells were rods but clearly different from *Lactobacillus* species in terms of size. While the cells of *Lactobacillus* species were rod shaped same as *Weissella*, the gram stain followed by microscopy indicated that *Weissella* species cells were by vision significantly shorter than those of *Lactobacillus* species

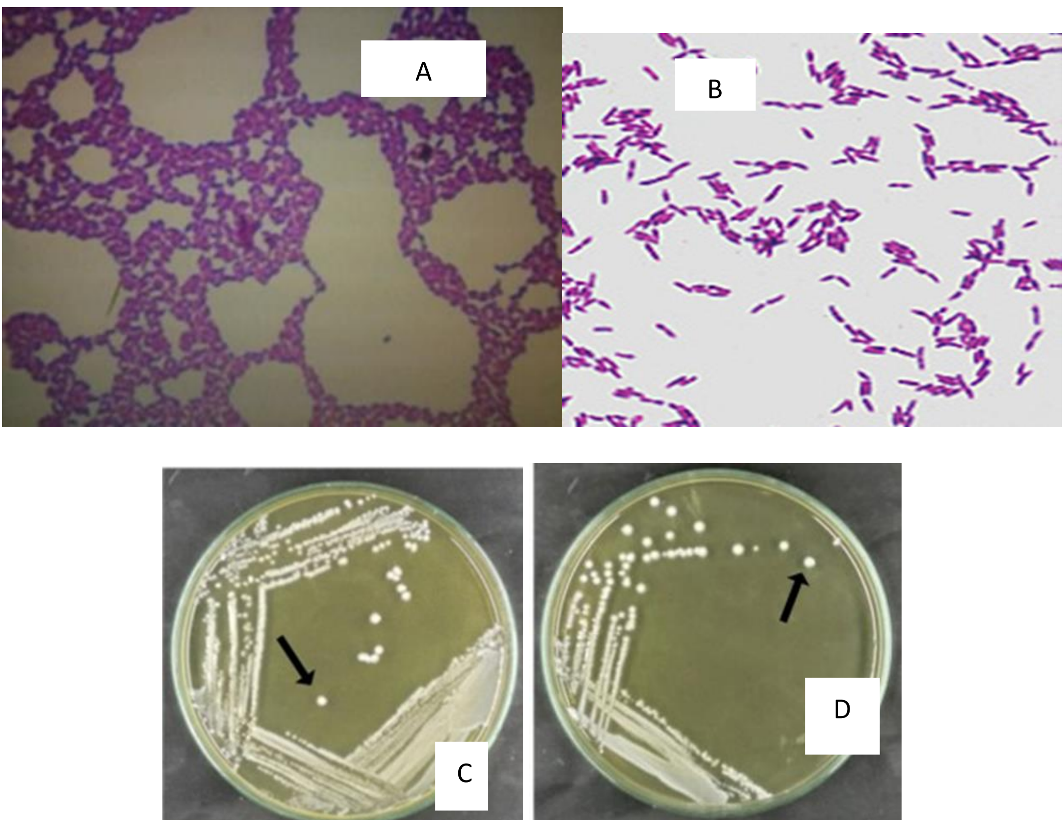



**Figure 4.3. A and B; Microscopy and Gram stain characteristic of *Weissella cibaria* and *Lactobacillus* strain. C and D; Purified *Lactobacillus* and *Weissella* colonies**

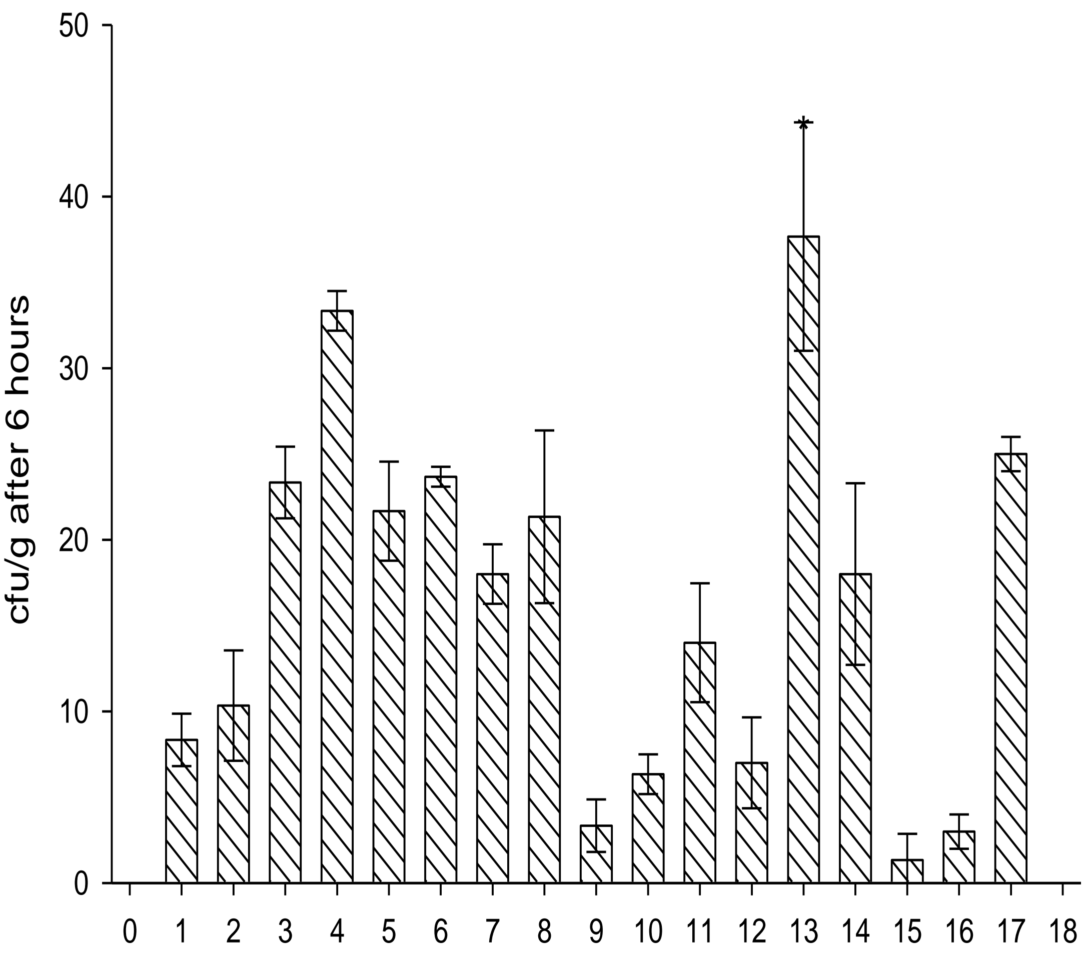



**Figure 4.4 Ability to survive in gastric conditions at pH2**

Key

1-NN1 2-NN3 3-NN6 4-NN7 5-NN8 6-NN9 7-NN11 8-NN13 9-NN14 10-NN16 11-NN18 12-NN19 13-NN20 14-NN21 15-NN22 16-NN26 17-GR1 as shown in Figure 4.1

### 4.3.2 PCR, Gel Electrophoresis and Identification of Strains

The gel electrophoresis methods indicated that the DNA extraction and polymer chain reaction process produced enough DNA profiles for sequencing (Figure 4.5). The C indicates the agarose gel ladder with a maximum pf 3000 base pairs and a minimum of 100 base pairs (bp). In the Figure 4.5, the results shows that the PCR process was successful. This is because the randomly selected

samples had an amplification from100 bp up to approximately 1000bp as indicated by bright regions at the bottom and middle (Figure 4.5)

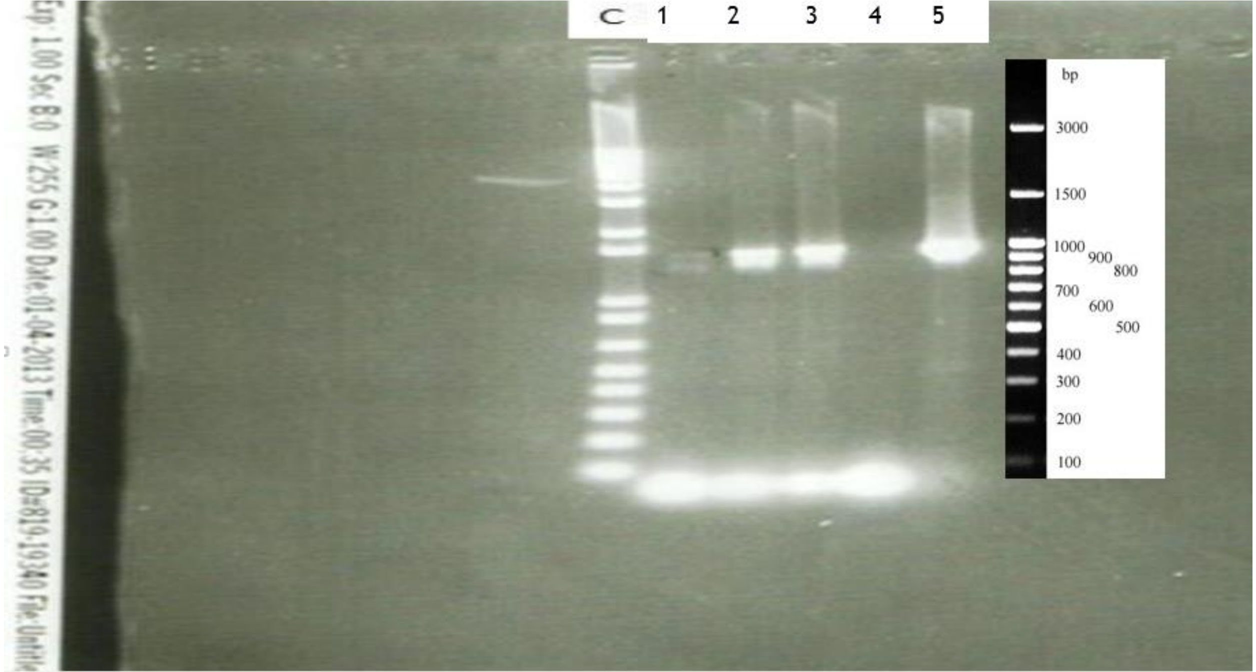



**Figure 4.5. X-ray film showing electrophoresis bands of amplified PCR products.**

Key C- ladder, 1-NN1, 7-NN7, 13-NN13, 20-NN20, and 26-NN26 6-NN6 (isolates in table 4.1)

In figure 4.5 it is clear that some strains did not amplify. For that reason including what has been mentioned earlier in the study of pure strains isolation, only 16 strains out of 300 were identified.

Out of the 16 strains 25% resembled *Lactobacilus fermentum.* 68.75% were similar to *Weissella cibaria*. Only one strain was *Weissella confusa* (Table 4.1).

**Table 4.1**. **Molecular DNA identification of the selected isolated strains**

| Strain/code | Possible Isolates Identity | % identified characteristics |
|---|---|---|
| NN1 | *Lactobacillus fermentum* | 98.9 % |
| NN3 | *Weissella cibaria* | 99.9 |
| NN6 | *Weissella cibaria* | 100 |
| NN7 | *Lactobacillus fermentum* | 99 |
| NN8 | *Weissella cibaria* | 100 |
| NN9 | *Weissella cibaria* | 99.9 |
| NN11 | *Weissella cibaria* | 99.9 |
| NN13 | *Lactobacillus fermentum* | 99.1 |
| NN14 | *Weissella cibaria* | 99.9 |
| NN16 | *Weissella cibaria* | 99.9 |
| NN18 | *Weissella cibaria* | 99.9 |
| NN19 | *Weissella cibaria* | 100 |
| NN20 | *Weissella cibaria* | 99.9 |
| NN21 | *Weissella cibaria* | 99.9 |
| NN22 | *Weissella confuse* | 99.9 |
| NN26 | *Lactobacillus fermentum* | 99.1 |
| GR1 | *Lactobacillus rhamnosus* | 99.9 |
| GR12 | Posted non-similarities | Not applicable |

### 4.3.3 AFB1 binding, survival in acidic condition

The highest binder of AFB1 was identified as strain code NN20 (*Weissella cibaria*). This strain bound 43.7% of available AFB1 (Table 4.2). The strain also survived better in acidic conditions compared to controls (Figure 4.6). In this case the two positive controls GR28 (traditional yoghurt), GR1 (known binder of environmental contaminant), shows less binding of AFB1 capacity as compared to the candidate strain NN20.

**Table 4.2. AFB1 binding levels by isolates after fermenting milk**

| Strain | ug/ml average AFB1 based on St curve | ug/ml Corrected average AFB1 | St dev | %free AFB1 | Bound %AFB1* |
|---|---|---|---|---|---|
| NN1 | 5.18 | 3.21 | 2.0 | 62.0 | 38.0 |
| NN3 | 4.92 | 2.84 | 2.3 | 57.8 | 42.2 |
| NN6 | 5.06 | 3.04 | 1.3 | 60.1 | 39.9 |
| NN7 | 5.23 | 3.28 | 7.8 | 62.8 | 37.2 |
| NN8 | 5.16 | 3.18 | 3.7 | 61.7 | 38.3 |
| NN9 | 5.05 | 3.03 | 5.7 | 60.0 | 40.0 |
| NN11 | 5.29 | 3.37 | 5.3 | 63.7 | 36.3 |
| NN13 | 5.00 | 2.95 | 6.4 | 59.1 | 40.9 |
| NN14 | 5.05 | 3.03 | 2.9 | 60 | 40.0 |
| NN16 | 5.01 | 2.97 | 2.3 | 59.3 | 40.7 |
| NN18 | 3.33 | 1.93 | 1.2 | 58.0 | 42.0 |
| NN19 | 5.14 | 3.15 | 6.4 | 61.3 | 38.7 |
| NN20 | 4.83 | 2.72 | 2.3 | 56.3 | 43.7 |
| NN21 | 5.04 | 3.01 | 0.3 | 59.7 | 40.3 |
| NN22 | 5.04 | 3.01 | 6.9 | 59.8 | 40.2 |
| NN26 | 5.02 | 2.98 | 2.0 | 59.4 | 40.6 |
| GR-28 | 4.99 | 2.94 | 2.0 | 58.9 | 41.1 |
| GR-1 | 5.22 | 3.27 | 3.6 | 62.7 | 37.3 |
| GR-12 | 5.62 | 5.01 | 1.5 | 89.1 | 10.9 |
| Blank | 6.29 | 6.27 | 8.6 | 99.84 | 0.16 |

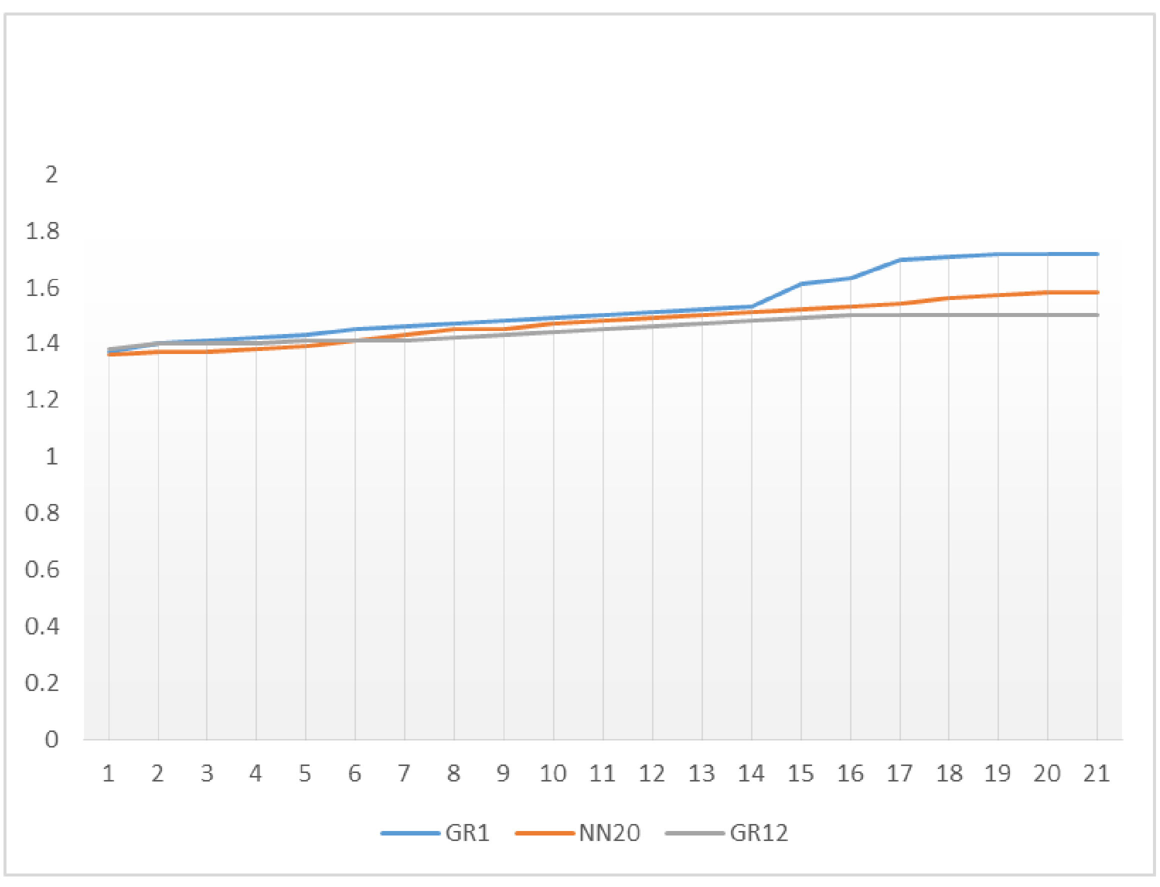



**Figure 4.6. Ability for *Weissella cibaria* NN20 isolate to survive in acidic condition compared to a known probiotic *Lactobacillus rhamnosus* GR-1 and a known negative control, *E.coli* GR12.**

Y- axis- Absorbance (ABS)
X- axis- Time (30mins interval)

### 4.3.4 Ability to Ferment Milk

All the 16 strains had ability to ferment milk but *Weissella cibaria* NN20 produced best quality product with viscosity and pH similar to the control ($P>0.5$) (Figure 4.7; Figure 4.8)

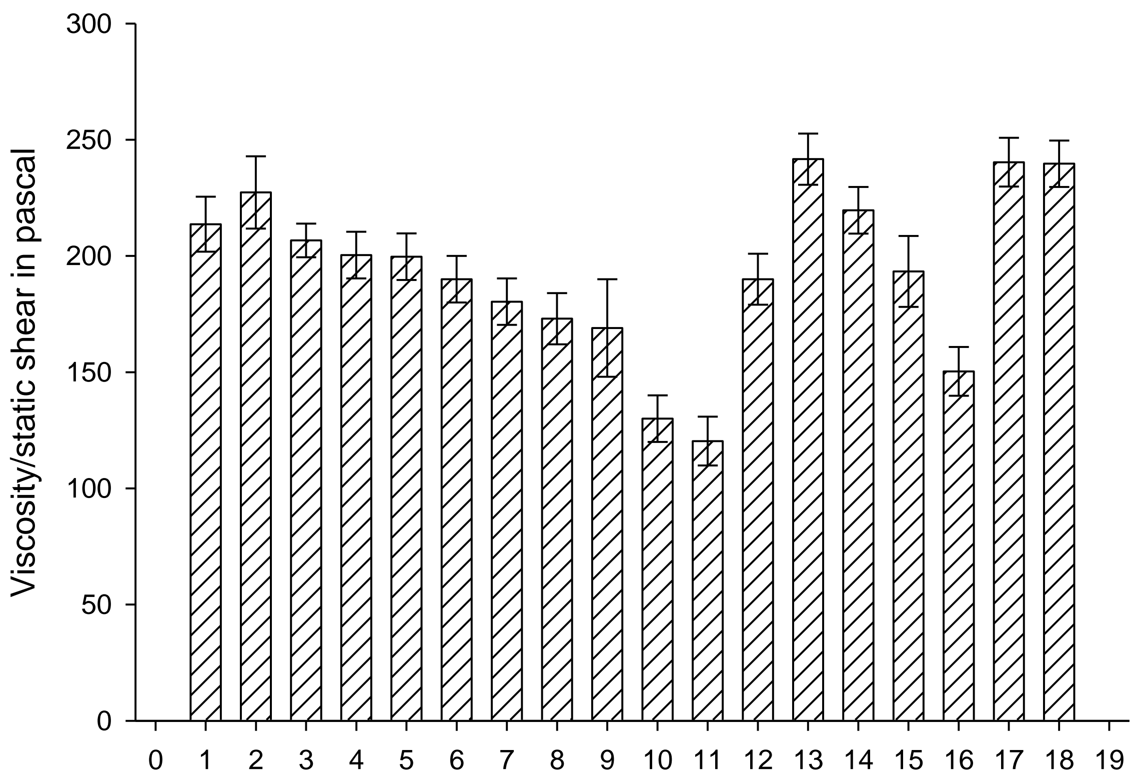



**Figure 4.7. Viscosities of probiotic yoghurts made by various isolated strains.**

X-axis- Yoghurt samples made by strains from 1 to 18 as indicated in the key

Key; 1-NN1 2-NN3 3-NN6 4-NN7 5-NN8 6-NN9 7-NN11 8-NN13 9-NN14 10-NN16 11-NN18 12-NN19 13-NN20 14-NN21 15-NN22 16-NN26 17-GR1 18-Control

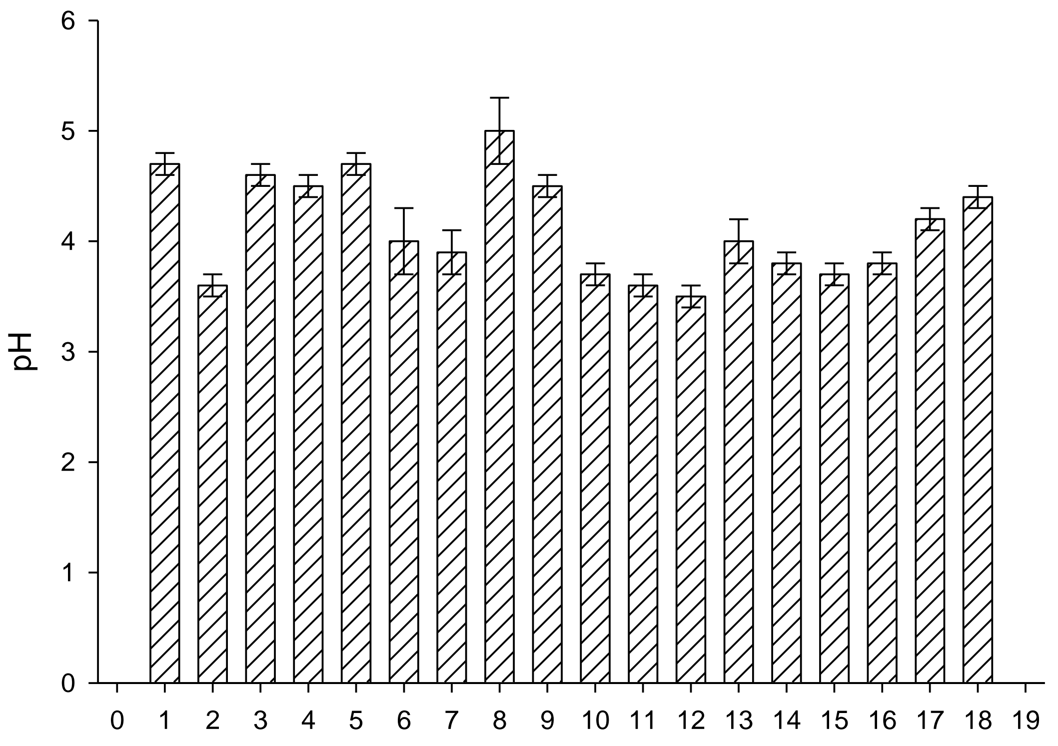
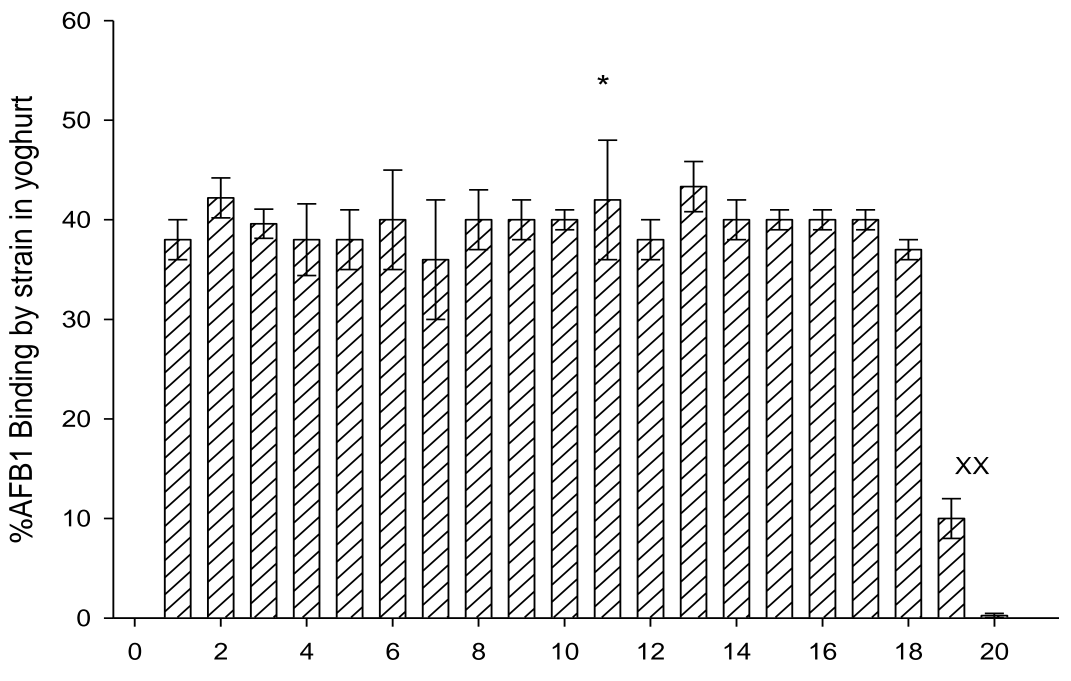



**Figure 4.8. pH of probiotic yoghurt made by various isolated strains.**

X-axis- yoghurt samples made by strains 1 to 18 as indicated in the key
Key; 1-NN1 2-NN3 3-NN6 4-NN7 5-NN8 6-NN9 7-NN11 8-NN13 9-NN14 10-NN16 11-NN18 12-NN19 13-NN20 14-NN21 15-NN22 16-NN26 17-GR1 18-Control (Isolates in figure 4.1)

The NN20 strain was the highest binder, 43.7±2.3 ppb, followed by NN3, 42.2±2.3 ppb. The least was NN7 at 37.2±7.8 ppb. The probiotic *Lactobacillus rhamnosus* GR-1 37.3 ±3.6ppb. There was no statistical difference between these results ($P>0.05$). A known non-binder *E. coli* GR12 bound 10.9±1.5ppb (Table 4.2, Figure 4.9)

Key




* Not significantly different among isolateed strains(appendix 24)

xx- significantly different (natural escherichia coli found in human gut) co
isolated strains (appendix 25)

X-axis- Strains 1 to 19 as indicated in the key

1-NN1 2-NN3 3-NN6 4-NN7 5-NN8 6-NN9 7-NN11 8-NN13 9-NN14 10-NN16 11-NN18 12-NN19 13-NN20 14-NN21 15-NN22 16-NN26 17-GR1 18-Control 19-Escherichea coli 20-Blank

*Not significantly different among isolated strains

xx- significantly different (natural *Escherichia coli* found in human gut) compared to isolated strains (appendix 10)

## 4.4 DISCUSSION

### 4.4.1 Isolation, Identification and Ability to Ferment Milk

Fermented food products in Kenya continue to draw significant interest in the dawn of reported increase of non-communicable diseases in SSA (Nduko *et al.,* 2017). This study identified strains of *Weissella cibaria* from *Kimere* as a dominant species. *Weissella* is a recently identified lactic acid bacteria whose classification is contested with some researchers identifying it as *Leuconostoc* and others as *Lactobacillus* genera (Abriouel *et al.,* 2015, Safika *et al.,* 2019). All the strains were able to survive the pH simulated conditions (Figure 4.4) but strain *Weissella cibaria* NN20 grew well (Figure 4.6). Ayeni *et al.,* (2011) reported that *Weissella* species survive gastrointestinal passage, providing another benefit to selecting NN20 for further study.

The isolates were identified by 16S rRNA region because 16S rRNA genes are ubiquitous. Bacterial ribosomes can't translate mRNA without their 16S rRNA component, so all bacteria have it. Because these genes are essential, they are also very highly conserved. That means it is possible to construct a tree of life linking together all known bacteria. This high conservation also makes it possible to construct universal primers that can amplify 16S rRNA genes from widely divergent bacteria.

In this study Helicase-dependent amplification (HDA) method of amplification was used as it utilizes a DNA helicase to generate single-stranded templates for primer hybridization and subsequent primer extension by a DNA polymerase. HDA does not require thermal cycling. In addition, it offers several advantages over other isothermal DNA amplification methods by having a simple reaction scheme and being a true isothermal reaction that can be performed at one

temperature for the entire process (Vincent *et al*., 2004). The dominant isolate identity was *Weissella cibaria.* Other isolates included *Lactobacillus fermentum* and *Weissella confusa*.

*Kimere* samples which provided the isolates were collected at optimum stage of fermentation that is 24 hours after wet milling. In spontaneous fermentation early stages are dominated by *Weissella* species while late stages are dominated by *Lactobacillus* species (Vuyst *et al*., 2016). It is therefore correct to say that the stage at which *Kimere* samples were collected contributed to the range of identified isolates. It is worthy to note the presence of *Weissella confusa* among the isolates. Even though this species has been mentioned in previous studies as a potential probiotic, it is worthy to mention that it has been found to be an opportunistic pathogen in spontaneous fermentations. Health problems associated with *Weissella confusa* include, sepsis and bacteraemia (Fair Fax *et al*., 2014; Kumar *et al*., 2011). In particular, *Weissella confusa* has been isolated from several clinical specimens in cases of polymicrobial infections (Lee *et al.,* 2011).

Selection of the best potential probiotic strains was based on how they bound to AFB1. The next criteria of selection was based on how they were able to ferment milk. All the strains were capable of fermenting milk. Even though there is no relationship between viscosity and pH during yoghurt formation, it is worthy to note that strain 13 (NN20) had better pH and viscosity of its yoghurt compared to control and known probiotics GR1 (strain 17).

#### 4.4.2 *In vitro* AFB1 binding

The study found reduced concentration of AFB1 when incubated with all the strains. This might reflect adsorption where toxins are physically attracted to the bacterial surface, or chemically

adsorbed by electrochemical bonding or by molecular sieves which traps toxins under pressure inside the bacteria in three ways (i) ion exchange reactions with peptidoglycan and teichoic acid, (ii) precipitation through nucleation reactions, and (iii) complexation with nitrogen and oxygen ligands (Monachese *et al*., 2012). The thicker the peptidoglycan and teichoic acid the better the binding (Monachese *et al*., 2012). This could explain why the negative control *E. coli* GR12 showed the lowest binding as Gram-negative bacteria have significantly less peptidoglycan and teichoic acid.

## 4.5 CONCLUSION

The study confirms *Weissella cibaria* NN20 as an AFB1 sequester/binder and that the said strain can be used to make yoghurt. Even though other strains were capable of binding AFB1, *Weissella cibaria* binding capacity was significantly higher than other isolates. The hypothesis that *Kimere* contains *Lactobacillus* and *Weissella* spp potentially able to reduce the risk of aflatoxicosis was confirmed. However, different strains have different capacities of binding.

# CHAPTER FIVE

# DETERMINATION OF THE CAPACITY OF *LACTOBACILLUS* AND *WEISSELLA* ISOLATES TO LOWER AFB1 METABOLITE (AFLATOXIN M1) IN URINE OF CHILDREN

## 5.1 Introduction

In both the developed and developing world, humans are continuously exposed to a plethora of environmental toxins, both in nature and as a result of anthropomorphic activity. Aflatoxin B1 is one of the most toxic substances known to humans, and can be found in our food, especially cereals which form over 70% of Kenyan staple diet. Limiting cereals as a solution to reduce aflatoxin uptake is not an option as it is one of the most plentiful and nutritious food sources in Kenya and is the primary source of dietary energy for many Kenyans.

### 5.1.2 Aflatoxicosis and Detoxification

Studies have shown that aflatoxicosis can be fatal as well as causative of the aetiology of hepatocellular carcinoma (HCC) (Figure13) (Sun *et al*., 1999). Further Sun *et al*., (1999) indicate that the risk of HCC is increased by 3.3 folds in detectable urinary AFM1 above 3.6ng/l and in people with chronic diseases such as hepatitis B virus (Figure 5.1) (Du *et al.,* 2017).

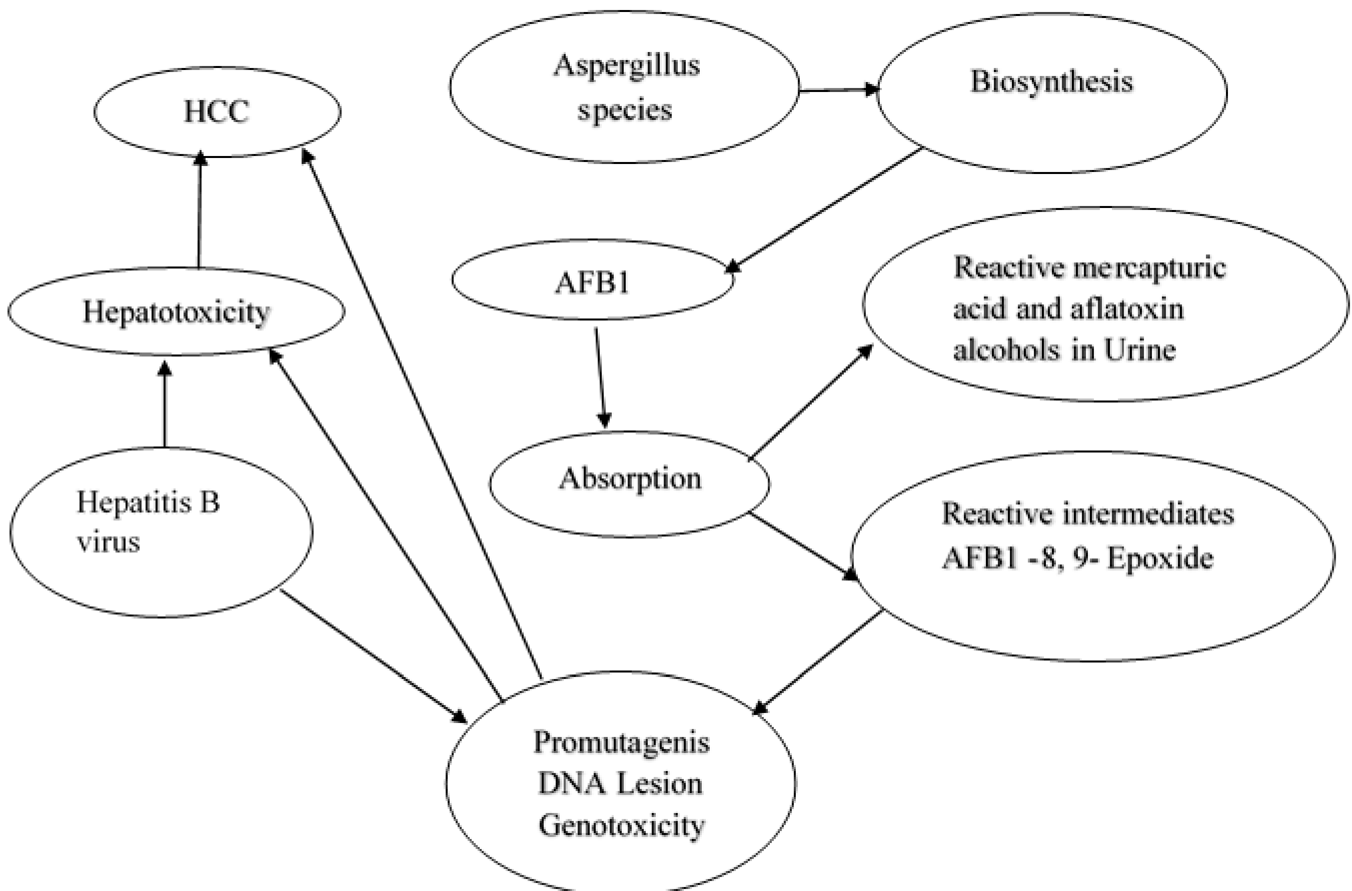



**Figure 5.1. Potential of AFB1 to cause HCC and relationship with chronic diseases as shown by various biomarkers**

(Bbosa *et al.,* 2013, Tuntiteerawit *et al,* 2019

AFB1 poisoning among Kenyan communities has been documented. The cases of poisoning (aflatoxicosis) is more prevalent in Eastern Kenya due to multifaceted reasons including climatic conditions and poor farming methods (Kenya Dairy 2010; Daniel *et al*. 2011).

No probiotic strain colonizes the gastrointestinal tract (Smith *et al.,* 2011) but their administration and passage through the gut could sequester the toxins and carry them out in the feaces (Dolan *et al.,* 2017). Such toxin binding mediated detoxification has been demonstrated in a murine model (Jansen *et al.,* 2006). In this current study *Weissella cibaria* NN20 was used to ferment milk. This strain was selected based on results adopted from previous part of this study.

## 5.2 MATERIALS AND METHODS

### 5.2.1 Ethical approval and location of study

To manage the *in vivo* trials using human subjects, ethics certificates were sought for and granted from Joint Ethics Certificates Committee of University of Nairobi and Kenyatta National Hospital Ethic Review Committee, certificate reference number P446/8/2013 (Appendix 11), the University of Western Ontario Ethics Review Board and registered in clinicalTrials.gov, Identifier NCT020416.

The field trial of *Weissella cibaria* NN20 was carried out at Ngenge primary school in Mbeere South in Embu County. Communities living within this site are cosmopolitan in nature. They include Kamba, Kikuyu, Mbeere, Embu, minority Tharaka a sub tribe of Meru, as well as pockets of other communities e.g. Luo who dwells along river Tana due to their adopted fishing activities for their livelihood. This mixed culture and society around Ngenge primary school, presented a good population where trial subjects were derived.

### 5.2.2 Probiotic Yoghurt Preparation at Site, Treatment Procedures and Quality Control

A powder was prepared containing *Weissella cibaria* NN20and *S. thermophilus* at $10^9$ colony forming units/ml per strain dried in skim milk. To achieve this, cells were cultivated in MRS agar for *Lactobacillus* or *Weissella* and M17 for *Streptococcus.* The cells were later inoculated in milk and fermentation achieved after 4 hours. Powder was prepared by lyophilizing the fermented milk in test tubes at minus 40 $^{o}$C for 12 hours. To prepare yoghurt, fresh milk, sourced from local Kenyan farmers, was boiled for 20 min and cooled to 25$^{o}$C then tested for viable pathogens and microorganisms using fast alcohol test. A mother culture was prepared by inoculating 1.5 gram of

the powdered bacteria into 3 liters of milk. Two 250ml cups of mother culture were transferred aseptically into 20 liters of pasteurized milk, held overnight and finally stirred after physically confirming consistency of the gelled product.

### 5.2.3 Sampling Eligibility and Recruitment

One hundred and twenty children from Ngenge primary school met the set criteria for inclusion, generated with the help of teachers and guardians after an authority was sought from local administrative leaders and area Education Officers. The sample size was calculated using Slovin's formula and subsequent screening done according to set criteria of inclusion and exclusion. Consenting, registration, enrolment and all individual study procedures for recruited subjects took place at Ngenge primary school. The criterion for individual inclusion was children of age 6 to 10 years. The age bracket is within the range of population who highly depend on maize as their staple food in addition they are highly vulnerable. A list of all the children either male or female and with equal chance of being included in the study was generated with the aid of teachers in the primary school. After passing the entry criteria, the children were further selected randomly by toss of a coin with a child allocated randomly head or tail. Whatever side that came on top was taken as a pass for the specific child and procedure was repeated until two balanced group of 20 children each was attained. Children with chronic disease, overly severe malnutrition, lactose intolerance and mental disability were excluded. For purposes of clear consenting to participate, the goal of the study was explained in local language to both the guardian and child. In total 21 boys and 19 girls participated in the study. The weight of each subject was taken to the nearest 0.1kg before and at the end of the treatment. The protocol is outlined in Figure 5.1.

After screening, children were further observed for two weeks to eliminate any chance of developed clinical observations outside criteria for inclusion. A Qualified Clinical officer helped in screening the 40 participants. After selection, participants were separated into 2 groups each with both males and females (n=20). One group was fed with yoghurt prepared by probiotic bacteria while the other was provided fresh milk for one week, followed by one-week break. The groups were similarly fed again at week-three. The reason for the one-week break was to monitor any development of adverse effects or compliance problems. In addition the study was designed to have double control. While placebo was meant for blank treatment the one week withdrawal would monitor the levels of AFM1 in urine during withdrawal period.

Participants were monitored throughout the trials for fitness and well-being every day by a Government registered Clinical officer. Thereafter they were monitored for 30 days after the completion of the trial.

Each participant received 200ml of either probiotic yoghurt or fresh milk in the morning in a calibrated cup with a label stating the participant's name (Table 5.1). Baseline urine samples were collected at the start of the trial, and thereafter every seven days. The urine samples were collected in the morning from the selected subject at the designated days. All containers were coded and placed in plastic ziplock plastic bags, and immediately placed in a -20C freezer at Ngenge primary school. A withdrawal period of seven days was observed after one week of treatment. Non-compliant was 25 subjects out of 40 participants. That is 25 subjects did not consistently follow the treatment routine to completion. While this would have affected the error terms statistically, it was taken care of by total quantification of Aflatoxins in every urine sample by use of LC-MS technique.

Eligible assessment of participant identified with help from Ngenge primary school teachers and frontline ministry of agriculture home economics extension officers n*=120

Not screened due to absenteeism n=11
Refused n=0
Migrated n=0
No baseline information n=0
Other n=0

Selected for baseline sampling (at start of treatment) and after 14

Randomized group 1 n=20

Randomized group 2 n=20

Treated with probiotic yoghurt for 7 days followed by 7 days withdrawal then 7 days further treatment

Treated with placebo (fresh milk) for 7 days followed by 7 days withdrawal then 7 days further

Migrated n=0

Non-compliant for daily treatment during the study n=25

Withdrawn at will n=0

Withdrawn after suspected lactose intolerance, n=1

Completed for each group and data for analysis
Group 1, n=7 Group 2, n= 7

**Figure 5.2. Study protocol for sampling, treatment allocation and completion of the study in vivo**

*n=sample size that is, number of children selected for screening

**Table 5.1. Routine treatment and samples collection plan**

| Day | Interventions | Sample collection |
|---|---|---|
| Day 1 | Start of treatment | 15ml of first morning urine (baseline samples) |
| Day 7 | End of treatment, seven days wash out period began | 15ml of first morning urine |
| Day 14 | Treatment resumed, seven days wash out period ended | 15ml of first morning urine |
| Day 21 | Treatments ended | 15ml of first morning urine |

The human pilot study was undertaken in children attending Ngenge Primary School, located 20 km south of Embu in Kenya. Almost half of the population in Embu live below the Kenyan poverty line. Embu town has modest infrastructure and piped clean water. This school is in Mbeere South, a semi-arid region with the majority of population depending on peasant farming. Maize forms the main source of energy and is complimented with vegetables and milk. Most (roughly 80%) of people around Ngenge primary school reside in a rural setting, higher than the Kenyan and sub-Saharan average (67.7%). Written informed consent was provided by the guardian, and individual written informed assent was provided by each child aged 6 years and above. The children had a basic understanding of the study purpose and study procedures. Fingerprints were used for guardians who were unable to sign. The consent and assent processes were documented through the use of consent and assent notes approved by the ethics review committees.

### 5.2.4 Enzyme Linked Immunosorbent Assay (ELISA)

Forty- 200g samples of maize were collected randomly from various homestead and analyzed for AFB1 along with urine samples from the subjects tested for AFM1. Every household provided a sample of maize from the collection that was being consumed by the family. In this part of Kenya, maize is purchased or harvested and stored in large consignments to be consumed for a stipulated period of time, in most cases two month. This can vary, for example with major drought or due to unavoidable reasons as is reported by Lewis *et al.,* (2004). The present study was undertaken during a bumper harvest of maize, thus the consignments tested for aflatoxin were from the same batch to those consumed for the 21 days of study. Before the sample collection, the consignment was first agitated to ensure homogeneity. The cobs were debrided and ground up the same day, then the liquid component was placed in sterile containers stored at -20$^{o}$C before thawing and processing.

An ELISA kit (Helica Biosystems, Santa Ana, California) was used, specifically for urine analysis, and an ELISA kit specifically for AFB1 (Helica Biosystems inc. cat log no 941BAFL01B1-96) in case of maize samples, following the manufacturer's instructions (Sabran *et al*., 2012). ELISA kits had aflatoxin-protein conjugate coated onto the microtiter plate. Samples or aflatoxin M1 standard (0.1-20ppb) were added to the micro wells followed by an aliquot of anti-aflatoxin antibody. The amount of antibody bound to the plate was detected by the addition of anti-rabbit IgG conjugated to alkaline phosphatase (ALP) followed by reaction with p-nitrophenyl phosphate to give coloured product. The resulting colour was then measured spectrophotometrically using microtiter plate reader (Neogen model) where the optical density of each micro well was read using a 450nm filter. The Limit of detection and upper limit of quantitation was 0.1-20ppb. Urine

samples from the subjects were tested for AFM1 using similar method to that described by Sulaiman *et al*., (2018). Using the Sulaiman method, the level of AFM1 was analyzed using an ELISA kit specifically designed for the determination of urinary AFM1. The debris and precipitate were removed through centrifugation at 3000× g for five minutes and supernatant was used for the determination of AFM1 according to manufacturer instructions. The protocol's washing step involved the use of an automated microplate washer. A microplate reader was used to measure absorbance at a wavelength of 450 nm. As for method validation, urine samples were spiked with 1.5 ng/mL AFM1 standard and the samples processed as per protocol.

### 5.2.5 Liquid Chromatography-Mass Spectrometry (LC-MS)

In total 114 urine samples were sent under dry ice from Kenya to Canada, stored at -80°C then thawed and prepared according to the methods of Warth *et al*., (2013). The 114 urine samples were from children that were available during urine sample collection. All the urine samples were analyzed, and results of detections recorded. Briefly, 200 ul urine was added to 800 ul of 1:9 Acetonitrile: $H_{2}0$. Samples were then centrifuged to pellet proteins, and 500 ul supernatant was transferred to LC-MS vials. Five microlitres of supernatant was injected into an Agilent 1290 Infinity HPLC coupled to a Q-Exactive Orbitrap mass spectrometer (Thermo Fisher Scientific) with a HESI source in positive ionization mode. Representative ions for each aflatoxin and their specific fragments were monitored as follows; AFB1 (313.07 -> 241.05 m/z), AFB2 (315.09 -> 259.06 m/z), AFG1 (329.07 -> 243.06 m/z), AFM1 (329.07 -> 273.07 m/z), AFG2 (331.08 -> 245.08 m/z), AFB1-N7-guanine (480.11 m/z). Aflatoxins were quantified in Xcalibur by integrating the area under the curve for fragments ions mentioned above, with the exception of

AFB1-N7-guanine, for which the parent M+H ion was used. Authentic standards were run with samples, with the exception of AFB1-N7-guanine, for which there was no standard available.

### 5.2.6 Data Management and Analysis Procedure

Unless stated, all analysis were conducted in triplicates experiments. All personal data was determined and stored at the Technical University of Kenya. Samples were coded and no names appeared on the labels as described in the letter of consent (Appendix 12). Relevant metadata pertinent to proper analysis e.g. sex, age, antibiotic use was digitalized and stored in a protected computer and a backup external memory. Means, mode median and range where applicable were calculated using Microsoft excel version. Standard deviation, Least Significance Difference (LSD) were calculated and analysis of variance conducted to ascertain differences of means using IBM software package of social sciences SPSS statistics version 21

### 5.2.7 Quality procedure

The feeding of subjects was done in collaboration with the Ministry of Agriculture (MoA) Home Economics Officers at Embu Agriculture Training Center (ATC)-an International Standards of Organization (ISO) accredited center- to ascertain quality assurance of the probiotic yoghurt. Feeding was done in the morning to avoid yoghurt contamination. Rapid alcohol test was conducted to confirm the quality of raw milk. Total counts was conducted in prepared yoghurt to ascertain the quality of colony forming unit per gram.

## 5.3 RESULTS

### 5.3.1 Reduction of AFB1 among Children in Eastern Kenya following consumption of Probiotic Yoghurt

The results presented here are for fifty-six samples from 14 children of which the samples were analyzed by ELISA, and by LC-MS. Due to limited resources not all samples from participants could be tested. There was no difference in consumption of maize by the yoghurt and milk groups P>0.05 which constituted 9.38±3.8 units over 21 days per child. The levels of aflatoxins in the milk was not assessed, but the same milk was used to produce the yoghurt, so it was assumed if any toxin was present it would equally affect both groups of children. While some regions in Kenya have cattle feeding on cattle feed made from maize and maize products that may be contaminated by AFB1, the milk used in this study was from cows that are grazed in jungle thus they generally feed on grass.

Of the 14 children who fully complied with the protocol, 7 were receiving probiotic yoghurt and 7 milk, and the mean age was 8 years 7 months. The mean urinary levels of AFM1 were higher in the probiotic group (6.3ppb) at baseline, but not statistically different (P> 0.05). Mean child weight was also similar for both groups (22 Kg) (P>0.05). Of those who did not comply fully with the study protocol, there was no evidence that adverse effects were the cause, and only one child withdrew due to lactose intolerance, although the guardian had failed to inform the study of this issue upon enrolment.

Out of the urine samples tested, some urine samples in each group were negative at the beginning of the study. Samples from these subjects were included in subsequence analysis because consumption of presumed contaminated staple food was not stopped during the study. The AFM1 urinary concentrations fell in the probiotic group by 51% over the study duration, based upon ELISA ($p<0.01$), despite cessation of yoghurt intake between days 7 and 14 (Figure 5.3). On the other hand, the AFM1 concentrations increased by 39% in the control milk group, with a steady rise of 1.2 ppb each week. This is illustrated by Figure 5.4. The effect was significantly different in favor of the probiotic treatment over milk ($p<0.01$) (Figure 5.3 and Figure 5.4). The large error bars indicate large standard deviation of means that could have been due to several inconsistencies of children present during feeding and sample collection.

To further confirm aflatoxin was present, LC-MS was used on 114 samples. The number of samples with aflatoxin detected pre-treatment was similar between groups (25% in probiotic vs 22% in controls), verifying exposure. The method was able to detect 5 ng/ml (ppb) in spiked samples. The trace amounts detected in samples were below quantifiable limit, possibly due to degradation with transportation. This may explain why LC-MS did not detect AFB1. Thus, the results are included only to confirm detection of the toxins not to compare with the ELISA data per se. For this reason only, percent detection for each aflatoxin was compared between groups. The percentage of samples post-treatment with aflatoxin was 21% for controls and 9% for those consuming the probiotic yoghurt. In both ELISA and LC-MS tests, high standard deviation was noted.

An *in vitro* binding assay showed that the strains *Weissella cibaria* NN20 and *Lactobacillus rhamnosus* GR-1 bound to aflatoxins (41.7±2.3% and 37.3±3.6% respectively) while the *E. coli* GR12 control essentially did not (3.9±1.5%).

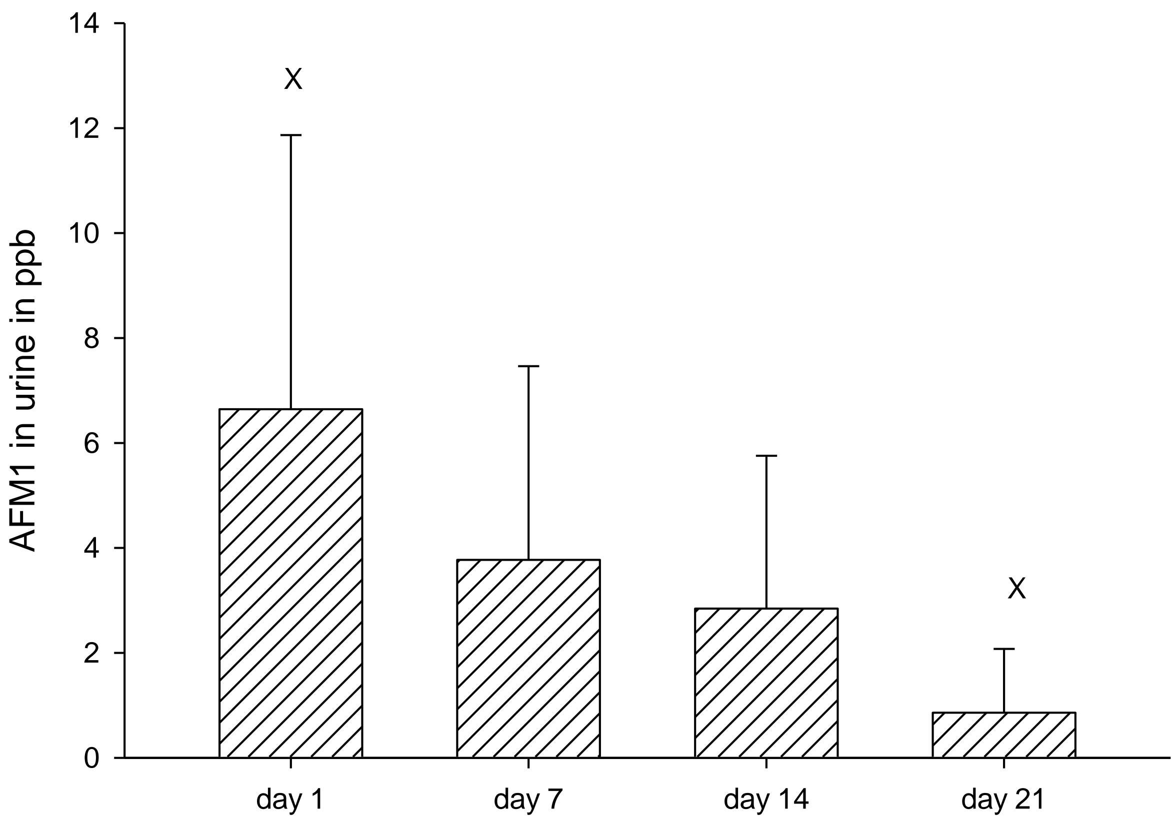



**Figure 5.3. AFM1 levels in Urine samples of children fed with probiotic yoghurt**

X-axis- Number of days after when the samples were collected

X- Significantly different at 95% confidence interval (appendix 13 and 14)

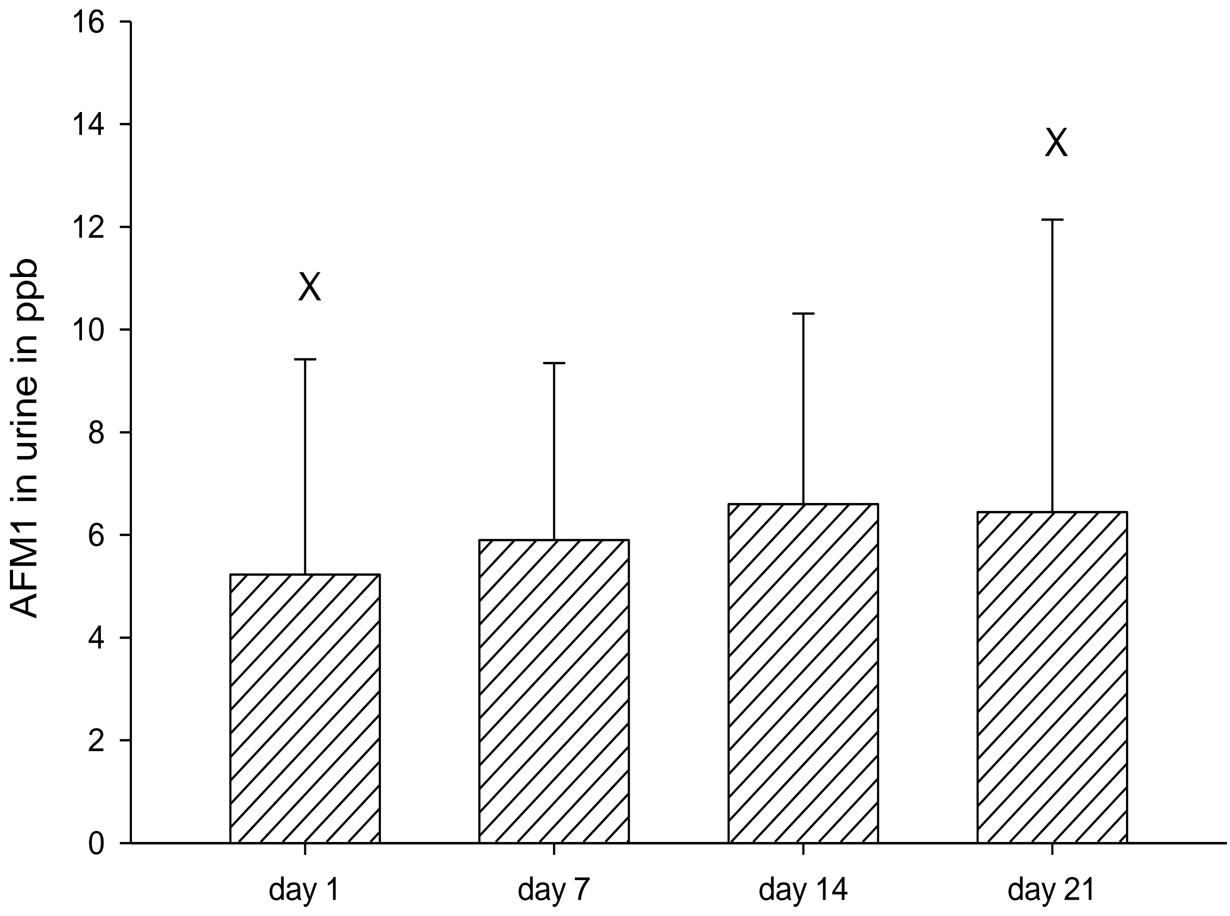



**Figure 5.4. AFM1 in urine of control group; children fed with fresh milk.**

X-axis- Number of days after when the samples were collected

X- Not significantly different at 95% confidence interval (appendix 15)

**Table 5.2. Mean percentages of urine samples with detectable aflatoxins as determined by LC-MS**

| | AFM1 | AFB1 | AFB2 | AFG1 | AFG2 | N7-Guanine | Any AFs |
|---|---|---|---|---|---|---|---|
| All samples | 1.25 | 0 | 8.75 | 1.25 | 0 | 6.25 | 16.25 |
| Probiotics | 0 | 0 | 6.06 | 0 | 0 | 3.03 | 9.09 |
| Control | 2.13 | 0 | 10.64 | 2.13 | 0 | 8.51 | 21.28 |

## 5.4 DISCUSSION

### 5.4.1 Reduction of AFM1 by *Weisella cibaria* in Urine

This pilot study shows that despite efforts to control the consumption of maize contaminated with AFB1 in Kenya, children continue to get exposed to this dangerous compound.

The intervention study shows a trend in favor of probiotic yoghurt to reduce intake of AFB1, assessed using ELISA and LC-MS, even though consumption was only for 14 days over a 21-day period. It is feasible that the probiotic lactic acid bacteria were retained in the gut in part or during all of the 7-day 'washout' period, thus maintaining the effects. The non-compliant subjects were 25. While this figure is high, it was noted that non-compliance was in consistency in treatments. In early stages on this study it was noted that the strains would survive in gastric high acidic condition. Thus, inconsistency of treatment would make reasonable difference in outcome. This was noted after analyzing total aflatoxins for the 100% compliant subjects as compared to the non-compliant (table 5.2).

In this study, AFM1 was used as the bio maker for AFB1. In most cases, biomarker is either the contaminant itself or a metabolite thereof (Mohd-Redzwan *et al.,* 2013). AFB1 is oxidized to AFM1 which later is excreted in the urine (Marchese et al., 2018). The target samples other than urine and blood include saliva, breast milk, feces and adipose tissue. Since there is no universal applicability in assessing the exposure to food contamination (McMullin *et al.,* 2015), the study looked at the metabolic behavior of AFB1 and selected urine body fluid for assessment. Several molecular biomarkers have been developed and they include, AFB1 metabolites and AFB1 macromolecular adducts (Moreno 2015). This study was particularly interested with AFB1 –n7 guanine and AFM1 both available in urine as AFB1 metabolites. In the data pertaining to ELISA

techniques, it was observed that AFM1 concentration in urine samples reduces considerably for the probiotic yoghurt treated subjects as compared to the fresh milk. The detection level using the otherwise more sensitive LC-MS was lower than the less sensitive ELISA technique. This can be explained by the behavior of the selected body fluid for analysis. Urinary AFB1 metabolites are less stable over a short period of time (Du *et al.,* 2017). However, the study chose not to use blood for the fact that AFB1- adduct bio maker in serum which has longer shelf life for it indicates longer term intake of AFB1 (Leroy *et al.,* 2019). Due to the short time, 21 days, the AFM1 was the most appropriate for this study. Moreover, the LC-LCMs confirmed that there was reduction of the both biomarkers AFM1 and AFB1-G7 guanine in the urine samples for the *Weissella cibaria* NN20 probiotic treated subjects.

The high standard deviations were due to the fact that some samples did not have detectable Aflatoxins concentrations. Although the study was not designed to determine mechanisms of action, the fact that the *Lactobacillus* GR-1 and *Weissella* NN20 strains bind well to aflatoxins suggests a mechanism to reduce toxin uptake in the host. Bacteria bind by (i) ion exchange reactions with peptidoglycan and teichoic acid, (ii) precipitation through nucleation reactions, and (iii) complexation with nitrogen and oxygen ligands (Monachese et al., 2012). The thicker the peptidoglycan and teichoic acid the better the binding (Monachese et al., 2012).

# CHAPTER SIX
# GENERAL DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

## 6.1 General Discussion

Maize is an important staple food in Kenya. However compared to other cereals it is more susceptible to molds which produce mycotoxins particularly aflatoxins. From the literature review, it is evident that maize is contaminated at various levels of growth during storage and handling. This makes it difficult to control the mold manifestation. As for nutritional composition maize is similar to other cereals consumed by the communities within Eastern Kenya. This study set out to isolate the *Lactobacillus* and *Weissella* from *Kimere* after confirming that indeed people were consuming contaminated maize further isolation of *Lactobacillus* and *Weissella* from *Kimere* was compounded by the fact that literature review indicated that while consumption of *Kimere* had declined aflatoxicosis had increased. The *Lactobacillus* and *Weissella* isolates were to be used in developing a probiotic yoghurt for aflatoxin control. While the first objective of this study basically to confirmed the levels of aflatoxins in consumed maize and maize flour, it further confirmed that there were differences in concentrations between regions and storage facilities. The study did not only confirm that there were high levels of aflatoxins contaminations but also that various levels of interventions were not sufficient. This is confirmed by the aflatoxin concentrations from various maize grains and flour samples as well as in baseline urine samples from children.

In general, the overall study was anchored in the fact that *Kimere* has been consumed for a long time among the eastern part of Kenyan communities. Aflatoxicosis was less prevalent when *Kimere* was consumed on daily basis. The study also shows that adopting maize consumption in regards to *Kimere* increased the cases of aflatoxicosis. . Indeed, *Kimere* consumption has reduced

almost to extinction. Preparation of *Kimere* is time consuming and involves a lot of body contact which can introduce contaminants. In addition, fermentation is spontaneous and thus uncontrollable. As evidenced by identification of *Weissella confusa* which has been reported to have some health disadvantages, spontaneous fermentation is not always ideal health wise. This study found that *Kimere* is rich in *Weissella* species. The probiotics tests that followed isolation confirmed that *Weissella species* were capable of developing a probiotic yoghurt and sequester aflatoxin B1 both *in vivo* and *in vitro.* Furthermore the study found that, probiotics characteristics including ability to grow and survive in high acidic gastric condition and the capacity of aflatoxins binding by strains is rather strain specific. At some point of the study a candidate strain was selected and tested for these characteristic against control (figure 4.6). During the test for high acidic gastric conditions where two environment thus, pH2 and pH3 with pH7 as neutral control were used, the study noted that at pH3 all the strains germinated to uncountable levels. This could be because the strains are acidophilic and none was acidophobic. Lowering the pH to 2 pushed the limit below minimum making it possible to notice the difference in acidic environment tolerance (figure 4.4). This study did not miss to notice that *Weissella* species is closely related to *Lactobacillus.* This is clearly shown by the fact that both species were isolated by use of same selective media and that they had similar characteristic during isolation process.

This knowledge provided a rationale to assess which in *Kimere* might be able to reduce the risk of aflatoxicosis. Fermented *Kimere* is known to contain *Lactobacillus* and *Weissella* strains in varying levels of concentrations.

The present study confirmed that isolated strains lowered the AFB1 *in vitro*, and one *Weissella cibaria* along with probiotic GR-1, survives the stomach's acidic condition, presumably allowing them to interact with the AFB1 that is ingested. The pilot human study that was used to confirm *in*

*vivo* performance of *Lactobacillus* and *Weissella,* indicated that the cells in the developed probiotic yoghurt did not lose their capacity to sequester aflatoxin B1 after yoghurt development.

### 6.2 General Conclusion

All four objectives were met. High levels of aflatoxins concentration are present in Kenyan maize and flour. However, the results confirms that eastern region is most vulnerable. While maize and maize flours were the target due to their consumption as staple food, it is worthy to mention peanut and sorghum that also can be source of aflatoxins. The fact that peanut and sorghum are also grown and consumed in those region, the can confidently predict that consumers also get some aflatoxins from these food crops. The results confirms that *Weissella cibaria* NN20 isolate from *Kimere* can bind aflatoxins both *in vivo* and in *vitro*; Further, *Lactobacillus* or *Weissella* isolates that were used proved that they can be used to produce probiotic yoghurt that can be used to control aflatoxins. The best overall results for yoghurt and aflatoxins binding was by *Weissella cibaria* NN20. Consumption of this yoghurt reduced aflatoxin levels in a pilot study of children inadvertently exposed to aflatoxins.

### 6.3 Recommendations

From the results the following recommendations are made.

1. That Maize grains in Kenya have aflatoxins concentrations above national required limits of 10ppb. In some cases even maize grain have alarming level of aflatoxins as recorded from Eastern Kenya maize samples in this study. It is from these findings this study recommends measures including use of probiotic yoghurt with *Weissella ciabria* NN20 to control the effect of the aflatoxins.

2. *Kimere* was found to have *Lactobacillus* and *Weissella* which could bind with Aflatoxins both *in vitro* and *in vivo.* This study recommend adoption of the Microorganism across Kenya. This will help consumers from the regions where *Kimere* is never known.
3. *Lactobacillus* and *Weissella* were found to ferment milk with characteristics same as yoghurt. The study therefore recommends that adoption of these microorganisms for production of yoghurt which will have an added health benefits.
4. *Weissella cibaria* NN20 could be added to yoghurt in Kenya to reduce aflatoxin uptake in school food programs. The binding capacity of *Lactobacillus* and *Weissella* to aflatoxins varies between strains.
5. Leong *et al*., (2013) noted that biomarkers in urine have a short shelf life. It is recommended that laboratories across Kenyan be supported with appropriate equipment to rapidly test urine from communities as a means of monitoring exposure to these lethal toxins.

## REFERENCES


Abriouel, H., Lerma, L. L., Casado Muñoz, M., Montoro, B. P., Kabisch, J., Pichner, R., & Benomar, N. (2015). The controversial nature of the *Weissella* genus: technological and functional aspects versus whole genome analysis-based pathogenic potential for their application in food and health. *Front. Microbiol.* 6, 1197.

Alshannaq, A., & Yu, J. H. (2017). Occurrence, toxicity, and analysis of major mycotoxins in food. *Int. J. Environ. Res. Public Health*, 14(6), 632.

Amils, R. (2011). Gram-Positive bacteria. *In Encyclopedia of Astrobiolgy*. (pp685). Berlin. Springer Heidelberg.

Andrade, P. D. & Caldas, E. D (2015). Aflatoxins in cereals: worldwide occurrence and dietary risk assessment. *World Myco J.* 8 (4):415 – 431.

Ashraf, A. & Shah, N. P. (2011). Selective and differential enumerations of *Lactobacillus delbrueckii* subsp. *bulgaricus, Streptococcus thermophilus, Lactobacillus acidophilus, Lactobacillus casei* and *Bifidobacterium* spp. in yoghurt — A review. *Inter. J. Food Microbiol*. 149(3):194-208.

Assamoi, A. A., Krabi, E. R., Ehon, A. F., Nguessan, G. A., Niamke, L. S. & Thonart, P. (2016). Isolation and screening of *Weissella* strains for their potential use as starter during attiéké production. *BASE* ISBN 1370-6233 1780-4507

Ayeni, F. A., Sanchez, B., Adeniyi, A. B., Reyes-gavilian, D. G. & Margolles, A., Ruas Madiedo, P. (2011). Evaluation of the functional potential of *Weissella* and *Lactobacillus*

isolates obtained from Nigerian traditional fermented foods and cow's intestine. *Inter. J. Food Microbiol*. 147(2):97-104.

Baere, S. D., Mil, T. D., Antonissen, G., Devreese, M., & Croubels, S. (2018). *In vitro* model to assess the adsorption of oral veterinary drugs to mycotoxin binders in a feed- and aflatoxin B1-containing buffered matrix. *Food Add. Contr. Part A.* 35(9)1728-1738.

Baraca A. (2019) Mycotoxins and human disease. In: Presterl E. (eds) Clinically Relevant Mycoses. *Springer, Cham* 213-225.

Bbosa S. G., Kitya, D., Lubega A., Ogwal, J. Anokbonggo W. W. & Kyegobe B. D. (2013a). Review of the biological and health effects of aflatoxins on body organs and body systems. Recent Advances and Future Prospects. Open access pear reviewed chapter. *Mehdi Razzaghi-Abyaneh, IntechOpen,* DOI: 10.5772/51201. Available from: https://www.intechopen.com/books/aflatoxins-recent-advances-and-future-prospects/review-of-the-biological-and-health-effects-of-aflatoxins-on-body-organs-and-body-systems

Bbosa, G. S., Kitya, D., Lubega, A., Ogwal-Okeng, J., Anokbonggo, W. W. & Kyegombe, D. B. (2013b). Review of the biological and health effects of aflatoxins on body organs and body systems. Open access pear reviewed chapter. *Mehdi Razzaghi-Abyaneh, IntechOpen,* DOI: 10.5772/51201. Available from: https://www.intechopen.com/books/aflatoxins-recent-advances-and-future-prospects/review-of-the-biological-and-health-effects-of-aflatoxins-on-body-organs-and-body-systems

Behnsen, J., Deriu, E., Sassone-Corsi, M., & Raffatellu, M. (2013). Probiotics: properties, examples, and specific applications. *Cold Spring Harb. Perspect. Med.1;* 3(3):a010074.

Bennett, J. W., & Klich, M. (2003). Mycotoxins. *Clin. Microbiol. Rev.* 16(3), 497–516.

Bezerra da Rocha, M. E., Freire, F., Maia, F. E. F., Guedes, Maria, I. F., & Rondina, D. (2014) Mycotoxins and their effects on human and animal health. *Food Cont.* 36(1):159-165.

Bisanz, J. E., Enos, K. M., Mwanga, J. R. (2014). Randomized open label pilot study of influence of probiotics and the gut microbiome on toxic metal levels in Tanzania pregnant women and school children. *mBio* 5(5) e01580-14 doi:101128 /mBio 01580-4

Boncompagni, E., Orozco-Arroyo, G., Cominelli, E., Gangashetty, P. I., Grando, S., Kwaku Zu, T. T., Sparvoli, F. (2018). Anti-nutritional factors in pearl millet grains: Phytate and goitrogens content variability and molecular characterization of genes involved in their pathways. *PloS One,* 13(6), e0198394.

Clotault, J., Thuillet, A. C., Buiron, M., De Mita, S., Couderc, M., Haussmann, B., Mariac, C., & Vigouroux, Y. (2012). Evolutionary history of pearl millet (*Pennisetum glaucum* [L.] R. Br.) and selection on flowering genes since its domestication. *Mol. Biol. Evol.* 29(4):1199-212.

Daniel, J. H., Lewis, L.W., Redwood, Y., Kieszak, S., Breiman, R. F., Flander, W. D., McGeehin, M. A. (2011). Comprehensive assessment of maize aflatoxin levels in Eastern Kenya, 2005-2007. *Environ. Health Perspect.* 119:1794-1999.

Darwish, W. S., Ikenaka, Y., Nakayama, S. M., & Ishizuka, M. (2014). An overview on mycotoxin contamination of foods in Africa. *J. Vet. Med. Sci.* 76(6):789–797.

De Baere, S., De Mil, T., Antonissen, G., Devreese, M., & Croubels, S. (2018). *In vitro* model to assess the adsorption of oral veterinary drugs to mycotoxin binders in a feed- and

aflatoxin B1-containing buffered matrix. *Food Add. Con. Part A Chem. Anal. Cont. Exp. Risk Assess.* 35(9):1728-1738.

Diaz, D. E., Hagler Jr, W. M., Blackwelder, J. T., Eve, J. A., Hopkins, B. A., Anderson, K. L., Jones, F. T., & Whitlow, L. W. (2004). Aflatoxin binders II: reduction of aflatoxin M1 in milk by sequestering agents of cows consuming aflatoxin in feed. *Mycopathologia,* 157(2):233-241.

Dolan, K. E., Pizano, J. M., Gossard, C. M., Williamson, C. B., Burns, C. M., Gasta, M. G., & Lipski, E. A. (2017). Probiotics and disease: a comprehensive summary-Part 6, Skin Health. *Integr. Med. (Encinitas).* 16(4), 32–41.

Drott, M. T., Debenport, T., Higgins, S. A., Buckley, D. H., & Milgroom, M. G. (2019). Fitness cost of aflatoxin production in *Aspergillus flavus* when competing with soil microbes could maintain balancing selection. *mBio* 10:e02782-18.

Du, X., Schrunk, D. E., Shao, D., Immerman, P. M., Wang, C., Ensley, S. M. & Rumeiha, K. W. (2017). Intra-laboratory development and evaluation of a quantitative method for measurement of aflatoxins B1, M1 and Q1 in animal urine by high performance liquid chromatography with fluorescence detection. *J. Anal.Toxicol.* 41(8):698-707.

Eduardo, A., Mary, O., Henry, N., Helen, A., George, L., Stephnie, K., Jack, N., Lorraine, B., Abdiker, M. D., Ambrose, M., Kevin, D., Carol, R. & the Kenyan aflatoxicosis investigation group. (2005). Case – control study of an acute aflatoxoscosis outbreak, Kenya, 2004. *J. Environ. Health Persp.* 113(12):1779-1783.

Ekpa, O., Natalia, P., Gideon, K., Vincenzo, F., Anita, R. L., (2019) Sub-Saharan African Maize-Based Foods - Processing Practices, Challenges and Opportunities. *Food Reviews International.* 35(7):609-639. DOI: 10.1080/87559129.2019.1588290

Ekpa, O., Natalia P. R., Gideon, K., Vincenzo F., Anita R. L. (2018). Sub-Saharan African maize-based foods: Technological perspectives to increase the food and nutrition security impacts of maize breeding programmes. *Global Food Security*. 17:48-56 https://doi.org/10.1016/j.gfs.2018.03.007

Fairfax M. R., Lephart, P.R. & Salimnia, H. (2014). *Weissella confusa*: problems with identification of an opportunistic pathogen that has been found in fermented foods and proposed as a probiotic. *Front. Microbiol*. Volume pages

FAO/Government of Kenya. 2018. Kenya Food Composition Tables. Nairobi, 254 pp. http://www.fao.org/3/I9120EN/i9120en.pdf

Franz, C. M., Huch, M., Mathara, J. M., Abriouel, H., Benomar, N., Reid G., Galvez, A., & Holzapfel, W. H. (2014). African fermented foods and probiotics. *Int. J. Food Microbiol. 3*(190):84-96.

Fusco, V., Quero, G. M., Cho, G. S., Kabisch, J., Meske, D., Neve, H., names Franz, C. M. (2015). The genus *Weissella*: taxonomy, ecology and biotechnological potential. *Front. Microbiol.* 6, 155.

Gallab A. (2013). *In vitro* test systems and their limitations. *EXCLI J.* 12:1024–1026.

Gao, L., Xu, T., Huang, G., Jiang, S., Gu, Y., & Chen, F. (2018). Oral microbiomes: more and more importance in oral cavity and whole body. *Protein & cell, 9*(5), 488–500. doi:10.1007/s13238-018-0548-1

Gonçalves, A., Gkrillas, A., Dorne, J. L., Dall'Asta, C., Palumbo, R., Lima, N., Battilani, P., Venâncio, A., & Giorni. P. (2019). Pre- and postharvest strategies to minimize mycotoxin contamination in the rice food chain. *Comp. Revs. Food Sci. Safety,* 18(2):441-454.

Goufo, P., & Trindade, H. (2014). Rice antioxidants: phenolic acids, flavonoids, anthocyanins, proanthocyanidins, tocopherols, tocotrienols, γ-oryzanol, and phytic acid. *Food Sci Nutr.* 2(2):75–104.

Guchi, E. (2015). Aflatoxin contamination in groundnut (*Arachis hypogaeam*L.) caused by *Aspergillus* species in Ethiopia. *J. Appl. Env. Microbiol. 3*(1): 11-19.

Gupta, R. K., Gangoliya, S. S., & Singh, N. K. (2013). Reduction of phytic acid and enhancement of bioavailable micronutrients in food grains. *J. Food Sci Technol.* 52(2), 676–684.

Hamid, A. S., Tesfamariam, I. G., Zhang, Y., & Zhang, Z. G. (2013). Aflatoxin B1-induced hepatocellular carcinoma in developing countries: Geographical distribution, mechanism of action and prevention. *Oncol. Lett.* 5(4), 1087–1092.

Hoffmann, V., Mutiga, S., Harvey, J., Nelson, R., Milgroom, M. (2013). Aflatoxin contamination of maize in Kenya: observability and mitigation behavior: annual meeting. *Agri. Appl. Econ. Ass.* August 4-6, 2013, Washington, D.C. 155024.

Huang, C. H., Li, S. W., Huang, L., & Watanabe, K. (2018). Identification and classification for the *Lactobacillus casei* group. *Front. Microbiol.* 9, 1974.

Ifesan, B., & Kolawole, A. O. (2018). Physicochemical properties of malted finger millet (*Eleusine coracana*) and pearl millet (*Pennisetum glaucum*). *Food Sci. Nutr.* 7(2), 476–482.

Inglin, R., Stevens, M., Meile, L., Lacroix, C., & Meile, L. (2015). High-throughput screening assays for antibacterial and antifungal activities of *Lactobacillus* species. *J. Microbiol. Methods,* 114: 15-30

International Agency for Research on Cancer, New report (2016). press release Number 242. https://www.iarc.fr/wp-content/uploads/2018/07/pr242_E.pdf

International Food Policy Research Institute, (2011). New Study Documents Spread of Aflatoxins in Kenya-Scientists Discuss Findings with Policymakers at International Workshop. *Press Release* JAN 13, 2011. https://www.ifpri.org/news-release/new-study-documents-spread-aflatoxins-Kenya

Isilay, L., Isil, V. (2017). The Control of Aflatoxin Contamination at Harvest, Drying, Pre-Storage and Storage Periods in Peanut: The New Approach, Aflatoxin - Control, Analysis, Detection and Health Risks, *Lukman Bola Abdulra'uf, IntechOpen,* DOI: 10.5772/intechopen.68675. Available from: https://www.intechopen.com/chapters/55153

Isleten, M., Karagul-Yuceer, Y. (2006). Effects of dried dairy ingredients on physical and sensory properties of nonfat yogurt. *J Dairy Sci.* 89(8):2865-72. doi: 10.3168/jds.S0022-0302(06)72559-0.

Janda, J. M. & Abott, L. S. (2007). *Mini review:* 16S rRNA Gene sequencing for bacterial identification in the diagnostic laboratory: pluses, perils, and pitfalls. *J. Clin. Microbiol*. *45*(9):2761-2764.

Jansen van Rensburg, C., Van Rensburg, C. E. J., Van Ryssen, J. B. J., Casey, N. H. & Rottinghaus, G. E. (2006). *In vitro* and *in vivo* assessment of humic acid as an aflatoxin binder in broiler chickens. *Poultry Sci.* 85(9):1576–1583.

Jimenez-Garcia, N., Garcia-Mier, L., Garcia-Trejo, J. F., Ramirez-Gomez, X. S., Guevara-Gonzalez R. G. & Feregrino-Perez, A. A. (2018). Fusarium mycotoxins and metabolites that modulate their production, Fusarium Plant Diseases, Pathogen Diversity, Genetic Diversity, Resistance and Molecular Markers, Tulin Askun, IntechOpen, DOI: 10.5772/intechopen.72874. Available from: https://www.intechopen.com/books/fusarium-plant-diseases-pathogen-diversity-genetic-diversity-resistance-and-molecular-markers/fusarium-mycotoxins-and-metabolites-that-modulate-their-production

Kalinina, S. A., Jagels, A., Cramer, B., Geisen, R., & Humpf, H. U. (2017). Influence of environmental factors on the production of penitrems A-F by *Penicillium crustosum*. *Toxins,* 9(7), 210.

Kamboj, K., Vasquez, A., & Balada-Llasat, J. M. (2015). Identification and significance of *Weissella* species infections. *Front. Microbiol*. 6:1204-1224.

Kang'ethe, E. K., Gatwiri, M., Sirma, A. J., Ouko, E. O., Mburugu-Musoti, C. K., Kitala, P. M., Nduhiu, G. J., Nderitu, J. G., Mungatu, J. K., Hietaniemi, V., Joutsjoki, V., Korhonen, H.

J. (2017). Exposure of Kenyan population to aflatoxins in foods with special reference to Nandi and Makueni counties. *Food Qual. Safety*, 1(2):131–137.

Kang'ethe, E. K., & Lang'a, K. A. (2009). Aflatoxin B1 and M1 contamination of animal feeds and milk from urban centers in Kenya. *Afr. Health Sci.* 9(4), 218–226.

Karlovsky, P., Suman, M., Berthiller, (2016). Impact of food processing and detoxification treatments on mycotoxin contamination. *Mycotoxin Res*. 32:179–205 https://doi.org/10.1007/s12550-016-0257-7

Kowalska, A., Walkiewicz, K., Kozieł, P., & Muc-Wierzgoń. M. (2017). Aflatoxins: characteristics and impact on human health. *Postepy Hig. Med. Dosw. (Online).* 5(71):315-327.

Kumar, A., Deepthi, A., Sudhindran, S., Anu, K., Kavitha, D., Sofwah, K. & Rosamma P. (2011). *Weissella confusa*: A rare cause of vancomycin resistant Gram-positive bacteraemia. *J. Med. Microbiol*. 60:1539-41.

Kumar, P., Mahato D. K., Kamle, M., Mohanta, T. K., Kang S.G. (2017). Aflatoxins: A Global Concern for Food Safety, Human Health and Their Management. Front Microbiol. 2017;7:2170. Published 2017 Jan 17. doi:10.3389/fmicb.2016.02170

Kumar, P., Mahato, D. K., Kamle, M., Mohanta, T. K., & Kang, S. G. (2017). Aflatoxins: a global concern for food safety, human health and their management. *Front. Microbiol*. 7:2170.

Kumar, V., Basu, M. S., and Rajendran, T. P. (2008). Mycotoxin research and microflora in some commercially important agricultural commodities. *Crop Prot*. 27, 891–905.

Kurt, A., Rosentrater, A. D., & Evers, (2018). Chapter 14 - Wet milling: Separating starch, gluten (protein) and fibre, Editor(s): Kurt A. Rosentrater, A.D. Evers, In Woodhead Publishing Series in Food Science. *Tech & Nutri. Kent's Tech. Cereals (Fifth Edition),* 839-860.

Lakkireddy, K., Kondapalli, K., & Sambasiva, R. K. (2014). Aflatoxins in food and feed: the science of safe food. Research & reviews: *J. Food Sc. and Tech.* (RRJoFST). 3. 6-11.

Le, B., & Yang, S. H. (2018). Isolation of *Weissella* strains as potent probiotics to improve antioxidant activity of salted squid by fermentation. *J. Appl. Biol. Chem.* 61(1), 93-100.

Lee, M. R., Huang, T. Y., Liao, C. H., Lai, C. C., Lee P. I. & Hsueh P. R. (2011). Bacteraemia caused by *Weissella confusa* at a university hospital in Taiwan, 1997–2007. *Clin. Microbiol. Infect.* 17(8): 1226-1231.

Leroy J., Wang, J. & Jones, K. (2019). Serum aflatoxin B1-lysine adduct level in adult women from Eastern Province in Kenya depends on household socio-economic status: A cross sectional study. *Social Sci. Med.* 146:104-110

Leslie J. F., Logrieco, A. (2014). Mycotoxin reduction in grain chains. *Books on google play, retrieved from* https://books.google.co.ke/books?id=9kXxBQAAQBAJ&pg=PA283&lpg=PA283&dq=maize+grain+is+more+susceptible+to+fungal+infections+than+millet&source=bl&ots=vmpFz3n69&sig=SM3sNAQmIWxXuQU4atxOBopVHvQ&hl=en&sa=X&ved=0ahUKEwjxwOSy5bDWAhVGshQKHcN4BLwQ6AEIKDAB#v=onepage&q=maize%20grain%20is%20more%20susceptible%20to%20fungal%20infections%20than%20millet&f=false

Leszczynka J., Maslowska J., Owczareka A., & Kucharska U. (2001). Determination of aflatoxin in foods products by the Elisa methods. *Czech J. Food Sci.* 19:8-12.

Lewis, L., Onsongo, M. and other the Kenya Investigation group. (2004). Aflatoxin contamination of commercial maize products during an outbreak of acute aflatoxicosis in Eastern and Central Kenya. *Env. Health Perspectives,* 113 Number 12. Pages?

Liew, W. P., & Redzwon, S.M. (2018). Mycotoxin: Its impact on gut health and microbiota. *Front. Cell. Infect. Microbial.* 8: 60

Liu, J., Lvhui S., Niya Z., Jiacai Z., Jiao G., Chong L., Shahid Ali R., Desheng Q. (2016) Effects of Nutrients in Substrates of Different Grains on Aflatoxin B1 Production by *Aspergillus flavus. BioMed Res. Inter.* vol. 2016, Article ID 7232858, 10 pages, 2016. https://doi.org/10.1155/2016/7232858

Logrieco, A. F., Miller, J. D., Eskola, M., Krska, R., Ayalew, A., Bandyopadhyay, R., Leslie, J. F. (2018). The Mycotox Charter: increasing awareness of, and concerted action for, minimizing mycotoxin exposure worldwide. *Toxins,* 10(4), 149.

López C., Laura, R., Lucía B., Silvana R., Fernanda R. (2002). Aflatoxin B1 content in patients with hepatic diseases. *Medicina (B Aires).*62(4):313-6.

Lopez-Martinez, L. X., & Garcia H. X. (2015). Chapter 39 - Processing of corn (maize) and compositional features. *Processing and Impact on Active Components in Food, Sci. Direct*, 329-336.

Luza, C., Saladinoa, F., Lucianob, F.B., Ma~nesa, J., & Meca, G. (2017). *In vitro* antifungal activity of bioactive peptides produced by *Lactobacillus plantarum* against *Aspergillus parasiticus* and *Penicillium expansum*. *LWT-Food Sci. Tech.* 81(2017):128-135.

Magnussen, A. & Parsi, M. A. (2013). Aflatoxins, hepatocellular carcinoma and public health. *World J. Gastroenterol.* 19(10), 1508–1512.

Mahato, D. K., Lee, K. E., Kamle, M., Devi, S., Dewangan, K. N., Kumar, P., Kang, S. G. (2019). Aflatoxins in Food and Feed: An Overview on Prevalence, Detection and Control Strategies. *Front Microbiol.* 2019; 10:2266. doi:10.3389/fmicb.2019.02266

Mahuku, G., Nzioki, H. S., Mutegi, C., Kanampiu, F., Narrod, C., & Makumbi, D. (2019). Pre-harvest management is a critical practice for minimizing aflatoxin contamination of maize. *Food Contr.* 96:219-226.

Manning, K., Pelling, R., Higham, T., Schwineger, J. L. & Fuller, D. Q (2011). 4500-Year old domesticated pearl millet (*Pennisetum glaucum)* from the Tilemsi Valley, Mali: new insights into an alternative cereal domestication pathway. *J. Arch. Sci.* 38(2): 312-322.

Marchese, S., Polo, A., Ariano, A., Velotto, S., Costantini, S., & Severino, L. (2018). Aflatoxin b1 and m1: biological properties and their involvement in cancer Development. *Toxins,* 10(6), 214.

Marco, M. L., Heeney, D., Binda, S., Cifelli, C. J., Cotter, P. D., Foligné, B., Gänzle, M., Kort, R., Pasin, G., Pihlanto, A., Smid, E. J., & Hutkins, R. (2017). Health benefits of fermented foods: microbiota and beyond. *Curr. Opin. Biotechnol.* 44:94-102.

Markowiak, P. & Slizews, K. (2017). Effects of probiotics, prebiotics, and synbiotics on human health. *Nutri. Rev.* 9(9)1021.

Mckevith, B. (2004). Nutritional aspect of cereals. *Nutrition Bulletin* *https://doi.org/10.1111/j.1467-3010.2004.00418.x*

McMullin, D., Mizaikoff, B., & Krska, R. (2015). Advancements in IR spectroscopic approaches for the determination of fungal derived contaminations in food crops. *Anal. Bioanal. Chem.* 407(3), 653–660.

Mehrzad, J., Bert D., Kim B., Eric C. (2014) Aflatoxin B1 interferes with the antigen-presenting capacity of porcine dendritic cells. *Toxicology in Vitro.* 28 (4):531-537 ISSN 0887-2333, https://doi.org/10.1016/j.tiv.2013.11.015

Microbeonline. (2013). *Bacterial cell wall*. Retrieved from microbeonline: https://i1.wp.com/microbeonline.com/wp-content/uploads/2013/08/Bacterial-cell-wall.jpg

Mohd-Redzwan, S., Jamaluddin, R., Abd-Mutalib, M. S., & Ahmad, Z. (2013). A mini review on aflatoxin exposure in Malaysia: past, present and future. *Front. Microbiol.* 4, 334.

Monachese, M., Burton, J. P. & Reid, G. (2012). Bioremediation and tolerance of humans to heavy metals through microbial processes: a potential role for probiotics. *Appl. Environ. Microbial*. 78: 186397-6404.

Moreno-carvajal, M. (2015). Metabolic changes of aflatoxin B1 to become an Active carcinogen and the control of this toxin. *Immunome Res*. 2015:11:2

Morris, A. L., Shamim, S. M. (2021). Biochemistry, Nutrients. *In: Stat Pearls* [Internet]. Treasure Island (FL): Stat Pearls Publishing; 2021 Jan-. Available from:

https://www.ncbi.nlm.nih.gov/books/NBK554545/Muriel, P. (2017). Chapter 1 - The Liver: General Aspects and Epidemiology, liver pathophysiology. *Academic Press, Mexico City, Mexico.* 3-22.

Mutegi, C. K., Cotty, P.J., & Bandyopadhyay, R. (2018). Prevalence and mitigation of aflatoxins in Kenya (1960-to date). *World Mycotoxin J.* 11(3): 341-357.

Mutiga, K., Morales, L., Angwenyi, S., Wainaina, J., Harvey, J., Das, B., & Nelson R. J. (2017). Association between agronomic traits and aflatoxin accumulation in diverse maize lines grown under two soil nitrogen levels in Eastern Kenya. *Field Crops Res.* 205:124-134.

Mwangi, L.W., Matofari, J.W. & Muliro, P.S. (2016). Hygienic assessment of spontaneously fermented raw camel milk (suusa) along the informal value chain in Kenya *Food Cont.* 3: 18.

Nduko, M. J., Matofari, W. J., Nandi O. Z. & Sichangi B. M. (2017). Spontaneously fermented Kenyan milk products: A review of the current state and future perspectives. *Afri. J. Food Sci.* 11(1):1-11.

Negash, D. (2018). A review of aflatoxin: occurrence, prevention, and gaps in both food and feed safety. *J. Nutr. Health Food Eng*. 8(2):190 – 197.

Njeru P. (2009) Molecular characterization and probiotic potential of Kimere lactobacilli. *Cuvillier Verlag, Inhaberin Annette Jentzsch-Cuvillier, Nonnenstieg* 8, 37075 Göttingen, Germany

Njeru, P. N., Rosch, N., Ghadimi, D., & Geis, A. (2010) Identification and characterization of lactobacilli isolated from Kimere, a spontaneously fermented pearl millet dough from Mbeere, Kenya (East Africa). *Benef. Microbes* 1(3):243-52.

Nuss, T., & Tanumihardjo, S.A. (2010). Maize: a paramount staple crop in the context of global nutrition. *Comp. Rev. Food Sci. Food Safety,* 9(4): 417-436.

Obonyo, M.A., Salano, E.N. (2018). Perennial and seasonal contamination of maize by aflatoxins in eastern Kenya. Food Contamination 5, 6 (2018). https://doi.org/10.1186/s40550-018-0069-y

Ochieng, P. J., Okun, D., Runo, S., Njage, N. J., & Murage, J. (2013). Public health strategies for preventing aflatoxin exposure. *BJC* 45(1):1661-8556.

Ognakossan K. E., Mutungi C. M. Otieno T. O., Affognon H. D., Sila D. N. & Owino, W. O. (2018). Quantitative and quality losses caused by rodents in on-farm stored maize: a case study in the low land tropical zone of Kenya," Food Security: the science, sociology and economics of food production and access to food. *Int. Soc. Plant Pathol.* 10(6):1525-1537.

Okaru, A. O., Abuga, K. O., Kibwage, I. O., Hausler, T., Luy, B., Kuballa, T., Rehm, J., & Lachenmeier, D. W. (2017). Aflatoxin contamination in unrecorded beers from Kenya – A health risk beyond ethanol. *Food Cont*. 79:344-348.

Okoth, S. A., & Kola, M. A. (2012). Market samples as a source of chronic aflatoxin exposure in Kenya. *Afri. J. Health Sci*. 20:56-61.

Ołdak, A., Zielińska, D., Rzepkowska, A., & Kołożyn-Krajewska, D. (2017). Comparison of antibacterial activity of *Lactobacillus plantarum* strains isolated from two different kinds of regional Cheeses from Poland: Oscypek and Korycinski Cheese. *BioMed Res. Inter.* 2017:6820369-682037.

Omotayo, O. P., Omotayo, A. O., Mwanza, M., & Babalola, O. O. (2019). Prevalence of mycotoxins and their consequences on human health. *Toxicol. Res.* 35(1), 1–7.

Owheruo, J. O., Ifesan, B., & Kolawole, A. O. (2018). Physicochemical properties of malted finger millet (*Eleusine coracana*) and pearl millet (*Pennisetum glaucum*). *Food Sci Nutr.* 7(2), 476–482.

Peraica, M., (2016). Chapter 5 – Mycotoxicosis; Environmental Mycology in Public Health. *Academic Press, city, country.* 45-49.

Petrova, M. I., Lievens, E., Malik, S., Imholz, N., & Lebeer, S. (2015). *Lactobacillus* species as biomarkers and agents that can promote various aspects of vaginal health. *Front. Physiol.* 6, 81.

Pitt, J. I., Taniwaki, M. H., & Cole, M. B. (2013). Mycotoxin production in major crops as influenced by growing, harvesting, storage and processing, with emphasis on the achievement of Food Safety Objectives. *Food Cont.* 32(1):205-215.

Pizzolitto, P., Armando, M. R., Salvano, M. A., Dalcero, A. M., & Rosa C. A. (2013). Evaluation of *Saccharomyces cerevisiae* as an anti aflatoxicogenic agent in broiler feedstuffs. *Poultry Sci.* 92:1655–1663.

Požrl, T., Mirela, K., Irena, K., Janez, H., Anja, J., & Marjan S. (2009). Phytate degradation during bread making: the influence of flour type and bread making procedures. *Czech J. Food Sci.* 27(1): 29–38.

Rawat, S. (2015). Food spoilage: microorganisms and their prevention. *Asian J. Plant Sci. Res.* 5(4):47-56.

Reid, G., Gadir, A. A., & Dhir, R. (2019). Probiotics: reiterating what they are and what they are tot. *Front. Microbiol.* 10:424.

Rizzello, C., & De Angelis, M. (2011). Lactic Acid Bacteria | *Lactobacillus* spp.: *Lactobacillus delbrueckii* group. *Ency. Dairy Sci*. volume? 119-124.

Robertson, C. C. (1997). Black, White, and Red All over: Beans, Women, and Agricultural Imperialism in Twentieth-Century Kenya. *Agricultural History society.* 71(3):259-299

Rocha, M. E. B., Freire, F., Maia, F., & Guedes, M. I. F. (2014). Mycotoxin and their effects on human and animal health. *Food Contr.* 36(1): 159-165.

Rosentrater, K. A., & Evers, A.D. (2018). Chapter 3 - Botanical aspects, Kent's Technology of Cereals (Fifth Edition). *Woodhead Publishing,* city, country 205-266.

Sabater-Vilar, M., Malekinejad, H., Selman, M. H., van der Doelen, M. A., & Fink-Gremmels, J. (2007). *In vitro* assessment of adsorbents aiming to prevent deoxynivalenol and zearalenone mycotoxicosis. *Mycopathologia,* 16*3*(2), 81–90.

Safika, S., Wardinal, W., Ismail, Y. S., Nisa, K. & Sari, W. N. (2019). *Weissella,* a novel lactic acid bacteria isolated from wild Sumatran orangutans (Pongo abelii). *Vet. World, 12*(7), 1060–1065.

Salvetti, E., Hugh, M. B., Harris, G. E., Felis, P., & O'Toole, W. (2018). Comparative genomics of the genus *Lactobacillus* reveals robust phylogroups that provide the basis for reclassification. *Appl. Environ. Microbiol.* 84 (17) e00993-18.

Salvetti, E., Torriani, S. & Felis, G.E. (2012). The Genus Lactobacillus: A Taxonomic Update. *Probiotics Antimicrob Proteins, 4*(4):217-26. Doi: 10.1007/s12602-012-9117-8.

Serna-Saldivar, S. O., & Carrillo E. P. (2019). Chapter 16 - Food uses of whole corn and dry-milled fractions. *Chem. Tech. Third ed. Sc. Direct,* 219:435-467. sheets/detail/natural-toxins-in-food

Sidhu, J. S., Kabir, Y., & Huffman, F. G. (2007) Functional foods from cereal grains. *Int. J. Food Properties,* 10(2):231-244.

Sirma, A.J., Lindahl, J.F., Makita, K., Senerwa, D., Mtimet, N., Kang'ethe, E.K., Grace, D. (2018).The impacts of aflatoxin standards on health and nutrition in sub-Saharan Africa: The case of Kenya. *Global Food Security.* 18(2018):57-61, ISSN 2211-9124. https://doi.org/10.1016/j.gfs.2018.08.001.

Smith, L. E., Prendergast, A. J., Turner, P. C., Humphrey, J. H., & Stoltzfus, R. J. (2017). Aflatoxin exposure during pregnancy, maternal anemia, and adverse birth outcomes. *Amer. J. Trop Med. Hyg,* 96(4):770–776.

Smith, V., Devani, D., Begley, C. & Clarke, M. (2011). Methodology in conducting a systematic review of systematic reviews of healthcare interventions. *BMC Med. Res. Method.* 15(2011).

Stepman, F. (2018). Scaling-up the impact of aflatoxin research in Africa. The role of social sciences. *Toxins,* 10(4), 136.

Sulaiman, S. H., Jamaluddin, R. & Sabran, M. R. (2018). Association between urinary aflatoxin ($AFM_1$) and dietary intake among adults in Hulu Langat District, Selangor, Malaysia. *Nutr.* 10(4), 460.

Suleiman, R., Rusentrater, K. A., Ben, C. (2013). Effects of Deterioration Parameters on Storage of Maize: A Review. *Journal of Natural Sc.* 3(9):147-165

Taheur, F. B., Mansour, C., Kouidhi, B., & Chaieb, K. (2019). Use of lactic acid bacteria for the inhibition of *Aspergillus flavus* and *Aspergillus carbonarius* growth and mycotoxin production. *Toxicon,* 166:15-23.

Tamang, J. P., Shin, D. H., Jung, S. J. & Chae, S. W. (2016). Functional properties of microorganisms in fermented Foods. *Front. Microbiol.* 7, 578.

Tao, F., Yao, H., Hruska, Z., Burger, L. W., Rajasekaran, K., & Bhatnagar, D. (2018). Recent development of optical methods in rapid and non-destructive detection of aflatoxin and fungal contamination in agricultural products. *TrAC Trends Anal. Chem.* 100:65-81.

Tournas, V., Michael, E., Stack, Philip, B., Missive, Herbert, A. K., Ruth, B. (2017) BAM Chapter 18: Yeasts, Molds and Mycotoxins. *US food and drug administration. Bacteriological Analytical Manual (BAM) Main Page* https://www.fda.gov/food/laboratory-methods-food/bam-chapter-18-yeasts-molds-and-mycotoxins

Tuntiteerawit, P., Singto, T., Anupon T. & Porasuphatana S. (2019). Modulation of Edible Plants on Hepatocellular Carcinoma Induced by Aflatoxin B<sub>1</sub> [Online First], IntechOpen, DOI: 10.5772/intechopen.87296. Available from: https://www.intechopen.com/online-first/modulation-of-edible-plants-on-hepatocellular-carcinoma-induced-by-aflatoxin-b1

Vekiru, E., Fruhauf, S., Rodrigues, I., Ottner, F., Krska, R., Schatzmayr, G., Ledoux, D. R., Rottinghaus, G. E., & Bermudez. A.J. (2015). *In vitro* binding assessment and *in vivo* efficacy of several adsorbents against aflatoxin B1. *World Mycotoxin J.* 8(4):477-488.

Velásquez, A. C., Christian, D. M. C., Shung, H. (2018). Plant and pathogen warfare under changing climate conditions. *Curr Biol.* 28(10): R619–R634. Doi: 10.1016/j.cub.2018.03.054

Vuyst, L. & Weckx, S. (2016). The cocoa bean fermentation process: from ecosystem analysis to starter culture development. *J. Appl. Microbiol.* 121(1):5-17.

Wacoo, A. P., Wendiro, D., Vuzi, P. C., & Hawumba, J. F. (2014). Methods for detection of aflatoxins in agricultural food crops. *J. Appl. Chem*. 2014:1-15.

Wagacha, J. M., Muthomi, J. W. (2008). Mycotoxin problem in Africa: current status, implications to food safety and health and possible management strategies, *Int. J. Food Microbiol*, 124(1):1–12.

Wambui, J. M., Karuri, E. G., Ojiambo, J. A., & Njage, P. M. (2017). Application of probabilistic modeling to quantify the reduction levels of hepatocellular carcinoma risk attributable to chronic aflatoxins. *Nutr. Cancer,* 69(1):1-13.

Wang, K., Wang, W., Ye, R., Liu, A., Xiao, J., Liu, Y., & Zhao, Y. (2017). Mechanical properties and solubility in water of corn starch-collagen composite films: effect of starch type and concentrations. *Food Chem.* 2016:209-216.

Warth, B., Sulyok, M., Berthiller, F., Schuhmacha, R. & Krska R. (2013). New insights into the human metabolism of the *Fusarium* mycotoxins deoxynivalenol and zearalenone. *Toxicol Lett.* 220(1):88-94.

Westerik, N., Wacoo, P. A., Anyimo, E., William, M., Reid, G., Kort, R. & Sybesma, W. (2019). Improving health and wealth by introduction of an affordable bacterial starter culture for probiotic yoghurt production in Uganda. *Challenges* 2019. 10(1), 2

WHO, Natural toxins in food 9 May 2018. https://www.who.int/news-room/fact-

William, J. H., Phillips, T. D., Jolly P. E., Stiles, J. K., Jolly, C. M. & Aggarwal D. (2004). Human aflatoxicosis in developing countries: a review of toxicology, exposure, potential health consequences, and interventions. *Amer. J. Clin. Nutr.* 80(5):1106–1122

Williams, J., Grubb, J., Davis, J., Wang, J. S., Jolly, P., Ankrah, N., Ellis, W., Afriyi, E., Johnson, N., Robinson, A. & Phillips, T. (2010). HIV and hepatocellular and esophageal carcinomas related to consumption of mycotoxin-prone foods in sub-Saharan Africa. *Amer. J. Clin. Nutr.* 92:154-60. 10.3945/ajcn.2009.28761.

Xing, F., Wang, L., Liu, X., Selvaraj, J. N., Wang, Y., Zhao, Y., & Liu, Y. (2017). Aflatoxin B1 inhibition in *Aspergillus flavus* by *Aspergillus niger* through down-regulating expression of major biosynthetic genes and AFB1 degradation by atoxigenic *A. flavus*. *Int. J. Food Microbiol*. 256:1-10.

Xu, Y., Gong, Y.Y., & Routledge, M.N. (2018). Aflatoxin exposure assessed by aflatoxin albumin adduct biomarker in populations from six African countries. *World Mycotoxin J.* 11(3):411-419.

Yuliana, R. T., Valentina, Lubomirova, C., Anton, Kolev, T., & Maria, S. A. (2014). Harmonize approaches to analysis and risk assessment of mycotoxins in foodstuffs. *Int. J. Adv. Res.*2(7):123-138 (ISSN 2320-5407).

## APPENDICES

### Appendix 1. Distribution of Pearl Millet Cultivation in the World

Source: Agropedia, http://agropedia.iitk.ac.in/content/area-and-distribution-pearl-milletassessed on 24/8/2016

**Appendix 2. Pearl Millet in Field and Grains after Threshing**

**Appendix 3. Comparison of Aflatoxins Concentrations in Stores among the Regions**

| **ANOVA** | | | | | |
|---|---|---|---|---|---|
| Comparison of Aflatoxin in Nairobi | | | | | |
| | Sum of Squares | Df | Mean Square | F | Sig. |
| Between Groups | 45.904 | 4 | 11.476 | 17.715 | .000 |
| Within Groups | 12.956 | 20 | .648 | | |
| Total | 58.860 | 24 | | | |
| Comparison of Aflatoxin in Western | | | | | |
| | Sum of Squares | Df | Mean Square | F | Sig. |
| Between Groups | 95.300 | 4 | 23.825 | 41.579 | .000 |
| Within Groups | 11.460 | 20 | .573 | | |
| Total | 106.760 | 24 | | | |
| Comparison of Aflatoxin in Eastern | | | | | |
| | Sum of Squares | Df | Mean Square | F | Sig. |
| Between Groups | 449.345 | 4 | 112.336 | 16.472 | .000 |
| Within Groups | 136.400 | 20 | 6.820 | | |
| Total | 585.745 | 24 | | | |

*The above table indicates that aflatoxin levels were significantly different among the five stores in Nairobi, Western and Eastern.

**Appendix 4. Post Hoc Analysis Table 1: Aflatoxins in Flour Samples per Brand from the Stores in Eastern**

| Comparison of Aflatoxin concentration in brand X in EasternTukey HSD | | | | | | |
|---|---|---|---|---|---|---|
| (I) Brand X_Eastern | (J) Brand X_Eastern | Mean Difference (I-J) | Std. Error | Sig. | 95% Confidence Interval | |
| | | | | | Lower Bound | Upper Bound |
| store1 | store2 | -1.10000* | .08165 | .000 | -1.3505 | -.8495 |
| | store3 | -1.90000* | .08165 | .000 | -2.1505 | -1.6495 |
| Store2 | store3 | -.80000* | .08165 | .000 | -1.0505 | -.5495 |
| | store2 | .80000* | .08165 | .000 | .5495 | 1.0505 |
| *. The mean difference is significant at the 0.05 level. | | | | | | |

| Comparison of Aflatoxin concentration in brand Y in Eastern_Tukey HSD | | | | | | |
|---|---|---|---|---|---|---|
| (I) Brand Y_Eastern | (J) Brand Y_Eastern | Mean Difference (I-J) | Std. Error | Sig. | 95% Confidence Interval | |
| | | | | | Lower Bound | Upper Bound |
| store1 | store2 | -.30000* | .08165 | .024 | -.5505 | -.0495 |
| | store3 | -1.10000* | .08165 | .000 | -1.3505 | -.8495 |
| Store2 | store3 | -.80000* | .08165 | .000 | -1.0505 | -.5495 |
| *. The mean difference is significant at the 0.05 level. | | | | | | |

**Appendix 5. Post Hoc Analysis Table 2: Aflatoxin in Maize Flour Samples from Western**

| Comparison of Aflatoxin concentration in brand x in Western_ Tukey HSD | | | | | | |
|---|---|---|---|---|---|---|
| (I) Brand X_Western | (J) Brand X_Western | Mean Difference (I-J) | Std. Error | Sig. | 95% Confidence Interval | |
| | | | | | Lower Bound | Upper Bound |
| store1 | store2 | -1.10000* | .08165 | **.000** | -1.3505 | -.8495 |
| | store3 | .20000 | .08165 | .109 | -.0505 | .4505 |
| Store2 | store3 | 1.30000* | .08165 | **.000** | 1.0495 | 1.5505 |
| *. The mean difference is significant at the 0.05 level. | | | | | | |

| Comparison of Aflatoxin concentration in Brand Y in Western_Tukey HSD | | | | | | |
|---|---|---|---|---|---|---|
| (I) Brand Y_Western | (J) Brand Y_Western | Mean Difference (I-J) | Std. Error | Sig. | 95% Confidence Interval | |
| | | | | | Lower Bound | Upper Bound |
| store1 | store2 | .30000* | .08165 | **.024** | .0495 | .5505 |
| | store3 | 1.10000* | .08165 | **.000** | .8495 | 1.3505 |
| Store2 | store3 | .80000* | .08165 | **.000** | .5495 | 1.0505 |
| *. The mean difference is significant at the 0.05 level. | | | | | | |

| Comparison of Aflatoxin concentration in Brand Z in Western_Tukey HSD | | | | | | |
|---|---|---|---|---|---|---|
| (I) Brand Z_Western | (J) Brand Z_Western | Mean Difference (I-J) | Std. Error | Sig. | 95% Confidence Interval | |
| | | | | | Lower Bound | Upper Bound |
| store1 | store2 | -.70000* | .08165 | **.000** | -.9505 | -.4495 |
| | store3 | .50000* | .08165 | **.002** | .2495 | .7505 |
| Store2 | store3 | 1.20000* | .08165 | **.000** | .9495 | 1.4505 |
| *. The mean difference is significant at the 0.05 level. | | | | | | |

*The P values in bold shows significance at 95% confidence interval

**Appendix 6. Post Hoc Analysis Table 3: Aflatoxins in Maize Grain Samples from Stores in Nairobi**

| Comparison of Aflatoxin in Nairobi_ Tukey HSD | | | | | | |
|---|---|---|---|---|---|---|
| | | | | | 95% Confidence Interval | |
| (I) Nairobi | (J) Nairobi | Mean Difference (I-J) | Std. Error | Sig. | Lower Bound | Upper Bound |
| 1st Store | 2nd Store | 1.08000 | .50904 | **.250** | -.4432 | 2.6032 |
| | 3rd Store | -1.10000 | .50904 | **.235** | -2.6232 | .4232 |
| | 4th Store | -3.00000* | .50904 | .000 | -4.5232 | -1.4768 |
| | 5th Store | -1.08000 | .50904 | **.250** | -2.6032 | .4432 |
| 2nd Store | 3rd Store | -2.18000* | .50904 | .003 | -3.7032 | -.6568 |
| | 4th Store | -4.08000* | .50904 | .000 | -5.6032 | -2.5568 |
| | 5th Store | -2.16000* | .50904 | .003 | -3.6832 | -.6368 |
| 3rd Store | 4th Store | -1.90000* | .50904 | .010 | -3.4232 | -.3768 |
| | 5th Store | .02000 | .50904 | **1.000** | -1.5032 | 1.5432 |
| 4th Store | 5th Store | 1.92000* | .50904 | .009 | .3968 | 3.4432 |
| *. The mean difference is significant at the 0.05 level. | | | | | | |

*Highlighted p-values shows similarity

**Appendix 7. Post Hoc Analysis: Aflatoxins in Maize Grain Samples from Stores in Western**

| Comparison of Aflatoxin in Western_ Tukey HSD | | | | | | |
|---|---|---|---|---|---|---|
| | | | | | 95% Confidence Interval | |
| (I) Western | (J) Western | Mean Difference (I-J) | Std. Error | Sig. | Lower Bound | Upper Bound |
| 1st Store | 2nd Store | -1.40000 | .47875 | **.057** | -2.8326 | .0326 |
| | 3rd Store | 1.30000 | .47875 | **.087** | -.1326 | 2.7326 |
| | 4th Store | -1.90000* | .47875 | .006 | -3.3326 | -.4674 |
| | 5th Store | -4.50000* | .47875 | .000 | -5.9326 | -3.0674 |
| 2nd Store | 3rd Store | 2.70000* | .47875 | .000 | 1.2674 | 4.1326 |
| | 4th Store | -.50000 | .47875 | **.832** | -1.9326 | .9326 |
| | 5th Store | -3.10000* | .47875 | .000 | -4.5326 | -1.6674 |
| 3rd Store | 4th Store | -3.20000* | .47875 | .000 | -4.6326 | -1.7674 |
| | 5th Store | -5.80000* | .47875 | .000 | -7.2326 | -4.3674 |
| 4th Store | 5th Store | -2.60000* | .47875 | .000 | -4.0326 | -1.1674 |
| *. The mean difference is significant at the 0.05 level. | | | | | | |

*Highlighted p-values shown similarities

**Appendix 8. One Way ANOVA of Viscosities of Probiotic Yoghurts made by Various Isolated Strains.**

| | | | | | | |
|---|---|---|---|---|---|---|
| NN7 | 3 | 601 | 200.3333 | 100.3333 | | |
| NN8 | 3 | 599 | 199.6667 | 100.3333 | | |
| NN9 | 3 | 570 | 190 | 100 | | |
| NN11 | 3 | 541 | 180.3333 | 100.3333 | | |
| NN13 | 3 | 519 | 173 | 121 | | |
| NN14 | 3 | 507 | 169 | 441 | | |
| NN16 | 3 | 390 | 130 | 100 | | |
| NN18 | 3 | 361 | 120.3333 | 110.3333 | | |
| NN19 | 3 | 570 | 190 | 121 | | |
| NN20 | 3 | 725 | 241.6667 | 121.3333 | | |
| NN21 | 3 | 659 | 219.6667 | 100.3333 | | |
| NN22 | 3 | 580 | 193.3333 | 233.3333 | | |
| NN26 | 3 | 451 | 150.3333 | 110.3333 | | |
| GR1 | 3 | 721 | 240.3333 | 110.3333 | | |
| CONTROL | 3 | 719 | 239.6667 | 100.3333 | | |
| | | | | | | |
| | | | | | | |
| ANOVA | | | | | | |
| *ce of Varic* | *SS* | *df* | *MS* | *F* | *P-value* | *F crit* |
| Between ( | 64817.93 | 17 | 3812.819 | 27.4048 | 1.306 | 1.915321 |
| Within Gr( | 5008.667 | 36 | 139.1296 | | | |
| | | | | | | |
| Total | 69826.59 | 53 | | | | |

**Appendix 9. One Way ANOVA of pH of Probiotic Yoghurt Made by Various Isolated Strains.**

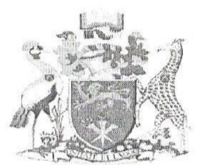
| | | | | | | |
|---|---|---|---|---|---|---|
| NN6 | 3 | 13.8 | 4.6 | 0.01 | | |
| NN7 | 3 | 13.5 | 4.5 | 0.01 | | |
| NN8 | 3 | 14.1 | 4.7 | 0.01 | | |
| NN9 | 3 | 12 | 4 | 0.09 | | |
| NN11 | 3 | 11.7 | 3.9 | 0.04 | | |
| NN14 | 3 | 15 | 5 | 0.09 | | |
| NN16 | 3 | 13.5 | 4.5 | 0.01 | | |
| NN18 | 3 | 11.1 | 3.7 | 0.01 | | |
| NN19 | 3 | 10.8 | 3.6 | 0.01 | | |
| NN20 | 3 | 10.5 | 3.5 | 0.01 | | |
| NN21 | 3 | 12 | 4 | 0.04 | | |
| NN22 | 3 | 11.4 | 3.8 | 0.01 | | |
| NN26 | 3 | 11.1 | 3.7 | 0.01 | | |
| GR1 | 3 | 11.4 | 3.8 | 0.01 | | |
| CONTROL | 3 | 12.6 | 4.2 | 0.01 | | |
| | | | | | | |
| | | | | | | |
| ANOVA | | | | | | |
| *ce of Varia* | *SS* | *df* | *MS* | *F* | *P-value* | *F crit* |
| Between ( | 10.58824 | 16 | 0.661765 | 28.84615 | 3.585 | 1.951566 |
| Within Gr | 0.78 | 34 | 0.022941 | | | |
| | | | | | | |
| Total | 11.36824 | 50 | | | | |

**Appendix 10. One Way ANOVA of NN20 and *Escherichia coli***

| SUMMARY | | | | |
|---|---|---|---|---|
| *Groups* | *Count* | *Sum* | *Average* | *Variance* |
| NN20 | 3 | 130 | 43.33333 | 6.333333 |
| Eschiriche | 3 | 30 | 10 | 4 |

| ANOVA | | | | | | |
|---|---|---|---|---|---|---|
| *rce of Varia* | *SS* | *df* | *MS* | *F* | *P-value* | *F crit* |
| Between ( | 1666.667 | 1 | 1666.667 | 322.5806 | 0.0333 | 7.708647 |
| Within Gr | 20.66667 | 4 | 5.166667 | | | |
| | | | | | | |
| Total | 1687.333 | 5 | | | | |

## Appendix 11. Ethics Certificates



KENYATTA NATIONAL HOSPITAL APPROVED 09 JAN 2014 ETHICS & RESEARCH COMMITTEE

UNIVERSITY OF NAIROBI
COLLEGE OF HEALTH SCIENCES
P O BOX 19676 Code 00202
Telegrams: varsity
(254-020) 2726300 Ext 44355

KNH/UON-ERC
Email: uonknh_erc@uonbi.ac.ke
Website: www.uonbi.ac.ke

KNH QUALITY HEALTH CARE
KENYATTA NATIONAL HOSPITAL
P O BOX 20723 Code 00202
Tel: 726300-9
Fax: 725272
Telegrams: MEDSUP, Nairobi

Ref: KNH-ERC/A/10 Link:www.uonbi.ac.ke/activities/KNHUoN 8th January 2014

Nicholas Nduti
Technical University of Kenya
NAIROBI

Dear Nicholas

RESEARCH PROPOSAL: INVESTIGATING THE EFFECTS OF PROBIOTIC YOGURT ON REDUCING THE LEVELS OF AFLATOXIN B1 TOXIN AMONG SCHOOL CHILDREN IN EASTERN KENYA (P446/08/2013)

This is to inform you that the KNH/UoN-Ethics & Research Committee (KNH/UoN-ERC) has reviewed and approved your above proposal. The approval periods are 8th January 2014 to 7th January 2015.

This approval is subject to compliance with the following requirements:

a) Only approved documents (informed consents, study instruments, advertising materials etc) will be used.
b) All changes (amendments, deviations, violations etc) are submitted for review and approval by KNH/UoN ERC before implementation.
c) Death and life threatening problems and severe adverse events (SAEs) or unexpected adverse events whether related or unrelated to the study must be reported to the KNH/UoN ERC within 72 hours of notification.
d) Any changes, anticipated or otherwise that may increase the risks or affect safety or welfare of study participants and others or affect the integrity of the research must be reported to KNH/UoN ERC within 72 hours.
e) Submission of a request for renewal of approval at least 60 days prior to expiry of the approval period. (*Attach a comprehensive progress report to support the renewal*).
f) Clearance for export of biological specimens must be obtained from KNH/UoN-Ethics & Research Committee for each batch of shipment.
g) Submission of an *executive summary* report within 90 days upon completion of the study
This information will form part of the data base that will be consulted in future when processing related research studies so as to minimize chances of study duplication and/or plagiarism.

For more details consult the KNH/UoN ERC website **www.uonbi.ac.ke/activities/KNHUoN.**

Protect to Discover

for: DISTRICT EDUCATION OFFICER
MBEERE SOUTH

Yours sincerely

PROF. M. L. CHINDIA
SECRETARY, KNH/UON-ERC

for: DISTRICT EDUCATION OFFICER
MBEERE SOUTH

c.c. Prof. A.N.Guantai, Chairperson, KNH/UoN-ERC
The Deputy Director CS, KNH
The Principal, College of Health Sciences, UoN
Assistant Director/Health Information, KNH
Supervisors: Dr. Patrisio Njeru, Technical University of Kenya
Dr. Mercy Mwaniki, Technical University of Kenya

Protect to Discover

**Appendix 12. Field Trial Subject Recruitments Forms**

**Care giver's Consent form**

**Investigating the effects of probiotic yoghurt on reducing the levels of aflatoxin B1 among school children in Eastern Kenya.**

By signing this form, I agree that:

I have read the letter of information, have had the nature of the study explained to me and I agree for my child to participate. All questions have been answered to my satisfaction.

Child's Name (please print): ____________________________________________

Parent/Guardian Name (please print): ____________________________________

Parent/Guardian Signature/Mark: ________________________________________

Date: ________________________________

Person Obtaining Informed Consent (please print): _________________________

Signature: ________________________________________________________

Date: ________________________________

**CARE GIVER QUESTIONNAIRE**

**Participant ID: ____________________________________**

**Investigating the effects of probiotic yoghurt on reducing the levels of Aflatoxin B1 among school children in Eastern Kenya**

**Instructions:** The following questions were designed to assess the eligibility of the participant based on the recruitment criteria for this study.

Please answer as honestly and clearly as possible.

Feel free to ask the study coordinator about any questions that may be unclear.

1. Please specify your child's birth year. (YYYY): ____________________

2. Does your child currently attend Primary School in eastern Kenya?

- o Yes
- o No

4. Is your child currently consuming fermented milk products, i.e. yoghurt?

- o Yes
- o No

5. Is your child lactose intolerant and/or allergic to dairy products?

- o Yes
- o No

12 Has your child taken any antimicrobials/antibiotics in the past 3 months?

- o Yes
- o No

Date stopped: ________________________________________________

**CARE GIVER'S LETTER OF INFORMATION**

**Investigating the effects of probiotic yoghurt on reducing the levels of aflatoxin B1 among school children in Eastern Kenya.**

**Invitation to participate in a research Study:**

Your child, for whom you are the primary caregiver, and you are being invited to participate in a research study being carried out for a trial to control aflatoxin. Up to 60 children and their caregivers are being invited to participate in a study that will examine how a probiotic yoghurt, that is a yoghurt containing specially selected health-promoting bacteria, can reduce levels of Aflatoxin B1 in children.

This letter contains information to help you decide whether or not your child and you wish to participate in this research study. It is important for you to understand why the study is being conducted and what it will involve. Please take the time to read this carefully and feel free to ask questions if anything is unclear or if there are words or phrases that you do not understand.

**Purpose of Study:**

In Kenya, and around the world, humans consume large quantities of harmful compounds in their diet. These compounds, termed environmental toxins, occur naturally and their consumption is often unavoidable. One example of these toxins is Aflatoxin B1. This toxin is associated with eating contaminated grains, like maize, and is linked to a range of diseases but is thought to particularly impact proper growth and development in children. We, and others, have discovered that the 'good-bacteria' in probiotic yoghurt, including *Weissella cibiria* NN20, interact with this toxin and have the potential to reduce the amount of toxin that is absorbed in the body.

The purpose of this study is to investigate the ability of a probiotic yoghurt to reduce the levels of aflatoxin B1 in the body. The study hypothesis that, information gained will help show the potential of probiotic bacteria to reduce levels of the toxic substances in children which could lead to new methods to improve health.

**Procedures:**

If you and your child agree to participate, your child will be given a daily portion (200g) of probiotic yoghurt or fresh milk for six (14) days free of charge. The study will last for 21 days in total. Your child will randomly be assigned into one of two groups. At the start of the study small urine samples (30ml) will be collected and there after 7 days until end of study. Your child will receive a probiotic Yoghurt at school. Samples will be shipped to the Lawson Health Research Institute in London, Ontario, Canada for analysis at the end of the study. Other than these urine samples and information from the questionnaire, no further information will be required from your child and you if you choose to participate.

**Inclusion Criteria:**

If your child is between the ages of 6 and 10 and enrolled at Primary School in Eastern Kenya, your child is eligible to participate in this study.

**Exclusion Criteria:**

If your child is already consuming fermenting milk products, for example yoghurt, or is lactose intolerant, they are ineligible to participate.

**Contacts:**

If you have any questions, feel free to contact:

Nicholas Nduti, PhD student - 0721335653

OR

Dr. Patrisio Njeru, Investigator - 0728712718

**Risks, Stress, or Discomfort:**

There is no expectation that your child will experience any risk by participating in this study.

**Voluntary Participation:**

Participation in this study is voluntary. You may refuse for your child to participate, refuse to answer any questions, or withdraw from the study at any time with no effect on your child's care or education. Samples will not be labeled with personal identification, so it will not be possible to withdraw your samples from the study once you have delivered them to the researchers.

**Confidentiality:**

Your child's participation in this study will be kept confidential. Your child's identity will only be known by the member(s) of the research team with whom you have direct contact, and will not be released to other members of the research team. The samples will not be labeled with personal information. When the results of this study are published, your child's name will not be used and no information that discloses their identity will be released or published. Study data and information is stored on computers equipped with encryption software to protect the data and are stored in locked cabinets with restricted access. Samples will be stored until all analysis has been completed. Data generated from this analysis is stored indefinitely.

**Benefits:**

Participation in this study may be of no direct benefit to you as the caregiver. You will be helping to understand how probiotic bacteria may affect toxin levels in children and their potential as a new method of improving children's health. The study long-term goal is the development of probiotic yoghurt specifically designed to protect against environmental toxins and lead to healthier children.

**Costs:**

There will be no cost to you for participating but by your child participating in this study, they will be receiving, probiotic yoghurt or milk for 21 days aiding in their nutrition.

**Consent:**

By consenting for the child, of which you are the primary caregiver, to participating this study, you give your consent that the information given by you and obtained from your child's samples can be compiled and analyzed in Kenya and Canada by the researchers participating in this study. If you are participating in another study, please let us know so that it can be determined if you should participate in this study.

**Your Rights:**

You do not waive any legal rights by signing the consent form.

**Copy of the Consent:**

You will be provided a photocopy of this letter and agreement once it has been signed.

## CHILDREN'S CONSENT FORM

**Investigating the effects of probiotic yoghurt on reducing the levels of aflatoxin B1 among school children in Eastern Kenya.**

I have read/been read the Letter of Information, have had the study explained to me and I agree to participate. All questions have been answered to my satisfaction.

Child's Name (please print): ______________________________________________

Child's Signature/Mark: ______________________________________________

Person Obtaining Informed Consent (please print):____________________________

Signature______________________________

Date:______________________________

## CHILDREN'S ASSENT FORM

**Investigating the effects of probiotic yoghurt in reducing the levels of aflatoxin B1 among school children in Eastern Kenya.**

**Why are we doing this study?**

In Kenya and around the world, we eat large amounts of bad compounds that are hidden in our food. We are testing if the good bacteria, or 'bugs' in yoghurt could help prevent these compounds from entering your body.

**What will happen to you?**

1. For 21 days, every day you will get either yoghurt or milk to eat at school.

2. At the beginning and end of the study, we will collect a small amount of urine.

**Will you get better if you are in the study?**

Eating this yoghurt may result in lower amounts of these bad compounds in your body. As well, both yoghurt and milk will help you grow and be healthy.

**What if you have any questions?**

You can ask questions any time, now or later. You can talk to the researchers, your family, or someone else.

**Do you have to be in the study?**

You do not have to be in the study. No one will be annoyed with you if you don't want to do this. If you don't want to be in this study, just say so. Even if you say yes now, you can change your mind later. It's up to you.

## Appendix 13. One Way ANOVA of AFM1 in Urine Samples of Treated Groups; Children Fed With Novel Yoghurt

SUMMARY

| *Groups* | *Count* | *Sum* | *Average* | *Variance* |
|---|---|---|---|---|
| Day 1 | 7 | 46.5 | 6.642857 | 27.27619 |
| Day 7 | 7 | 26.4 | 3.771429 | 13.60571 |
| Day 14 | 7 | 19.9 | 2.842857 | 8.472857 |
| Day 21 | 7 | 6 | 0.857143 | 1.47619 |

ANOVA

| *rce of Varia* | *SS* | *df* | *MS* | *F* | *P-value* | *F crit* |
|---|---|---|---|---|---|---|
| Between ( | 121.5514 | 3 | 40.51714 | 3.188384 | 0.041854 | 3.008787 |
| Within Gr | 304.9857 | 24 | 12.70774 | | | |
| | | | | | | |
| Total | 426.5371 | 27 | | | | |

**Appendix 14. One Way ANOVA of Mean Percentage of Urine Samples with Detectable Aflatoxins as Determined by LC-MS**

| Anova: Single Factor | | | | | | |
|---|---|---|---|---|---|---|
| | | | | | | |
| SUMMARY | | | | | | |
| *Groups* | *Count* | *Sum* | *Average* | *Variance* | | |
| AFM1 | 2 | 2.13 | 1.065 | 2.26845 | | |
| AFB1 | 2 | 0 | 0 | 0 | | |
| AFB2 | 2 | 16.7 | 8.35 | 10.4882 | | |
| AFG1 | 2 | 2.13 | 1.065 | 2.26845 | | |
| AFG2 | 2 | 0 | 0 | 0 | | |
| N7-Guanir | 2 | 11.54 | 5.77 | 15.0152 | | |
| Any AFs | 2 | 30.37 | 15.185 | 74.29805 | | |
| | | | | | | |
| | | | | | | |
| ANOVA | | | | | | |
| *ce of Varic* | *SS* | *df* | *MS* | *F* | *P-value* | *F crit* |
| Between ( | 389.4049 | 6 | 64.90082 | 4.354159 | 0.037562 | 3.865969 |
| Within Gr( | 104.3384 | 7 | 14.90548 | | | |
| | | | | | | |
| Total | 493.7433 | 13 | | | | |

**Appendix 15. One Way ANOVA of AFM1 in Urine of Control Group; Children Fed with Fresh Milk**

| *Groups* | *Count* | *Sum* | *Average* | *Variance* | | |
|---|---|---|---|---|---|---|
| day 1 | 7 | 36.6 | 5.228571 | 17.56905 | | |
| day 7 | 7 | 41.3 | 5.9 | 11.87333 | | |
| day 14 | 7 | 46.2 | 6.6 | 13.76 | | |
| day 21 | 7 | 45.1 | 6.442857 | 32.42952 | | |
| | | | | | | |
| | | | | | | |
| ANOVA | | | | | | |
| *rce of Varic* | *SS* | *df* | *MS* | *F* | *P-value* | *F crit* |
| Between ( | 8.077143 | 3 | 2.692381 | 0.142394 | 0.933538 | 3.008787 |
| Within Gr( | 453.7914 | 24 | 18.90798 | | | |
| | | | | | | |
| Total | 461.8686 | 27 | | | | |

**Appendix 16. One Way ANOVA of Binding Capacities of Strains in the Yoghurt and *Escherichia Coli***

| Anova: Single Factor | | | | | | |
|---|---|---|---|---|---|---|
| | | | | | | |
| SUMMARY | | | | | | |
| *Groups* | *Count* | *Sum* | *Average* | *Variance* | | |
| NN1 | 3 | 114 | 38 | 4 | | |
| NN3 | 3 | 126.6 | 42.2 | 4 | | |
| NN6 | 3 | 118.8 | 39.6 | 2.17 | | |
| NN7 | 3 | 114 | 38 | 13 | | |
| NN8 | 3 | 114 | 38 | 9 | | |
| NN9 | 3 | 120 | 40 | 25 | | |
| NN11 | 3 | 108 | 36 | 36 | | |
| NN13 | 3 | 120 | 40 | 9 | | |
| NN14 | 3 | 120 | 40 | 4 | | |
| NN16 | 3 | 120 | 40 | 1 | | |
| NN18 | 3 | 126 | 42 | 36 | | |
| NN19 | 3 | 114 | 38 | 4 | | |
| NN20 | 3 | 130 | 43.33333 | 6.333333 | | |
| NN21 | 3 | 120 | 40 | 4 | | |
| NN22 | 3 | 120 | 40 | 1 | | |
| NN26 | 3 | 120 | 40 | 1 | | |
| GR28 | 3 | 120 | 40 | 1 | | |
| GR1 | 3 | 111 | 37 | 1 | | |
| GR12 | 3 | 30 | 10 | 4 | | |
| | | | | | | |
| | | | | | | |
| ANOVA | | | | | | |
| *ce of Varia* | *SS* | *df* | *MS* | *F* | *P-value* | *F crit* |
| Between ( | 2656.931 | 18 | 147.6073 | 16.94551 | 6.39E-13 | 1.882603 |
| Within Gr | 331.0067 | 38 | 8.710702 | | | |
| | | | | | | |
| Total | 2987.937 | 56 | | | | |

**Appendix 17. ANOVA Comparison of Three Brands in Respective Regions**

| **ANOVA** | | | | | |
|---|---|---|---|---|---|
| Comparison of Aflatoxin level in Brand X among Nairobi, Western & Eastern | | | | | |
| | Sum of Squares | Df | Mean Square | F | Sig. |
| Between Groups | .260 | 2 | .130 | .203 | .822 |
| Within Groups | 3.840 | 6 | .640 | | |
| Total | 4.100 | 8 | | | |
| Comparison of Aflatoxin level in Brand Y among Nairobi, Western & Eastern | | | | | |
| | Sum of Squares | Df | Mean Square | F | Sig. |
| Between Groups | .016 | 2 | .008 | .021 | .979 |
| Within Groups | 2.180 | 6 | .363 | | |
| Total | 2.196 | 8 | | | |
| Comparison of Aflatoxin level in Brand Z among Nairobi, Western & Eastern | | | | | |
| | Sum of Squares | Df | Mean Square | F | Sig. |
| Between Groups | .743 | 2 | .371 | .724 | .523 |
| Within Groups | 3.077 | 6 | .513 | | |
| Total | 3.820 | 8 | | | |

*Each brand had significantly similar aflatoxin levels among the three areas $p>0.05$

**Appendix 18. ANOVA Comparison of Brand X among the Three Leading Stores in Eastern***

| **ANOVA** | | | | | |
|---|---|---|---|---|---|
| Comparison of Aflatoxin concentration in brand X in Eastern | | | | | |
| | Sum of Squares | Df | Mean Square | F | Sig. |
| Between Groups | 5.460 | 2 | 2.730 | 273.000 | .000 |
| Within Groups | .060 | 6 | .010 | | |
| Total | 5.520 | 8 | | | |
| Comparison of Aflatoxin concentration in brand Y in Eastern | | | | | |
| | Sum of Squares | Df | Mean Square | F | Sig. |
| Between Groups | 1.940 | 2 | .970 | 97.000 | .000 |
| Within Groups | .060 | 6 | .010 | | |
| Total | 2.000 | 8 | | | |
| Comparison of Aflatoxin concentration in brand Z in Eastern | | | | | |
| | Sum of Squares | Df | Mean Square | F | Sig. |
| Between Groups | 3.620 | 2 | 1.810 | 181.000 | .000 |
| Within Groups | .060 | 6 | .010 | | |
| Total | 3.680 | 8 | | | |

*The above table indicates that aflatoxin levels were significantly different among the three stores compared to each of the three brands investigated in Eastern.

**Appendix 19. ANOVA, Differences in Concentrations of aflatoxin in grains among Store for Various Brand in Western**

| **ANOVA** | | | | | |
|---|---|---|---|---|---|
| Comparison of Aflatoxin concentration in brand x in Western | | | | | |
| | Sum of Squares | Df | Mean Square | F | Sig. |
| Between Groups | 2.940 | 2 | 1.470 | 147.000 | **.000** |
| Within Groups | .060 | 6 | .010 | | |
| Total | 3.000 | 8 | | | |
| Comparison of Aflatoxin concentration in Brand Y in Western | | | | | |
| | Sum of Squares | Df | Mean Square | F | Sig. |
| Between Groups | 1.940 | 2 | .970 | 97.000 | **.000** |
| Within Groups | .060 | 6 | .010 | | |
| Total | 2.000 | 8 | | | |
| Comparison of Aflatoxin concentration in Brand Z in Western | | | | | |
| | Sum of Squares | Df | Mean Square | F | Sig. |
| Between Groups | 2.180 | 2 | 1.090 | 109.000 | **.000** |
| Within Groups | .060 | 6 | .010 | | |
| Total | 2.240 | 8 | | | |

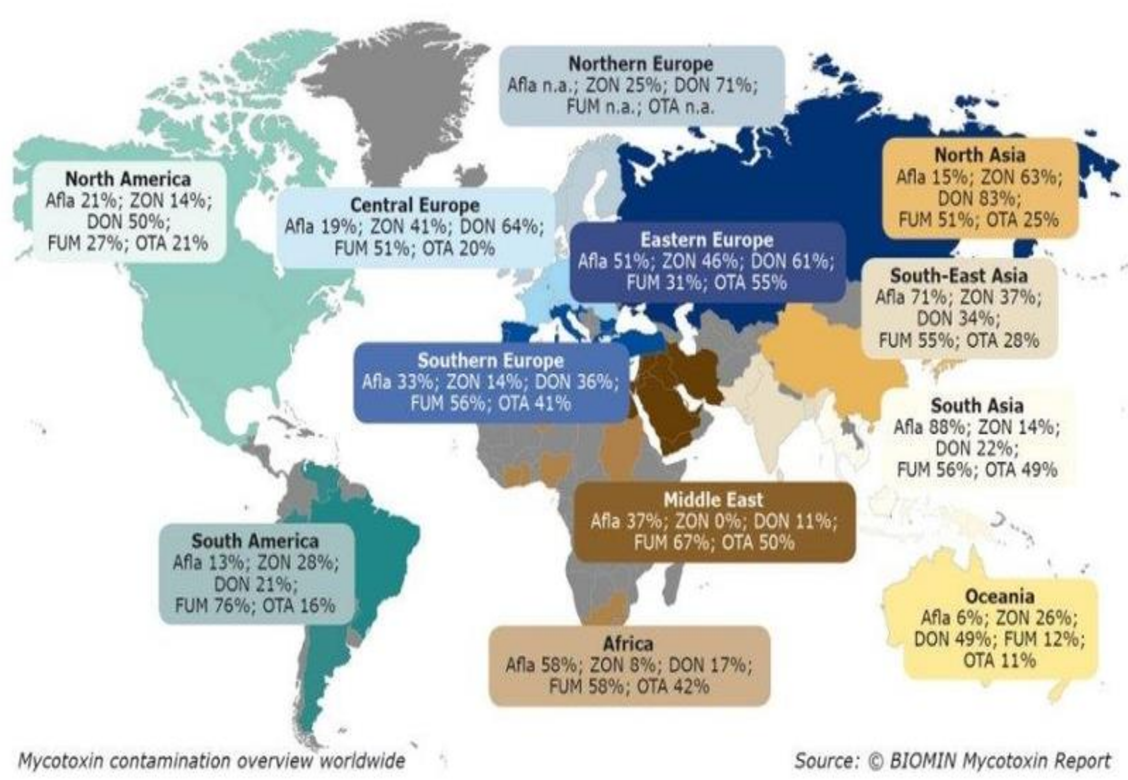
$P<0.05$ thus concentrations are significantly different

**Appendix 20. Post Hoc Analysis: Aflatoxins in Maize Grain Samples between Stores in Western**

| Comparison of Aflatoxin in Eastern_Tukey HSD | | | | | | |
|---|---|---|---|---|---|---|
| (I) Eastern | (J) Eastern | Mean Difference (I-J) | Std. Error | Sig. | 95% Confidence Interval | |
| | | | | | Lower Bound | Upper Bound |
| 1st Store | 2nd Store | 1.08000 | 1.65167 | **.964** | -3.8624 | 6.0224 |
| | 3rd Store | -7.04000* | 1.65167 | .003 | -11.9824 | -2.0976 |
| | 4th Store | -5.83000* | 1.65167 | .016 | -10.7724 | -.8876 |
| | 5th Store | -10.01000* | 1.65167 | .000 | -14.9524 | -5.0676 |
| 2nd Store | 3rd Store | -8.12000* | 1.65167 | .001 | -13.0624 | -3.1776 |
| | 4th Store | -6.91000* | 1.65167 | .004 | -11.8524 | -1.9676 |
| | 5th Store | -11.09000* | 1.65167 | .000 | -16.0324 | -6.1476 |
| 3rd Store | 4th Store | 1.21000 | 1.65167 | **.946** | -3.7324 | 6.1524 |
| | 5th Store | -2.97000 | 1.65167 | **.402** | -7.9124 | 1.9724 |
| 4th Store | 5th Store | -4.18000 | 1.65167 | **.123** | -9.1224 | .7624 |
| *. The mean difference is significant at the 0.05 level. | | | | | | |

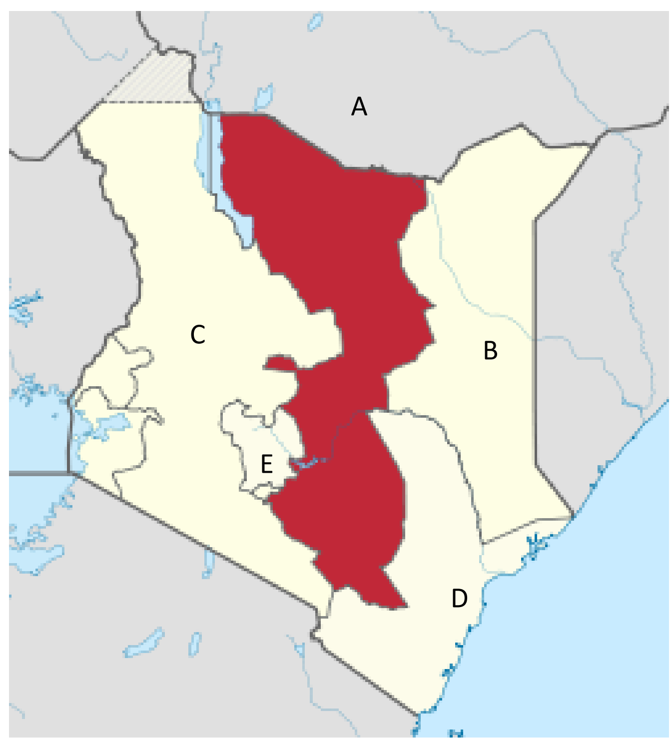
*Highlighted p-values shown similarities

**Appendix 21. Prevalence of Mycotoxins across the World between January and December 2011**




Afla-aflatoxins; DON-deoxynivalenol; FUM-fumonisins; OTA-ochratoxins; ZON-zearalenone.
Source: (Wambui et al, 2017, Petkova sheva et al 2014, Hamid *et al.,* 2013)

**Appendix 22. Eastern Kenya Geographical Map**




Key

A – Ethiopia
B – North eastern Kenya
C- Rift valley
D- Coast
E-central

**Appendix 23. List of Publications**


1. Nduti, N., McMillan, A., Seney, S., Sumarah, M., Njeru, P., Mwaniki, M. & Reid, G. (2016). Investigating probiotic yoghurt to reduce an aflatoxin B1 biomarker among school children in Eastern Kenya: Preliminary study. *International Dairy Journal,* 63: 124-129.
2. Nduti, N., Njeru, P., Mwaniki, M. & Reid. G. (2017). Aflatoxin variations in Maize flour and grains collected from various regions of Kenya. *African Journal of Food, Nutrition and Development, 17*(1):11743-11756.
3. Nduti, N. N., Reid, G., Sumarah, M., Hekmat, S., Mwaniki, M. & Njeru, P. N. (2018). *Weissella Cibaria* NN20 Isolated from fermented kimere shows ability to sequester AFB1 *in vitro* and ferment milk with good viscosity and pH in comparison to yoghurt. *Food Science and Nutrition Technology*, *3*(1): 000137-000149.

**Appendix 24. Conference Abstracts**

1. Nduti, N., Reid, G., Sumarah, M. & Hekmat. S. (2016). *Weissella cibiria* NN20 isolated from fermented *Kimere* product in Kenya: its ability to sequester AFB1 *in vitro* and ability to prepare probiotic Yoghurt. *The Third TU-K International Conference on Innovative Technologies for Development,* Nairobi, February, 23-25, 2016.
2. Nduti, N., McMillan, A., S., Seney, Sumarah M., Njeru, P. & Mwaniki, M. Reid. G. (2016). Investigating probiotic Yoghurt to reduce an aflatoxin B1 biomarker among school children in Eastern Kenya: Preliminary study. *Society for the Advancement of Science in Africa,* Nairobi, August 22-26.
3. Nduti, N., Njeru, P., Mwaniki, M. & Reid, G. (2016). Aflatoxin variations in maize flour and grains collected from various regions of Kenya. *Fostep-K conference,* Nairobi, March 28-31.