HYGIENE PRACTICES OF VENDORS AND QUALITY OF GRASSHOPPER (*Ruspolia differens*) PRODUCTS SOLD IN OPEN MARKETS OF UGANDA

By

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DEPARTMENT OF FOOD SCIENCE, NUTRITION, AND TECHNOLOGY

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DECLARATION

This dissertation is my original work and to the best of my knowledge has not been presented for an award in any other institution

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DEDICATION

I dedicate this study to my ever loving and supportive family. My parents, Mr. and Mrs. Mugo, my siblings, Carol, Lee and JohnMark and to my husband Steve and daughters, Wambui and Njoki.

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LIST OF ACRONYMS AND ABBREVIATIONS

EI – Edible insects

WHO – World Health Organization

FAO – Food and Agriculture Organization

UN- United Nations

LAB – Lactic Acid Bacteria

TVC- Total Viable Count

EFSA - European Food Safety Authority

FDA –Food and Drug Administration

FSANZ- Food Standards Australia NewZealand

MDG –Millenium Development Goals

PCR – Polymerase chain reaction

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ABSTRACT

The edible grasshopper, *Ruspolia differens* Serville locally known as "*Nsenene*" is a major delicacy in Uganda, providing food and income to many households. Grasshoppers are harvested from the wild, mainly in the Central and Western parts of Uganda and transported to urban markets for processing and sale. Both fresh and cooked grasshoppers are processed and sold along the streets, in open air environments that make them prone to contamination. The aim of this study was to investigate the characteristics of the grasshopper vendors, the microbial status of the marketed grasshoppers and to conduct a shelf life study of boiled and dried grasshoppers. In the first objective, cross-sectional data on vendor characteristics were collected from 74 grasshopper vendors, in 12 major markets in Kampala and Masaka districts of Uganda. For the second objective, 25 samples of fresh, deep-fried and boiled grasshoppers sold by the respective vendors were collected for microbial analysis using standard plating techniques and molecular tools. As the third objective, an additional 3kg of raw unprocessed grasshoppers was obtained from vendors and these were boiled for 10, 15 and 20 minutes and further oven and sun-dried. The samples were then packaged in Kraft paper bags with a viewing window and their shelf life monitored against the following parameters: Total viable counts (TVC), oxidative rancidity and sensory characteristics.

Our research findings showed that 62% of the vendors were mobile street vendors while 38% were stationary market vendors. Of these, 68% of the vendors were women, half of whom had studied up to primary school level. Furthermore, 85% of the vendors did not have a public health food handler's certificate. Vendors also scored poorly on personal and food hygiene practices examined (48 and 52% for street and market vendors, respectively) Microbial analysis of marketed grasshoppers (raw, deep-fried and boiled) showed high counts of total aerobic bacterial load (4.3-9.5 Log cfu/g), Enterobacteriaceae (4.6-9.3 Log cfu/g) and

yeasts and molds (3.5-7.9 Log cfu/g). These levels were above the acceptable limits for ready-to-eat marketed foods which stand at < 5 logcfu/g.

Molecular characterization of bacteria and fungi colonies isolated from the grasshoppers revealed 7 pathogenic species of bacteria, 3 of which are known pathogens capable of causing illness in otherwise healthy individuals, (*Bacillus cereus, Staphylococcus aureus, Staphylococcus haemolyticus).* In addition 2 types of mycotoxin producing mold were isolated and these included *Aspergillus fumigatus* and *Aspergillus neobridgeri*. These results confirm that there is a risk of foodborne illness after consuming grasshoppers from the streets of Uganda.

For the shelf life study, results show that boiling for 10, 15 and 20 minutes was sufficient to reduce the TVC load to undetectable levels. Accelerated shelf life analysis for 6 days at 55ºC showed that the sample boiled for 20 minutes and oven-dried had a significantly ($p<0.05$) better microbial quality, with a plate count of < 5 log cfu/g up to the 5^{th} day. The Thiobarbituric acid test results showed high lipid oxidation of >1.0 mg MDA/kg of grasshoppers after day 1 of storage. Sensory scores for odour, appearance and general acceptability had mean scores of 4.48±1.446, 4.03±1.464, and 4.31±1.400 respectively, indicating that the panelists neither liked nor disliked the samples during the course of storage. These findings demonstrate that boiling for 20 minutes and oven drying is the most preferred processing method to ensure microbial safety of grasshoppers for up to an equivalent of approximately 6 months. In conclusion, there is a dire need for improvement of the microbial quality of processed and unprocessed *R. differens* grasshoppers marketed in Uganda. This study recommends the training of grasshopper vendors on good food hygiene practices as well as on optimum processing techniques and the creation of specific local and national policies as well as regulations that governs food safety in the edible "*nsenene*" subsector in order to provide safe insect-based food products to consumers.

CHAPTER ONE: INTRODUCTION

1.1 BACKGROUND

Entomophagy is a term used to describe the dietary consumption of insects by any organism, but it is commonly used to refer to the consumption of insects by humans (Shockley *et al*., 2014). Over 1900 species of insects are consumed in many countries of the World. The insects are largely collected from the wild during their swarming seasons (Van Huis, 2003; Raheem *et al*., 2018; Kelemu *et al*., 2015) but recently domesticated rearing of insects is gaining traction in many countries of the World.

The Longhorn grasshopper *Ruspolia differens* Serville (Orthoptera: Tettigoniidae) is a major delicacy in the Lake Victoria regions of Uganda, Tanzania and Kenya (Mmari *et al.,* 2017). In Uganda where this study is focused the insect is locally known as '*nsenene'*. The insect is harvested from the wild during the swarming seasons of April to June and November to January, mainly in the Central and Western parts of the country. During the swarming seasons, the insects create a profitable microenterprise involving harvesting, processing and marketing of fresh, and processed insects (Ndimubandi *et al.*, 2018). The processing and vending are done in open air markets by street food vendors where the insects are exposed to environmental contaminants that may lead to microbial pathogens that easily act as vectors of food-borne illnesses.

As with other fresh foods with high water contents, fresh harvested grasshoppers are highly perishable with very short shelf-life of about 12 to 48 hours at ambient temperatures (Wilfred, 2017). As a result, different processing methods are used by the vendors to preserve them , including deep-frying, sun-drying, boiling and pan-frying (Biryomumaisho, 2012). These processing and preservation are based on cultural experiences and thus, they may lack the scientific knowledge with regard to technological and hygiene practices (Mmari *et al.*, 2017).

Preparation and processing, and selling in open market and in streets makes the products prone to contamination with soil, debris, and microorganisms.

This study was therefore designed to establish the characteristics of vendors, their knowledge and practice of hygiene, product diversity and the microbial status of the edible grasshopper sold in Uganda.

1.2 PROBLEM STATEMENT

Studies carried out previously show that insects carry a significant amount of micro flora on them. This is evidenced by a study carried out in Botswana which revealed the presence of mycotoxin producing mold species such as *Aspergillus*, *Fusarium* and *Penicillium* spp. in sundried mopane worms (Lepidoptera: Saturniidae) as well as Aflatoxins (Mpuchane, 1996). Another study carried out in Nigeria on degutted, washed, spiced, roasted and sun-dried *Bunaea alcinoe* larvae (Lepidoptera: Saturniidae) reported the presence of *Pseudomonas* and *Proteus* spp. as well as toxigenic *Staphylococcus aureus*, *Bacillus cereus* and *Escherichia coli* (Braide *et al*., 2011). In addition, some grasshoppers are also known to be vectors of some tapeworm species and horsehair worms which have been known to infect humans (Hill *et al*., 2012).

These findings imply the possibility of inadequate processing, as well as improper handling and careless exposure of the insects products to environmental contaminants by street food vendors (Banjo *et al*., 2006), leading to post-processing contamination such as *Staphylococcus* spp. reported in heat-processed insects in Nigeria (Opara, 2012). Since the grasshoppers understudy are wild harvested, little is known about the food hazards that they carry. In addition, there is a risk of microbial contamination of insect products as a result of the open air and the road side environment where they are marketed as well as lack of proper food safety knowledge by vendors, as seen in previous studies by Banjo *et al*., (2006).

This study therefore investigated the vendor characteristics and microbial hazards associated with wild harvested grasshoppers (*R. differens*) which are widely processed, marketed and consumed in Uganda. The study also evaluated different boiling and drying methods currently used, to establish how effective they are in reducing microbial load of grasshoppers and prolonging the shelf life. Given the potentially diverse use of insects as food in combating nutritional deficiencies and food insecurity in East Africa and their current social and economic importance to communities, the scientific assessment of postharvest measures to ensure their safety along the value chain is crucial.

1.3 JUSTIFICATION

This study will be useful to policy makers, consumers and all the stakeholders in the *nsenene* industry because it will highlight the status of food hygiene and sanitation among the vendors, the microbial safety of the ready-to-eat grasshoppers and molecular analysis will also give a first insight on the microbial diversity of processed edible grasshoppers. This information will form a basis for training on good hygiene and sanitation practices among the vendors in Uganda where the insect is widely consumed and commercialized, but will also be useful to other countries where *nsenene* consumption is gaining popularity. Establishing the optimum processing techniques that will ensure microbial safety of insect products and prolong their shelf life, will not only preserve the health of the consumer, but also enable vendors to sell processed grasshoppers for a longer period of time during the season thus becoming a valuable source of extra income.

1.4 OBJECTIVES

1.4.1 Overall Objective

The overall objective is to investigate the hygiene knowledge and practices of vendors, to assess the microbial status of edible grasshoppers currently commercialized in Uganda and the shelf life of processed insect products.

1.4.2 Specific Objectives

1. To determine the socio-demographic characteristics of the vendors of *nsenene* in Uganda, their practices on post-harvest insect handling and, food hygiene and sanitation practices.
2. To evaluate the microbial characteristics of the marketed *nsenene* products using classical plating techniques coupled with molecular tools.
3. To investigate the boiling and drying techniques that will lead to the highest microbial quality of edible grasshoppers and result in the longest shelf life.

1.5 Research Questions

1. What are the vendors' socio-demographic characteristics and what are their general practices in post-harvest handling and sanitation of insects?
2. What are some of the food-borne pathogens present in marketed edible grasshoppers?
3. How do these different processing techniques i.e. boiling and oven drying and boiling and sun drying, affect the shelf life and sensory characteristics of the edible grasshoppers?

CHAPTER TWO: LITERATURE REVIEW

2.1 EDIBLE INSECTS AND THEIR CONSUMPTION

2.1.1 A Global Perspective

The practice of eating insects has been going on for hundreds of years in many parts of the world, as a delicacy/part of a diet or as emergency food in times of scarcity (FAO, 2010). As many as 3071 ethnic groups in 130 countries utilize insects as essential elements of their diet (Ramos-elorduy *et al.*, 2009). Entomophagy is the term used to refer to dietary consumption of insects by humans or any other organism. (Dossey, 2014).

The traditional use of insects as food is widespread in Africa, Asia, and South America while Europe is slowly gelling up to the idea. In the countries where Entomophagy is common, the practice continues to be widespread as it provides significant nutritional, economic, and ecological benefits for some rural communities (Dossey, 2014).

Insects are widely accepted as a delicacy in Africa, Asia, and South America. However, in the European society, edible insects have long been rejected as food and their safety has been questioned (Raheem *et al.*, 2018), probably due to limited scientific research along their value chain.

2.1.2 The Legal Status of Edible Insects in the World

According to Reverberi (2017), the European Food Safety Authority (EFSA) considers edible insects (EI) as a novel food source (Raheem *et al*., 2018), which should then be subject to approvals that may take up to three years. Some countries, however, do not subscribe to this and allow regulated rearing and/or marketing of EI. These countries include Netherlands, Denmark, Belgium, Norway, Switzerland, Denmark, and Britain. Countries such as Germany and Italy still have zero tolerance for EI.

In America, there are no set standards yet, but the Food and Drug Administration (FDA) allows the marketing of insects specifically bred for consumption. They must, however,

follow the standards required by the FDA for bacteriological tests and Good Manufacturing Practices (GMP) certification. (Reverberi, 2017)

In Australia and New Zealand, the shared food safety agency, FSANZ, have not put any consumption limits for super mealworm (*Zophobus morio*) (Bettle; Darkling Bettle) the domestic cricket (*Acheta doemsticus*) (Orthoptera; cricket), and the meal worm larvae (*Tenebrio molitor*) (Bettle; Darkling Bettle). They are not considered novel foods and no food safety issues have been encountered with their consumption (Reverberi, 2017).

In non-western countries particularly Asia, Entomophagy is traditionally practiced, but there are no regulations present regarding their marketing and consumption. Thailand being the largest cricket breeder in the world, it is set to work on the first breeding guidelines for crickets. China is well known for silkworm (Endopterygota; Bombycidae) production and the silkworm pupae were in 2014 added to the list of foods allowed by the Ministry of Health (Reverberi, 2017).

2.1.3 Diversity of Insects Consumed

More than 1900 insect species are reported to be consumed globally (van Huis, 2013). About 679 species are recorded in North and South America, 524 species in Africa, 349 species in Asia, 152 in Australia and 41 in Europe (FAO, 2010). The highest numbers of edible insects are consumed in Mexico followed by Thailand, Congo, India, Australia, China and Zambia (Blásquez *et al.*, 2012). The species predominantly consumed belong to the Order Coleoptera (beetles) which make up 40 % of all insect species and 31% of all species consumed. Second in line are insects of the order Lepidoptera (caterpillars) which make up 18% of the edible insects' species. Hymenoptera consisting of bees, ants, and wasps come in third at 14% followed by Orthoptera consisting of grasshoppers, locusts and crickets at 13%. Following these we have Hemiptera (scales insects, planthopper and leafhoppers) making up 10% of

species consumed, Isoptera (termites) at 3%, Odonata (dragonflies) at 1%, Diptera (flies) at 2% and others at 5%. (Cerritos, 2009; Agbidye *et al.,* 2009; van Huis, 2013).

Different insects are consumed at different stages of maturity. Lepidopterans are consumed as caterpillars while Hymenopterans are harvested and eaten mostly in their larval or pupal stages. Coleopterans are eaten at both adult and larval stages, while the Orthoptera, Isoptera and Hemiptera orders are eaten as mature adults (Raheem *et al.,* 2018).

2.1.4 The Edible insect *Ruspolia Differens* Serville

The edible grasshoppers *R. differens* Serville found in Uganda, scientifically known as *Homorocoryphus nitidulus vicinus,* is a long-horned grasshopper of the Tettigoniidae family (Paul *et al.,* 2016; FAO, 2013). *Ruspolia differens*, locally known as '*nsenene* or *senene'* have long been part of the diet culture for communities residing along the shores of Lake Victoria in Kenya Uganda and Tanzania (FAO, 2013). It is also considered as a delicacy in central and Southern Africa (van Huis, 2003). Other widely eaten insects in Uganda include termites, white ants, and crickets (Agea *et al.,* 2008).

Harvesting and the mode of collection depend on the behavior of the insect which is influenced by environmental factors such as temperature as reported by van Huis (2003). *Nsenene* is mainly sold fresh hence they have a short shelf life (Agea *et al.,* 2008), hence the importance of conducting the proposed research study to potentially extend its shelf life. These insects are usually abundant during the rainy seasons. This is because grasshopper eggs only develop in wet conditions (Wilfred, 2017). Traditionally, grasshoppers are collected early in the morning by the women and children and majority of the time consumed by men although this has since changed and all women and people of all age groups are allowed (Wilfred, 2017). In Uganda, grasshoppers contribute about 16,100 Kcal and 513 g of protein per person per annum (Mbabazi, 2011).

2.2 NUTRITIVE VALUE OF EDIBLE INSECTS

According to a report by FAO on the state of food security and nutrition (Resilience *et al.*, 2017), food insecurity seems to be on the rise again with 815 million people estimated to be undernourished in 2016 up from 777 million in 2015. Wasting continues to threaten the lives of almost 52 million children (8 percent) and almost one-third (33 percent) of women of reproductive age worldwide suffer from anemia.

The United Nations (UN) has placed heavy emphasis on alleviating hunger and malnutrition in children as was elaborated in the Sustainable Development Goals (SDGs) which were set to be achieved by 2015. The first SDG is to "eradicate extreme poverty and hunger," and the fourth is to "reduce child mortality rates'' and number 7 on the SDG list is "ensuring environmental sustainability'' (Dossey, 2013). For this reason, FAO has taken the initiative and proposed a program of feeding people with alternative food sources, including insects (Gahukar, 2014).

Studies have shown that edible insects in general and species from the order Orthoptera (grasshoppers, crickets, locusts) in particular are rich in proteins and represent a valuable alternative protein source (Rumpold and Schlüter, 2013a). Research by Kinyuru (2010) on grasshoppers (*Ruspolia differens)* showed high protein content of 43.1% for green morphotype and 44.3% for brown morphotype in comparison to common lean red meat as reported by (William, 2007), whereby beef contains 23.2% protein, 24.8% for veal and 21.5% for mutton.

The protein quality of insects, as measured in terms of chemical score, protein digestibility, protein efficiency ratio (PER) compared favorably to casein and soy but has variations and can be improved by the removal of the chitin. In addition, most edible insects provide satisfactorily the required essential amino acids (Rumpold and Schlüter, 2013a).

Feeding trials of spent silkworm pupae indicate a higher chemical score regarding food intake, weight gain, protein digestibility, PER, and net protein utilization (NPU). The chemical score of the spent silkworm pupae protein was 60 in comparison to 100 for whole egg protein (Rao, 1994). In evaluating the protein quality of different cricket meals fed to rats, it was observed that proteins from both cricket meals tested (*Acheta domesticus* and *Anabrus simplex*) were equal or superior to soy protein as an amino acid source. All insect orders are generally found to meet the requirements of the WHO for amino acids. High values have been obtained for phenylalanine + tyrosine and some insects are rich in tryptophan, lysine, and threonine.

Most edible insects show high zinc contents. Especially species of the order Orthoptera (grasshoppers, crickets, locusts) implying it could function as zinc supplementing food (ingredients). The cricket *Onjiri mammon* and several termites from Kenya are high in iron (van Huis, 2013). Kinyuru (2009) confirmed iron was the most abundant trace mineral in the termite with a value of 11.52 ± 0.92 mg/100g. Insects could partially contain much more iron and calcium than beef, pork and poultry (FAO, 2010). However, more research is still required on the bioavailability of iron in edible insects (Rumpold and Schlüter, 2013a).

The fat content of food insects is variable among species, but the highest values are found in termites and palm weevil larvae. The saturated/unsaturated fatty acid ratio of most edible insects is less than 40%, comparing favorably with poultry and fish, although the content of polyunsaturates, linoleic and linolenic acids, is higher in insects (DeFoliart, 1991).

Grasshoppers, locusts, crickets, and beetles are particularly rich in folic acid but are not an efficient source of Vitamin A, C, niacin, and thiamine. Furthermore, EI are generally rich in Riboflavin, Pantothenic acid, and biotin (Rumpold and Schlüter, 2013a).

Howwever, edibe insects have been shown to contain some antnuutrients and allergens which could be a potential risk. Pupae of the African silkworm (*Anaphe venata*) for example,

contain a heat-resistant thiaminase and can cause thiamine deficiency (Rumpold and Schlüter, 2013b). This has been linked to the seasonal annual thiamine deficiency that has plagued Nigeria for the past 40 years. Four types of edible insects were analyzed for the antinutrients hydrocyanide, oxalate, phytate, and tannin but were found to be generally far below the toxic levels for human consumption (Ekop, 2010). Other studies also on anti-nutritional components of *Cirina forda* (Westwood) yielded low levels of oxalate and phytic acid within nutritionally accepted values and in no tannin (Omotoso, 2006).

It has been reported that insects can cause allergic reactions (Phillips, 1995) and can contain toxic substances (Berenbaum, 1993). Furthermore, it has been discovered that insects just like other arthropods (e.g., shellfish) can cause allergic reactions. These are caused by injectant allergens (bees, wasps, and ants), contact allergens, inhalant allergens, and/or ingestion. Contact and inhalant allergens are common in insect rearing industry where people have been reported to suffer from rhinitis, asthma, and dermatitis (Paul *et al.,* 2016).

2.2.1 The Potential of Insect consumption to improve protein intake in Africa

Apart from consuming insects in their whole and recognizable form, they can be processed into insect products such as powders and or extracts such as protein isolates (Klunder *et al.*, 2012). These insect powders or extracts have the potential to be used for the enrichment of protein-deficient foods and feed as an alternative to soy or animal protein. Termite flour, for example, has been used in the enrichment of sorghum flour in Kenya and other countries as well (Klunder *et al.,* 2012). *Ruspolia differens* has been useful in increasing heme iron and retinol when used in the enrichment of sweet potato-based complementary foods (Wilfred, 2017).

2.3 MICROBIAL CONTAMINATION OF INSECT PRODUCTS

2.3.1 Bacteria and Fungi Contamination

Insects are processed in many different ways but the majority of the time they have their gut intact during processing and this may affect the microbiological quality of the food (Klunder *et al.,* 2012). Several studies have shown that insects carry an appreciable amount of microflora and these include the presence of mycotoxin producing mold pathogenic species such as *Aspergillus, Fusarium* and *Penicillium* spp. in sundried mopane larvae, as well as Aflatoxins in Botswana (Mpuchane, 1996). Another study carried out in Nigeria on degutted, washed, spiced, roasted and sun-dried *Bunae alcinoae* larvae reported the presence of *Pseudomonas* and *Proteus* spp. as well as toxigenic *Staphylococcus aureus*, *Bacillus cereus,* and *Escherichia coli* (Braide *et al.,* 2011). These findings imply inadequate processing, improper hygiene, careless exposure to the environment and consequently shorter shelf life of insects even when their gut is removed.

Apart from the pathogenic microorganism (PMOs), spoilage microorganisms were also found to be present in grasshoppers. The bacterial community was mainly dominated by two species of Lactic acid bacteria (LAB); *Weissella* and *Lactococcus* spp. and one of the Enterobacteriaceae (*Yersinia*/*Rahnella*). Additionally, LAB (*Enterococcus*) and the Enterobacteriaceae (*Klebsiella*/*Enterobacter*), were found to be abundantly present in grasshoppers. Altogether, LAB and Enterobacteriaceae were found to represent more than 88.5% of the bacterial sequences obtained in grasshoppers (Stoops *et al.,* 2016).

Screening that was done on fresh Black Soldier Fly (Endopterygota; soldier fly) and crickets were positive for *Escherichia coli, Salmonella typhi, Staphylococcus aureus,* fecal coliforms, yeasts, and molds. Similar results were obtained when boiled at 96^{o} C for 1 or 2 minutes or toasted at 15^{o} C for 1 minute. However, when boiled for 5 min or more or toasted for 2 min and above, the insect materials were free from microbes (Fiaboe and Nakimbugwe, 2017).

This shows that an optimum cooking level should be determined for different edible insect species.

Both processed and unprocessed EI carry a significant amount of bacterial and fungal pathogens as depicted in studies by (Banjo *et al.*, 2006; Klunder *et al.*, 2012; Haubruge *et al.*, 2017; Mpuchane, 1996) and more. Due to the presence of these bacteria and fungi, it is likely that the toxins associated with them are also present in these EI. However, to the author's knowledge, limited studies have been carried out to establish the presence of bacterial toxins. As for aflatoxins and other fungal toxins, they are rarely detected in fresh insects as was reported in a study conducted by Fiaboe and Nakimbugwe (2017) on 13 insect samples. Since fungal toxins usually occur during long term storage in temperature and moisture abused conditions, they are likely to be a risk in EI that have been dried and stored for future consumption.

2.3.2 Parasitic Microorganisms in Edible Insects

'Some species of grasshoppers serve as intermediate hosts to several avian parasites and horsehair worms, including several species that have been reported as accidentally infesting humans' (Fink, 2004). Some North American, South American and Caribbean species of grasshoppers are known as vectors of *Tholera americana* (Lepidoptera: Noctuidae) through ingestion of feces of avian hosts which carry the eggs. These become infective 42 days after entering the grasshopper's system and are distributed in all body parts of the insect. This is, therefore, a problem when grasshoppers are used as poultry feed since the parasite can cause anemia and severe weight loss in the birds (Paul *et al.*, 2016). In addition, horse hairworms develop as parasites in grasshoppers and may cause illness in humans who consume them (Hill, 2012).

CHAPTER THREE: STUDY DESIGN AND METHODOLOGY

3.1 STUDY DESIGN

This study adopted a descriptive cross-sectional approach with an analytical component, and was carried out in three phases:

Phase 1: Baseline field survey

This was done to establish vendors' socio-demographic characteristics, post-harvest handling techniques and food hygiene and sanitation practices.

Phase 2: Microbial Analysis

Grasshoppers in the categories of: fresh, boiled, and deep fried were analyzed both qualitatively and quantitatively for: Total viable count (TVC), Enterobacteriaceae, and Yeasts and molds. Further, molecular identification of resulting colonies was carried out.

Phase 3: Processing and Shelf Life Evaluation

A modification of the boiling and drying methods used by *nsenene* vendors was simulated in the lab to establish the method that resulted in the insect's products with the lowest microbial contamination followed by an accelerated shelf life analysis to establish the shelf stability of the insects.

3.2 METHODOLOGY

3.2.1 Study Setting

This study was carried out during the period of June – July 2018 in Kampala and Masaka districts of Uganda, where *nsenene* swarming was observed. Masaka district was purposively selected for this study as it is the main swarming region, but also grasshoppers marketed along the main road by street vendors. Similarly, Kampala was purposively selected because it is the main marketing city for *nsenene* that are collected from the major swarming areas. A total of 11 market locations in Kampala and 1 market in Masaka were selected for the study on the basis of presence of edible grasshopper vendors, according to informant recommendations. These markets are Nakasero, Busega, Bwaise, Old Taxi Park, Ndeeba, Nateete, Katwe, Karlewe, Namugoona, Kibuye and Owino in Kampala and Ngendo market in Masaka (Figure 1). Kampala is the national and commercial capital city of Uganda lying between geographical coordinates of 1°00'N, 32°00'E. The city covers an area of 181 Km^2 and stands at an elevation of 1,190 m above sea level and has a projected population of 1.65 million in 2019 (Uganda National Bureau Of Statistics, 2017).

Masaka town is situated in Central Uganda on the West of Lake Victoria, about 140 Km from Kampala. It is close to the equator with coordinates of 0°20'28.0"S, 31°44'10.0"E and within a latitude of -0.341111 and Longitude of 31.736111. The town had a population of 297,004 in the 2014 Uganda national census and covers an area of 1298 Km2 (National Population and Housing Census, 2017). It is the major swarming area for edible long-horned grasshoppers because it is one of the wettest districts in Uganda with an average annual rainfall of 1174mm.

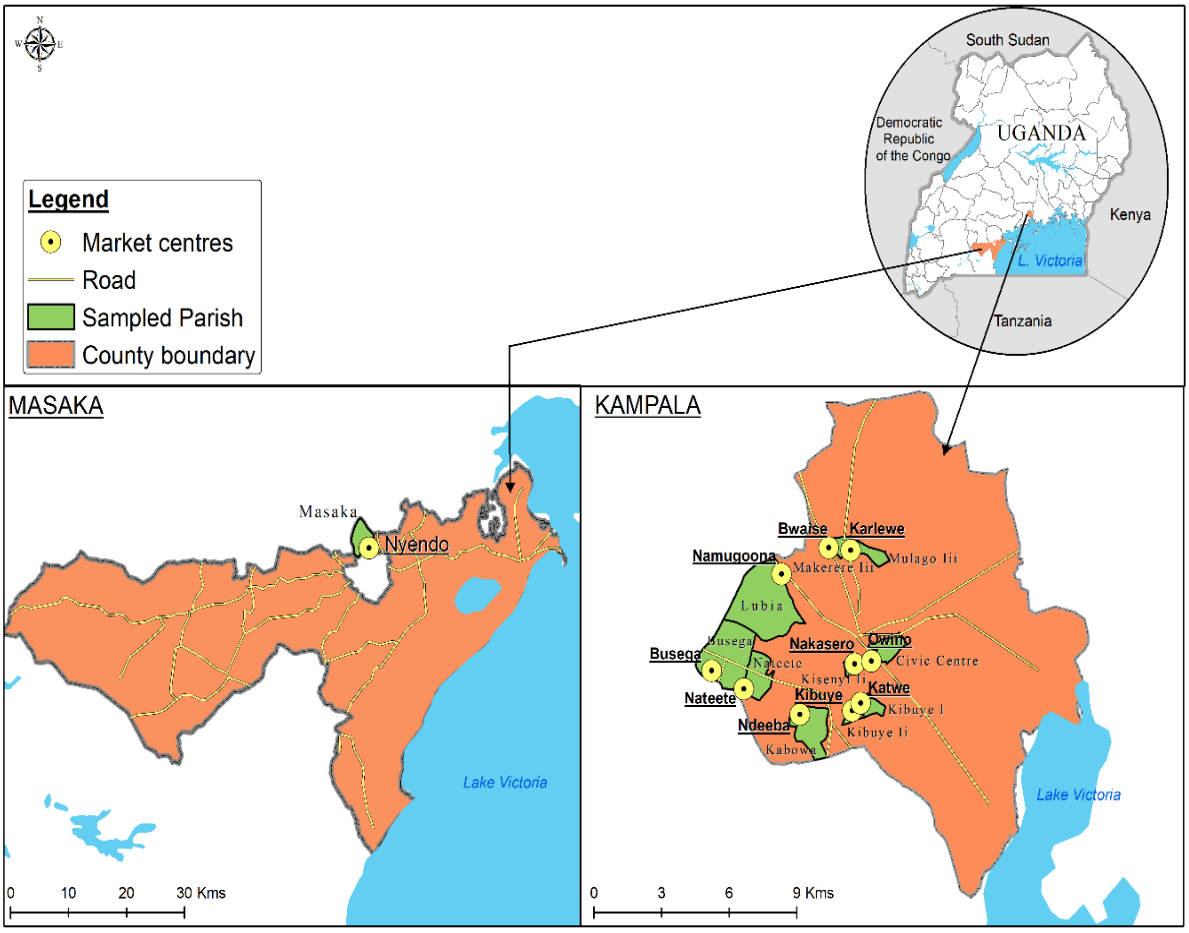

Figure 1: Map showing markets in Kampala and Masaka

Source: GPS Coordinates

3.2.2 Phase 1: Baseline Field Survey

The descriptive component involved an interview-based survey of grasshopper vendors to assess their knowledge of food and personal hygiene and sanitation in insect processing and marketing. Sampling of vendors was exhaustive as the population was less than 100 vendors. A census of all the vendors present per market location was conducted and all the vendors present were interviewed as illustrated in Figure 2. A total of 74 respondents who processed and sold edible grasshoppers along the streets or in stationary market stalls were selected for this study.

A semi-structured, pre-tested questionnaire (Appendix 2) was used as the data collection tool in the descriptive study. The questionnaire was divided into three sections. The first section

focused on the socio-demographic characteristics of the vendors, i.e. age, gender, level of education and length of time in the grasshopper vending business and the ownership of the business. The second section contained specific questions to test the respondents' knowledge on food safety aspects such as quality and hygiene factors considered when purchasing raw grasshoppers, cleaning and sorting of grasshoppers, preservation and storage and frequency of changing deep frying oil as well as their knowledge on foodborne illnesses. The third section was an observation checklist to score the personal and food hygiene and sanitation practices of vendors and their working environment. The practice score was an index between 0 and 1 constructed from 13 indicator variables in the checklist. For every good practice, a score of 1 was assigned while 0 was given for poor practice.

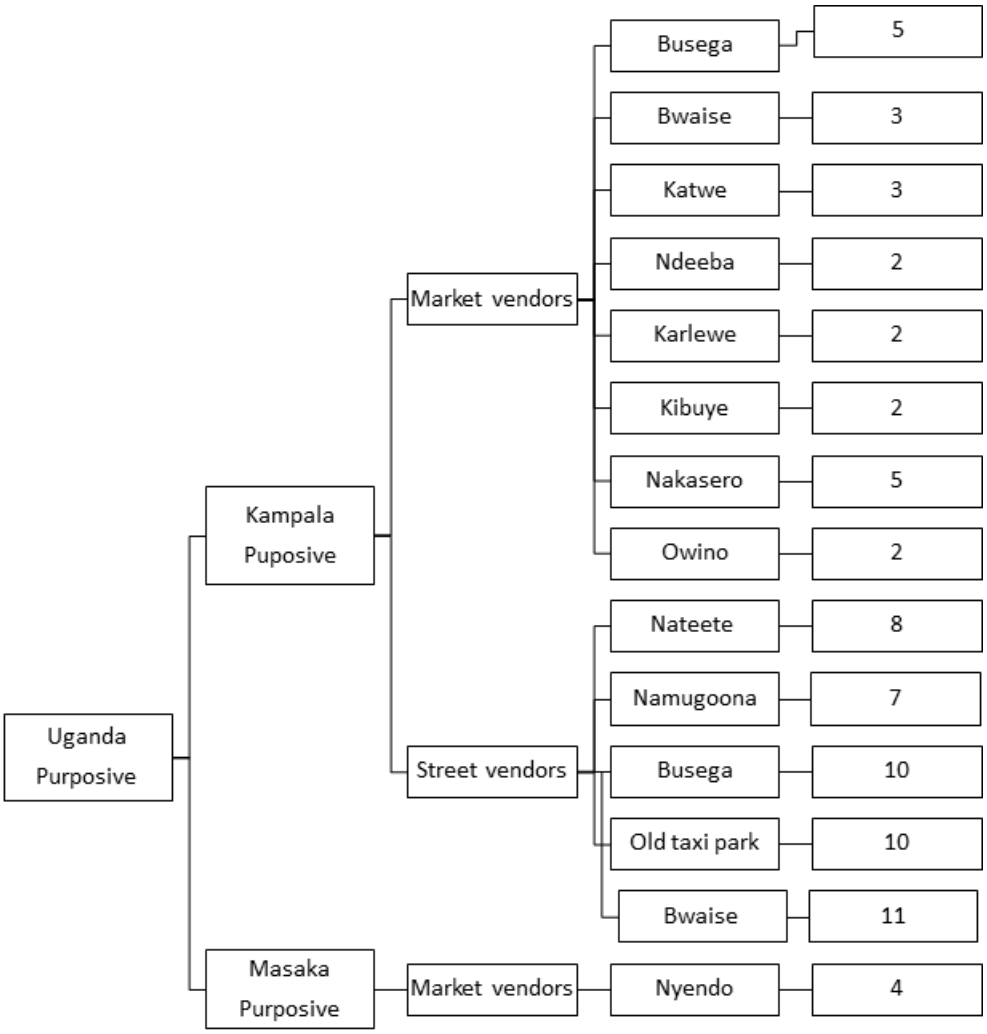

Figure 2: Distribution of vendors in different market locations

3.2.3 Phase 2: Collection of insects samples and microbial analyses

In each market, approximately 250g of either fresh or deep fried or boiled grasshoppers were obtained from all the vendors present in each location. Sampling was therefore done exhaustively in each market from all the vendors present and they were pooled together and categorized according to the length of time they had been stored, the type of vendors that sold them, and the market location. Samples were placed in sterile plastic containers with a seal and transported in dry ice (-20ºC) to the laboratory and transferred in a freezer until time of analyses.

3.2.3.1 Microbial culturing

For the determination of microbial load, plate count methods were used according to food microbiology ISO standards summarized by (Dijk, 2007). About 5g of the sample were weighed using an analytical scale then crushed with a pestle and mortar in mixture with 45ml of sterile distilled triton water (0.05%), to make a 10^{-1} dilution. A 10-fold serial dilution series was done up to 10^{-6}. The samples were analysed in duplicate for three categories of microorganism; Total viable count, Enterobacteriaceae and Yeast and molds. Total viable count (TVC) was determined using Luria Bertani (LB) media which contained 10g Tryptone, 5g Yeast Extract agar, 5g NaCl and 15g Agar per liter of media. These were incubated at 37ºC for 24h. Enterobacteriaceae was determined using Violet red bile glucose agar (VRBGA, Oxoid), incubated at 37 ºC for 24h. Yeasts and molds were determined using Potato Dextrose Agar incubated at 25ºC for 5 days. Using spread plate technique, 100 microliters of sample was spread with a sterile spreader, over media that had solidified on a petri dish and incubated in the respective temperatures. Contamination results were expressed in Log cfu/g.

3.2.3.2 Molecular identification of colonies

Bacterial and fungal colonies obtained through culturing were purified by repeated streaking to obtain 136 pure colonies in total. Each pure bacterial culture obtained was further grown in 10ml of Nutrient Broth (Oxoid, UK), to obtain enough cell quantities for DNA extraction. For fungal DNA extraction, Isolate II Plant DNA extraction Kit (Bioline, UK) was used while for bacterial DNA extraction Isolate II genomic DNA extraction kit (Bioline, UK) was used as per manufacturer's instructions. The extracted bacteria and fungi DNA was quantified using a NanoDrop 2000/2000c Spectrophotometer (Thermo Fisher Scientific, Wilmington, USA). Bacteria and fungi DNA samples were stored at -20°C awaiting Polymerase Chain Reaction (PCR) analysis. For bacterial colonies, an approximately 1450 base pair fragment of the 16SrRNA gene was amplified using 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-TACCTTGTTACGACTT-3') primers (Lane, 1991). Isolated bacterial DNA was amplified in 10µl PCR mix containing 5.65µl PCR water, 2µl My Taq Buffer (Bioline, UK), (5mM dNTPs, 15mM $MgCl_2$, stabilizers and enhancers), 0.5µl of each primer, 0.25µl of 25mM $Mgcl_2$ (Thermo scientific, USA), 0.1µl 1 unit My Taq DNA polymerase (Bioline, UK) and 15ng/l of DNA template. The reaction was set up in a Mastercycler Nexus Gradient thermocycler (Thermo scientific, USA) using conditions as follows: Initial denaturation at 95°C for 2 minutes followed by 40 cycles of denaturation at 95°C for 30 seconds, annealing at 51.9°C for 45 seconds and primer elongation at 72°C for 1 minute. The final extension step lasted for 10 minutes at 72°C. For fungal isolates, an approximately 600 base pair fragment of the internal transcribed spacer region was amplified using ITS4 (5'-GGAAGTAAAAGTCGTAACAAGG-3') and ITS5 (5'-TCCTCCGCTTATTGATATGC-3') primers (Glass and Donaldson, 1995). Isolated fungi DNA was amplified in 10µl PCR mix containing 5.65µl PCR water, 2µl My Taq Buffer (Bioline, UK), (5mM dNTPs, 15mM MgCl2, stabilizers and enhancers), 0.5µl of each primer, 0.25µl of 25mM Mgcl2 (Thermo

scientific, USA), 0.1µl 1 unit My Taq DNA polymerase (Bioline, UK) and 15ng/l of DNA template. The reaction was set up in a Mastercycler Nexus Gradient thermocycler (Thermo scientific, USA) using conditions as follows: Initial denaturation at 95°C for 1 minute followed by 35 cycles of denaturation at 95°C for 30 seconds, annealing at 59°C for 40 seconds and primer elongation at 72°C for 1 minute. The final extension step lasted for 10 minutes at 72°C. PCR amplicons of 16S and 18S rRNA were verified by visualization in 1% (w/v) agarose gel. Successfully amplified 16S and 18S regions were purified using Isolate II PCR and Gel Kit (Bioline) as per the manufacturer's instructions and sent to a commercial sequencing facility (Macrogen Europe BV, Amsterdam, the Netherlands) for Sanger sequencing.

3.2.4 Phase 3: Shelf life Evaluation of the Products

Sample preparation for shelf life evaluation

Approximately 3 kg of fresh, raw *Ruspolia differens* grasshoppers were obtained from collectors around Ndeeba region of Kampala and transported back to the lab in dry ice (-20ºC). They were kept in a freezer until the time of processing. As shown in Figure 3, processing started by washing the grasshoppers 3 times thoroughly in twice the volume of clean potable water each time. Grasshoppers were then boiled in 3 batches of 720g. One batch was boiled at 100ºC for 10 minutes, the second batch for 15 minutes and the third batch for 20 minutes. To each 720g batch, 1% salt was added during boiling. The grasshopper samples were then drained and cooled and the batches were divided into two sub-batches for each boiling treatment (Figure 3). One sub batch was oven-dried at 55 ºC for 24 hrs. (Klunder *et al.,* 2012), while the other sub-batch for each boiling treatment was sun-dried inside a screen house at temperatures of 27-32 ºC for 36-48 hours (Mmari *et al.,* 2017). After both sun drying and oven drying, the grasshoppers were packaged in waxed Kraft paper bags with a viewing window. The samples were then assessed for shelf life using the accelerated shelf

life technique. They were stored at 55 ºC whereby one day at 55 ºC represents 37 days at ambient temperature when calculated using the Arrhenius equation, as described in (Shema, 2018).

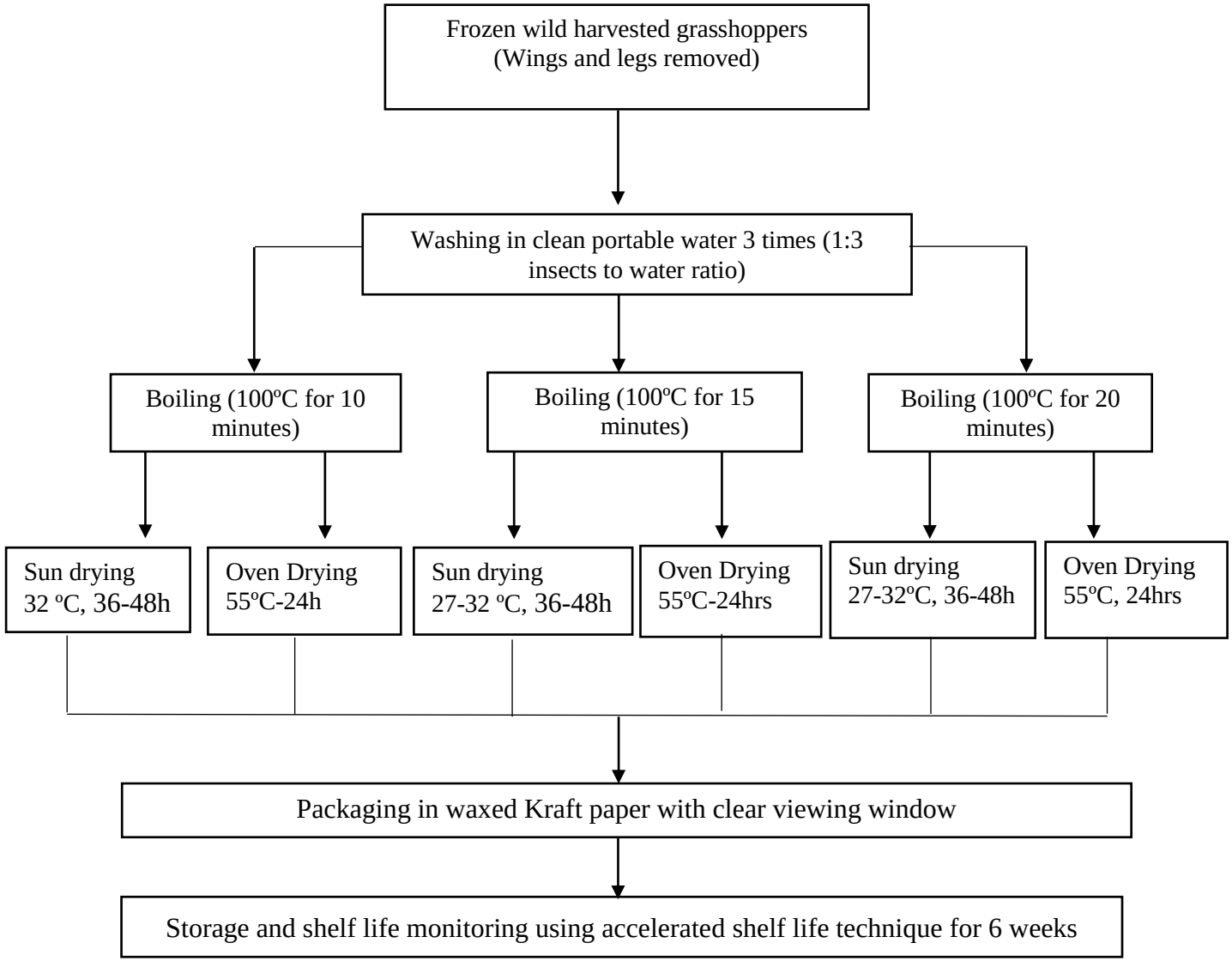

Figure 3: Framework for Grasshopper processing, packaging and shelf life monitoring

Shelf life stability monitoring

3.2.4.1 Microbial analysis

Total viable count (TVC) was determined using plate count methods according to food microbiology ISO standards summarized by (Dijk, 2007). About 5g of the sample were weighed using an analytical scale then crushed with a pestle and mortar in mixture with 45ml of sterile distilled triton water (0.05%), to make a 10^{-1} dilution. Total viable count (TVC) was determined using Luria Bertani (LB) media which contained 10g Tryptone, 5g Yeast Extract agar, 5g NaCl and 15g Agar per liter of media. These were incubated at 37ºC for 24h. Using spread plate technique, 100 microliters of sample was spread with a sterile spreader, over media that had solidified in a petri dish and incubated. All samples were analyzed in duplicate and results expressed in Log cfu/g.

3.2.4.2 Thiobarbituric Acid Reactive Substances analysis

The Thiobarbituric acid reactive substances (TBARS) values for the oven and sun-dried grasshoppers was done using methods described in (Rababah *et al.*, 2006;D U Ahn *et al.*, 1998;W. Vyncke, 1970) with slight modifications. 20g of grasshopper samples was homogenized with 100ml of 7.5% Trichloroacetic acid (TCA) solution and 1% Ethylenediaminetetraacetic acid (EDTA) antioxidant for 1 minute and filtered using Whatman No. 1 filter paper. The filtrate was transferred to test tube with screw caps and 5 ml of TBA reagent which is a solution of 0.02M 2-Thioburbituric acid in distilled water was added to the filtrate. The test tubes were then put in a boiling water bath for 90 minutes. After color development, the test tubes were cooled under running tap water for 10 minutes, then centrifuged for 15 min at 2000 x g. The absorbence of the resulting supernatant was read at 538nm against a blank containing pure TCA solution. Malondialdehyde standard curves were prepared by using 1,1,3,3-tetra-ethoxypropane (Witte and Bailey, 1970). The TBARS

numbers were calculated from the standard curve and were expressed as milligrams malondialdehyde (MDA) per kg of grasshoppers.

3.2.4.3 Storage and Sensory evaluation

Grasshopper samples that were boiled for 20 minutes and both sun and oven-dried, which proved to have the longest keeping quality from microbial tests, were further evaluated for sensory acceptability. Each sample was evaluated by a panel of 10 untrained members for odor, appearance and overall acceptability on a 7-point hedonic scale where 1 represents dislike extremely, 2 represents dislike moderately, 3 represents dislike slightly, 4 represents neither like nor dislike, 5 represents like slightly, 6 represents like moderately and 7 represents like extremely.

3.3 STATISTICAL ANALYSIS OF DATA

Phase 1

For the descriptive data, Stata statistical software (Stata Corp 2013) was used for the analysis of data. Descriptive statistics were used to obtain means and standard deviations. Significance of ≤ 0.1 was used for the data on the survey.

Phase 2

R statistical software (R Core Team, 2018) was used to perform statistical analyses. Descripti ve statistics were used to obtain means and standard deviations. ANOVA was used to determi ne the effect of type of vendor, market location, product status and storage on Enterobacteriac eae load, yeast and molds load and Total viable counts. Mean separation was achieved using Tukey's test with ≤ 0.05 set as the significance level.

Phase 3

R statistical software (R Core Team, 2018) was used to perform statistical analyses. Descripti ve statistics were used to obtain means and standard deviations. ANOVA was used to determi ne the effect of boiling time and method of drying on the TVC load after processing and duri

ng storage. Mean separation was achieved using Tukey's test with ≤0.05 set as the significanc e level.

CHAPTER FOUR: RESULTS AND DISCUSSION

4.1 BASELINE FIELD SURVEY

4.1.1 Socio-demographic Characteristics of the Vendors

Socio-demographic characteristics of the vendors are presented in Table 1. The results show that the majority of the vendors were mobile street vendors (SV) (62%) of which 74% were females. The stationary market vendors (MV) were only 38%, with women accounting for 61%. Majority of the market vendors and street vendors, were between the ages of 25-35 years with male vendors making up a bigger proportion (45% and 50% respectively.) This suggests a greater participation of male youth in the insect-based food enterprise. Majority of both market and street vendors (57 and 43%, respectively) had only primary school level education with female vendors having a greater proportion of respondents who had attained primary school education. A greater proportion of the male vendors in both categories of vendors were married monogamously. Several studies carried out in Uganda (Muyanja *et al.*, 2011), in Philippines (Alamo-tonelada *et al.*, 2018) and in Nigeria (Andy *et al.*, 2015) support these observation, which demonstrated similar demographic attributes for both street insect-based food vendors. Low levels of education and the seemingly low socio-economic class of vendors can be associated with the poor knowledge of food handling practices which are likely to increase the occurrence of food contamination and foodborne illnesses.

Table 1: Socio-demographic characteristics of the vendors

Characteristics	Market vendors (N = 28)		Street vendors (N = 46)	
	Male	Female	Male	Female
	%	**%**	**%**	**%**
Gender	39	61	26	74
Age group (years)				
18 - 24	18	6	8	0
25 - 35	45	31	50	41
36 - 45	18	19	8	35
46 - 55	27	25	25	21
56 - 70	0	19	8	3
Education level				
No education	9	6	0	18
Primary	55	59	33	47
Ordinary level	27	24	25	32
Advance level	9	6	25	0
Tertiary level	0	6	17	3
Marital status				
Never Married	36	0	33	3
Married monogamous	54	17	50	44
married polygamous	0	18	17	15
Divorced/Separated	0	18	0	21
Widow/widower	9	47	0	18

4.1.2 Ownership of the Business and Years of Experience

Table 2 and Table 3 show the relationship of the respondents to the business and their years of experience in vending *nsenene* respectively. Majority of the respondents were individual owners of the grasshopper businesses as mentioned by 86% MV and 85% SV. Both market and street vendors had been in the business between 9 and 12 years suggesting that grasshopper vending has been a permanent source of income and longstanding employment option for the youth especially women. Relative to male vendors, females had been more engaged in market vending for longer period than in street vending. This is possibly due to the women's ability to identify accessible and target market places for the business that is more convenient and lucrative as opposed to the high mobility that is characteristic of street vending. Unlike market vending, street vending tends to be insecure and restrictive (Bhowmik *et al.*, 2012), thus a deterrent for women.

Table 2: Relationship of the respondent to the business

Characteristics	Market vendors (N = 28)		Street vendors (N = 45)	
	Frequency	Percentage (%)	Frequency	Percentage (%)
Individual	24	85.71	39	86.67
Family Business	1	3.57	2	4.34
Association/Group	3	10.71	4	8.69

Table 3: Experience in years of vendors

	Market vendors (N = 28)		Street vendors (N = 45)	
	Male	Female	Male	Female
Mean number of years in grasshopper vending business (mean±SD)	9.4±7.74	10.2±9.88	12.1±11.1	9.3±7.22

4.1.3 Diversity of insect products sold by vendors

The results on the vendors' most preferred processing technique of edible grasshoppers are presented in Table 4. The information obtained indicated that the majority of both street and market vendors sold raw, unprocessed *Nsenene*, which consisted of 56% of MV and 44% of SV. Most vendors (>90%), however claim that unprocessed grasshoppers have a short shelf life of less than 24hrs (Table 4) hence they fry them to prolong the shelf life. Deep-fried grasshoppers were the second most traded product as indicated by the vendors (22% SV, 31% MV) and the most widely available throughout the season. These results are in line with studies done in Tanzania and Uganda whereby deep-fried grasshoppers are the most common and most preferred particularly among the younger consumer (Biryomumaisho, 2012; Mmari *et al.,* 2017). Boiled grasshoppers are also present in the market (Table 4), but these also have a short shelf life of less than 24hrs, hence have to be sold the same day or deep-fried or sun-dried to preserve them further. These findings are in line with other studies conducted in Uganda on the marketing and shelf life of *R. differens* (Ndimubandi *et al.,* 2018; Ssepuuya *et al.,* 2016). Pan-fried grasshoppers tend to be the least popular in Uganda but remain popular in other grasshopper consuming regions such as Tanzania. In Tanzania toasting or pan-frying is common because it uses less oil which is seen as a more nutritious and cheaper practice (Mmari *et al.,* 2017).

Table 4: Diversity of insect's products sold by different vendors

	Market vendors (N = 28)		Street vendors (N = 46)	
Processing methods	Frequency	Percentage (%)	Frequency	Percentage (%)
Raw	23	56	31	44
Deep-Fried	9	22	22	31
Pan-fried	1	2	5	7
Boiled	8	20	13	18

Table 5: Preparation techniques of the different types of grasshoppers marketed

Grasshoppers sold	Processing Techniques
Deep- Fried	These are washed in water, drained and deep fried in edible oil for approx. 10 minutes until brown and crunchy. They are seasoned with salt and onions for sale.
Boiled	Vendors begin by washing, then boiling in water with salt for approx. 25 minutes. They are then drained and seasoned with salt and onion for sale.
Pan-fried	These are fried in their own oil while stirring for approx. 20 minutes until brown and crunchy. These are also seasoned with salt and onion for sale.

4.1.4 Health and safety compliance of vendors

4.1.4.1 Issuance of food handler's certificate

Our results revealed that only 5% of the vendors had a food handlers' certificate. The Kampala capital city authority (KCCA) is the body that is charged with enforcing the public health act by ensuring and monitoring food hygiene practices among food handlers. However, this authority only follows up on food hygiene practices of formally registered food businesses such as hotels, bars and restaurants (KCCA, 2019). This shows a gap in the regulation of informal vending businesses, which represents over 80% of ready-to-eat foods, sold in the informal markets (Delia and Roesel, 2014). These are often left unregulated and vendors are not made aware of good food handling practices, thus exposing a large number of consumers at risk of food-borne illnesses.

4.1.5 Food Safety and Quality Awareness Among Vendors

4.1.5.1 Quality attributes considered by vendors when purchasing raw grasshoppers

Majority of the vendors (>90%) interviewed were keen to buy insects that are still alive despite the higher cost involved compared to dead insects, which were mostly considered to

be of bad conditions and less fit for consumption (Figure 4). Other attributes considered include clean and well-aerated packaging (reported by 62% of the vendors), and the cleanliness of the collectors and cleanliness of the delivery van (by 41%). *Nsenene* vendors appear to pay little attention to cleanliness of *nsenene* themselves because they sort to remove most of the dirt, dust and other insects caught together with the grasshoppers. However, vendors may not be able to control the contaminants introduced during collection and transportation. For instance, collectors smear grease and oils that may not necessarily be edible, in trapping drums, to prevent insects from escaping thus contaminating the grasshoppers. It is therefore prudent to ensure that raw grasshoppers are handled in the most hygienic way possible before they reach the market to ensure that the end product is of equally good quality and safe to eat.

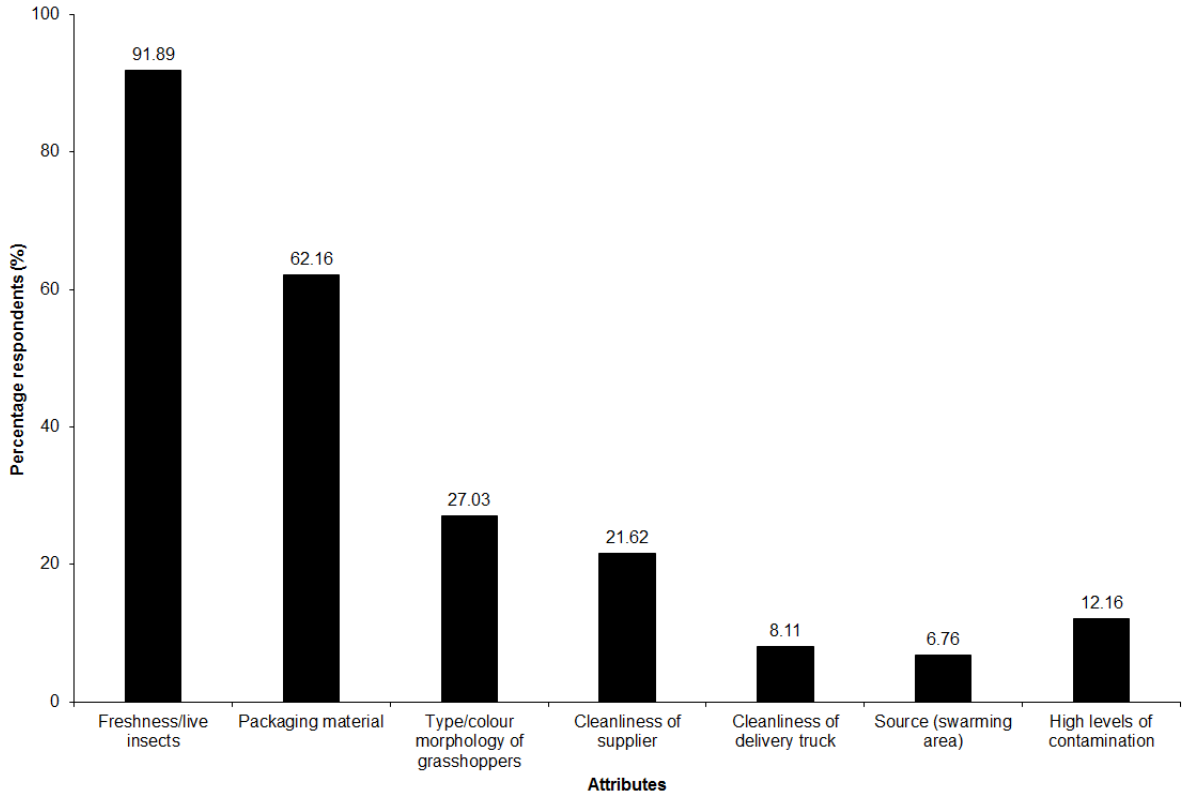

Figure 4: Quality attributes considered by vendors when purchasing raw grasshoppers

4.1.6 Sorting of raw grasshoppers before processing

The study established that a key first step in grasshopper processing is the removal of appendages which are the legs, wings and, ovipositor because they are likely to cause harm to the consumers. The study, therefore, sought to find out if the vendors understood the practice as one that promoted food safety, and the results are presented in Table 5. Although more than 80% of the vendors indicated that they remove the grasshopper appendages, only 43.4% of them knew that they are physical hazards to consumers. They noted that if not removed, appendages may cause choking and constipation, especially in children. About 9.4% of the vendors did not remove the appendages and did not know they were hazardous.

Table 6: Respondents' knowledge vs. practice in the removal of grasshopper appendages

Knowledge: Why do you remove appendages	Practice: Do you remove grasshopper appendages? (n=53)			
	Yes		No	
	Frequency	Percentage (%)	Frequency	Percentage (%)
Hazard	23	43.4	3	5.6
Not a hazard	22	41.5	5	9.4

4.1.7 Frequency of use of deep frying oil

The quality of oil used for deep frying nsenene may influence their safety and quality as food. In this study, respondents' were asked how often they change their deep-frying oil as a proxy for the quality of the oil. While most of the street vendors (45.5%) reported that they use fresh oil for deep frying on a daily basis majority of the market vendors (66.7%) never changed the oil but topped up old oil with fresh oil. This result probably may be attributed to fewer volumes grasshoppers handled by street vendors relative to their market counterparts. The data indicate that street vendors purchased an average of 1189 kg of grasshoppers per month in April-June season of 2018 which is significantly less ($p=0.074$, $\alpha=0.1$) than the 2936kg purchased by market vendors in the same season. From a nutritional standpoint, repeatedly using the same deep frying oil as market vendors do, causes a chain of oxidative reactions that lead to the formation of free radicals, acrylamides, and trans-fats which cause cancers and

cardiovascular diseases (Goswami *et al.,* 2015) thus putting consumers at risk.

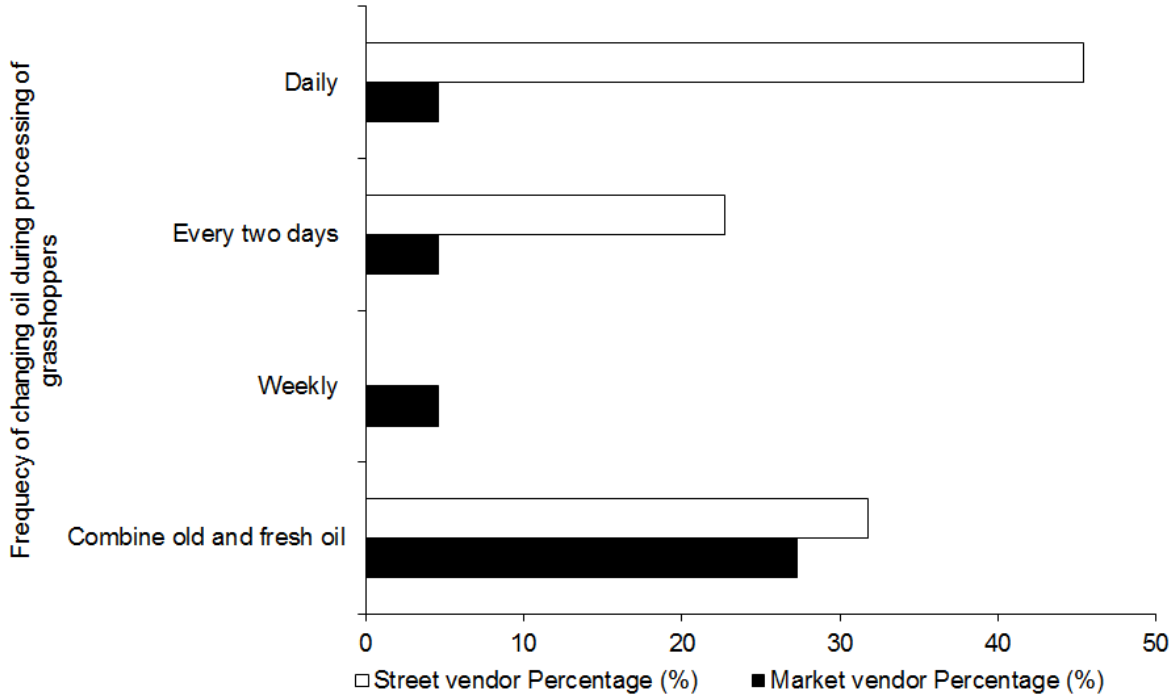

Figure 5: Frequency of use of oil in deep frying grasshoppers

4.1.8 Vendors' perception on shelf life and preservation of processed and unprocessed grasshoppers

Due to lack of legislation in the nsenene subsector, there are currently no standards that give the maximum shelf life of processed and unprocessed grasshoppers. Vendors were therefore asked what they perceive to be the length of time that unprocessed and processed grasshoppers remain for processing and consumption respectively. About 50% of both street and market vendors agreed that raw nsenene remain fresh only for 1 day after which almost all insects die and begin to decompose. The two categories of vendors, however, reported different perceptions of shelf life of deep-fried nsenene. About 50% of market vendors concluded that deep-fried *nsenene* remained fit for sale and consumption for 60 days, whereas street vendors argued that they can stay fit for human consumption even up to 90 days; beyond which they develop undesirable properties such as hardening and off-odors. It was observed that unprocessed grasshoppers are usually stored spread out on a sack on the ground or on a raised surface where it is shaded and airy. According to the vendors the cool

environment reduces the rate of death and decomposition before *nsenene* is sold or processed. Deep-fried grasshoppers are generally stored in opaque carton boxes at ambient temperatures for long term storage while awaiting sale. During sale of nsenene, it was observed that deep-fried ones are deliberately exposed on a tray in order to be seen by buyers, a practice which exposes them to contamination from the environment. It was common practice to have streets vendors keep the Nsenene on charcoal warmers placed in their pushcarts, a practice that keeps them at danger zone temperatures (5-60ºC) enabling rapid growth of bacteria. The respondents were also asked about the use of refrigeration as a potential strategy to preserve nsenene. Although studies such as Ssepuuya *et al.,* (2016) show that refrigeration can reduce the rate of spoilage and extend shelf life of nsenene, only a mere 16% of market vendors and only 5% of street vendors practise refrigeration. Vendors believe that refrigeration reduces the quality of the raw grasshoppers making the insects watery and mushy. This is possible probably due to thawing damage which occurs when food is frozen slowly resulting in large ice crystals which rapture the tissues during thawing. Thawing damage could also occur due to temperature abuse during freezing whereby frozen food is repeatedly thawed and refrozen (Archer, 2004).

Table 7: Vendors' perception of the shelf life of raw and deep fried grasshoppers (days)

Shelf-life in days	Market vendors (N=28)		Street vendors (N=46)	
	Mean± SD	Median	Mean± SD	Median
Raw	1.01±0.75	1	1.08±0.51	1
Deep-fried	77.9±84.6	60	158±134.6	90

4.1.9 Hygiene and sanitation practices of grasshopper vendors

In this study, hygiene and sanitation practices of vendors were observed against a checklist. As can be seen in Table 7 edible grasshopper vendors surveyed had poor knowledge of personal hygiene. Only 50% of the market vendors had a mean score of 0.48 slightly lower than the mean score for the street vendors (0.52). Similar findings are reported by other studies such as (Baş et al., 2006). Some of the positive observations made were; a majority of the vendors, (71.4% MV and 73.9% SV) kept their fingernails clean and short during food handling (Table 6). The majority were observed not to sneeze or cough over food and did not blow air into the polythene bags used to package food. However, many vendors did not exhibit proper hand washing during food handling. Washing of hands every time they got contaminated was practiced by only 18% of MV and 2% of SV. About 64% of MV, 50% of SV reported that they washed their hands with soap and water after visiting the toilet. During packaging, it was a common practice among 35% of MV and 45% of SV to use spoons to package processed grasshoppers, while they used their bare hands to package the unprocessed ones. This poses a risk of cross-contamination from raw to cooked insects. In the case of food hygiene practices, we learned that it was not common practice to wash the utensils used in the Nsenene trade on a daily basis. Only 17.1% MV and 21.7% SV washed their utensils every day. Due to having leftovers, the storage containers and the spoons for scooping stayed without washing for several days until the batch of insects was sold out. It was not apparent, therefore from our study how well utensils were washed and whether they used soap and hot water since grasshoppers are greasy food. Failure to wash storage containers increases the probability of microbial loading and transfer from the outside of the containers to the inside due to careless handling, posing a risk of foodborne pathogens growing in the food and causing illness.

Table 8: Hygiene practices of grasshopper vendors

Personal hygiene	Positive observations			
	Market Vendors (N = 28)		Street vendors (N = 46)	
	Frequency	Percentage (%)	Frequency	Percentage (%)
Wearing clean apron/overcoat	5	17.85	9	19.56
Short Clean fingernails	20	71.42	34	73.90
Washing hands after handling money or raw grasshoppers to avoid cross-contamination to processed ones	5	17.85	1	2.17
Hair covered	6	21.40	12	26.00
Washes hands with soap and water after visiting the toilet	18	64.30	23	50.00
Blowing air into polythene bags before packaging grasshoppers	2	7.14	0	0
Smoking while handling grasshoppers	0	0.00	0	0
Coughing/Sneezing over food	1	3.57	0	0
Scratching/itching of hair and body while handling grasshoppers	2	7.14	3	6.52
Handling of food while visibly sickly i.e. suffering from a cold	4	14.20	4	8.69
Hygiene of preparation environment				
Cleaning of utensils everyday with soap and water	5	17.85	10	21.70
Type of preparation surface				
Wood	15	53.50	32	69.60
Cardboard	10	35.70	8	17.40
Concrete	3	10.70	6	13.00
Type of packaging				
Use spoons for scooping	10	35.70	21	45.65
Serve in recycled newspaper wrapping	17	60.70	27	58.70
Serve in store-bought disposable containers	16	57.10	26	56.50

4.2 MICROBIAL CONTAMINATION OF THE MARKETED INSECT PRODUCTS

4.2.1 Microbial Contamination of marketed edible grasshoppers

Out of the 4 unprocessed grasshopper samples analyzed, Enterobacteriaceae, aerobic mesophilic bacteria and yeasts and molds were detected in all. Out of the 21 processed samples, 2 of which were boiled and 19 deep-fried, Enterobacteriaceae was detected in all but 2 of the deep-fried samples, while aerobic mesophilic bacteria and yeasts and molds were detected in all. The mean bacterial population of the samples analyzed is presented in Table 8. These results suggest very high levels of contamination beyond acceptable limits for ready-to eat-foods and minced meat (FASFC, 2014; FSAI, 2016). For the fresh unprocessed samples, the Enterobacteriaceae count averaged 8.61±0.73 log cfu/g, total viable count averaged 8.39±0.803 log cfu/g and yeast and mold count averaged 6.09±1.42 log cfu/g. For deep-fried samples, Enterobacteriaceae count averaged 6.65±1.28 log cfu/g, total viable count averaged 7.74±1.67 log cfu/g and yeast and molds 5.5±2.2 log cfu/g. As for the boiled samples, Enterobacteriaceae counts averaged 5.4±0.44 log cfu/g, total viable count averaged 8.84±0.58 log cfu/g and yeasts and molds averaged 5.91±0.3 log cfu/g. These findings are consistent with others observed in grasshoppers by Stoops et al., (2016) and Ssepuuya et al., (2018) for TVC, Enterobacteriaceae, Yeasts, and molds and are comparable to those observed in other insects by Klunder *et al.,* (2012) for TVC and Enterobacteriaceae.

Statistically, there was a significant difference ($p<0.05$) in contamination levels of Enterobacteriaceae among the three categories of fresh, deep-fried and boiled. Furthermore, there was a relatively strong association between the Enterobacteriaceae load and the status (Fresh, boiled, deep-fried) of the samples ($r=0.058$, $p>0.01$). There were also significant differences ($p<0.05$) in levels of contamination of all the three categories of microorganisms tested among the different market locations (Table 8), but there were no significant differences ($p>0.05$) in levels of contamination between street vendors and market vendors.

However, there was no correlation between the age of the samples and the Enterobacteriaceae load ($r=0.008$, $p=0.96$) indicating that the age of the samples did not have an influence on the Enterobacteriaceae load. This may be attributed to poor food handling practices regardless of the length of storage of the samples. There was a weak significant correlation between TVC load and the age of the samples ($r=0.37$, $p=0.008$). In most samples, older ones did tend to have a higher microbial load than younger ones implying that length of storage did have an effect on the microbial load. For yeasts and mold contamination, there was also no significant correlation between the age and the fungal load ($p=0.415$).

Due to lack of food safety and hygiene guidelines for edible insects, general principles of food hygiene are proposed for use by the Codex Alimentarius Commission (CAC), as well as process hygiene guidelines for minced meat. These have been used as a benchmark in previous studies on microbial contamination of insects (Stoops et al., 2016). According to these guidelines, the results in this study far exceed the set upper limits for process hygiene for minced meat and other ready-to-eat foods (FASFC, 2014; FSAI, 2016), although this study did not consider the recommended 5 samples per batch criteria. Both the Enterobacteriaceae and total viable counts load, which are used as process hygiene indicator were on average higher than the recommended level (3 log cfu/g for Enterobacteriaceae and 5 log cfu/g for TVC) for all samples except two.

Typically, such unsatisfactory plate count results for hygiene indicators point to a likelihood of the presence of food-borne pathogens and toxins that can potentially be harmful to consumers (FSAI, 2016). These findings, therefore, suggest poor hygiene and sanitation during Nsenene handling, fecal contamination, and temperature abuse during storage, or inadequate heat treatment during deep frying and a generally unhygienic environment in the nsenene preparation area. Therefore, there is need for further investigation on foodborne

pathogens in edible grasshoppers marketed on the streets and training of vendors on proper hygiene and sanitation during food handling to address these food safety concerns.

Table 9: Microbial load of marketed *nsenene* from the different market locations

Market location	Type of vendor	Product status	Storage time (Days)	Enterobacteriaceae (Log cfu/g)	TVC (Log cfu/g)	Yeasts and Moulds (Log cfu/g)
Busega	MV	Deep fried	1	nd^{a}	4.81±0.04^{b}	4.74±0.67^{cd}
Owino	MV	Deep fried	0	nd^{a}	5.74±0.11^{c}	6.07±0.02^{fg}
Nyendo	MV	Deep fried	7	4.688±0.24^{b}	6.29±0.05^{d}	4.78±0.00^{cd}
Katwe 1	MV	Deep fried	2	4.871±0.15^{bc}	8.58±0.14^{ghi}	5.63±0.03^{ef}
Ndeeba	MV	Deep fried	4	4.999±0.04^{bcd}	5.48±0.00^{c}	6.75±0.02^{hi}
Busega	SV	Boiled	1	5.023±0.06^{cd}	9.34±0.06^{mn}	5.99±0.09^{fg}
Busega	SV	Deep fried	1	5.404±0.06^{bcde}	4.33±0.06^{a}	4.97±0.05^{fg}
Nyendo	MV	Boiled	1	5.778±0.02^{cdef}	8.34±0.04^{fg}	5.83±0.43^{fg}
Nateete	SV	Deep fried	21	5.903±0.03^{cdef}	9.01±0.00^{jklm}	3.54±0.08^{a}
Bwaise	SV	Deep fried	21	5.961±0.07^{def}	8.78±0.09^{hij}	4.45±0.23^{bc}
Katwe 3	MV	Deep fried	1	5.99±0.04^{defg}	9.53±0.07^{n}	6.76±0.02^{hi}
Namugoona	SV	Deep fried	14	6.342±0.07^{efg}	9.24±0.05^{lmn}	4.82±0.04^{cd}
Karlewe	MV	Deep fried	21	6.429±0.19^{efg}	9.36±0.06^{mn}	5.07±0.09^{de}
Katwe 2	MV	Deep fried	2	6.724±0.06^{fgh}	7.14±0.06^{e}	3.66±0.07^{a}
Nakasero	MV	Deep fried	0	7.00±0.02^{ghi}	8.35±0.06^{g}	6.77±0.02^{hi}
Old park	SV	Deep fried	1	7.56±0.02^{hij}	8.99±0.11^{jklm}	4.78±0.00^{cd}
Busega	MV	Fresh	1	7.84±1.19^{ijk}	8.49±0.13^{fg}	7.22±0.74^{ij}
Bwaise	SV	Deep fried	30	7.91±0.07^{ijk}	8.82±0.06^{hijk}	7.94±0.07^{k}
Nakasero	MV	Deep fried	2	7.94±0.08^{ijk}	7.97±0.10^{f}	5.58±0.09^{ef}
Nyendo	MV	Deep fried	6	7.99±0.20^{ijkl}	6.56±0.08^{d}	7.69±0.06^{jk}
Kibuye	MV	Deep fried	4	8.27±0.00^{jkl}	9.93±0.06^{ijkl}	5.65±0.41^{fg}
Nakasero	MV	Fresh	0	8.43±0.03^{jklm}	7.32±0.06^{e}	3.89±0.12^{ab}
Busega	MV	Fresh	0	8.83±0.03^{klm}	8.30±0.06^{fg}	7.22±0.07^{gh}
Namugoona	SV	Deep fried	21	9.00±0.04^{lm}	9.18±0.28^{klmn}	4.84±0.06^{cd}
Ndeeba	MV	Fresh	0	9.33±0.02^{m}	9.43±0.03^{n}	7.05±0.09^{i}

***MV –market Vendors**
***SV – Street Vendors**
***nd – Not detected**

4.2.2 Microbial diversity of processed and unprocessed edible grasshoppers

Using culture-dependent PCR assay, 73 isolates were successfully identified from the GeneBank reference with a similarity index of ≥ 95%.

4.2.2.1 Bacterial species identified

A mixed flora of gram-positive and gram-negative bacteria, as well as yeasts and molds, were isolated from the marketed grasshoppers (Table 9). Out of 57 samples, 8 contained pathogenic bacteria majority of which belonged to the family Bacilli. The species of pathogenic bacteria isolated include; *Bacillus cereus* which is a gram-positive bacteria adapted to grow in the gut of insects and mammals (Stenfors *et al.,* 2008). It was isolated from two samples, fresh grasshoppers that were less than 24 hours old and deep-fried grasshoppers that had been stored for 21 days. *B. cereus* is known to produce an enterotoxin in the small intestines when viable cells or spores are ingested, causing emetic food poisoning and bloody diarrhea (Guinebretière *et al.,* 2002). In some cases, it causes systemic infections in such as pneumonia, meningitis, and endophthalmitis in patients (Bottone, 2010; Kamar *et al.,* 2013). *Hafnia alvei* the only named species of the genus *Hafnia* was isolated from a 30-day old sample of deep-fried grasshoppers from a street vendor.

Serratia marcescens, a gram-negative bacteria of the Enterobacteriaceae family was also isolated from two samples, fresh grasshoppers that were a few hours old and deep-fried grasshoppers from a street vendor that were 1 day old. *S. marcescens* is classified as a Class I pathogen of both grasshoppers and humans. It is therefore pathogenic to non-crustacean arthropods (NCA) such as the grasshoppers under study and humans alike (Grabowski and Klein, 2017). *S. marcescens* is an opportunistic emerging human pathogen that causes nosocomial infections such as urinary tract infections, respiratory tract infections, wound infections, septicemia, pneumonia and eye infections (Castelli *et al.,* 2008). Recently most strains of *S. marcescens* have shown multiple-antibiotic resistance representing growing

public health concern (Haddy *et al.*, 1996). Its presence in both raw and cooked forms of grasshoppers indicate that it can be transmitted through oral route to consumers thereby presenting a food-borne risk of traditional Entomophagy.

In the Micrococcaceae family, *Staphylococcus aureus, Staphylococcus xylosus, Staphylococcus sciuri, and Staphyloccocus haemolyticus* were isolated. *S. aureus* was isolated from one sample of deep-fried grasshopper that was collected only two hours after deep frying. *S. aureus* is a gram-positive bacteria that is part of the natural human flora, commonly found in the nasal cavity, mouth, and skin of healthy individuals (Lowy, 1998). If allowed to enter the bloodstream or internal tissues through ingesting contaminated food, these bacteria may cause Staphylococcal Food Poisoning (SFD), one of the most common foodborne diseases known today (Taylor and Unakal, 2019; Hennekinne *et al.*, 2012). Improper food handling is the main cause of SFD as the bacteria enters food either from direct contact or sneezing and coughing into food by handlers. Food kept at danger zone temperatures (5-60ºC) presents a conducive environment for the bacteria to multiply and form staphylococcal enterotoxin (SE) which when ingested causes gastrointestinal illness (Kadariya *et al.*, 2014).

S. sciuri was also isolated from one deep-fried sample that was 10 days old while *S. xylosus* was isolated from 3 samples that were 0, 4 and 21 days old respectively. Although the two species are generally non-pathogenic, it has emerged that some strains can carry the enterotoxin gene ((Udo *et al.*, 1999; Rodríguez *et al.*, 1996). S. xylosus *S. sciuri* have also been shown to have the ability to adhere to abiotic food surfaces and cause biofilm formation which enables them to contaminate food and ultimately cause food poisoning (Marino *et al.*, 2011). These two species have also shown multiple antibiotic resistance and their biofilms are resistant to some sanitizers (Marino *et al.*, 2011) thus posing risk in the food preparation

environment. *S. sciuri* was also isolated in fresh, unplucked *R. differens* according to a study by Ssepuuya *et al.,* (2019).

Staphylococcus haemolyticus was the last of the pathogenic species in the Micrococcaceae family that was isolated from a 21-day old deep-fried sample. Unlike other staphylococci, *S. haemolyticus* is known to be the second most frequently isolated bacteria in blood cultures and is notorious for being highly antibiotic-resistant (Takeuchi *et al.,* 2005). It is also found in the normal skin flora of healthy individuals but once in the bloodstream, it may cause septicemia, peritonitis, otitis, and urinary tract infections (Takeuchi *et al.,* 2005).

Pseudomonas aeruginosa was also isolated from one sample of deep-fried grasshoppers which was collected only a few hours after deep frying. It is a versatile gram-negative bacterium that is ubiquitous to the environment. It is found most commonly in soils (Green et al., 1974), some animal hosts and also has the ability to multiply to high numbers in some foods (Hardalo and Edberg, 1997). It is one of the top three causes of opportunistic human infections, causing disease only in immunocompromised individuals, particularly burn victims, pneumonia and cancer patients (Stover *et al.*, 2000). *Ps. aeruginosa* of food origin can be a major risk to consumers as a consequence of its antibiotic-resistance and resistance to some disinfectants when it forms biofilms (Taylor et al., 1999). Since this bacteria is favored in environments such as wounds on the body (Hardalo and Edberg, 1997), food handlers with wounds can possibly be carriers of this bacteria ad can transmit it to food if careless.

Some spoilage bacteria such as *Kurthia gibsonii* were isolated from grasshopper samples and are responsible for spoilage of meat and meat products. Others such as *Ps. protegens*, *Ps. Putida* are plant growth promoters while *Ps. Marginalis* are plant pathogens possibly transmitted to the grasshoppers through contact with vegetation during feeding or in the markets whereby most vendors sell grasshoppers alongside fruits and vegetables.

4.3.2 Fungal species identified

Thirty-nine isolates of pathogenic and non-pathogenic yeasts and molds species were also isolated from the grasshopper samples. Some mycotoxins producing species of Aspergillus mold were also isolated and these included *Aspergillus fumigatus* and, *Aspergillus neobridgeri. A. fumigatus* causes Invasive Aspergillosis (IA), one of the most common mold infections worldwide, capable of causing fatalities among immunocompromised patients (Lin *et al.,* 2001). It is also known to produce various mycotoxins including gliotoxin, which has cytotoxic and immunosuppressive properties (Bauer et al., 1989), verruculogen and fumitremorgin A,B & C which are tremorgenic mycotoxins known to affect the Central Nervous System (CNS), causing tremors (Land *et al.,* 1987) and fumigaclavines A,B and C. Interestingly, Fumigaclavine C which is an indole alkaloid have been observed to inhibit growth, migration and induce apoptosis of MCF-7 breast cancer cells (Li *et al.,* 2013). Despite it being a human pathogen, it is a pathogen of some insects in the orders of Hymenoptera and Blattodea (Schlüter *et al.,* 2017). *Aspergillus neobridgeri* is one of the species of *Aspergillus* section circumdati that produce penicillic acid and Xanthomegnis mycotoxins which are believed to have a synergistic toxic effect. Among the toxigenic fungi, *Penicillium brevicompactum* was also isolated from a 1 day old boiled sample. This fungus is a weak pathogen of a variety of fruits and root crops such as apples, grapes, cassava, potatoes, and ginger(Pitt, 2006). It produces a mycotoxin known as mycophenolic acid which is claimed to be potent immunosuppressant in some studies (Overy and Frisvad, 2005) but a weak mycotoxin in others (Pitt, 2006), demonstrating that further studies regarding human consumption of this metabolite need to be done.

Meyerozyma guillermondii is a yeast-like fungus found is 6-day old deep-fried samples. It has been isolated from various sources such as flowers, fruits, insects' frass, and food products. It is an opportunistic pathogen in humans and animals known to cause numerous human

infections of cutaneous origin, largely in immunosuppressed individuals (Corte *et al.,* 2015; Riviera *et al.,* 2009; Molnár, *et al.,* 2008) ***Papiliotrema laurentii (Cryptococcus Laurentii)*** was isolated in a one day old deep-fried sample. It is a yeast that has been implicated in human infections such as fungemia, meningitis and cutaneous infections such as keratitis in immunocompromised hosts. It has been reposted to occur in soil and products contaminated with pigeon excreta and can be transmitted by inhalation of fomites (Haider *et al.,* 2013). *Penicillium decumbens* is ubiquitous fungi isolated from a wide variety of foods such as meat products, rice, flour, dried legumes and fresh vegetables (Pitt *et al.,* 2009). It is an opportunistic pathogen that can potentially cause fatal human infection in immunocompromised individuals(Alvarez, 1990). Its presence in grasshoppers could be due to cross-contamination since vendors sell these insects alongside other foods as well. *Rhodotorula mucilaginosa* is a yeast that is commonly isolated from foods and beverages It has been isolated from fruits, fruit juices, edible mollusks, cheese, and sausages (Tournas *et al.,* 2006; Eklund *et al.,* 1965). This species has also been isolated from air, seawater and freshwater environments (Nagahama *et al.,* 2006). It is an emerging pathogen of susceptible humans and animals such as sheep, goats, and chicken (Wirth and Goldani, 2012). Several *Candida* species were isolated from the grasshopper samples as well. *Clavispora lusitaniae (Candida lusitaniae)* and *Candida catenulata* were isolated in a 1 day old boiled sample from a street vendor while *Candida intermedia* yeast was isolated from a one a day old deep-fried sample. The candida species are known to be the most common cause of human fungal infections (Rajkowska and Kunicka-Styczyńska, 2018). *C. lusitaniae* is a known cause of disseminated candidiasis, including septicemia and pyelonephritis. It was first isolated from the digestive tract of warm-blooded animals and environmental isolations have been made from cornmeal, citrus peel, fruit juices, and milk from cows with mastitis (Turner and Butler, 2014). Similarly, *C. catenulata* has been found to be a contaminant of dairy

products(Delavenne et al., 2011) but has also been isolated from environmental dust (Janke *et al.,* 2013). It is known to cause both superficial and invasive infections in humans (O'Brien *et al.,* 2018) and thus can be a food safety risk in the consumption of grasshoppers. Its presence in environmental dust could explain how it found its way into grasshoppers because most vendors expose them for consumers to see. *C. intermedia*, on the other hand, is a rare human pathogen but has been implicated in cases of fungemia in at-risk groups of people (Ruan *et al.,* 2010).

Table 10: Microbial diversity of fresh, boiled and deep-fried edible grasshoppers from different market locations and stored for different lengths of time

Day	Sample	Market location	Bacteria	Accession number	Fungi	Accession number
0	Fresh	Nakaero	Serratia marcesens	KM099142.1	Didymella anserina strain	MH855074.1
0	Fresh	Nakasero	Bacillus sp	MH547258.1	Cladosporium tenuissimum	KX999700.1
0	Fresh	Ndeeba	Staphylococcus sp	EU784844		
0	Fresh	Nyendo	Bacillus cereus	MK088304.1		
0	Fresh	Ndeeba	Staphylococcus sp	EU784844		
0	Fresh	Busega			Wickerhamomyces anomalus	MF442419.1
0	Fresh	Busega			Ascomycota sp	MK267731.1
0	Boiled	Nyendo	Bacillus cereus	MK088304.1		
0	Deep fried	Owino	Staphylococcus xylosus	MK414862.2	Purpureocillium sp	MK120858.1
0	Deep fried	Nakasero	Pseudomonas sp	MF144536.1		
0	Deep fried	Nakasero	Psedomonas marginalis	MG972908.1		
0	Deep fried	Owino	Staphylococcus aureus	MK780062.1		
0	Deep fried	Naksero	Marococcus sp	AB859243.1		
0	Deep fried	Naksero	Kurthia gibsonii	MK414929.1		
0	Deep fried	Owino	Pseudomonas aeruginosa	DQ294293		
0	Deep fried	Katwe	Kurthia gibsonii	MK414929.1		
1	Fresh	Busega	Pseudomonas putida	MF952434.1		
1	Boiled	Nyendo			Ramichloridium apiculatum	EU041792.1
1	Boiled	Nyendo			Cladosporidium sp	LC433822.1
1	Boiled	Nyendo			Penicilluium brevicompactum	KY401086
1	Deep fried	Old taxi park	Pseudomonas poae	MK883127.1		
1	Deep fried	Old taxi park	Serratia marcescens	MK961214.1		
1	Deep fried	Old taxi park	Pseudomonas sp	LC420171.1		
1	Deep fried	Busega			Cladosporium tenuissimum	MK957180.1

Day	Sample	Market location	Bacteria	Accession number	Fungi	Accession number
1	Deep fried	Busega			Aspergillus fumigatus	JQ767180.1
1	Deep fried	Katwe			Cladosporium tenuissimum	MG669138.1
1	Deep fried	Busega SV			Cladosporium tenuissimum	MK957180.1
1	Boiled	Busega SV			Clavispora lusitaniae	LC413208.1
1	Boiled	Busega SV			Candida catenulata	
1	Deep fried	Katwe			Candida intermedia	KM246246.1
1	Deep fried	Katwe			Papiliotrema laurentii	KY104470.1
1	Deep fried	Old taxi park			Wickerhamomyces anomalus	MF442419.1
1	Deep fried	Old taxi park			Cryptococcus laurentii	EF521207.1
2	Deep fried	Nakasero			Debaryomyces fabryi	MK394103
2	Deep fried	Nakasero			Debaryomyces hansenii	LC412703
2	Deep fried	Old Taxi Park			Aspergillus micronesiensis	KP987080.1
2	Deep fried	Katwe			Wickerhamomyces anomalus	MK998688.1
4	Deep-fried	Kibuye	Pseudomonas sp	MK414951.1	Penicillium lanosocoeruleum	NR163541.1
					Penicillium decumbens	MK267667.1
4	Deep-fried	Ndeeba	Macroccocus caseolyticus	KJ555014.1	Wickerhamomyces anomalus	MK630211
4	Deep fried	Ndeeba	Kurthia zopfii	MK253317.1		
4	Deep fried	Ndeeba	Staphylococcus xylosus	HM816680.1		
6	Deep fried	Nyendo			Meyerozyma guillermondii	MF940125.1
7	Deep fried	Nyendo	Pseudomonas protegens	MK615142	Aspegillus neobridgeri	MK600510.1
7	Deep fried	Nyendo			Aspergillus sclerotiorum	KP006347.1
7	Deep fried	Nyendo			Beauveria bassiana	MH922796.1
7	Deep fried	Nyendo			Cladosporium aciculare	NR152294.1
10	Deep fried	Nyendo	Staphylococcus sciuri	MK414794.1		
21	Deep fried	Bwaise	Staphylococcus xylosus	MK414862.1		
21	Deep fried	Bwaise	Staphylococcus haemolyticus	MK446926.1		
21	Deep fried	Namugoona	Pseudomonas putida	MK737106.1		

Day	Sample	Market location	Bacteria	Accession number	Fungi	Accession number
21	Deep fried	Namugoona	Pseudomonas sp	MK414951.1		
21	Deep fried	Nateete SV	Staphylococcus sp	KU598984.1		
21	Deep fried	Nateete SV	Bacillus cereus	KM248381		
30	Deep fried	Bwaise SV	Pseudomonas poae	MK883127.1		
30	Deep fried	Bwaise SV	Hafnia alvei	MH620746.1		

4.4 OPTIMIZING PROCESSING AND EVALUATION OF SHELF LIFE OF PROCESSED *R. DIFFERENS* GRASSHOPPERS

4.4.1 Effect of Boiling Time and Method of Drying on Microbial Load of *R. differens* grasshoppers

The microbial quality of boiled and dried grasshoppers was monitored using TVC and the results are presented in Table 10. Boiling for 10, 15 and 20 minutes resulted in decrease of aerobic bacteria to undetectable levels. However, after both oven and sun drying, the samples boiled for 10 and 15 minutes showed elevated levels of TVC load while the sample boiled for 20 minutes remained with undetectable microbial levels. Boiling treatments of 10 and 15 minutes may have therefore been insufficient to inactivate spores, which then germinate to active bacterial cells during drying.

The 20-minute boiled and dried sample which showed lowest microbial loads was further monitored for changes in microbial quality during storage over a period of 6 days at 55ºC (Accelerated Shelf-life Testing). The TVC load recorded was < 5 log cfu/g up to the 5^{th} day of storage (Table 10). The recommended TVC load for ready-to-eat marketed foods is an upper limit of 5 log cfu/g (FSAI, 2016) suggesting that if grasshoppers are boiled for 20 minutes and oven-dried they can remain safe to eat for up to an equivalent of 6 months when stored at ambient temperatures as calculated according to (Shema, 2018).

4.4.2 Changes in Lipid Oxidation and sensory acceptability of dried grasshoppers during storage

The results for TBA test for lipid oxidation are expressed in Table 11. Due to lack of rancidity standards for edible insects, standards for meat and meat products were used. At day 0, both oven and sun dried samples that were boiled for 20 minutes were within the acceptable limits of TBA for rancidity in beef (1.0 mg MDA/kg) (Rahman *et al.,* 2015).

Sensory acceptability scores of both oven and sun-dried samples during the 6 weeks are presented in Tables 12. The overall score for Appearance was 4.03±1.464, Odor was

4.48±1.446, and Acceptability was 4.31±1.400. There were no significant differences between oven and sun-dried samples ($p>0.05$) with regards to sensory parameters. These results implied that the panelist neither liked nor disliked the odor, appearance and general acceptability of grasshopper samples. According to Table 12, the odor scores increased with storage from day 0 to day 6 with no significant difference recorded ($p>0.05$). This suggests that the untrained panelist could not detect rancidity in edible grasshoppers through a sniff test, although TBA test showed rancidity of lipids after day 1 of storage. Sensory acceptability and the appearance scores of the grasshoppers did not also change significantly ($p>0.05$) during the storage period.

High TBA values as seen in this study, suggest lipid peroxidation, which is one of the primary mechanisms of spoilage of fatty foods such as grasshoppers (Rahman *et al.,* 2015). The grasshopper *R. differens* is dominated by polyunsaturated fatty acids which are highly susceptible to oxidation (Kinyuru, 2009) and are also rich in trace minerals such iron which is a major active catalyst of the lipid oxidation process (Fombong, 2017;Ahn *et al.,* 2005). Lipid oxidation leads to the formation of primary and secondary oxidation products which affect sensory characteristics such as flavor, texture, aroma and color/appearance of foods (Ahn, 2005). However, from this study, chemical analysis shows high levels of rancidity through the TBARS score but there was no particular detection of rancidity in the odor and appearance of our grasshopper samples in the sensory evaluation. This suggests that an untrained panel is not effective in detecting rancidity in foods as some studies have shown that some panelists even prefer rancid odors in food and may score it favorably (Addis *et al.,* 1985)

Table 11: Effect of storage on the TVC load of dried grasshoppers

		Storage Time						
Drying Method	**Boiling Time (Min)**	**Day 0**	**Day 1**	**Day 2**	**Day 3**	**Day 4**	**Day 5**	**Day 6**
Oven drying	20	0.00^{a}	0.00^{a}	4.90 ± 0.00^{b}	4.50 ± 0.14^{a}	4.70 ± 0.42^{a}	4.55 ± 0.07^{a}	5.80 ± 0.28^{c}
Sun Drying	20	0.00^{a}	3.55 ± 0.07^{b}	4.30 ± 0.00^{a}	5.10 ± 0.00^{b}	5.05 ± 0.07^{ab}	4.95 ± 0.07^{ab}	5.10 ± 0.00^{b}

*Values with different letters along a column are significantly different at $p<0.05$
*Results are mean ± Standard deviation

Table 12: Effect of storage on lipid oxidation measured as Thiobarbituric acid reactive substances (mg of manoaldehyde/kg grasshoppers on dry weight basis) values

Drying Method	**Boiling Time (Min)**	**Day 0**	**Day 1**	**Day 2**	**Day 3**	**Day 4**	**Day 5**	**Day 6**
Oven drying	20	0.90 ± 0.01^{b}	1.58 ± 0.03^{d}	1.45 ± 0.06^{b}	1.43 ± 0.05^{b}	1.77 ± 0.03^{c}	1.72 ± 0.01^{cd}	1.55 ± 0.01^{bc}
Sun Drying	20	0.72 ± 0.03^{a}	1.38 ± 0.01^{b}	1.44 ± 0.21^{b}	1.29 ± 0.01^{a}	1.24 ± 0.01^{a}	1.35 ± 0.04^{a}	0.92 ± 0.03^{a}

*****Values with different letters along a column are significantly different at $p<0.05$
*Results are mean ± Standard deviation

Table 13: Sensory evaluation scores of dried grasshoppers during accelerated storage at 55ºC

Parameter	Boiling time (min)	Drying method	Storage time (days)						
			Day 0	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6
Appearance	**20**	**Oven dried**	3.33±1.11	5.11±1.453	4.11±1.453	4.56±1.23	4.22±0.97	4.44±1.33	4.00±1.581
Odor			3.78±1.481	5.67±1.225	4.67±1.225	4.89±1.691	4.44±1.236	4.556±1.51	4.67±1.323
Overall Acceptability			3.67±1.0	5.44±1.33	4.78±1.302	4.67±1.5	4.33±1.323	4.33±1.581	4.56±1.236
Appearance	**20**	**Sun dried**	3.44±1.014	4.50±1.79	4.11±1.452	4.00±1.32	3.22±1.86	3.56±1.42	3.44±1.24
Odor			4.56±1.59	4.67±1.534	4.67±1.22	4.56±1.01	3.67±1.41	3.77±1.394	4.11±1.62
Overall Acceptability			3.67±1.225	4.67±1.790	4.77±1.301	4.56±0.726	3.67±1.581	3.67±1.225	3.67±1.00

*No significant differences noted
*Results are mean ± Standard deviation

CHAPTER FIVE: CONCLUSIONS AND RECOMMENDATIONS

5.1 CONCLUSIONS

The socio demographic characteristics of vendors show that, women less than 35 years of age dominate the business of "*nsenene*" and majority have low levels of education (primary education). In terms of hygiene practices, both street and market vendors scored poorly in areas related to food handling (*i.e.* personal and food hygiene practices). Market and street vendors prepare and sell processed grasshopper in unhygienic conditions that could create enabling environment for pathogens to grow and multiply to levels that could cause infections in humans. The sector also lacks regulation such as testing and provision of food vendors with a medical food handlers' certificate.

The microbial contaminant levels are considerably higher than the acceptable limits for ready-to-eat food products (FSAI, 2016) implying a great likelihood for presence of food pathogens which was confirmed through molecular biology techniques. The microbial diversity shows the presence of bacterial pathogens that can cause potentially fatal foodborne diseases in healthy individuals such as *B. cereus, S. aureus, S. haemolyticus*. There is also presence of several emerging and opportunistic pathogens such as *S. marcesens, Ps. aeuruginosa, S. sciuri, and s. xylosus,* which can cause illness in immunosuppressed individuals. Among the fungal isolates, there is presence of mycotoxin producing mold such as *Aspergillus fumigatus* and, *Aspegillus neobridgeri*his study also confirms the presence of pathogenic fungi such as *Clavispora lusitaniae (Candida lusitaniae),* emerging fungal pathogens such as *Rhodotorula mucilaginosa* and several opportunistic pathogens such as *Papiliotrema laurentii (Cryptococcus Laurentii)* and *Meyerozyma guillermondii*. The sources of the microorganisms isolated in this study span from air, waste-water, human skin and

mucosa, the gut of mammals, birds, and humans as well as plant sources within the environment. Since grasshoppers are wild harvested and largely marketed in the open air, it goes to show that they are likely to accumulate microbes from all these different sources and therefore are an important source of environmental pathogens.

Processing and shelf life analysis reveals that boiling for 20 minutes, oven drying and packaging in Kraft paper bags with a viewing window gave the longest microbial quality of grasshoppers. The grasshoppers last for an equivalent of 6 months under accelerated storage conditions with a TVC load of <5 log cfu/g. However, the grasshoppers are highly rancid with TBARS values of >1.0mgMDA/kg after day 1 of storage. Sensory scores for odor, appearance, and general acceptability indicated that panelists neither liked nor disliked the grasshopper samples.

5.2 RECOMMENDATIONS

This study shows that there is an urgent need for food safety training in the areas of handling fresh grasshoppers, processing of grasshoppers i.e. proper heat treatment, storage and packaging as well as training on personal and food hygiene practices, prevention of cross-contamination, and suitable cleaning procedures. This will likely improve hygiene and the microbial status of the insect products and preserve the consumers' health.

Given the economic and nutritional importance of grasshoppers, this study recommends that government and policy makers develop standards that govern the processing of these edible insects to guide the stakeholders on how best they can process, package and market them to ensure that they are safe to eat. In addition, we recommend that public health departments of the government ensure that insect vendors' are tested periodically and issued with a food

handlers certificate to ensure that they are not carriers of food borne pathogens that might put consumers at risk.

The study also recommend that sensory panels for novel foods such as grasshoppers and other edible insects should be trained in order to better detect rancid odors in insect products. Further research into incorporation of an antioxidant during processing should also be done which could potentially reduce the lipid oxidation as observed in this study.

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APPENDICES

Appendix 1: Consent Form

Volunteer Agreement Form

Title: Food Safety Knowledge and Hygiene Practices of Vendors of *Nsenene* in Uganda

General Information about the Research

The study will investigate food safety knowledge and practices of vendors of *Nsenene* in Uganda.

Potential benefits and discomforts to the vendor

The immediate benefit is that the investigator will purchase a cup of the different categories of *Nsenene* that the vendor is selling after the study is done. There are no risks associated with this study. The only inconvenience might be the time you will spend answering the questions provided. The information gathered from this study will be used for developing a Master's Dissertation.

Confidentiality

Your identity and participation in this study will be kept confidential. The information we will obtain from you will not be shared with anybody, except the study investigators. Your identity remains secret since your personal information will only be designated by a unique participant number. Your name will not appear in any reports or publications resulting from this study. After the study is completed, you may request information about the study outcomes.

Voluntary Participation and Right to Leave the Research

Your participation in this study is entirely voluntary. You have the right to refuse to participate in the study. You also have the right to stop your participation in the study at any

time, even after you have signed the informed consent form. The withdrawal of your consent will not cause any disadvantage or loss of privileges.

Volunteer agreement

I have read all the information provided regarding this study, and all my questions and concerns have been addressed. I accept to truthfully and to the best of my knowledge answer the questions provided to me. I agree to participate as a volunteer.

Signature:……..... Date: …….

Appendix 2: Questionnaire

Food Safety Knowledge and Hygiene Practices Questionnaire

General Information

Questionnaireno……. Vendor number……..Collector number……. Date……

Market name……Town/City……...

Section A: Socio-demographic Characteristics

1. **Gender** **M [ ]** **F [ ]**
2. **Age** **18-25 [ ]** **26-25 [ ]** **36-55 [ ]** **Above 55 [ ]**
3. **Highest level of education attained**

No education [ ] Primary [ ] Secondary [ ] Tertiary [ ]

4. **Marital Status**

Never Married [ ] Married monogamous [ ] Married Polygamous [ ] Divorced/Separated[ ] Widowed [ ]

5. **Have you ever received any training on food safety, hygiene and sanitation?**

Yes [ ] No [ ]

6. **Length of time in the job/ business**

Less than 1 year [ ] 1-5yrs [ ] 6-10yrs [ ] 10-15yrs [ ] 16-20yrs [ ] above 20yrs [ ]

Section B: Food safety Knowledge

I. Grasshopper processing

1. **What type of vendor are you?**

Market vendor [ ] Street Vendor [ ]

2. Where do you prepare your *nsenene* from

Market stall [ ] Home [ ] Other (Specify)…….

3. What type of processed grasshopper do you specialize in

Fresh [ ] Boiled [ ] Fried without oil [ ] Deep fried [ ]

4. What do you consider when buying *nsenene* from the collectors

Freshness [ ] Price [ ] Type of packaging [ ] Cleanliness of collector/Transport Vehicle [ ]

5. Do you think *nsenene* is clean when you receive it from the collector or does it need sorting and cleaning

Clean [ ] Requires Sorting/ Cleaning [ ]

6. Which parts are removed during the cleaning/ sorting process

Wing [ ] Hind legs [ ] Others (specify)……

7. Why is it important to remove these parts/ Why are they not eaten?

Can cause injury to consumer [ ] Not liked by consumer [ ]

Other (specify)…….....

8. Are the insects washed with water before cooking

Yes [ ] No [ ]

9. How many times should insects be washed in order to be completely clean

Once [ ] Twice [ ] Change washing water until it comes out clear [ ]

10. What is your source of water supply?

Public tap [ ] Bring from home [ ] Buy from water vendors [ ] Private property i.e. Church [ ]

11. During cooking, how long are the grasshoppers,

a) **Boiled ……**

b) **Deep-fried…….**

c) **Pan-fried without oil……..**

12. How often do you change the oil

Daily [ ] Weekly [ ] Monthly [ ] other (specify).....

13. How long can cooked *nsenene* stay without getting spoilt without refrigeration

Less than 24hrs [ ] 24hrs [ ] 24-36hrs [ ] 36-48hrs [ ] More than 48 hrs [ ]

14. How long does the fresh *nsenene* last without getting spoilt without refrigeration

Less than 24hrs [ ] 24hrs [ ] 24-36hrs [ ] 36-48hrs [ ] More than 48 hrs [ ]

15. How is the food stored while vending

Open air [ ] In covered containers [ ] Cooler box/refrigerator [ ]

16. Is there a chance of mixing fresh and processed grasshoppers

Yes [ ] No [ ]

17. Type of surface used by the vendor for preparing food

Wood [ ] Cardboard [ ] Aluminum [ ] Concrete [ ] Other (specify)……

18. Type of packaging used to sell *nsenene*

Plastic paper [ ] Newspaper wrapping [ ] Kraft Paper(brown) [ ] Other (Specify)…….

19. How do you handle leftover *nsenene*

Throw away [ ] Store for selling next day [ ] Take home to eat [ ] No leftovers [ ]

20. How are leftovers stored

Room temperature [ ] Refrigerator [ ]

21. If stored to be sold later, are they?

Sold first as they are [ ] Mixed with new stock [ ] Re-cooked and sold separately [ ]

II. Food Borne Illnesses

1. Do you have a valid food handlers' certificate?

Yes [ ] No [ ]

2. Have you ever suffered any kind of illness after consuming *nsesene*

Yes [ ] No [ ]

3. Have any of your customers ever complained of falling sick after consuming *nsenene*

Yes [ ] No [ ]

4. How would you describe the illness suffered (By either you or the customer)

……

……

……

5. Did you or the customer suffer any of the following symptoms

Diarrhea [ ] Vomiting [ ] Stomach Pain [ ] Itchy skin/ Rashes [ ] Difficulty breathing [ ]

Swollen face/lips [ ]

Section C: Hygiene and sanitation

I. Toilet Facilities

1. Access/use of toilet

Yes [ ] No [ ]

2. Toilet used

Public toilet [ ] Private property i.e. church [ ] Street [ ]

3. Hand washing after toilet

Running tap water within the facility [ ] Water in a container within facility [ ] Water in container at the vendors selling point [ ]

Is there soap where they wash their hands

Yes [ ] No [ ]

II. Personal Hygiene

The interviewer will tick what they observe in the checklist below

Use a tick ✓ for YES and an X for No

Talking while working with food	
Handling food and money without washing hands in between	
Dry sweat in a way that can contaminate food	
Handles *nsenene* without gloves / any hand covering	
Vendor is wearing a clean apron or overcoat	
Vendor has short clean finger nails	
Smoking while handling the insects	
Vendor's hands are clean	
He/she is not wearing any jewelry	
His/her hair is covered	
Blows air into polythene bag before use	
Vendor is coughing/sneezing over food	
He/she is itching hair/body while working with food	
Vendor looks like he/she is suffering from a cold while working	

Section D: Information to be filled for collectors only

1. What type of grasshoppers are usually available for collection

Green [ ] Brown [ ] Purple [ ]

2. Which one is mostly preferred by vendors and consumers

Green [ ] Brown [ ] Purple [ ]

3. a) Are the equipment used from grasshopper collection usually cleaned

Yes [ ] No [ ]

b) How often

Daily [ ] Twice a week [ ] Weekly [ ] Never for the season [ ]

4. Are there any parts of the grasshoppers that are removed during collection

Yes [ ] No [ ]

5. Which parts are removed during this process

Wing [ ] Hind legs [ ] Others

(specify)……

6. Why is it important to remove these parts?

To prevent grasshoppers from escaping [ ] Can cause injury to consumer [ ] Not liked by consumer [ ] Other (specify)…….....

7. How are the grasshoppers stored for transportation to the market?

Sisal sacks [ ] Plastic sacks [ ] Drums/Containers [ ]

8. Do the nsenene make it to the market alive or dead?

Alive [ ] Dead [ ] half dead, half alive [ ]

9. How do the vendors prefer them

Alive [ ] Dead [ ] Any can do [ ]

10. How long does it take after collection for the insects to reach the market?

24 hrs [ ] 36 hrs [ ] 48 hrs [ ] More than 48 hrs [ ]

11. How long do the grasshoppers remain fresh to eat and process after collection

12. 24 hrs [ ] 36 hrs [ ] 48 hrs [ ] More than 48 hrs [ ]

Section E: Section to be filled for Consumers only

1. What influences choice of vendor that you buy nsenene from

Cleanliness of vendor and stall [ ]
Freshness [ ]
Type of cooking/Processing [ ]
Price [ ]

2. Which type of grasshopper do you prefer to buy

Fresh [ ] Boiled [ ] Pan fried without oil [ ] Deep fried [ ]

3. What do you do with the *nsenene* once you get home

Eat as is [ ] Cook again to preferred dish [ ]

4. How long does fresh *nsenene* last before it spoils and cannot be eaten

Less than 24hrs [ ] 24hrs [ ] 24-36hrs [ ] 36-48hrs [ ] More than 48 hrs [ ]

5. How long does cooked nsenene last before it spoils and cannot be eaten

Less than 24hrs [ ] 24hrs [ ] 24-36hrs [ ] 36-48hrs [ ] More than 48 hrs [ ]

6. Have you ever suffered any kind of illness after consuming *nsesene*

Yes [ ] No [ ]

7. How would you describe the illness suffered

……

……

……

8. Did you suffer any of the following symptoms

Diarrhea [ ] Vomiting [ ] Stomach Pain [ ] Itchy skin/ Rashes [ ] Difficulty breathing [ ]

Swollen face/lips [ ]

End