MOLECULAR IDENTIFICATION AND SAFETY ASSESSMENT OF WILD HARVESTED LONG-HORNED GRASSHOPPERS (*RUSPOLIA* SPP.), AND THE EFFECT OF FEED SUPPLEMENTATION ON GROWTH AND SAFETY OF FARMED EDIBLE CRICKETS (*ACHETA DOMESTICUS* AND *GRYLLUS BIMACULATUS*)

JEREMIAH KINUTHIA NG'ANG'A

DOCTOR OF PHILOSOPHY

(Food Science and Technology)

JOMO KENYATTA UNIVERSITY OF

AGRICULTURE AND TECHNOLOGY

2022

Molecular Identification and Safety Assessment of Wild Harvested Long-horned Grasshoppers (*Ruspolia* spp.), and the Effect of Feed Supplementation on Growth and Safety of Farmed Edible Crickets (*Acheta domesticus* and *Gryllus bimaculatus*)

Jeremiah Kinuthia Ng'ang'a

A Thesis Submitted in Partial Fulfilment of the Requirements for the Degree of Doctor of Philosophy in Food Science and Technology of the Jomo Kenyatta University of Agriculture and Technology

2022

DECLARATION

This thesis is my original work and has not been presented for a degree in any other university.

Signature…… Date….....

Jeremiah Kinuthia Ng'ang'a

This thesis has been submitted for examination with our approval as university supervisors:

Signature…… Date….....

Dr. John Kinyuru, PhD

JKUAT, Kenya

Signature…… Date….....

Dr. Samuel Imathiu, PhD

JKUAT, Kenya

Signature…… Date….....

Prof. Jozef Vanden Broeck, PhD

KU Leuven, Belgium

DEDICATION

I dedicate this thesis to my loving wife Rosemary, daughter Tasha, to my late dad Mr. Edward Ng'ang'a, to my mother Mrs. Ruth Ng'ang'a, brothers and sisters for their encouragement, moral support, prayers and love. Finally, to men and women who make big contributions in the world of science.

ACKNOWLEDGEMENT

First and foremost, I wish to thank the almighty God for the strength and good health during the period of my study. This study was made possible by the material and moral support from my supervisors Dr. John Kinyuru, Dr. Samuel Imathiu and Prof. Jozef Vanden Broeck. I wish to express my utmost gratitude to 'Vlaamse Interuniversitaire Raad voor Universitaire Ontwikkelingssamenwerking' (VLIR-UOS) for awarding me a study scholarship.

I am grateful to my colleagues in the Department of Food Science and Technology for the support. I specially thank the technical staff in Food Microbiology Laboratory Ms. Jennifer Wambugu for her guidance in various microbiological works.

I am grateful to the head of Molecular Developmental Physiology and Signal Transduction lab, KU Leuven, Prof. Jozef Vanden Broeck for hosting me and laboratory colleagues for the support. I specially thank Mr. Forkwa Fombong for the moral support and brilliant ideas he shared to make this study a success. My sincere appreciation goes to the technical staff Darron Cullen and Evert Bruyninckx for their guidance in various molecular analyses.

My sincere appreciation goes to the Lab4Food team, KU Leuven. I specially thank the head of the laboratory Prof. Leen Van Campenhout for permitting me to carry out part of this research work in her laboratory. I am grateful to Enya Wynants and Ann Borremans for their guidance in various ISO methods of food analysis and metagenetics in Lab4Food.

The love, moral support and encouragement from my wife Rosemary and daughter Tasha; my parents Mr. and Mrs. Ng'ang'a, and brothers, sisters, and friends is highly appreciated.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF APPENDICES

LIST OF ABBREVIATIONS

AIL	Azadirachta Indica Leaf
ANOVA	Analysis of Variance
AOAC	Association of the Official Analytical Chemist
AV	Average Gain
BW	Body Weight
CAR	Central Africa Republic
CFU	Colony Forming Unit
COII	Cytochrome c Oxidase II
DNA	Deoxyribonucleic acid
DRC	Democratic Republic of Congo
EFSA	European Food Safety Authority
FAO	Food and Agriculture Organization
FCR	Feed Conversion Ratio
FI	Feed Intake
ISO	International Organization for Standardization
ITS	Internal transcribed spacer region
JKUAT	Jomo Kenyatta University of Agriculture and Technology
KBS	Kanzania Bureau of Standards
LAB	Lactic Acid Bacteria
MOL	Moringa Oleifera Leaf
MRS	de Man Rogosa Sharpe medium

NVWA Netherlands Food and Consumer Product Safety Authority

OUT Operational Taxonomic Unit

PCA Plate Count Agar

PCR Polymerase Chain Reaction

RAD-Seq Restriction site associated DNA markers

RBA Rose-Bengal Chloramphenicol Agar

S+W Starter chicken feed supplemented with fresh Wandering Jew weeds

SHC and FASFC Superior Health Council and Federal Agency for Safety of the Food Chain

SO Starter chicken feed only

SS Salmonella Shigella agar

SSA Sub-Saharan Africa

TBS Tanzania Bureau of Standards

TVC Total Viable Count

VRBG Violet Red Bile Glucose medium

VRBLA Violet Red Bile Lactose Agar

ABSTRACT

The rapid growth of the world population and the continuous need to ensure future food security has led to a global surge in demand for affordable, alternative and sustainable food sources. In this perspective, increased use of insects as food and feed to ensure future food security has been promoted. However, the majority of the edible insects, especially in Africa, are harvested from the wild with exception of a few insect species that are being reared and thus, their utilization is hampered by seasonal availability and food safety risks. This study aimed at molecular identification and safety assessment of wild harvested edible long-horned grasshoppers (of the genus *Ruspolia*) that exist(s) in East Africa, and evaluate the effect of feed supplementation on growth and safety of farmed edible crickets (*Acheta domesticus* and *Gryllus bimaculatus*). Three loci *i.e.,* 18S ribosomal subunit (18S rDNA), 28S ribosomal subunit (28S rDNA) and cytochrome oxidase subunit II (COII) were used for molecular identification of *Ruspolia* spp. In order to assess the microbial load of long-horned grasshoppers, samples were collected in two districts, *i.e.,* Bukoba rural and Muleba within the Kagera region, Tanzania. In farmed crickets, *G. bimaculatus* were supplied with two types of feed, *i.e.,* Starter chicken feed only (SO), and Starter chicken feed supplemented with fresh Wandering Jew weeds (S+W). In a separate experiment, starter chicken feed was supplemented with *Moringa oleifera* leaf powder (MOL: 5% or 10%), and *Azadirachta indica* leaf powder (AIL: 5% or 10%) and fed to crickets (*A. domesticus* and *G. bimaculatus*). Besides, the efficacy of common processing methods (toasting, boiling, deep-frying and smoking) in reducing microbial load was evaluated. All microbial analyses were conducted using standardized ISO methods. The levels of non-essential metals (Pb and Cd) and essential metals (Cu, Cr, Mn and Zn) were also investigated in farmed crickets (*A. domesticus* and *G. bimaculatus*), and in wild harvested long-horned grasshoppers using AOAC method 1995.01. The 18S rDNA partial sequences obtained from long-horned grasshoppers sampled in E. Africa were in agreement with the 18S rDNA sequence of *Ruspolia differens* (Orthoptera: Tettigonidae) that was already available in GenBank. On the contrary, it was impossible to reliably distinguish *R. differens* from other related tettigoniids based on two other genetic markers (28S rDNA and Cytochrome Oxidase subunit II), due to the absence of the corresponding gene sequences from *R. differens* in GenBank. The microbial analysis of wild harvested *R. differens* revealed high microbial counts including total viable count (TVC: 7.1 – 7.5 log cfu/g), *Enterobacteriaceae* (5.4 – 5.6 log cfu/g), lactic acid bacteria (LAB: 6.1 – 7.0 log cfu/g), bacterial endospores (3.0 – 3.3 log cfu/g), and yeasts and moulds (4.7 – 5.0 log cfu/g), exceeding the recommended process hygiene criteria of comparable food matrices, such as raw minced meat. After feeding *G. bimaculatus* for four weeks, the final body weight differed significantly ($P = 0.026$) between crickets fed with SO (1.11 g) and with S+W (1.39 g). Although high microbial counts were observed in both experimental groups, bacterial endospore counts of S+W fed crickets (2.7 log cfu/g) were significantly lower ($P = 0.021$) than those of the SO fed ones (3.9 log cfu/g). Metagenetic analyses revealed a few Operational Taxonomic Units (OTUs) with potential food pathogens that included *Clostridiaceae*, *Staphylococcus* and *Enterobacteriaceae*. The supplemented feeds (10% MOL or 10% AIL) significantly decreased ($P < 0.05$) the body weights in both *A. domesticus* and *G. bimaculatus* while supplementation with 10% MOL and AIL

(5% or 10%) significantly increased ($P < 0.05$) the mortality in both cricket species. Although high microbial counts were observed, significantly lower counts ($P < 0.05$) of LAB and bacterial endospores in treatments containing 10% MOL or 10% AIL in both cricket species were recorded. After processing long-horned grasshoppers and crickets, significant reductions ($P < 0.001$) of microbial counts (TVC, *Enterobacteriaceae*, LAB, yeasts and moulds) were observed, but bacterial endospores were not completely eliminated. In both grasshopper and cricket samples, Cu and Zn were consistently the most abundant metals while Pb and Cd were not detected. The findings of this study illustrate that raw edible insects can contain a significantly high microbial load, above the recommended hygiene criteria. However, feeding crickets with either S+W or with supplemented feeds containing 10% MOL or 10% AIL significantly reduced spore-forming bacteria. Besides, the inclusion of Wandering Jew weeds significantly increased the body weight of farmed crickets, and thus, it should be tapped in insect feed formulation. Therefore, greater focus should be directed towards sustainable farming of edible insects in controlled environments, so that it could be easy to identify the sources of the hazards, such as those associated with the feeds and farming environments. It is also evidenced that farmed crickets and wild harvested edible grasshoppers may not pose a food safety health risk in relation to heavy metal contamination when used as food or feed.

CHAPTER ONE

INTRODUCTION

1.1 Background information

The rapid growth of the world population, currently at 2.3% per year in Sub-Saharan Africa (SSA) (FAO, 2013), has led to a steady increase in demand for food, and agricultural productivity can hardly keep pace with. This has prompted re-evaluation of other sources of food that are affordable, sustainable, nutritious, and with a low environmental footprint. In this perspective, an initiative to use insects as food and feed was proposed in order to ensure future food security (van Huis et al., 2013a). The initiative on the use of insects as a source of proteins and other nutrients for human and animal consumption was based on the publication "*Potential of Insects as Food and Feed in Assuring Food Security*" (van Huis, 2013b). Since then, edible insects have been considered as suitable alternatives to animal sources of food, such as chicken and beef in addressing food and nutrition insecurity (Belluco et al., 2013; Nowak et al., 2016).

The choice of insects as food source is justified by the fact that their nutritional composition (relative amount of proteins, fat, vitamins, minerals, and calories) compares favourably with that of meat and fish (Belluco et al., 2013; Nowak et al., 2016; Rumpold and Schlüter, 2013a). In addition, edible insects have a higher feed-conversion efficiency than conventional livestock, *i.e.*, insects need less feed for the production of 1 kilogram of biomass than livestock does (Lundy and Parrella, 2015; Nakagaki and DeFoliart, 1991), have a much higher fecundity level, *i.e.*, produce more than one generation during a single season, and several species are omnivorous, *i.e.*, they could be raised on various organic waste materials (Caparros Megido et al., 2015; Kinyuru and Kipkoech, 2018). This translates into a much better ecological footprint of mass-produced insects compared to livestock, with significantly lower greenhouse gas emissions, water and land requirements (Oonincx et al., 2010).

Although consumer acceptance of edible insects and insect-derived products is still limited in western societies (Caparros Megido et al., 2014; Mancini et al., 2019; Orsi

et al., 2019), in many regions of Africa, Asia, and Latin America, the culture of eating insects (entomophagy) by various ethnic groups has been part of traditional food systems and diets for decades (Christensen et al., 2006; Kelemu et al., 2015; van Huis, 2013b). Worldwide, over 2,000 species are known to be edible, and consumed by approximately 2 billion people (Kelemu et al., 2015; van Huis, 2013b). Africa alone is reported to consume approximately 500 different species of insects at some stages of their life cycle (Belluco et al., 2013; Kelemu et al., 2015).

Up to date, majority of the edible insects, especially in Africa and Latin America, are harvested from the wild with exception of a few insect species that are being reared on either large scale for commercial use or small scale for experimental purpose (van Huis, 2013b). The consumption of wild harvested insects is usually hindered by seasonal availability, sustainability issues, high perishability, and food safety risks (Murefu et al., 2019; Ssepuuya et al., 2019). In this context, in an effort to overcome the above challenges, the development of insect farming has been proposed to (i) reduce the collection of insects from the wild and, thus, relieve the pressure on natural populations and habitats, (ii) generate a continuous supply of edible insects, and consequently, (iii) increase households' protein consumption and improve the safety (Caparros Megido et al., 2015; Halloran et al., 2018).

The edible insects in this study included commonly consumed long-horned grasshoppers found in East Africa, *i.e., Ruspolia differens* (Serville) (Orthoptera: Tettigoniidae), and crickets, *i.e.*, the house cricket *Acheta domesticus* (Orthoptera: Gryllidae) and the field cricket *Gryllus bimaculatus* (Orthoptera: Gryllidae). Within the genus *Ruspolia*, two species, *i.e.*, *R. differens* and *R. nitidula* (Scopoli) in the family Tettigoniidae (long-horned grasshoppers, also designated as katydids or bush crickets), are morphologically very similar and often indistinguishable (Matojo and Hosea, 2013a; Matojo, 2017). In reference to previous studies, the two species have been widely interchanged, misdiagnosed, or incorrectly named due to a lack of clear-cut information on their differences (Matojo, 2017).

In the literature, the long-horned grasshoppers harvested in Tanzania were referred to as *R. differens* (Matojo & Hosea, 2013a; Mmari et al., 2017), while those collected in

Uganda were described as *R. nitidula* (Agea et al., 2008; Ssepuuya et al., 2017). This misconception led to confusion in research studies, treating *R. differens* and *R. nitidula* as two sympatric species in E. Africa. Therefore, the current knowledge on the identity of long-horned grasshoppers found in E. Africa is poor. The use of molecular techniques, such as DNA barcoding, in species identification could aid in resolving confusion and possible contradiction (Flook & Rowell, 1998). Up to date, long-horned grasshoppers found in E. Africa are harvested in the wild during the swarming seasons, which coincide with the rainy seasons of March - May and October - December (Agea et al., 2008; Ssepuuya et al., 2017). Therefore, in regard to long-horned grasshoppers, this study aimed to (i) identify the *Ruspolia* (sub)species that exist(s) in E. Africa using molecular techniques and (ii) determine the microbial load along the food chain, and heavy metal contamination.

On the other hand, cricket farming is gaining momentum in many parts of the world particularly in low- and middle-income countries. A greater attention has been put on the house cricket (*A. domesticus*) and the field cricket (*G. bimaculatus*) because of the ease of their domestication. More so, these species can feed on different organic wastes, and can be reared with significantly shorter life cycles (Caparros Megido et al., 2015; Halloran et al., 2016). It has been demonstrated that the cricket rearing is simple, requires less technical support and capital expenditure, and simple harvesting techniques (Caparros Megido et al., 2015; Halloran et al., 2016; Halloran et al., 2018). Although cricket farming is making progress, research on how to improve and optimize cricket farming systems for increased production is still on-going. Some of the challenges hindering their mass production is unavailability of affordable feeds and lack of an understanding on the effect of various feeds on the nutrition and safety of insects intended for human consumption. As one way of addressing this, this research study aimed at exploring the use of locally available plant resources as feeds in the rearing of both *A. domesticus* and *G. bimaculatus*, and to assess the effect of various feeds on body growth parameters, microbial load, and heavy metal levels in insects intended for human consumption.

1.2 Problem statement

Worldwide, there were 821 million chronically undernourished people in 2018, up from 811 million the previous year (UNICEF and WHO, 2019). According to this report, one in nine people in the world now faces hunger, and Africa remains the continent with the greatest prevalence of hunger in the world, where one in five people faces hunger. Most of the conventional sources of animal proteins like beef, poultry, fish and pork are too expensive for the majority of the people in developing countries. In this perspective, as the number of hungry people continues to rise, this has necessitated the need to re-evaluate alternative sources of foods that are affordable and sustainable.

In Africa, entomophagy has been practiced for decades based on wild collection *e.g.*, *Ruspolia* grasshoppers (identity of exact spp. in E. Africa not clear and ought to be identified using molecular techniques). Thus, high perishability of the harvested insects is a huge challenge associated with this practice (Murefu et al., 2019). In addition, little information is available on the food safety issues associated with consumption of edible insects and scientific data are scarcely available, as indicated in the European Food Safety Authority opinion report (EFSA, 2015). Furthermore, previous studies (Belluco et al., 2013; Klunder et al., 2012) have demonstrated that edible insects, just like meat and meat-derived products, are rich in nutrients and moisture, thus, providing a favourable environment for growth of pathogenic microbes.

For instance, edible insects collected in natural environments were found to be infected with pathogenic micro-organisms, including bacteria, viruses, fungi and protozoa (Belluco et al., 2013) while Schabel (2008) reported some cases of botulism and other food-borne illnesses linked with the consumption of wild harvested insects in Africa. On the other hand, although insect farming is gaining momentum in many parts of the world, the safety of organic waste streams considered as the potential rearing substrate is the crucial factor, since it might introduce safety risks such as microbial hazards and bioaccumulation of chemical residues, *e.g.*, toxic metals in reared insects. The presence of pathogenic micro-organisms and chemical residues in

insects used as food and feed are therefore of public health concern warranting this study.

1.3 Justification

Edible insects are well-appreciated food with over 2,000 species known to be consumed by about 2 billion people worldwide (Christensen et al., 2006; Kelemu et al., 2015; van Huis, 2013b). Edible insects are highly nutritious with vital nutritional components such as proteins, fats, vitamins and trace elements comparing favourably with that of meat and fish (Belluco et al., 2013; Nowak et al., 2016). However, the majority of edible insects are still collected in natural environments, limiting their consumption when they are not seasonally available. In order to relieve the pressure on wild collections and continuously supply edible insects to the consumers, insect farming has been proposed by FAO (2013). Sustainable insect farming could contribute substantially to increased food security, especially in areas where the prevalence of undernourishment is common (van Huis et al., 2013a).

Furthermore, farming of insects in controlled environments has been advocated as one way of reducing the food safety risks associated with consumption of wild harvested insects. More so, during the farming of insects, it could be easy to control the hazards mostly emanating from the feeds. For instance, during the rearing of mealworm larvae (*Tenebrio molitor*), Klunder et al. (2012) observed that insect farming can allow greater control over hygienic practices and safe feed sources, thus, mitigating potential microbiological hazards. In this regard, this study sought to evaluate the effect of supplementing feed using locally available plant materials on body growth parameters and food safety aspects (microbial and heavy metal hazards) of farmed crickets. Also, microbial and heavy metal hazards of wild harvested long-horned grasshoppers were also examined. Additionally, crucial information on the efficacy of conventional processing methods in reducing microbial load in farmed crickets and in wild harvested grasshoppers was evaluated.

1.4 Objectives

1.4.1 General objective

To conduct molecular identification and safety assessment of wild harvested long-horned grasshoppers (*Ruspolia* spp.), and evaluate the effect of feed supplementation on growth and safety of farmed edible crickets (*Acheta domesticus* and *Gryllus bimaculatus*)

1.4.2 Specific objectives

1. To conduct molecular identification of wild harvested long-horned grasshoppers (*Ruspolia* spp.) from different out-breaking populations in E. Africa
2. To assess the microbial load along the food chain of wild harvested long-horned grasshoppers (*Ruspolia* spp.)
3. To determine the effect of dietary inclusion of Wandering Jew (*Commelina sinensis*) weed on growth and bacterial community composition of farmed crickets (*Gryllus bimaculatus*)
4. To evaluate the effect of dietary supplementation with powder derived from drumstick tree (*Moringa oleifera*) and neem tree (*Azadirachta indica*) leaves on growth and microbial load of farmed crickets (*Acheta domesticus* and *Gryllus bimaculatus*)
5. To quantify heavy metal levels in wild harvested long-horned grasshoppers (*Ruspolia* spp.) and in farmed crickets (*Acheta domesticus* and *Gryllus bimaculatus*)

1.5 Hypotheses

Ho_1: The wild harvested long-horned grasshoppers (*Ruspolia* spp.) from different out-breaking populations in E. Africa do not differ

Ho_2: The microbial load along the food chain of wild harvested long-horned grasshoppers (*Ruspolia* spp.) do not differ

H_{O3}: The dietary inclusion of Wandering Jew (*Commelina sinensis*) weed has no significant effect on growth and bacterial community composition of farmed crickets (*Gryllus bimaculatus*)

H_{O4}: The dietary supplementation with powder derived from drumstick tree (*Moringa oleifera*) and neem tree (*Azadirachta indica*) leaves does not significantly affect the growth and microbial load of farmed crickets (*Acheta domesticus* and *Gryllus bimaculatus*)

H_{O5}: The heavy metal levels in wild harvested long-horned grasshoppers (*Ruspolia* spp.) and in farmed crickets (*Acheta domesticus* and *Gryllus bimaculatus*) are not significantly different

CHAPTER TWO

LITERATURE REVIEW

2.1 Insects for food and feed

Edible insects are widely consumed food throughout the world. It is estimated that over 2000 insect species contribute to the traditional diets of over 2 billion people especially in South America, Africa and Asia (van Huis, 2013b). Of the 2000 species, about 500 species are consumed in Africa (Kelemu et al., 2015; van Huis, 2013b). The majority of the edible insects, especially in Africa and Latin America, are collected from their natural environments (van Huis, 2013b). In developed regions such as Europe and some parts of America, insects are not traditionally consumed (Mancini et al., 2019). In these temperate regions, edible insects are not available throughout the year or during the predicted times of the year as is the case in tropical regions such as in Africa (van Huis, 2013b). As a result, many western citizens have a negative attitude towards entomophagy and are reluctant to try novel foods such as edible insects and insect-derived products (Mancini et al., 2019; Orsi et al., 2019).

In general, edible insects are highly nutritious. Nutritional profiles based on dry matter consisting of 236 edible insect species were established (Rumpold & Schlüter, 2013a). Although significant variation was found in the compiled data, the nutritional composition (relative amount of proteins, fat and calories) that meets humans' requirements was established. In addition, high levels of monounsaturated and /or polyunsaturated fatty acids, and some essential metals including copper, iron, magnesium, manganese, phosphorous, selenium and zinc were reported (Rumpold & Schlüter, 2013a). These nutritional benefits, put together with the social-cultural and economic perspectives, have led entomophagy being practiced as a traditional heritage particularly in Africa (Christensen et al., 2006; Kelemu et al., 2015; van Huis, 2013b). Insects are mostly consumed in their entirety, but they can also be processed into different products such as pastes or powders. More so, the extraction of proteins, fats, chitin, minerals and vitamins from insects for other uses has attracted a lot of attention (FAO, 2013).

In Africa, a single community has been reported to consume different kinds of insect species in different stages of their life cycles. For instance, Malaisse and Latham (2014) reported 30 species consumed among the Bemba people in northern Zambia, southern Democratic Republic of Congo (DRC) and north-eastern Zimbabwe, while Obopile and Seeletso (2013) identified 27 edible insect species consumed as traditional diets in Botswana. In DRC, Takeja (1990) reported 21 species consumed by the Ngandu community predominantly getting their edible insects from the nearby forests. More so, in the city of Kinshasa, DRC, an average household consumed approximately 300 g of caterpillars per week and 96 tonnes of caterpillars were harvested and consumed in the city annually as a major source of protein and other nutrients (Vantomme et al., 2004).

The Mbunda people in Angola, Zambia and Namibia have been reported to consume about 31 species of edible insects (Silow, 1976). In the Central African Republic, 95% of people who lived in the nearby forests heavily depended on edible insects as the only sources of protein, fats, vitamins, and minerals (van Huis et al., 2013a). In Uganda, grasshoppers commonly known as “nseenene” or “senene” and the winged termites commonly known as “nswa” are the most commonly consumed insects and main sources of protein intake particularly in rural areas (Mbabazi et al., 2012). In Kenya, insect species such as lake flies, black ants, crickets, grasshoppers, and large alate termites commonly known as ‘agoro’ in Luo or ‘chiswa’ in Luhya, form part of traditionally consumed meals particularly in Lake Victoria region and western Kenya (Ayieko et al., 2011; 2012). However, there is no conclusive inventory of all edible insects in each country in Africa despite several attempts to revise it (Kelemu et al., 2015).

The use of insects for feed is traditionally constricted mainly to fish bait, pet birds and reptiles (van Huis et al., 2013a). Most insect species have the added advantage of a proportionally higher protein content compared to the conventional fish and soybean meals that are commonly used as feed (Anand et al., 2008). In addition, the amino acid compositions derived from most insect proteins are superior to those from plant supplements in poultry feed formulations (Bukkens, 2005; Ravindran & Blair, 1993). Recently, the larva or prepupa of the black soldier fly (BSF) (*Hermetia*

illucens) has gained a lot of attention as potential insect species reared for animal feed (De Smet et al., 2019). The larvae of *H. illucens* are generalist detritivores and may be reared on a variety of organic waste streams (Makkar et al., 2014). Furthermore, the high feed conversion factors of most insects (Nakagaki & DeFoliart, 1991; Oonincx et al., 2015), make them a promising commodity to be promoted for feed.

2.2 Commonly consumed insect species in East Africa

Although about 500 species are reported to be consumed in Africa (Kelemu et al., 2015; van Huis, 2013a), in E. Africa the most consumed insects include black ants, termites and long-horned grasshoppers (Ayieko et al., 2011; 2012; Mbabazi et al., 2012).

2.2.1 Black ants

Within the Lake Victoria basin, it is estimated that there are over 200 species of black ants, of which only one, *i.e., Carebara vidua* Smith is edible in the community (Ayieko et al., 2012). *Carebara vidua* also known as 'onyoso' in Luo and Luhya ethnic languages in Kenya, are considered a delicacy by the communities within the Lake Victoria region (Ayieko et al., 2012). *Carebara vidua* usually build mounds underground and under rocks, and their emergence is not easily noticeable on the ground. Often, the insects emerge and quickly fly away unnoticed because they look just like other black ants of less value (Ayieko et al., 2012). Nevertheless, the local communities manage to collect some of these insects using traditional methods, such as hand-picking, for their own consumption. The ants are rarely sold in the open markets due to the small harvest. Notably, *C. vidua* has a favourable nutritional value, and in particular its oil gives it a unique rich flavor (Ayieko et al., 2012). Of late, *C. vidua* is one of the edible insect species facing danger of extinction within the Lake Victoria region due to environmental degradation (Ayieko et al., 2012).

2.2.2 Termites

In Sub-Saharan Africa (SSA), edible termites are thought to constitute about 10% of all animal biomass, and up to 95% of soil insect biomass (van Huis, 2017). In E. Africa, termites form an important part of the food culture within the Lake Victoria region (Defoliart, 1999; van Huis, 2017). Within the winged termites (*Macrotermes* spp), several species exist including *Macrotermes nigeriensis*, *Macrotermes notalensis*, *Macrotermes subhylinus* and *Macrotermes bellicosus*. However, the large alate termite (*M. subhylinus*) is the mostly consumed within the Lake Victoria region. These termites tend to emerge during the onset of long rains between the months of March to May and October (Ayieko et al., 2011; Kinyuru et al., 2013), but these emergence patterns are changing with the climate changes (van Huis et al., 2017).

Termites are normally wild-collected using traditional methods, which varies from species to species in different parts of the region (Ayieko et al., 2011). For instance, *M. subhylinus* normally emerge in large numbers at night and are collected by the locals by introducing a light source inside a bucket lined with wet slippery banana leaves, placed near an active mound (Ayieko et al., 2011). Another common collection technique includes placing a bowl of water under a light source. Attracted to the light, the termites fall into the water bowl, cannot fly away, and can be easily collected (Ayieko et al., 2011). In other instances, when the population of emerging termites is small, the hand-picking is practiced but this usually results in small harvests (Ayieko et al., 2011; Kinyuru et al., 2013).

The burrowing termites are normally found nesting in pairs or more under loose covers on the ground such as stones, fallen leaves, and dry woody materials. Thus, the harvesters have to unearth them from these temporary nests or tunnels in their early stages of forming colonies (Ayieko et al., 2011). The catch in this stage is tedious, slow and not ideal for collecting sizeable amounts to make a meal (Ayieko et al., 2011; Christensen et al., 2006; Kinyuru et al., 2013). Other species of termites emerge only at night while others come out between mid-mornings to late afternoon, while some emerge early evenings. However, these variations in emergence times

normally pose a challenge to the collectors. Although traditional methods of harvesting termites may result into sizeable catch, they could also pose a danger to the harvesters because of likeliness of coming into contact with their dangerous predators such as snakes (Ayieko et al., 2011; Kinyuru et al., 2013).

In an attempt to improve the harvesting methods of termites, Ayieko and co-workers (2011) designed a light trap (consisting of the trap stand, a light source to attract the insects, and a receptacle basin) specifically for collecting *M. subhylinus* at night based on it's behavior and characteristics (Figure 2.1). Although the authors observed some improved efficiency in the collection of *M. subhylinus*, a call for further research into the characteristics exhibited by this termite species was advocated.

Figure 2.1: The termite harvester showing light source, collector basin and stand. Source: Ayieko et al. (2011).

2.2.3 Long-horned grasshoppers

In E. Africa, long-horned grasshoppers are considered a delicacy by many tribes. In particular *Ruspolia differens* is known to be non-destructive, as it causes no damage to crops and vegetation and this distinguishes it from migrating locusts (Kelemu et al., 2015). In addition, *R. differens* is known to exhibit swarming behavior during certain periods of the year (Bailey & McCrae, 1978). During the swarming seasons, most of the swarms are concentrated on streetlights because the swarming adult

grasshoppers are normally attracted to light. During this period, the local communities collect them using baskets, polythene bags, and drums (Kinyuru et al., 2010; Ssepuuya et al., 2016). These grasshoppers are usually wild harvested bi-annually during the months of March-May and October-December, and consumed as a traditional snack by many tribes in the region (Kinyuru et al., 2010; Mmari et al., 2017; Ssepuuya et al., 2016).

In order to increase the catch of the grasshoppers especially during the dense swarming, locals have designed their own traditional methods of harvesting. During the swarming seasons, harvesters set up open barrels (150 to 200 litres) on top of which are metal sheets inclined at an angle between 45^{o} and 75^{o} (Figure 2.2). Then, at night, high voltage lights are switched on, attracting grasshoppers towards the lights in front of the metal sheets. The grasshoppers land on the metal sheets, and slide down into the barrels. Although it is difficult for grasshoppers to fly vertically out of the barrel, the walls of the barrels are deliberately made slippery with either cooking oil/ industrial grease or maize flour as a precaution, especially when the barrel is almost full to capacity and harvesters are not within (Ssepuuya et al., 2016).

Figure 2.2: Long-horned grasshopper harvesting trap using drums as a recipient. Source: https://www.bbc.com/news/world-africa-46357020

However, this mass harvesting technique of grasshoppers could be expensive and challenging to many due to the inputs required for the set-up. This include barrels, electric supplies (bulbs and wires), generators in areas where there is no electricity, poles for making the platforms on which the barrels sit or lean, and packaging materials (Agea et al., 2008). After catching the grasshoppers, they are removed from the drums, and or plucked (removal of legs, wings, antennae, ovipositor, and other appendages), and later packed in aerated polythene or nylon bags of various capacities depending on the quantities harvested, and delivered to the households/ markets.

2.3 Mass rearing, harvesting and technological upscaling of edible insects

In the past, rearing of insects has mainly focused on commercially valuable products derived from insects such as silk and honey. Recently, the potential of insects to be reared for food and feed has gained increased attention. The rearing of insects as replacement for wild capture has a number of advantages, such as relieving the pressure on natural populations and habitats as well as enabling a more consistent quality control to ensure consumers food safety (Caparros Megido et al., 2015; Halloran et al., 2018; van Huis et al., 2013a). Using valuable traditional knowledge and food culture, together with modern science, contributions aimed at scaling up of mass rearing technologies of edible insects across the world are gaining momentum.

In general, based on EFSA (2015), Rumpold and Schlüter (2013b) and the Superior Health Council and Federal Agency for Safety of the Food Chain (SHC and FASFC, 2014) the rearing process for most insects can be sub-divided as follows: (1) rearing phase, (2) harvesting phase and (3) post-harvest phase. During the rearing phase, insects can be reared in small containers or cages, depending on the species. Then, the feed is supplied; this can be, *e.g.*, commercial chicken feed and water. During the feeding period, insects are either kept inside their feeds (*e.g.*, black soldier fly larvae), or feed is provided in a separate container (*e.g.*, crickets). Environmental conditions are controlled, maintaining temperatures most often between 25 °C and 31 °C. Relative humidity is mostly controlled in the range of 50% to 70%. During harvesting, some species are harvested in their final juvenile stage (*e.g.*, black soldier

fly larvae), and other species in the adult stage (*e.g.*, crickets and grasshoppers). Harvesting involves the separation of the insects (juvenile stage or adult stage) from the containers/cages and from the residues, and may be performed manually or by use of an automated system. Post-harvest phase may include treatments such as cleaning, killing by submerging them in hot water, drying and grinding.

Commonly commercially farmed insects for human consumption include crickets and palm weevils in Africa, the giant water bug and palm weevils in Thailand, and water beetles in China (Halloran et al., 2018; Hanboonsong et al., 2013; van Huis et al., 2013a). In low and middle income countries, such as Thailand, Cambodia, Kenya, and Lao People's Democratic Republic, cricket farming is becoming increasingly popular in improving the livelihood of local people (Halloran et al., 2018; Hanboonsong et al., 2013). In these countries, cricket rearing is normally carried out using basic cages and occupying little space, and feeding them mainly with household food wastes (Caparros Megido et al., 2015; Halloran et al., 2015; Halloran et al., 2018).

In developed regions such as Europe, with the increasing attention for insects as food and feed, commercial rearing companies are being established and some are already producing insects for food and feed. The species mostly reared for food include the yellow mealworm larvae (*Tenebrio molitor*), the lesser mealworm larvae (*Alphitobius diaperinus*), the house cricket (*Acheta domesticus*), and the tropical house cricket (*Gryllodes sigillatus*) (EFSA, 2015; van Huis, 2013b). In regard to animal feed, large scale rearing companies are producing house fly larvae (*Musca domestica*) and black soldier fly (*H. illucens*) larvae and/or prepupae grown on organic waste streams used for livestock and aquaculture feed (EFSA, 2015). Up to date, the available legislation allows companies in Europe to focus mainly on the use of whole living insects and insect-derived fats and proteins, from seven insect species namely *H. illucens, M. domestica, T. molitor, A. diaperinus, A. domesticus, G. sigillatus* and *G. assimilis* in feed for aquaculture animals (European Commission, 2017). However, these insects are not allowed as a feed ingredient for other farmed animals, like pigs or poultry. In developing countries particularly in Africa, there appears to be a lack of specific regulatory frameworks for using insects as food.

There are some exceptions, for instance mopane caterpillars (*Imbrasia belina*) are considered edible according to Botswana's food law (Grabowski et al., 2020). Recently, the Kenya Bureau of Standards (KEBS) approved National Standard that will guide the primary production and handling of insects for food and feed (KS 2921:2020).

2.4 Safety issues associated with consumption of edible insects

Little is known on the food safety aspects of edible insects and this could be of critical importance to meet the society's approval, especially if people are not accustomed to eating insects. Despite a plethora of studies on edible insects, most of the studies have mainly focused on nutritional composition, studies on the safety of the edible insects are still limited or missing, especially in Africa. Just like vertebrates, safety concerns associated with the consumption of edible insects, such as microbiological, chemical and allergenic agents, can present a health threat to consumers (Garofalo et al., 2019; Imathiu, 2020; van der Fels-Klerx et al., 2018).

The safety of edible insects is of greatest concern in most African countries because the majority of these insects are collected from their natural environments (Murefu et al., 2019; van der Fels-Klerx et al., 2018). This makes it difficult to control the hazards emanating from the wild feeds the insects consume. However, in some developed countries where insects are farmed in controlled environments, and good handling and production techniques are applied, it could be easy to control some levels of hazards (Rumpold & Schlüter, 2013b). According to EFSA (2015), prevalence and concentration of contaminants in insects and insect-derived foods is majorly influenced by the production method, insect species, insect stage of harvest and substrate used in the rearing process. Thus, to improve the food safety aspect of edible insects, the shift from wild harvesting to farming in controlled environments is advocated, and the above stated factors have to be controlled and regulated during the rearing process.

2.4.1 Microbial hazards associated with edible insects

2.4.1.1 Bacteria

Studies about microbial contamination of edible insects and insect-derived products are limited in the scientific literature particularly in Africa. However, the available data indicate that edible insects either wild-collected or reared may be infected with spoilage and pathogenic microorganisms (Garofalo et al., 2019; Imathiu, 2020; Murefu et al., 2019; Rumpold & Schlüter, 2013b; van der Fels-Klerx et al., 2018). More specific studies on the microbiological safety comparing reared versus wild harvested insects for food or feed are rare or missing in the literature. At present, there are no specific microbial criteria laid down for edible insects or insect-derived products. However, some national food safety authorities (EFSA, 2015; SHC & FASFC, 2014) and some authors (Caparros Megido et al., 2017; Grabowski & Klein, 2017a) proposed the total viable counts (TVC) in minced meat as an indicator of food safety for edible insects.

High numbers of spoilage micro-organisms and some pathogenic micro-organisms have been reported in some edible insects. For instance, pathogenic bacteria including *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Bacillus cereus* were isolated in three rhinoceros beetles (*Oryctus Monoceros*, *Oryctus boas*, and *Oryctus owariensis*) that are commonly consumed in Nigeria (Banjo et al., 2006). Wild harvested raw grasshoppers (*R. differens*) from Uganda were found to be highly contaminated by *Enterobacteriaceae*, lactic acid bacteria (LAB), and spore-forming bacteria (Ssepuuya et al., 2019). Furthermore, the authors observed the possibility of *R. differens* harboring potentially dangerous genera of bacteria, such as *Campylobacter*, *Bacillus*, *Staphylococcus*, *Neisseria*, *Pseudomonas* and *Clostridium*.

When two commonly consumed edible insect species (*A. domesticus* - farmed and *R. differens* – wild - harvested) and two species reared as animal feed (*H. illucens* and *Spodoptera littoralis*) in E. Africa were analyzed, although each species revealed varied levels of microbial contamination, they were found to contain high microbial counts (TVC, Lac+ enteric bacteria, *S. aureus*) exceeding the recommended criteria

for raw minced meat (Nyangena et al., 2020). The authors further observed the presence of *Salmonella* in all raw insect samples.

When farmed mealworm larvae (*T. molitor*) and crickets (*A. domesticus*) were subjected to different processing conditions (*i.e.,* fresh; boiled; roasted; fresh and stored; boiled and stored) and analyzed for microbial load, high levels of contamination with *Enterobacteriaceae* and spore-forming bacteria were observed in fresh insects (Klunder et al., 2012). In addition, when the same insects samples were pulverized, a further increase in contamination levels was observed and attributed to the release of gut microbiota (Klunder et al., 2012). High levels of microbial contamination were also observed in fresh *T. molitor* larvae and locusts (*Locusta migratoria migratorioides*) intended for human consumption (Stoops et al., 2016).

In a different study, when *A. domesticus, G. sigillatus*, and *T. molitor* samples from seven different insect rearing companies were analyzed, high counts of *Enterobacteriaceae* ranging from 6.8 to 8.3 log cfu/g in *T. molitor*, and from 7.3 to 8.3 log cfu/g in crickets were found (Vandeweyer et al., 2017a). In an effort to determine the microbial community in processed edible insects in Thailand markets, metagenetic analyses revealed presence of many potentially human pathogenic bacterial genera including *Vibrio*, *Streptococcus*, *Staphylococcus*, C*lostridium* and *Bacillus* (Osimani et al., 2017).

A microbiological survey involving 38 processed samples of *A. domesticus*, *Gryllus assimilis, G. bimaculatus, L. migratoria, Blabtica dubia, Galleria mellonella, Chilecomadia moorei, Pachnoda marginata, T. molitor, Zophobas atratus,* and *Apis mellifera* was carried out. Although each sample type revealed varied levels of microbial contamination, higher microbial counts were observed in dried and powdered samples than in cooked ones. In addition, *Bacillus cereus, Staphylococcus aureus*, *Escherichia coli*, *Salmonella*, *Shigella,* and *Campylobacter* were isolated (Grabowski & Klein, 2017b). In a risk assessment study on the microbiological status of 55 insect products (locusts, lesser mealworms, mealworms and a mealworm snack) that had undergone no treatment apart from freeze-drying, the Netherlands Food and Consumer Product Safety Authority (NVWA, 2014) found that the TVC

and *Enterobacteriaceae* counts of more than 60% of the insect products exceeded the process hygiene criterion recommended for raw meat.

These studies suggest the likelihood for both raw and processed ready-to-eat insects in contributing to microbial foodborne illnesses to the edible insects' consumers. Thus, there is need to ensure hygienic handling of edible insects during production, processing, distribution and preservation in order to reduce the risks of foodborne illnesses associated with them.

2.4.1.2 Fungi and mycotoxins

Foodborne fungi can be responsible for food spoilage through product quality deterioration and nutritional losses. Insects can be affected by fungi whose presence and extent of the effect depends on environment they inhabit. To date, only a few studies have been carried out in edible insects to determine the presence of fungi and levels of contamination with mycotoxins. Mycotoxins are secondary metabolites produced by many phytopathogenic and food spoilage moulds of mainly three genera, *i.e.*, *Fusarium*, *Aspergillus* and *Penicillium* (Kachapulula et al., 2018). Out of all the mycotoxins detected or quantified in edible insects, aflatoxins are of the greatest health concern particularly in Africa. In humans, a condition called aflatoxicosis, a primary hepatic disease associated with aflatoxin is common (Dhanasekaran et al., 2011).

In the literature, moulds associated with the production of mycotoxins have been isolated from fresh edible insects, as well as from dried or processed insects, and probably are a result of poor handling and processing practices (Mbata & Chidumayo, 2003; Mpuchane et al., 2000). Several authors (Caparros Megido et al., 2017; Grabowski & Klein, 2017a; Ssepuuya et al., 2019; Vandeweyer et al., 2017a; Wynants et al., 2018) found high counts of yeasts and moulds in raw edible insects surpassing the levels recommended for good production and processing practices set for raw meat. Furthermore, Grabowski and Klein (2017a) found high counts of fungi in dead crickets compared to living ones.

In Botswana, the inner flesh of wild harvested mopane caterpillar (*Imbrasia belina*) disintegrated due to mould growth with fungal isolates of the genera *Aspergillus*, *Penicillium*, *Fusarium*, *Cladosporium* and *Phycomycetes* isolated (Mpuchane et al., 2000). A study in Belgium involving reared crickets (*G. sigillatus*) yielded fungal isolates from the genera *Aspergillus*, *Candida*, *Kodamaea*, *Lichtheimia*, *Terapisipora*, *Trichoderma* and *Trichospora.* In addition, mycotoxin-producing fungi from *Aspergillus* spp. and *Penicillium* spp. were recovered from the feed, substrate and in *G. sigillatus* (Vandeweyer et al., 2018). The presence of moulds in these insects is of major concern because when the conditions are conducive (temperature and relative humidity), the toxigenic moulds can grow, leading to the production of mycotoxins.

The growth of fungi and accumulation of aflatoxin B1 in edible stink bugs (*E. delegorguei*) that were wild harvested in Zimbabwe, and transported using traditionally woven baskets previously used to store other produce was reported (Musundire et al., 2016). When commonly consumed insects (*Gynanisa maja, Gonimbrasia zambesina,* and *Macrotermes falciger*) in Zambia were analyzed for aflatoxin contamination, the average aflatoxin concentrations exceeded regulatory limits for that country (10 µg/kg). More so, when same samples were subjected to simulated poor storage, aflatoxins increased to unsafe levels in caterpillars (mean of 4,800 µg /kg) (Kachapulula et al., 2018). This is a clear indication that some toxigenic fungal species could germinate and produce mycotoxins when conditions are conducive presenting a health threat to edible insect consumers.

2.4.2 Chemical hazards associated with edible insects

Like products from other animals, insects and insect-derived foods and feed products may contain hazardous chemical residues.

2.4.2.1 Heavy metals

Contamination of edible insects and insect-derived products by heavy metals can cause adverse health effects, both acute and chronic to the consumers (Devkota and Schmidt, 1999). Heavy metal contaminants may be present in food and feed

originally derived from insects, and they occur as a result of the presence of the particular contaminant in the environment and/or the substrate (van der Fels-Klerx et al., 2018). However, the main exposure route is through the substrate on which the insects are fed and, therefore, the metal(s) of concern will vary accordingly (van der Fels-Klerx et al., 2018). The few studies available indicate that insects, whether wild harvested or reared could bioaccumulate heavy metals when they are exposed and the risk may be transferred further along the food chain (Diener et al., 2015; van der Fels-Klerx et al., 2018). Bioaccumulation of heavy metals in edible insects may depend on many factors including insect species, growth phase, feed substrate and metal element involved (van der Fels-Klerx et al., 2018). Heavy metals originate from both natural sources and human pollution, and the most common heavy metals reported in edible insects include cadmium, lead, mercury and arsenic (Devkota & Schmidt, 1999; Poma et al., 2017).

In a recently published study in Uganda involving *R. differens*, concentrations of lead (39.1 – 455.6 ppm) were detected, with significantly higher levels in grasshoppers harvested in peri-urban areas than those in rural areas (Kasozi et al., 2019). The authors attributed high concentrations of lead in peri-urban areas to plenty of forages during swarming seasons in close proximity to heavy industrial discharges. In the same study, the concentration of chromium and cadmium in grasshoppers harvested in both areas were low (Kasozi et al., 2019). A study on the yellow mealworm larvae (*T. molitor*) and black soldier fly (*H. illucens*) showed that both insects bioaccumulated cadmium, lead, and arsenic when they were fed on contaminated substrates such as organic matter in soils that contain these metals (Vijver et al., 2003).

When concentrations of heavy metals along the food chain (food plants and grasshoppers) were analyzed, the bioaccumulation of lead was very low compared to cadmium (Devkota and Schmidt, 1999). The authors hypothesized that the higher absorption of cadmium by grasshoppers was due to a higher chemical activity compared to lead. Poma et al. (2017) found low concentrations of arsenic, cadmium and lead (all < 0.03 mg/kg) in *L. migratoria* grasshoppers. Similarly, low concentrations of arsenic, cadmium and lead (all mean levels < 0.70 mg/kg) were

reported in edible grasshoppers from Korea and considered safe for human consumption (Hyun et al., 2012).

In a risk assessment study following an outbreak of lead poisoning, Handley et al. (2007) found a high lead content in chapulines (dried grasshopper) from Oaxaca (Mexico) and associated it to grasshoppers feeding in highly polluted mine areas. Low inorganic mercury and methyl mercury levels in the migratory locust (*L. migratoria manilensis*) and bordeaux cricket (*Acheta chinensis*), and in the feed were reported (Sheng et al., 2008). Similarly, when grasshoppers and locusts were exposed to mercury, low mean concentrations of 10-620 ng/g dry body weight were found (Rimmer et al., 2010).

Recently, low levels of mercury, lead, cadmium and arsenic were detected in four edible insect species (mulberry silkworm, scarab beetle, house cricket and bombay locust) consumed in Thailand and deemed safe for human consumption (Köhler et al., 2019). Although these few available studies suggest that edible insects may be generally regarded as safe in regard to heavy metal accumulation, there is a likelihood for edible insects, particularly those wild harvested ones, to contribute to unsafe foods in future.

2.4.2.1 Pesticide residues

Pesticides used in agricultural production may be present in plant materials and in agricultural by-products, which are used as a substrate for insect production. Wild harvested edible insects are of great concern when it comes to presence of pesticide residues (Poma et al., 2017). This is because the kind of materials they feed on in the wild is not controlled and sometimes they may feed on pesticide-sprayed vegetation or crops, which may potentially lead to accumulation of the residue, particularly for the case of swarming or migrating grasshoppers (van der Fels-Klerx et al., 2018). Thus, consumers relying on wild harvested edible insects are particularly at risk of pesticide food poisoning. It is a real problem in some developing countries, where even dead insects, mainly locusts and grasshoppers, are collected and consumed after insecticide treatment.

According to a study in Kuwait, after spraying crops to control pests, the collected locusts contained chlorinated and organophosphorus pesticide residues. In this research work, some of the analyzed locusts from the markets contained as high as 49.2 μg/kg and 740.6 μg/kg of chlorinated and organophosphorus pesticides, respectively (Saeed et al., 1993). Similarly, in Thailand, pesticide contaminated insects were found in the markets endangering lives of the consumers (DeFoliart, 1999). In a study consisting of composite samples of several species of edible insects (greater wax moth, migratory locust, mealworm beetle, buffalo worm) and four insect-based food items currently commercialized in Belgium, the organophosphate pirimphos-methyl (an insecticide used to control pests and mites in stored grain), was identified at a highest level of confidence (Poma et al., 2017).

In an investigation to detect the presence of pesticide residues in fly larvae, out of 393 pesticide residues analyzed, chlorpyrifos was detected in one sample of *M. domestica* reared on milk powder and sugar from China, and piperonyl butoxide was detected in one sample of *Calliphora vomitoria* farmed in the United Kingdom (Charlton et al., 2015). In another study, when *T. molitor* were exposed to carrots spiked with a pesticide containing twelve active ingredients at different concentrations, it was observed that the pesticide with the highest active ingredient concentrations appeared to bioaccumulate, while that with the lowest concentrations was readily excreted by the insects, though further studies were advocated (Houbraken et al., 2016).

Although the data available from the few studies in the literature have shown that the pesticide residues are generally below recommended maximum concentrations suggested by supranational organizations, such as the European Commission and the World Health Organization, more studies are needed.

2.4.3 Allergens

Globally, food allergy is an emerging public health concern. Differences in geographical food traditions can result in differences in food allergy risk. A wide range of foods containing protein can cause allergic reactions to sensitive people (Imathiu, 2020). Since edible insects contains great amount of protein, it is possible

that some insects and insect-derived foods are potential allergen sources. Some specific proteins present in edible insect including arginine kinase, α-amylase and tropomyosin are considered potential allergens (Murefu et al., 2019).

To date, few studies have been published on allergic reactions due to insect consumption. A study carried out in Thailand revealed cases of anaphylaxis related to cricket and grasshopper consumption but could not identify the species responsible for the allergic reaction (Piromrat et al., 2008). It has also been reported that allergic reactions induced by grasshoppers are more common and more severe than those induced by crickets (Ji et al., 2009). Although 0.1 to 5.7% of European adults have food allergies (Nwaru et al., 2014), a study carried out in Belgium revealed that 19% of persons were sensitized by skin prick tests prepared with grilled *A. domesticus* and *T. molitor* insect samples (Francis et al., 2019), suggesting high risk of developing allergic reactions to insects' consumers.

In China, silkworm pupa is the most commonly consumed insect type, and it is estimated that at least one thousand of the consumers experience allergic reactions with at least fifty of these consumers requiring hospitalization (Ji et al., 2008). Other studies revealed that 7.6% of insects' consumers in Laos exhibited allergic reactions (Barennes et al., 2015), while insect food allergy has been reported for mealworm, silkworm, sago worms, caterpillars, grasshopper, locust, bee, cicada, *Bruchus lentis* and *Clanis bilineata* (de Gier & Verhoeckx, 2018). In the scientific literature, most insect food allergies reported are from developed countries where edible insects are considered 'novel food' as opposed to developing countries where they are largely consumed. Nevertheless, with edible insects considered as food for the future, there is a potential for their increased consumption with the possibility of increasing insect food allergy prevalence particularly in developing countries. Thus, the potential for insects and insect-based products to cause allergenicity to consumers need to be established through further research.

CHAPTER THREE

MOLECULAR IDENTIFICATION OF LONG-HORNED GRASSHOPPERS (*RUSPOLIA* SPP.; TETTIGONIIDAE; ORTHOPTERA) FOUND IN EAST AFRICA

3.1 Abstract

In East Africa, the long-horned grasshoppers are source of food to many communities. However, the identity of long-horned grasshoppers in E. Africa is mainly inferred based on the morphological and behavioural characters with which their taxonomic status was delineated. This study adopted molecular analysis to identify long-horned edible grasshoppers in Kenya, Tanzania and Uganda. The 18S rDNA, 28S rDNA and Cytochrome Oxidase subunit II genes were used for grasshopper DNA analysis.

Based on 18S rDNA partial sequences, the sampled long-horned grasshoppers in E. Africa were identified as *Ruspolia differens* (Orthoptera: Tettigonidae). However, the genetic distance in all samples analysed and other related tettigoniids from the BLAST search was low. On the other hand, the genetic markers (28S and COII) were incapable of identifying *R. differens* from other related tettigoniids due to the absence of the corresponding gene sequences from *R. differens* in the GenBank. In conclusion, there is very limited genomic information on the long-horned grasshoppers occurring from E. Africa. Therefore, it is recommended that the extant populations would be analyzed in more detail via molecular genetic methods based on deep sequencing methods, such as restriction site associated DNA markers (RAD-Seq), to identify the (sub)species that occur in the area. To enable comparisons, sampling of morphologically well-described individuals from various regions in E. Africa would then be required.

3.2 Introduction

Ruspolia spp. are very widespread throughout the world, including islands of the Indian ocean as shown in Figure 3.1 (Hemp et al., 2010; McCrae, 1982; Matojo &

Hosea, 2013a). The geographical distribution of these long-horned grasshoppers is particularly linked with the need for a fairly even rainfall with seasonal peaks at regular intervals (Hemp et al., 2010; McCrae, 1982; Matojo & Yarro, 2010a). In E. Africa, the *Ruspolia* grasshoppers are a source of food to many communities, and are mostly consumed in Uganda, parts of western Kenya and Tanzania (Kinyuru et al., 2010; Mmari et al., 2017; Ssepuuya et al., 2016).

Within the genus *Ruspolia*, two species, *i.e., Ruspolia differens* and *Ruspolia nitidula*, in the family tettigoniidae are morphologically very similar and often indistinguishable (Matojo and Hosea, 2013a; Matojo, 2017). In his review of African species of the genus *Ruspolia*, Bailey (1976) stressed that *R. differens* could be composed of at least three subspecies that possibly overlapped with three other species based on main areas of occurrence. In reference to recent studies in E. Africa, the two species have been widely interchanged, misdiagnosed, or incorrectly named due to a lack of clear-cut information on their differences (Matojo, 2017). This situation has led to possible contradictions in the research literature, which seems to treat *R. differens* and *R. nitidula* as two sympatric species in this region.

Figure 3.1: Geographical distribution of *Ruspolia* spp. (maroon shadings) in (a) East Africa and islands of the Indian Ocean and, (b) West and North Africa, Europe and Asia. Source: MacCrae et al. (1982).

Nevertheless, the morphological and behavioral characteristics of *R. differens* and *R. nitidula* have been described in the literature. It is reported that *R. differens* is native in SSA including the E. African region while *R. nitidula* is a Palearctic species restricted to northern Africa, Asia and Europe (Bailey & McCrae, 1978; Matojo & Njau, 2010b; McCrae, 1982). The available data also indicates that *R. differens* exhibits unique colour polymorphism with six morphs (green, brown, purple-stripped green, purple-stripped brown, purple suffused green and purple suffused brown), while *R. nitidula* essentially consists of a green morph (Bailey, 1975; Bailey & McCrae, 1978; Matojo & Yarro, 2010a). Furthermore, *R. differens* is known to exhibit swarming behaviour during the wet seasons, while a non-swarming phase occurs during dry seasons. On the other hand, *R. nitidula* is known to be a non-swarming species (Bailey & McCrae, 1978; Brits & Thorton, 1981; Matojo & Njau, 2010b; McCrae, 1982).

In addition, sex dimorphism has been observed in *R. differens* whereby the male adults have much longer antennae (about 1.5 times) than the females, and have a pair of tongue-like metathoracic flaps located at the dorsal side of the proximal base of the hind wing while the females only have paired bud-like nodules appearing

somewhat as underdeveloped equivalents of the male flaps, and these features distinguish *R. differens* from other tettigoniids including *R. nitidula* (Bailey, 1975; Matojo & Yarro, 2013b; Matojo & Yarro, 2010a; McCrae, 1982).

Historically, morphological and behavioral traits have been used to examine the relationships of closely related taxa with global distributions. However, the use of such traits sometime may be misleading and may not provide the resolution needed to distinguish various hypotheses that may explain the distribution patterns of closely related taxa. One such taxon may include the genus *Ruspolia*. The relationships among this genus have seldomly been inferred based on molecular data (Legendre et al., 2010), and thus *Ruspolia* spp. lack apparent diagnostic features, hence their taxonomy largely requires molecular evidence. Molecular characterization and DNA barcoding has emerged as a tool for discovering previously unknown biodiversity, getting insight into speciation processes, and phylogenetic analyses (Legendre et al., 2010; Flook & Rowell, 1998). In addition, DNA barcoding can be used to discover cryptic species, *i.e.,* closely related and morphologically similar (Legendre et al., 2010).

In previous studies, DNA barcoding has been used to establish phylogenetic relationships among closely related taxa. For instance, Flook and Rowell (1998) and Flook et al. (1999) established the phylogenetic relationships among various Orthoptera groups while Dian-feng et al. (2008) described the diversity existing among members of the family Acrididae (short-horned grasshoppers). In the literature, molecular data of various *Ruspolia* spp. have been reported in various parts of the world including Asia, Europe and Australia (Danley et al., 2007; Flook & Rowell, 1998; Pratt et al., 2008). However, there are only limited or scanty published data from E. Africa, in which there is an immense abundance of *Ruspolia* grasshoppers (Matojo & Hosea, 2013a). Therefore, the existing molecular data are insufficient, as they do not convey comprehensive phylogenetic relationship of the *Ruspolia* spp. populations in E. Africa. In this study, attempts were made to infer the phylogenetic relationships of *Ruspolia* spp. found in E. Africa based on sequence comparison of their 18S rDNA, 28S rDNA and Cytochrome Oxidase subunit II genes.

3.3 Materials and Methods

3.3.1 Study sites and sample collection

Samples of *Ruspolia* spp. were collected from different out-breaking populations in E. Africa. During the months of March - May in 2017, swarms were observed in some villages in the Kagera region, Tanzania (1°45′S and 32°40′E) and in the Mbarara district in Uganda (0°36' S and 30°39'E), as well as in Jomo Kenyatta University of Agriculture and Technology (JKUAT), Kenya (1°08′S and 37°02′E). Also, in the months of March-May in 2018, swarms were observed in Nandi Hills, Kenya (0°06'N and 35°10'E). In this study, a total of 53 live adults (Kagera = 30; Mbarara = 8; JKUAT = 4; Nandi Hills = 11) of *Ruspolia* spp. were randomly sampled for molecular identification using DNA barcoding. Immediately after collection, the grasshoppers were sedated, preserved by submerging them in 95% ethanol and stored at 4 °C until DNA extractions were performed. The insects were also identified by their key morphological characteristic (colour polymorphism) following the description by Bailey (1975) for the genus *Ruspolia*.

3.3.2 DNA extraction, PCR amplification and sequencing

DNA extraction was conducted in the Molecular Developmental Physiology and Signal Transduction Lab, University of Leuven, Belgium. *Ruspolia* samples were transported to Belgium under dry ice and further stored at -18 °C. Following the thawing process, DNA extraction was conducted using a Qiagen DNeasy Blood and Tissue kit (Qiagen, Valencia, California, USA) according to the protocol supplied by the manufacturer. Whole genomic DNA was extracted from the leg muscle tissue or from the thoracic region. Three loci (two rRNA genes and one mitochondrial gene) commonly used in insect phylogenetic studies, *i.e.*, 18S ribosomal subunit (18S rRNA), 28S ribosomal subunit (28S rRNA) and cytochrome oxidase subunit II (COII) were used for this analysis (Svenson & Whiting, 2004; Whiting, 2002).

The expected sizes of PCR product were 850 bp for both 18S and 28S, and 1250 bp for COII, and the adopted primer sequences are shown in Table 3.1a. Polymerase chain reaction (PCR) protocols as previously developed for 18S and 28S (Whiting,

2002) and COII (Svenson & Whiting, 2004) were followed. The primer sequences and amplification conditions are given in Table 3.1a and Table 3.1b respectively. PCR was performed in a 25-µL volume, containing (i) 22 µL mixture of distilled water and puReTaq solution composed of 0.2 mM of dNTP, 0.33 µL Biotaq DNA polymerase, 1.5 µL 10x NH_4 reaction buffer, and 1.5 mM $MgCl_2$, (ii) 1 µL forward primer, (iii) 1 µL reverse primer, and (iv) 1 µL DNA template that was diluted in sterilized distilled water to 10-fold. PCR reactions were performed in a Veriti TM 96 - well Thermal Well Cycler (ABI, USA).

Table 3.1a: Primer sequences

Gene	Primer	Sequence	Reference
18S rDNA	a0.79	5'- TTAGAGTGCTYAAAGC -3'	Whiting, 2002
	a0.7	5'- ATTAAAGTTGTTGCGGTT -3'	
28S rDNA	Rd4.2a	5'- CTAGCATGTGYGCRAGTCATTGG -3'	Whiting, 2002
	Rd4.5a	5'- AAGTTTCCCTCAGGATAGCTG -3'	
COII	9b	5'- GTACTTGCTTTCAGTCATCTWATG -3'	Svenson and Whiting, 2004
	R-lys	5'- GAGACCAGTACTTGCTTTCAGTCATC -3'	

Table 3.1b: Amplification profiles

Gene	Hot start	Denature	Anneal	Extension	Final extend	Cycles
18S rDNA	95° (12 min)	94° (30 s)	52° (30 s)	72° (1 min 15 s)	72° (3 min)	40
28S rDNA	95° (12 min)	94° (30 s)	54° (30 s)	72° (1 min 15 s)	72° (3 min)	40
COII	95° (12 min)	94° (1 min)	52° (1 min)	72° (1 min 15 s)	72° (1 min)	40

The amplified DNA fragments were separated on 1.5% (w/v) agarose gel (1 × TAE buffer), stained and visualized under UV light to confirm amplification and test for contamination. A wide-range molecular weight DNA marker (100 - 1000 bp ladder) was used on each gel as the standard reference. PCR products were purified with GenElute™ PCR Clean-Up Kit (Sigma-Aldrich, Inc., St Louis, Missouri, USA)

following the manufacturer's instructions. Products were sequenced with BigDye chain terminator chemistry and fractioned on an AB13730xl (Applied Biosystems) at the Hamburg University DNA Sequencing Center (Hamburg, Germany).

3.3.3 Sequence alignment and phylogenetic analyses

Contigs were concatenated, proofed and edited using Geneious v6.1.5 (Kearse et al., 2012). Primer regions were trimmed from the ends of the concatenated sequences. Edited sequences were compared with various complete, nearly complete, and partial sequences that were widely deposited in GenBank. For the case of COII gene, protein coding sequences were translated to amino acid sequences using MEGA 7 (Kumar et al., 2008). Once the proper reading frame was established, the sequences were aligned using the MUSLCE plug-in under default parameters found in MEGA.

The resultant combined dataset of the aligned nucleotide sequences were selected and entered into phylogenetic analysis. Phylogenetic relationship between *Ruspolia* and other tettigoniids was inferred in MEGA 7 based on the nuclear 18S rDNA, 28S rDNA and COII gene sequences of these insects and those of their relatives that inferred high similarity in the GenBank. The phylogenetic trees were reconstructed using the Neighbor-Joining method (Saitou & Nei, 1987; Tamura et al., 2011).

3.4 Results and Discussion

3.4.1 Morphological characterization – colour polymorphism

Based on cuticular coloration, three morphs were identified, *i.e.,* green, brown and purple which occurred at a ratio of 64:34:2, respectively. The observed colour morphs are shown in Figure 3.2. *Ruspolia* spp. and related tettigoniids are known to exhibit unique colour polymorphism. The color forms range from completely green to completely brown with a range of intermediates that have purple either striped or superimposed on the greens and browns (Matojo and Yarro, 2010a). In a study conducted in Uganda, Owen (1965) observed six distinct sympatric colour forms in *Homorocoryphus nitidulus* later referred as *R. differens,* namely: green, brown, green with-purple stripes, green-with-purple head, brown-with-purple head, and brown-

with-purple stripes. In this work, the green and brown morphs constituted 63% and 34%, respectively, of the population, leaving only 3% of the remaining four colour forms. In addition, Owen (1965) established that the colour forms were cryptic and that the colour combinations and the grasses in which the insects live could be ranked with a fair chance of certainty. Olembo (1970) reported similar colour morphs with greens and browns constituting about 60% and 36%, respectively, of the total population leaving barely 4% to make up the remaining four colour forms.

Figure 3.2: The major colour polymorphs of *Ruspolia* grasshoppers from E. Africa

According to Matojo and Yarro (2010a), *R. differens* collected in Tanzania exhibited unique colour polymorphism with six morphs, *i.e.,* green (65%), brown (31.8%), purple-striped green (2.8%), while purple-striped brown, purple suffused green and purple suffused brown morphs constituted less than 1%. On the other hand, Nyeko et al. (2010) in central Uganda identified eight colour morphs of *R. differens*, *i.e.,* green, green striped purple, purple light green, brown striped purple, light green, pale green, light brown and brown. The green (59.7%) and brown (36.9%) morphs were the most dominant followed by the purple light-green morph (2%). The other remaining morphs constituted less than 2% of the total individuals examined.

In general, from what was observed in this study and compared to what is reported in the literature, greens and browns are dominant while other colour morphs occur in relatively low abundance. Nevertheless, seasonal weather alterations have been evidenced to alter the relative frequency and sex-ratio biasness of these morphs. During the dry season the equilibrium of the colour polymorphism favours the frequency of browns and the population is chiefly male-biased, while during the wet seasons the greens are favoured and the population is female-biased (Matojo and Yarro, 2010a). It is worth noting that the grasshoppers examined in this study and those referred in the literature were collected during the rainy seasons, which may explain the dominance of green morphs. Thus, these grasshoppers are very well adapted to vary the frequency of its colour morphs in order to camouflage themselves with changes in the environmental conditions. This may imply that morphological traits are directly governed by environmental influences and do not always reflect the genetic information. Therefore, in section 3.4.2, DNA barcoding has been employed in an attempt to differentiate these cryptic species within the genus *Ruspolia* found in E. Africa.

3.4.2 Molecular identification of long-horned grasshoppers

The DNA extraction, PCR, and sequencing of long-horned grasshoppers collected in E. Africa were successful. The quality of PCR products was checked using 1.5% (w/v) agarose gel (demonstrated using 18S rDNA: Figure 3.3). NCBI BLAST produced a list of previously published ribosomal 18S and 28S nuclear sequences, and COII sequences significantly aligning with the sequences of *Ruspolia* found in E. Africa. The sequences were reported together with the respective Accession numbers, similarity scores (%), and references as shown in Table 3.2, Table 3.3 and Table 3.4.

The 18S barcode sequences for long-horned grasshoppers analyzed in this study, showed a sequence identity of 99 - 100% with *R. differens, R. nitidula, R. lineosa, R. consobrina* and *R. dubia* from BLAST search (Table 3.2).

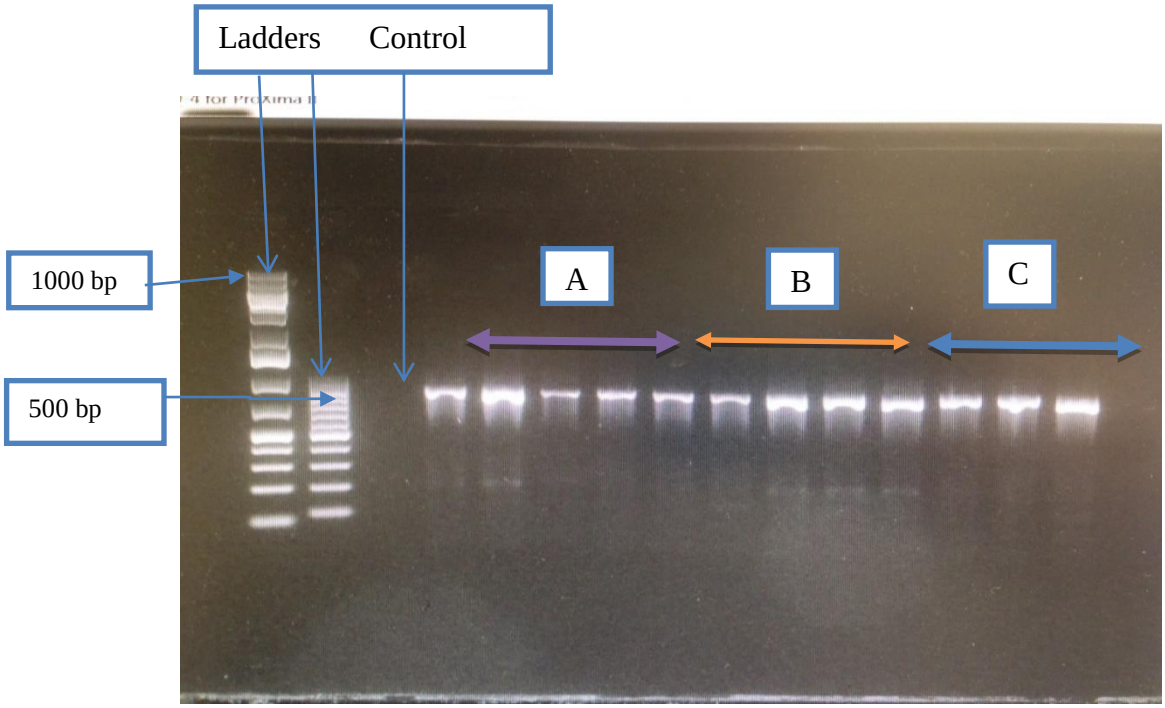


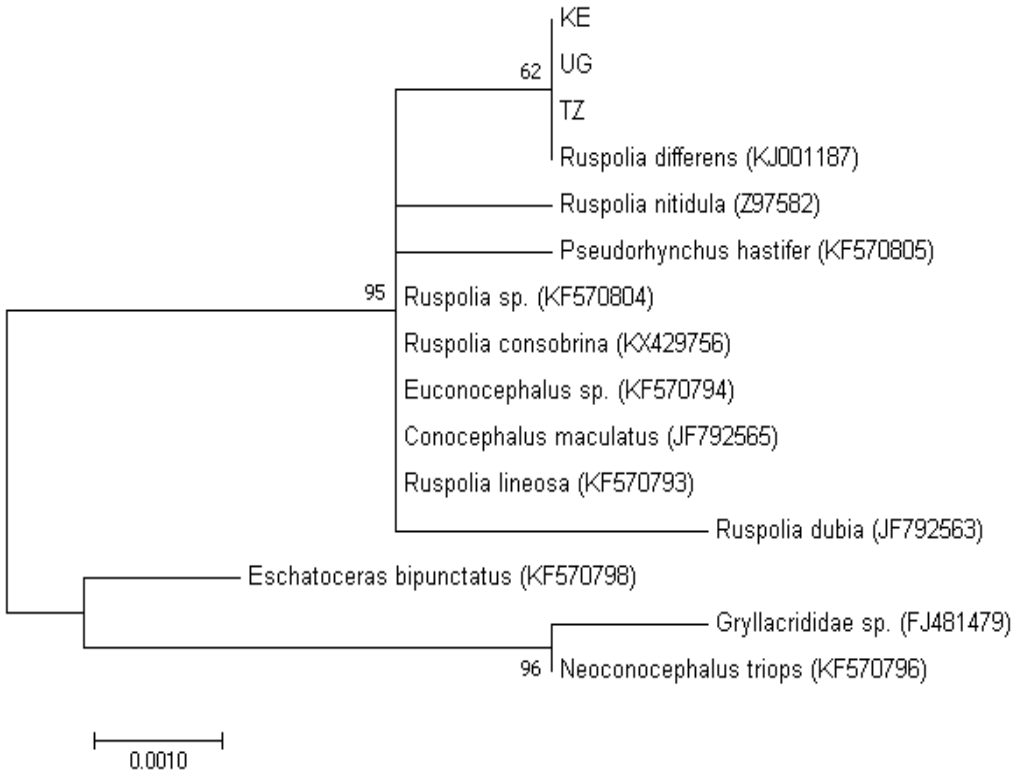
Figure 3.3: Gel photo showing PCR products of partial 18S rDNA of *Ruspolia* collected in E. Africa. DNA fragments of known sizes were used the ladders while PCR grade water was the negative control. DNA fragments of long-horned grasshoppers collected in Kenya (A), Uganda (B) and Tanzania (C).

The phylogenetic tree of long-horned grasshoppers using 18S branched into two main branches. The first branch included samples from all sites analyzed in this study, *R. differens*, *R. nitidula*, *R. consobrina*, *R. lineosa*, *P. hastifer* and *C. maculatus*; whereas the second branch comprised the rest of GenBank accessions (Figure 3.4). The first branch was subdivided into two clades; the first comprised all samples involved in this study and *R. differens*, and the second included *R. nitidula* (Z97582.1) and *P. hastifer* (KF570805.1) GenBank accessions. The branch that had other GenBank accessions of the *Ruspolia* sequences was also subdivided into two Clades, whereby the first comprised *R. consobrina* and *R. lineosa*, and the second involved *R. dubia*. The clade with the samples in this study was separated into two sub-clusters; the first cluster included *R. differens* (KJ001187) and the second sub-cluster comprised the samples in this study analyzed regardless of their colour morph (Figure 3.4).

Table 3.2: NCBI BLAST sequences matching with 18S rDNA sequence (partial) of *Ruspolia* found in E. Africa

Accession number	Nearest taxon	Similarity score	Reference
KJ001187	*Ruspolia differens*	100%	Matojo and Hosea 2013a
KF570804	*Ruspolia* sp.	100%	Mugleston et al. 2013
KF570793	*Ruspolia lineosa*	100%	Mugleston et al. 2013
JF792564	*Ruspolia lineosa*	99%	Wang et al. 2011
KX429756	*Ruspolia consobrina*	99%	Mugleston et al. 2016
Z97582	*Ruspolia nitidula*	99%	Flook and Rowell 1998
JF792563	*Ruspolia dubia*	99%	Wang et al. 2011
KF570794	*Euconocephalus* sp.	99%	Mugleston et al. 2013
JF792565	*Conocephalus maculatus*	99%	Wang et al. 2011
KF570805	*Pseudorhynchus hastifer*	99%	Mugleston et al. 2013
KF570798	*Eschatoceras bipunctatus*	99%	Mugleston et al. 2013
KF570796	*Neoconocephalus triops*	99%	Mugleston et al. 2013
FJ481479	*Gryllacrididae* sp.	99%	Pratt et al. 2008

The 18S phylogram has indicated that the long-horned grasshoppers analyzed are closely related to *R. differens* (GenBank accession), with *R. nitidula* being closest species while the closest genera to genus *Ruspolia* are *Conocephalus* and *Pseudorhynchus*. The genetic distance of 18S gene observed in all samples analyzed



was low, with an overall mean of 0.004, which is within the acceptable range for intraspecific variation (Virgilio et al., 2010). Although the 18S barcode sequences showed a sequence identity of 99 - 100% from BLAST search (Table 3.2), the Neighbour-Joining model-based phylogenetic analysis showed that the long-horned grasshoppers analyzed clustered separately from the closely related species *R. nitidula* and other species confirming the identity of edible long-horned grasshoppers in E. Africa as *R. differens* (Bailey, 1975; Matojo & Hosea, 2013a), and not *R. nitidula* as suggested by Agea et al. (2008). However, with identity score so close (99 – 100%) from the BLAST search, this study sought to explore further the identity of long-horned grasshoppers using other genetic markers (28S rDNA and COII gene).

Figure 3.4: Neighbor-Joining tree showing the evolutionary relationship of 18S rDNA gene of *Ruspolia* samples from E. Africa inferred by MEGA7. The tree is drawn to scale, and the branch lengths are in the same units as the evolutionary distances. Bootstrap values based on 1,500 replicates are indicated at the branches. Numbers in brackets are GenBank accessions. KE = grasshopper samples from Kenya, UG = grasshopper samples from Uganda, TZ= grasshopper samples from Tanzania

The 28S barcode sequences for long-horned grasshoppers produced a sequence identity of 99% with two main species, *i.e.*, *R. lineosa* and *R. consobrina* (Table 3.3), although this included samples from Uganda and Tanzania only (Figure 3.5). The quality of the DNA after PCR amplification from the Kenyan samples did not allow further sequencing.

Table 3.3: NCBI BLAST sequences matching with 28S rDNA sequence (partial) of *Ruspolia* found in E. Africa

Accession number	Nearest taxon	Similarity score	Reference
KX429800	*Ruspolia consobrina*	99%	Mugleston et al. 2016
KF570924	*Ruspolia* sp.	99%	Mugleston et al. 2013
KF570923	*Ruspolia lineosa*	99%	Mugleston et al. 2013
KF570922	*Pseudorhynchus cornutus*	99%	Mugleston et al. 2013
KF570926	*Euconocephalus* sp.	99%	Mugleston et al. 2013
KF570925	*Pseudorhynchus hastifer*	99%	Mugleston et al. 2013
KF570927	*Belocephalus subapterus*	99%	Mugleston et al. 2013
KF570929	*Nicsara trigonalis*	98%	Mugleston et al. 2013
KF570930	*Macroxiphus sumatranus*	98%	Mugleston et al. 2013
KF570931	*Oxylakis* sp.	98%	Mugleston et al. 2013
KX429799	*Copiphora hastata*	98%	Mugleston et al. 2016
KF570920	*Sphyrometopa femorata*	98%	Mugleston et al. 2013
KF570917	*Acantheremus colwelli*	98%	Mugleston et al. 2013

The phylogenetic tree of long-horned grasshoppers using 28S branched into three main branches. The first branch included samples from all sites analyzed in this study, *R. consobrina*, *R. lineosa*, *P. hastifer* and *B. subapterus*; whereas the second and third branches comprised the rest of GenBank accessions (Figure 3.5). The first branch was subdivided into three clades; the first comprised all samples involved in this study, *R. consobrina* and *R. lineosa*, whereas the second and third clades comprised the rest of GenBank accessions (Figure 3.5). The 28S phylogram has indicated that the long-horned grasshoppers analyzed are closely related to *R. consobrina* and *R. lineosa* (GenBank accessions) with closest genera to genus *Ruspolia* being *Euconocephalus* and *Pseudorhynchus*.

Regardless of the colour morph, the genetic distance of 28S gene observed in all samples analyzed was low, with an overall mean of 0.051, which is within the acceptable range for intraspecific variation (Virgilio et al., 2010). However, it was difficult to fix the identity of *R. differens* with the 28S DNA barcodes, due to the absence of corresponding 28S gene sequences of *R. differens* in the GenBank. In perspective, this sequence will be deposited in GenBank to serve as reference DNA barcode in subsequent studies.

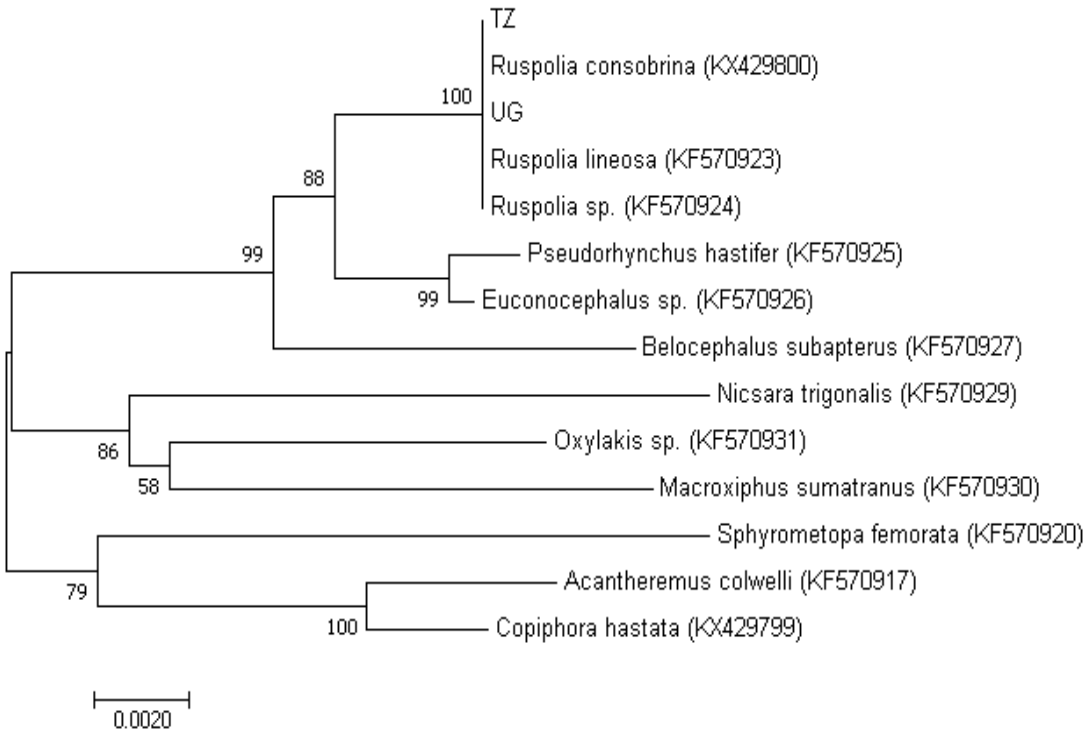


Figure 3.5: Neighbor-Joining tree showing the evolutionary relationship of 28S rDNA gene of *Ruspolia* samples from E. Africa inferred by MEGA7. The tree is drawn to scale, and the branch lengths are in the same units as the evolutionary distances. Bootstrap values based on 1,500 replicates are indicated at the branches. Numbers in brackets are GenBank accessions. UG = grasshopper samples from Uganda, TZ= grasshopper samples from Tanzania

The COII barcode sequences showed identity score of 97 – 98% with *R. consobrina* and *R. lineosa* (partial sequences; Table 3.4). In regard to this gene, partial and complete sequences were observed from the BLAST search. However, a huge difference in identity score was observed for the same species depending on whether the sequence is partial or complete, *e.g.*, *R. lineosa* (partial = 97% and complete = 88%).

Table 3.4: NCBI BLAST sequences matching with COII sequence of *Ruspolia* found in E. Africa

Accession number	Nearest taxon	Nucleotide sequence	Similarity score	Reference
KX429851	*Ruspolia consobrina*	Partial	98%	Mugleston et al. 2016
KX429852	*Ruspolia* sp.	Partial	97%	Mugleston et al. 2016
EF583824	*Ruspolia dubia*	Complete	94%	Zhijun et al. 2007
KF570987	*Ruspolia lineosa*	Partial	97%	Mugleston et al. 2013
KX057729	*Ruspolia lineosa*	Complete	88%	Zhou et al. 2017
KF571061	*Euconocephalus* sp.	Partial	90%	Mugleston et al. 2013
KX057717	*Ruspolia* sp.	Complete	87%	Zhou et al. 2017
KF571026	*Neoconocephalus triops*	Partial	87%	Mugleston et al. 2013
KF571011	*Pseudorhynchus hastifer*	Partial	87%	Mugleston et al. 2013
KF571072	*Pseudorhynchus cornutus*	Partial	86%	Mugleston et al. 2013
KF571012	*Nicsara trigonalis*	Partial	85%	Mugleston et al.

				2013
KF571035	*Nicsara trigonalis*	Partial	85%	Mugleston et al. 2013
KX057724	*Conanalus pieli*	Complete	83%	Zhou et al. 2017
KF570986	*Odontolakis virescens*	Partial	84%	Mugleston et al. 2013
KF571017	*Lirometopum coronatum*	Partial	84%	Mugleston et al. 2013

The phylogenetic tree of long-horned grasshoppers using COII branched into three main branches. The first branch included samples from all sites analyzed in this study, *R. consobrina*, *R. lineosa*, *R. dubia*, *N. triops*, *P. hastifer* and *P. cornutus*; whereas the second and third branches comprised the rest of GenBank accessions (Figure 3.6). The first branch was subdivided into two clades; the first comprised all samples involved in this study, *R. consobrina, R. lineosa* and *R. dubia*, whereas the second comprised the rest of GenBank accessions (Figure 3.6). The COII phylogram has indicated that the long-horned grasshoppers analyzed are closely related to *R. consobrina* and *R. lineosa* (GenBank accessions), and closest genera to genus *Ruspolia* are *Euconocephalus* and *Pseudorhynchus*.

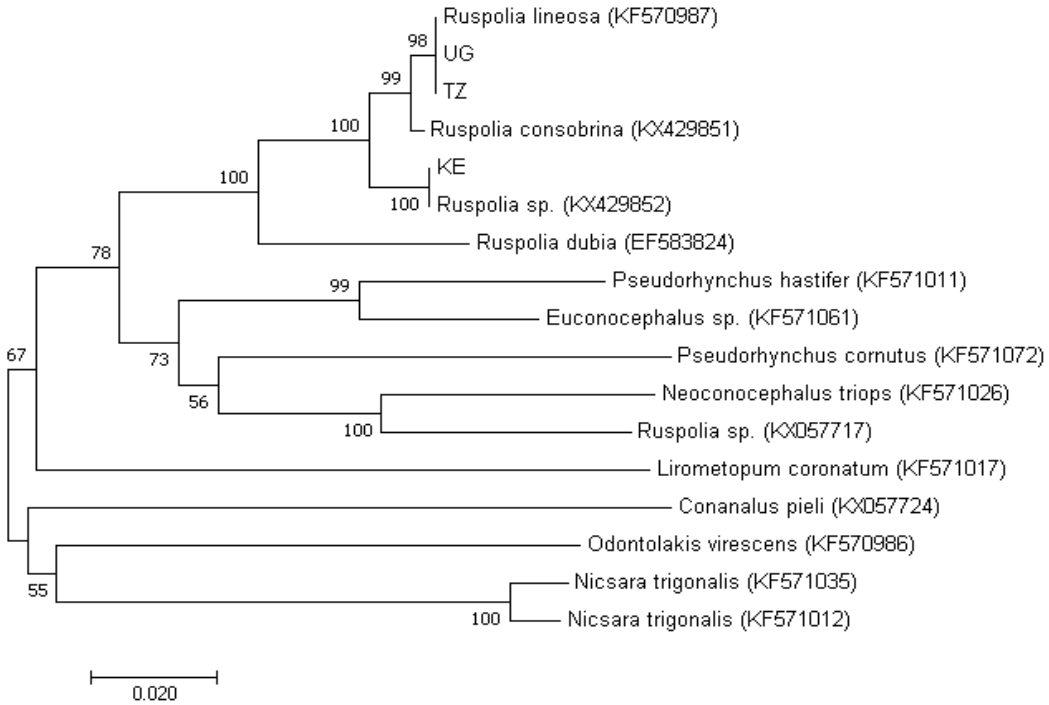


Figure 3.6: Neighbor-Joining tree showing the evolutionary relationship of COII gene of *Ruspolia* samples from E. Africa inferred by MEGA7. The tree is drawn to scale, and the branch lengths are in the same units as the evolutionary distances. Bootstrap values based on 1,500 replicates are indicated at the branches. Numbers in brackets are GenBank accessions. KE = grasshopper samples from Kenya, UG = grasshopper samples from Uganda, TZ= grasshopper samples from Tanzania

The genetic distance of COII gene observed in all long-horned grasshoppers samples analyzed was low, with an overall mean of 0.047, which is within the acceptable range for intraspecific variation (Virgilio et al., 2010). Nevertheless, similar to 28S rDNA, it was difficult to fix the identity of *R. differens* with the COII DNA barcodes, due to the absence of previous COII sequences of *R. differens* available in the GenBank. Recently, *R. differens* samples from Uganda were identified using COI gene region and linked them to *R. nitidula* (JQ824865.1) as closest species, however only at 96 – 97% similarity (Leonard et al., 2020).

In general, molecular data of various *Ruspolia* spp. have been reported in various parts of the world including Asia, Europe and Australia (Danley et al., 2007; Flook & Rowell, 1998; Pratt et al., 2008). However, there are only limited or scanty published data from E. Africa (Matojo & Hosea, 2013a). Therefore, this would support the findings in this study where in both cases (28S and COII genes); there was absence of previous sequences available in the GenBank matching with *R. differens* from E. Africa. In the literature, Bailey (1978) reported global distributions of other closely related species to *R. differens* (in brackets being their chief geographical distribution), *i.e., R. dubia* (Asia), *R. consobrina* (Europe), *R. nitidula* (Europe and North Africa), *R. lineosa* (Asia) and *C. maculatus* (Europe), and this would support why much of the molecular works have been conducted in Europe and Asia.

3.5 Conclusion

Based on 18S rDNA partial sequences, this study suggests that the sampled long-horned grasshoppers in E. Africa were *R. differens* and its closest relative is *R. nitidula*. However, there was only one GenBank accession (KJ001187) of *R. differens* from Tanzania demonstrating there is limited molecular data in regard to this genetic marker. The genetic markers 28S and COII did not enable identifying *R. differens* from other related tettigoniids due to the absence of previous sequences of *R. differens* in the GenBank. Although a recent study by Leonard et al. (2020) has identified *R. differens* using COI gene region, this was still based on partial sequence. Overall, in regard to genus *Ruspolia*, there is very limited molecular data particularly from E. Africa. In this perspective, the use of deep sequencing based molecular methods for population genetic analysis, such as Restriction site associated DNA markers (RAD-Seq), would be recommended when limited genomic information/ or no genomic information is available for the studied taxa.

Although the identification of long-horned grasshoppers found in E. Africa using 28S and COII genes was not fully conclusive due to scarcity of molecular data, in subsequent chapters in this dissertation they will be referred as *R. differens* based on the 18S rDNA sequence comparison.

CHAPTER FOUR

MICROBIAL LOAD OF EDIBLE LONG-HORNED GRASSHOPPERS *RUSPOLIA DIFFERENS* (SERVILLE) (ORTHOPTERA: TETTIGONIIDAE): FROM WILD HARVESTING TO FORK IN THE KAGERA REGION, TANZANIA

4.1 Abstract

In East Africa, edible grasshoppers *Ruspolia differens* is a source of food to many tribes but still harvested from the natural environments. In this perspective, little is known about the microbiological load of wild harvested *R. differens*. This study was conducted to evaluate the microbiological load of wild harvested *R. differens* and assess the effectiveness of common processing methods in reducing microbial load. Two districts (Bukoba rural and Muleba) within the Kagera region, in Tanzania were purposively selected for the study. Sampling was done along the *R. differens* food chain as follows: (1) at harvest points in the villages, (2) after transportation to the market and plucking of wings and legs, (3) after cleaning with potable tap water and (4) after processing using conventional methods (deep-frying, toasting, and smoking).

In general, high microbial counts including total viable count (TVC), *Enterobacteriaceae*, lactic acid bacteria (LAB), bacterial endospores, and yeasts and moulds were observed in *R. differens* samples collected at the harvest points. Following transportation and plucking, a significant increase ($P < 0.05$) in TVC, bacterial endospores, and yeasts and moulds was observed. After processing using conventional methods, a statistically significant reduction in all types of counts ($P < 0.05$), with the exception of bacterial endospores was observed. Foodborne pathogens including *Salmonellae*, *Listeria monocytogenes*, and *Escherichia coli* were not detected in all processed samples analyzed.

Although improved handling is advocated along the *R. differens* food chain in order to reduce the food safety risks, the focus should be on domestication and sustainable farming of *R. differens* in controlled environments where it could be easy to identify

the sources of the hazards such as those associated with the feeds and farming environments.

4.2 Introduction

The world population's constant increase and the continuous need to ensure future food security have led to a global surge in demand for affordable, alternative and sustainable food sources. In particular, human diet is evolving with a decreased inclusion of proteins from animal sources. In this perspective, the Food and Agriculture Organization of the United Nations (FAO) encouraged the increased use of insects as food and feed to ensure future food security (van Huis, 2003b). In many developing countries, insects are an important food source for many local cultures from ancient times to the present (Christensen et al., 2006; van Huis, 2013b). However, in western societies, *e.g.*, in Europe, acceptance of edible insects and insect-derived products is still limited (Mancini et al., 2019; Orsi et al., 2019). In Africa, the majority of the consumed insects are still collected from the wild environments. Therefore, consumption of these insects usually have many challenges such as sustainability issues, pathogenic risks and food safety issues (Murefu et al., 2019; Ssepuuya et al., 2016; Ssepuuya et al., 2019).

In E. Africa, local communities have developed the skills and techniques of harvesting, preparing, and preserving edible insects. For instance, harvesting and consumption of the locally occurring *Ruspolia differens* form a major part of food culture and constitutes about 5-10% of protein intake of the rural and urban population (Kinyuru et al., 2010; Ssepuuya et al., 2016). *R. differens* is a swarming grasshopper and during certain times of the year, usually in the months of March - May and October - December, huge swarms of *R. differens* are observed coinciding with short or long rainy seasons (Mmari et al., 2017; Ssepuuya et al., 2016). Most of the swarms are usually concentrated on streetlights in urban areas (they are attracted to the light in the evenings), on grasses and bushes (Agea et al., 2008; Ssepuuya et al., 2016). The local communities normally harvest, prepare and consume these edible grasshoppers at the households' level contributing to increased protein intake

in their diets. In addition, the surplus grasshoppers are traded at the nearby markets, thus a source of income to the households (Agea et al., 2008).

In Tanzania, *R. differens* are traditionally consumed as boiled, toasted, deep-fried or smoked products, with a few people reported to eat raw grasshoppers while in the fields depending on the season (Mmari et al., 2017). However, the pathogenic risks of consuming raw insects compared to cooked ones are immense (Klunder et al., 2012; Vandeweyer et al., 2017b). More so, wild harvested insects are likely to contain more spoilage and pathogenic micro-organisms compared to those that are reared in controlled environments (Klunder et al., 2012). For instance, in a microbiological study involving wild collected edible rhinoceros beetle species in Nigeria, Banjo et al. (2006) reported the presence of the pathogenic bacteria including *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Bacillus cereus*. In a different study assessing the presence of chemical hazards, Musundire et al. (2016) identified the presence of human carcinogen aflatoxin B1 associated with fungal contamination at low levels in edible stink bugs (*Encosternum delegorguei*) that were wild harvested in Zimbabwe. The presence of these pathogenic bacteria and chemical hazards represents a risk factor for food-borne illnesses and diseases that could be associated with consumption of these insects.

Although the nutritional composition of various insects has been studied and reported (*e.g.*, (Belluco et al., 2013; Caparros Megido et al., 2018; Fombong et al., 2017; Kinyuru et al., 2010; Rumpold & Schlüter, 2013; Ssepuuya et al., 2016), the microbiological data on edible insects is scarcely available, as indicated in the European Food Safety Authority opinion report (EFSA, 2015). Only a few studies are available containing microbiological data of wild harvested edible insects and insect-derived products particularly in Africa, although this is a growing area of research (EFSA, 2015). In addition, the scientific data assessing the microbial load of edible insects along the food chain is weak, sporadic, or missing; searching the literature reveals the scarcity of the data.

In this perspective, the microbiological load of edible insects needs to be assessed along the entire farm-to-fork food chain (production, harvesting, handling,

processing, and consumption). Therefore, this study was conducted to assess the microbial load and common foodborne pathogens along the food chain of wild harvested *R. differens* during harvesting, handling (transportation to the market, plucking of wings, legs, ovipositors, and appendages), and cleaning. In addition, the efficacy of conventional processing methods in reducing microbial loads was assessed.

4.3 Materials and Methods

4.3.1 Study sites and samples collection

The study was conducted in the northwestern corner of Tanzania in the Kagera region (one of Tanzania's 30 administrative regions), situated between 1°45′S and 32°40′E. The Kagera region shares borders with Uganda to the north, Rwanda and Burundi to the west and Lake Victoria to the east. Two districts (Bukoba Rural and Muleba) within Kagera region were purposively selected during the months of March - May in 2017, when *R. differens* swarms were observed.

During the swarming seasons, the local communities in Kagera region have developed their own harvesting techniques as described in section 2.2. Briefly, the harvesters set up open barrels (150 to 200 litres) on top of which are metal sheets inclined at an angle between 45^{o} and 75^{o}. Then, at night, high voltage bulbs are switched on, attracting grasshoppers towards the lights in front of the metal sheets. The grasshoppers land on the metal sheets, and slide down into the barrels (Figure 2.2). Using this technique, and when there are dense swarms, large quantities of grasshoppers are harvested within a short time. After *R. differens* harvesting was conducted, the random sampling at harvest points was done as follows: sampling within 4 - 5 villages in each district was conducted. At harvests sites, 27 unplucked samples of *R. differens* weighing approximately 0.5 kg each were sampled (Bukoba Rural =14 and Muleba =13). Flowchart showing food chain of wild harvested *R. differens* and sampling points indicated is shown in Figure 4.1.

The surplus *R. differens* is traded in the nearby local markets, and then processed immediately due to high perishability. As observed by Mmari et al. (2017), the locals

usually use traditionally woven baskets and used woven polypropylene bags to transport *R. differens* to the markets. In addition, plucking is usually done on bare ground or sometimes on old woven polypropylene bags spread on the ground. After transportation to the local markets, plucking is done first, a job traditionally assigned to women (Mmari et al., 2017).

In the local markets, where surplus *R. differens* from the same batches that were previously sampled at harvest points were traded, random sampling was done as follows: after transportation and plucking process, in total 21 plucked samples of *R. differens* (Bukoba Rural =11 and Muleba =10). Then, after rinsing *R. differens* using potable tap water, a total of 21 samples (Bukoba Rural =11 and Muleba =10) were collected. Cleaning involved washing with portable tap water and draining.

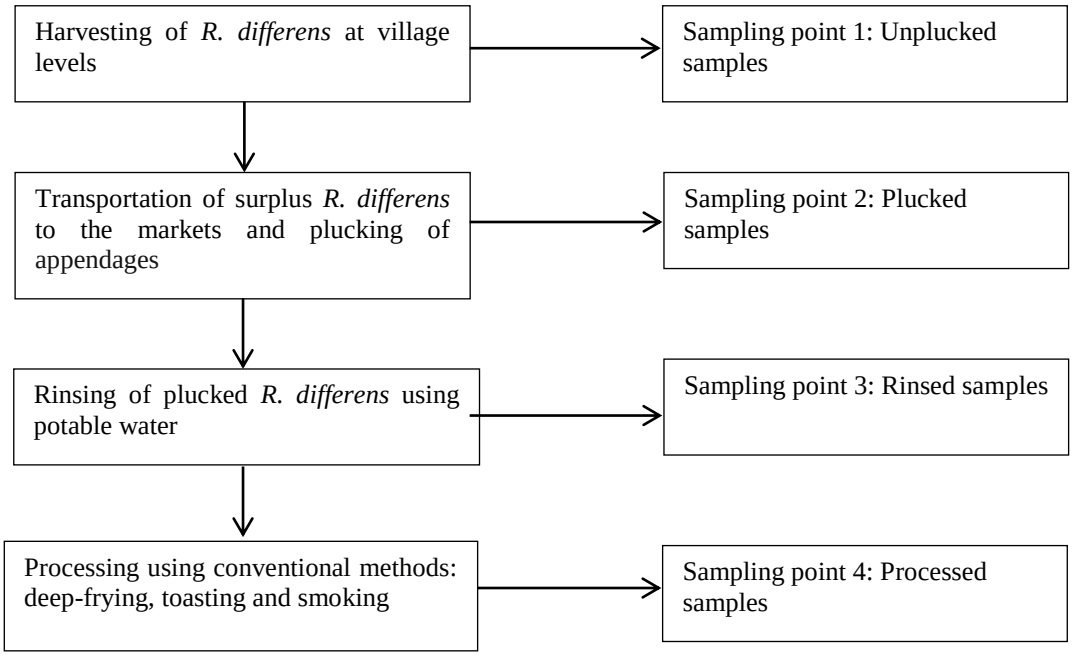


Figure 4.1: Flowchart showing food chain of wild harvested *R. differens* and sampling points indicated

The efficacy of conventional processing methods (*i.e.*, deep frying – rinsed raw *R. differens* were placed in boiling cooking oil for ten minutes until they turned deep-

brown in color; toasting - rinsed raw *R. differens* were placed on a hot pan, stirred for ten minutes until they turned deep-brown; smoking – involved a brief boiling step of raw *R. differens* for five minutes, draining them, and rolling boiled *R. differens* in a fresh banana leaves and placed on metal sieves where smoke was produced by burning woods). From the previously sampled batches, sampling was done as follows: Bukoba Rural (deep fried =8, toasted =6 and smoked =6) and Muleba (deep fried =10, toasted =7 and smoked =5) each weighing approximately 0.5 kg.

The samples were then packed in cool boxes and transported to the Food microbiology laboratory at JKUAT for microbiological analysis which commenced within 24 hours of collection.

4.3.2 Culture dependent microbiological analysis of *R. differens*

Standardized ISO methods for microbial analyses of food were used to determine bacterial counts (Dijk et al., 2007). Twenty grams of the *R. differens* was pulverized prior to analysis as described by Stoops et al. (2016). Then, an aliquot of 10 g of pulverized insect sample was aseptically transferred into a sterile stomacher bag (Bagmixer® 400 W, Interscience, St Nom, France) and 90 ml peptone was added. The mixture was homogenized for one minute in a stomacher. A ten-fold serial dilution series of 1 ml was plated on different media using the pour plate method except for moulds and yeasts. Total viable aerobic counts (TVC) were determined on Plate Count Agar (PCA, Biokar Diagnostic) incubated at 30°C for 72 hours, *Enterobacteriaceae* on Violet Red Bile Glucose medium (VRBG, Biokar Diagnostic) with an overlay of the same medium and incubated at 37 °C for 24 hours, lactic acid bacteria (LAB) on De Man Rogosa Sharpe medium (MRS, Biokar Diagnostic) with an overlay of the same medium and incubated at 30 °C for 72 hours. For yeasts and moulds, 0.1 ml was plated using the spread plate method on Dichrolan Rose-Bengal Chloramphenicol Agar (RBA, Biokar Diagnostic) and incubated at 25 °C for 120 hours. Bacterial endospores were determined by giving the 10^{-1} dilution a heat shock treatment (10 minutes at 80 °C), followed by serial dilution, plating using the pour plate method onto PCA and incubation at 37 °C for 48 hours. All microbial counts were expressed as log cfu/g.

4.3.3 Foodborne pathogens detection

The presence of *Salmonella* was assessed according to ISO 6579-1:2007 (absence in 25 g). Briefly, 25 g of *R. differens* sample was added in 225 ml of buffered peptone water and incubated at 37°C for 24 hours. Then 0.1 ml of the pre-enrichment culture was added to 10 ml of tetrathionate broth and incubated at 37°C for 24 hours. Then, loopful inoculums were streaked into *Salmonella Shigella* agar and incubated at 37°C for 24 hours. The presence of black-centered colonies was checked. Further confirmation test of these black-centered colonies involved incubation in triple sugar-iron agar at 37°C for 24 hours.

The presence of *Escherichia coli* was assessed according to ISO 7251-1: 2005. Ten-fold serial dilution series of *R. differens* was plated on violet red bile lactose agar using the pour plate method and incubated at 37°C for 24 hours, and presence of colonies examined. *Listeria monocytogenes* was assessed according to ISO 11290-2:2004. Briefly, 25 g of *R. differens* sample was added in 225 ml of buffered peptone water and incubated at 30°C for 24 hours. Then 0.1 ml of the pre-enrichment culture was added to 10 ml of fraser broth and incubated at 37°C for 48 hours. Then, loopful inoculums were streaked into agar Listeria Ottovani and Agosti and incubated at 37°C for 24 hours. Gram stain, catalase and motility were conducted as confirmation tests. The pathogens detection was only assessed on processed (deep-fried, toasted, and smoked) *R. differens* samples.

4.3.4 Statistical analysis

All microbial counts were expressed as log cfu/g. Statistical analyses were performed using Stata SE version 12 (StataCorp LP, Texas, USA). One-way ANOVA was performed to determine significant differences in individual microbial counts at harvest points (unplucked samples), after transportation and plucking (plucked samples), and after rinsing (rinsed samples); and among different conventional processing methods under investigation. For all analyses, means were separated using Bonferroni adjustment at 95 % confidence level.

4.4 Results and Discussion

4.4.1 Microbial load of raw *R. differens* samples

Table 4.1 summarizes the results of microbial counts of *R. differens* at harvest points (unplucked samples), after transportation and plucking (plucked samples) and rinsing (rinsed samples). For unplucked samples, high microbial counts, *i.e.,* the TVC, *Enterobacteriaceae,* LAB, bacterial endospores, and yeasts and moulds were observed. However, no statistical differences for individual counts were observed between the two districts for the unplucked samples analyzed (all *P*-values > 0.05). Although there are no specific microbiological criteria for edible insects and insect-derived products, some authors (Caparros Megido et al. 2017; Grabowski and Klein 2017a) and European Food Safety Authority (EFSA, 2015) proposed the total viable counts (TVC ≤ 6.7 log cfu/g) in minced meat as an indicator of food safety hygiene for edible insects and derived products. In this study, the TVC numbers observed exceeded this recommended limit.

Nevertheless, the values obtained in this study compare closely to numbers found for wild harvested *R. differens* from Uganda TVC > 8.0 log cfu/g (Ssepuuya et al., 2019) and TVC = 9.1 log cfu/g (Nyangena et al., 2020). When different batches of grasshoppers (*L. migratoria migratorioides*) bought from an eco-shop in Belgium were analyzed, TVC ranging between 7.8 and 8.6 log cfu/g were reported (Stoops et al., 2016). Published studies on edible insects collected from the wild have also revealed high microbial counts particularly the TVC and possibility of presence of pathogenic micro-organisms (Banjo et al., 2006; Klunder et al., 2012; Mpuchane et al., 2000; Murefu et al., 2019).

A significant increase in microbial counts (TVC, bacterial endospores, and yeasts and moulds) after transportation to the local markets and plucking of wings and legs (plucked samples) was observed (Table 4.1). Traditionally, *R. differens* appendages, ovipositors and wings are not generally consumed and are usually plucked off and discarded (Mmari et al., 2017). When insects come in direct contact with the soil or stored in uncleaned bags, this could lead to further contamination (Banjo et al., 2006; Klunder et al., 2012). For instance, in a study assessing the food taboos and cultures

along the food chain of *R. differens* in Tanzania, Mmari et al. (2017) observed that after wild harvesting and transportation (usually done using containers and sacks previously used to store other produce such as maize), plucking (done on bare ground in contact with the soil) was very unhygienic. These unhygienic handling practices could be associated with increase in these particular microbial counts in this study. Similarly, Musundire et al. (2016) reported increased fungal growth in edible stink bugs (*E. delegorguei*) that were wild harvested and stored in woven baskets and gunny bags that were previously used to store other produce.

Table 4.1: Microbial counts (log cfu/g) of raw *R. differens* at harvest points (unplucked), after transportation and plucking (plucked), and rinsing (rinsed).

Microbial counts (log cfu/g)	**Bukoba Rural**			**Muleba**		
	Unplucked	**Plucked**	**Rinsed**	**Unplucked**	**Plucked**	**Rinsed**
Total viable aerobic counts	7.1 ± 0.4^{a}	8.1 ± 0.4^{b}	6.9 ± 0.3^{a}	7.5 ± 0.3^{ab}	8.5 ± 0.4^{b}	7.0 ± 0.3^{a}
Enterobacteriaceae	5.6 ± 0.4^{ab}	6.1 ± 0.2^{ac}	5.8 ± 0.2^{ab}	5.4 ± 0.2^{ab}	6.3 ± 0.2^{c}	5.6 ± 0.2^{a}
Lactic acid bacteria	6.1 ± 0.4^{a}	6.8 ± 0.5^{a}	6.5 ± 0.3^{a}	7.0 ± 0.5^{a}	6.3 ± 0.2^{a}	6.9 ± 0.3^{a}
Bacterial endospores	3.0 ± 0.1^{a}	3.6 ± 0.1^{b}	2.8 ± 0.1^{a}	3.3 ± 0.1^{a}	3.8 ± 0.1^{b}	3.2 ± 0.1^{a}
Yeasts and moulds	4.7 ± 0.1^{a}	5.6 ± 0.2^{b}	4.3 ± 0.1^{a}	5.0 ± 0.2^{a}	5.4 ± 0.2^{b}	5.0 ± 0.2^{a}

Data are the means with three replicates ± standard errors (Unplucked n= 11, Plucked n= 10, Rinsed n= 10 for each district)

^{a,b,c,} Means with the same superscript within the same row do not differ significantly ($P > 0.05$)

No significant increase was observed in the microbial counts of *Enterobacteriaceae* and LAB after transportation and plucking (plucked samples) although the log counts increased slightly in both districts (Table 4.1). These two classes of bacteria have been reported to emanate mostly from the insects feed (Klunder et al., 2012), and after feeding, these micro-organisms are usually concentrated in the gastrointestinal

tract. However, the slight increase in these two microbial counts could possibly be due to unhygienic handling environments, improperly cleaned hands and handling equipment (Mmari et al., 2017; Ssepuuya et al., 2016).

Washing is a technique routinely applied in most food industries to aid in the decontamination process of most produce before further processing (Huffman, 2002). For edible insects, just like any other food products, washing is a crucial procedure before processing. In Tanzania, the standard requirements of drinking water (piped) and bottled drinking water are specified by Tanzania Bureau of Standards (TSZ 789:2003), albeit the quality of water used was not ascertained in this study. After cleaning of *R. differens* samples, a significant reduction in individual microbial counts (TVC, bacterial endospores, and yeasts and moulds) was observed while log counts of *Enterobacteriaceae* and LAB did not change in both districts (Table 4.1). These findings are supported by Wynants et al. (2017), who assessed the quality of the water before and after rinsing mealworm larvae (*T. molitor*). The authors observed a significant reduction in TVC after rinsing, indicating that the procedure is capable of reducing considerate amount of micro-organisms.

4.4.2 Microbial load of processed *R. differens* samples

Table 4.2 summarizes and compares the microbiological quality of ready-to-cook (rinsed) and ready-to-eat (after processing, *i.e.*, deep-frying, toasting, and smoking). Processing *R. differens* resulted in a statistically significant reduction of all types of microbial counts except bacterial endospores. For the total TVC, and yeasts and moulds, reductions of about 6.1 and 2.6 log cfu/g respectively were observed after heat treatment (Table 4.2). Klunder et al. (2012) found low TVC for boiled house cricket (*A. domesticus*) with significant reductions of about 3.1 log cfu/g. Similarly, low TVC were observed after examining blanched mealworms and house crickets from European farms, and smoked termites from Congolese market (Caparros Megido et al., 2017).

Enterobacteriaceae and LAB were completely eliminated or reduced to a level below the detection limit when *R. differens* samples were processed using any of the three, methods. These groups of bacteria are usually heat sensitive (Klunder et al., 2012) and that fact is supported by this study, where either method under investigation involved heating. In a different study, when mealworm larvae (*T. molitor*) were blanched for 10, 20, and 40 seconds, *Enterobacteriaceae* and LAB were not detected in investigated samples (Vandeweyer, et al., 2017b).

Table 4.2: Microbial counts (log cfu/g) of ready-to-cook (rinsed) and ready-to-eat (processed) of *R. differens*

	Ready-to-cook *R. differens*		**Ready-to-eat *R. differens***					
	Bukoba Rural	**Muleba**	**Bukoba Rural**			Muleba		
Microbial counts (log cfu/g)	Rinsed	Rinsed	Deep-fried	Toasted	Smoked	Deep-fried	Toasted	Smoked
Total viable aerobic counts	6.9 ± 0.3^{a}	7.0 ± 0.3^{a}	2.1 ± 0.1^{b}	2.0 ± 0.1^{b}	2.2 ± 0.1^{b}	2.4 ± 0.3^{b}	2.2 ± 0.1^{b}	2.4 ± 0.2^{b}
Enterobacteriaceae	5.8 ± 0.2^{a}	5.6 ± 0.2^{a}	$<1\pm0.0^{b}$	$<1\pm0.0^{b}$	$<1\pm0.0^{b}$	$<1\pm0.0^{b}$	$<1\pm0.0^{b}$	$<1\pm0.0^{b}$
Lactic acid bacteria	6.5 ± 0.3^{a}	6.9 ± 0.3^{a}	$<1\pm0.0^{b}$	$<1\pm0.0^{b}$	$<1\pm0.0^{b}$	$<1\pm0.0^{b}$	$<1\pm0.0^{b}$	$<1\pm0.0^{b}$
Bacterial endospores	2.8 ± 0.1^{a}	3.2 ± 0.1^{a}	2.2 ± 0.1^{b}	2.0 ± 0.2^{b}	1.9 ± 0.1^{b}	2.1 ± 0.1^{b}	2.7 ± 0.2^{a}	2.3 ± 0.2^{b}
Yeasts and moulds	4.3 ± 0.1^{a}	5.0 ± 0.2^{a}	2.0 ± 0.2^{b}	2.1 ± 0.1^{b}	2.6 ± 0.2^{c}	2.1 ± 0.2^{b}	2.0 ± 0.1^{b}	2.8 ± 0.2^{c}

Data are the means with three replicates ± standard errors (Rinsed n= 10, Deep-fried n= 8, Toasted n= 6, Smoked n= 5 for each district)

[a,b,c] Means with the same superscript within the same row do not differ significantly ($P > 0.05$)

On the contrary, bacterial endospores were slightly reduced, thus common conventional processing methods mostly applied in this region are incapable of eliminating them entirely. Researchers (Klunder et al., 2012; Ter Beek and Brul, 2010), have demonstrated that spore-forming bacterial species, such as *Bacillus* spp. are not entirely inactivated despite the heat treatment; and thus could cause spoilage on foods representing health risks to consumers. In a similar study, Vandeweyer et al. (2017b) found no significant change in bacterial endospores counts after blanching mealworm larvae (*T. molitor*) for 10, 20, and 40 seconds. Bacterial endospores in the insects can be introduced from direct contact with the soil, unhygienic handling, or storing edible insects in uncleaned bags (Banjo et al., 2006; Klunder et al., 2012). However, SHC and FASFC (2014) and EFSA (2015) recommended that, the source of insects' feeds and the farming environments could have a great impact on the insects' microflora, but controlling these factors would be difficult for the case of wild harvested insects. In regard to *R. differens*, the focus should be domestication and sustainable farming in controlled environments, thus, it could be easy to identify the sources of the hazards such as those associated with the feeds and farming environments.

4.4.3 Major foodborne pathogens

In this study, the preference of major pathogens analyzed was based on international standards used to describe food quality and food safety of foodstuffs containing animal protein (Grabowski and Klein, 2017a). Traditionally, within the Haya tribe in Tanzania, *R. differens* are not consumed raw but they are processed using different conventional methods (Figure 4.1) before consumption (Mmari et al., 2017). In this context, the current study focused on the safety of processed *R. differens* in the markets ready for consumption. All processed samples analyzed were free of *Salmonellae*, *L. monocytogenes*, and *E. coli*. In insects, the majorities of these bacteria originates from the gastrointestinal tract and are usually highly sensitive to heat (Klunder et al., 2012). The absence of these pathogens in ready-to-eat samples may suggest that the identified methods of processing *R. differens* (deep-frying, toasting and smoking) are effective, and processed *R. differens* may not pose health risks to consumers.

Similar results were reported by Grabowski and Klein (2017b) after deep frying processed edible insects' products. Although there are no specific microbiological criteria for insects used as human food, food safety criteria for minced meat that include *Salmonellae* (absence in 25 g), *L. monocytogenes* (<2.0 log cfu/g), *E. coli* (most probable number < 230/100 g) (Grabowski and Klein, 2017a) could give useful indication. In a recently updated circular from the Belgian SHC and FASFC (2016), products available on the market should be periodically tested for the presence of *Salmonella* and *L. monocytogenes* pathogens to assess the effectiveness of handling. In this study, comparing the obtained results with the current recommendations showed that food hygiene criteria for major foodborne pathogens of processed *R. differens* were met.

4.5 Conclusion

This study examined the microbial counts of *R. differens* along the food chain. Generally, high microbial counts exceeding the recommended criteria were observed in *R. differens* samples collected at harvesting sites. Furthermore, the handling of *R. differens* samples along the value chain (transportation and plucking) increased the contamination. However, the commonly applied processing methods eliminated most of the microflora examined except bacterial endospores. Thus, when edible insects such as *R. differens* are processed, bacterial spores and their survival may still need special attention, and practical procedures will be needed in order to eliminate or inactivate them and reduce the risks associated with them.

CHAPTER FIVE

EFFECT OF DIETARY INCLUSION OF WANDERING JEW (*COMMELINA SINENSIS*) WEED ON GROWTH AND BACTERIAL COMMUNITY COMPOSITION OF FARMED CRICKET (*GRYLLUS BIMACULATUS*)

5.1 Abstract

Farming of edible insects has been proposed as a means to reduce current practices of harvesting from the wild, and generate endless supply of edible insects to the consumers. However, the high cost of commonly used commercial chicken feeds is still a challenge to many farmers. Therefore, this study was conducted to evaluate whether the partial replacement of commercial chicken feed with the farm weed, Wandering Jew (*Commelina sinensis*), would have an impact on body weight, mortality and microbial load of farmed field cricket (*Gryllus bimaculatus*). The study experimentally compared two types of feed: (1) Starter commercial chicken feed only (SO), and (2) Starter commercial chicken feed supplemented with fresh Wandering Jew weeds (S+W). The feeding period was thirty days.

The final body weight differed significantly (P = 0.026) between crickets fed with SO and with S+W, which averaged at 1.11 g and 1.39 g, respectively. In regard to microbial numbers, high counts of total aerobic, *Enterobacteriaceae*, lactic acid bacteria, bacterial endospores, yeasts and moulds were observed in both experimental groups. However, bacterial endospore counts of S+W fed crickets (2.7 log cfu/g) were significantly lower (P = 0.021) than those of the SO fed ones (3.9 log cfu/g). Metagenetic analyses revealed that Proteobacteria, Firmicutes and Tenericutes were dominant phyla in both experimental groups. Members of the family Coxiellacea, and the genera *Lactobacillus*, and *Spiroplasma* were the most abundant Operational Taxonomic Units (OTUs) in both experimental groups though with small variation. In regard to food safety concerns, a few OTUs were identified with potential food pathogens that included *Clostridiaceae*, *Staphylococcus* and *Enterobacteriaceae*. In conclusion, the inclusion of fresh Wandering Jew weeds in commonly used commercial chicken feed produced crickets with increased body weight and improved microbial load.

5.2 Introduction

Over the past several years, the potential of edible insects to address food and nutrition security has been on the increase (van Huis, 2013b). In most regions of Africa, Latin America and Asia, edible insects already are part of the traditional food systems and diets (Christensen et al., 2006; Kelemu et al., 2015; van Huis, 2013b). In Kenya for instance, consumption of different kinds of insects such as termites, crickets, grasshoppers, ants, lake flies by various ethnic groups is well documented (Ayieko et al., 2012; Kelemu et al., 2015). However, most of these edible insects are collected from natural environments and seasonally available, thus, not sustainable to communities who rely on them as foods.

In this context, the development of insect farming has been proposed to reduce the collection of insects from the wild and, thus, relieve the pressure on natural populations and habitats; and consequently generate a continuous supply of edible insects to households (Caparros Megido et al., 2015; Halloran et al., 2016; Kinyuru & Kipkoech, 2018). Although several research works are being carried out in an attempt to domesticate most of the edible insects, a greater attention has been put on the crickets, *i.e.*, house cricket (*Acheta domesticus*) and field cricket (*Gryllus bimaculatus*) because of ease of domestication and can be reared with significantly shorter life cycles (Caparros Megido et al., 2015; Halloran et al., 2016). Previous studies have shown that *A. domesticus* and *G. bimaculatus* can mature and be harvested after 60 to 75 days (Caparros Megido et al., 2015; Kinyuru & Kipkoech, 2018).

Cricket farming has been successfully implemented in parts of Asia *e.g.*, Thailand, Cambodia, and Lao People's Democratic Republic (Halloran et al., 2016), and in Africa *e.g.*, Kenya and the Democratic Republic of the Congo (Ayieko et al., 2016; Halloran et al., 2018; Kinyuru & Kipkoech, 2018). However, one of the challenge to many farmers is the relatively high cost of the local commercial feeds, such as commercial chicken feeds that is commonly used as source of protein to crickets (Caparros Megido et al., 2015; Durst & Hanboonsong, 2015). Nevertheless, some studies have suggested the possibility of replacing the commonly used commercial

feeds with agricultural farm weeds and waste vegetable (Choo et al., 2017; Kinyuru & Kipkoech, 2018; Miech et al., 2016), or agricultural by-products (Caparros Megido et al., 2015; Magara et al., 2019; Orinda et al., 2017) with some promising results. In these studies, the nutritional compositions of such farmed insects were mostly documented, but data on microbial safety is limited or never reported. Furthermore, it is worth noting that insect feeds and farming environments are important factors that eventually will influence the insects' microbial composition (Dillon & Dillon, 2004; EFSA, 2015). In this regard, the microbial dynamics of edible insects meant for human consumption depends on the rearing process (Wynants et al., 2018).

In Kenya, a country that heavily relies on agriculture, farm weeds are common in farmlands, as well as in some natural environments. Kinyuru and Kipkoech (2018) examined the acceptance level of five common farm weeds by *A. domesticus*, (level of acceptance was categorized into high, medium or low. Consumption of at least 75% of the farm weed was considered high acceptance), the Wandering Jew (*Commelina sinensis*) had highest acceptability score, over 75%. From this perspective, an experiment was designed to assess whether a partial replacement of commercial chicken feed with farm weeds (Wandering Jew) would have an impact on the body weight, mortality and microbial community composition of farmed field cricket (*G. bimaculatus*).

5.3 Materials and Methods

5.3.1 Study site, rearing containers and experimental treatments

Cricket rearing was carried out at the Jomo Kenyatta University of Agriculture and Technology (JKUAT) insect farm, situated between 1°08′S and 37°02′E, located 1519 m above sea level. The experiment was designed as an exact replica of what many small-scale farmers rearing crickets in Kenya are currently practicing. The eggs were collected from the stock colony of *G. bimaculatus* using moist cotton balls. The cotton balls containing the eggs were thinly spread-out and placed in sterilized 2 Litres transparent rectangular plastic containers (21×14×15 cm) (Kenpoly Manufacturers Ltd., Nairobi, Kenya) and incubated in a controlled environmental

chamber maintained at 30 °C, 70% relative humidity, and a photoperiod of 12 hours :12 hours light: dark until hatched. The emerging nymphs were transferred into 50 Litres plastic containers, and fed until when they were 30 days old.

The experiment was conducted in 50 Litres plastic containers with a top diameter of 56.4 cm, a bottom diameter of 48.5 cm and a height of 33.4 cm (Kenpoly Manufacturers Ltd., Nairobi, Kenya). Cardboard egg cartons (29 × 29 cm) were placed horizontally inside the plastic containers to increase the insect living areas and to provide the shelter. In the experiment, two types of feeds were used as experimental treatments: (1) Starter commercial chicken feed only (SO), and (2) Starter commercial chicken feed + fresh Wandering Jew weeds (S+W). Eight rearing plastic containers containing cardboard egg cartons were arranged randomly in the rearing room, and 40 nymphs, 30 days old were added in each of them. Then, for each feed (n = 4 replications) was administered randomly as follows: SO = 20 g/rearing container/2 days while S+W = S (10 g/rearing container/2 days) + W (10 g/container/2 days). Then, 16 cm diameter saucers containing wet cotton wools (changed after every 2 days) were placed on each rearing container to provide water to crickets. The rearing containers were cleaned after every 2 days to remove the substrates, feaces and dead crickets. The rearing containers were covered by a 2 mm net to keep the insects inside and prevent entry of predators. The rearing was carried out for 30 days during the months of June and July, 2018.

5.3.2 Determination of body weight and mortality

The body weight was determined at the onset of the experiment, as well as after 15 and 30 days; while the mortality rate was assessed at 15 and 30 days of rearing. During each sampling period, 30 crickets per experimental feed condition were randomly selected and used for body weight determination. After 15 days, the rearing containers for each experimental feed condition were randomly selected, and two cardboard egg cartons per rearing container were lifted and shaken into a clean container, and the homogenized samples obtained used for body weight determination. During this sampling period, after obtaining the samples for body weight determination, all cardboard egg cartons in each feed condition were

removed, and live and dead crickets recorded. Similarly, at the end of 30 days of rearing, the live and dead crickets were recorded. Then, the crickets were harvested and four homogenized samples per feed condition were obtained. One sample per feed condition was used for body weight determination. Also, separate body weights for both female and male crickets were recorded. To sedate the insects, samples for each feed condition were refrigerated for 24 hours. Then, samples for microbial plate counts were withdrawn and analysis commenced after 24 hours. The remaining samples were stored at -18° C awaiting metagenetics analysis.

5.3.3 Culture dependent microbiological analysis of crickets

Culture dependent analysis (Total aerobic viable counts, *Enterobacteriaceae*, lactic acid bacteria, bacterial endospores, and yeasts and moulds) of two experimental feed conditions was conducted at the end of 30 days rearing period and determined as described in section 4.3.2.

5.3.4 Metagenetic analyses

To study the bacterial community of the crickets, following the thawing process, 20 g of pulverized samples per feed condition were subjected to high-throughput 16S rRNA gene sequencing (V4 region, 250 bp) using the Illumina MiSeq platform. The microbial genomic DNA was extracted from each homogenized sample per feed condition (0.2 g) in duplicate following the protocol of the PowerSoil DNA isolation kit (MO BIO Laboratories, Carlsbad, California, USA). DNA quality and concentrations were assessed using a Nanodrop Spectrophotometer (mySPEC, VWR), and all extracted DNA were stored at -20°C for further downstream processing.

PCR amplification was performed using the dual index strategy as described by Kozich et al. (2013). The DNA extracts were subjected to a PCR assay using barcode-labelled versions of the primers 515F (5'-GTGCCAGCMGCCGCGGTAA-3') and 805R (5' GGACTACHVGGGTWTCTAAT-3') (Caporaso et al., 2011), amplifying the V4 region (250 bp) of the 16S rRNA gene. The PCR amplification (20 µl) contained 5 ng / µl genomic DNA, 1.5 µl of dNTP mix (0.15 mM each), 0.5

µl of each primer (0.5 µM), 2 µl of Titanium Taq PCR buffer, 0.4 µl of Taq DNA polymerase (Clontech, Saint-Germain-en-Laye, France), and 14.1 µl of sterile milli-Q water. The PCR amplification protocol consisted of an initial denaturation at 95 °C for 2 minutes, then, 30 cycles of denaturation at 95 °C for 45 seconds, followed by primer annealing at 59 °C for 45 seconds and and elongation at 72 °C for 45, and a final extension step at 72°C for 10 minutes.

Following the amplification, the PCR products were purified by Agencourt AMPure XP beads (Beckman Coulter, Brea, CA, USA), quantified using the Qubit fluorometer (HS reaction kit, Invitrogen, Carlsbad, CA, USA), combined into a library in equimolar concentrations and subjected to an ethanol precipitation. As a final quality control, the pooled library was subjected to electrophoresis on a agarose gel (1.5 %), after which amplicons were cut out and purified with the NucleoSpin gel and PCR cleanup kit (Machery-Nagel, Düren, Germany). Finally, the library was diluted to 2 nM concentrations and sequenced at the Center of Medical Genetics Antwerp (University of Antwerp, Belgium).

The sequences were received in the format of a de-multiplexed FASTQ file. Paired-end reads were merged with a maximum of five mismatches using USEARCH (v.9.2.64) (Edgar, 2013) to form consensus sequences and truncated at the 250th base. Shorter reads or reads with a total expected error threshold above 0.10 for all bases after truncation were discarded. Next, the “classify.seqs” and “remove.lineage” commands in Mothur (v1.39.3; Edgar, 2013) and the Silva database (v123; Gurevich et al., 2013) were used to identify and remove potential mitochondrial, chloroplast, archaeal and eukaryote contaminants. Samples that yielded too few sequences were discarded from further analysis. Remaining sequences were grouped into operational taxonomic units (OTUs) based on a 3% sequence dissimilarity cut-off using the UPARSE greedy algorithm in USEARCH, during which chimeric sequences were also removed (Edgar, 2013), as the global singletons (i.e., OTUs representing only a single sequence in the entire dataset), minimizing the risk of retaining sequences from sequencing errors (Brown et al., 2015; Waud et al., 2014). The taxonomic origin of each OTU was determined with the SINTAX algorithm implemented in USEARCH (Edgar, 2016) based on the Silva database v123 (LTP v123). Taxonomic

assignments were considered reliable when bootstrap confidence values exceeded 0.80.

5.3.5 Statistical analysis

Stata SE version 12 (StataCorp LP, TX) was used for the analysis. Microbial counts were expressed as log cfu/g. Chao1 and Shannon-Wiener diversity indices were calculated using the R-packages Vegan (v.2.5-5) (R Development Core Team, 2013). Independent t-test was performed to determine significant differences in individual microbial counts (Total aerobic viable counts, *Enterobacteriaceae*, lactic acid bacteria, bacterial endospores, and yeasts and moulds), the weight gain, mortality, and alpha diversity indices (OTUs richness, Chao1, Shannon-Wiener, coverage, and evenness) between the two feed conditions in the experiment. All tests considered 0.05% significance level.

5.4 Results and Discussion

5.4.1 Body weight and mortality

Table 5.1 summarizes the crickets' body weight, weight gain and the mortality within 30 days rearing period. During the onset of the experiment, the cricket nymphs' weight per experimental feed condition did not differ significantly $t(58) = 0.372$, $P = 0.626$ (Table 5.1). However, a steady increase in the weight gain was observed between the two feeds during 30 days of rearing, as shown in Table 5.1. Similar trends were reported by Caparros Megido et al. (2015) on oriental ground crickets (*Teleogryllus testaceus*) that were fed on agricultural side streams and by Kinyuru and Kipkoech (2018) on house cricket (*A. domesticus*) reared using farm weeds. Although there was small variation in average body weight within the first 15 days of rearing, the mean weights between the two experimental feeds did not differ significantly $t(58) = 0.211$, $P = 0.715$. Studies have shown that young cricket nymphs' have little preference to household wastes and farm weeds compared to the adult crickets (Kinyuru and Kipkoech, 2018; Orinda et al., 2017), and a similar observation was made in this study.

The final body weights of adult *G. bimaculatus* ranged from 0.89 g to 1.5 g and differed significantly between the two experimental feed conditions (Table 5.1). In a different study, Orinda et al. (2017) reported slightly lower body weights (0.68 g – 0.99 g) of adult *G. bimaculatus* fed with agro-byproducts, while Caparros Megido et al. (2015) recorded higher body weights (1.8 – 3.1 g) of *T. testaceus* that were fed agricultural side streams.

In this study, the weight of adult *G. bimaculatus* fed with S+W was the highest (average 1.39 g), and as demonstrated previously (Kinyuru & Kipkoech, 2018), the fresh farm weeds, such as Wandering Jew weeds, are more palatable, and tend to increase the crickets' feed consumption capability, and hence, their high body weight. In addition, it was observed that fresh farm weed in experimental feed condition (S+W) also acted as source of water to the crickets. This finding has demonstrated that small-scale farmers rearing crickets can partially replace the commonly used commercial chicken feeds with naturally occurring farm weeds and even produce crickets with higher sellable weight. Similarly, when native broiler finisher was substituted with agricultural side streams and fed on *T. testaceus*, crickets with higher sellable weight were produced (Caparros Megido et al., 2015).

Table 5.1: Body weight, weight gain and mortality of *G. bimaculatus* fed Starter chicken feed only (SO) and Starter chicken feed + Wandering Jew weeds (S+W) for 30 days

Duration (days)	**Body weight (g)**		**Mortality (%)**	
	SO	S+W	SO	S+W
0	0.39 ± 0.1^{a}	0.37 ± 0.1^{a}	-	-
15	0.88 ± 0.1^{a}	0.83 ± 0.2^{a}	5.62 ± 1.9^{a}	7.50 ± 1.6^{a}
30	1.11 ± 0.2^{a}	1.39 ± 0.2^{b}	17.50 ± 2.9^{a}	15.00 ± 2.6^{a}
	Weight gain (g)			
0-15	0.49	0.46	-	-
15-30	0.23	0.56	-	-

Data are the means ± standard deviations

[a,b,] Means with the same superscript within the same row do not differ significantly ($P > 0.05$).

Table 5.2 summarizes the body weight and weight gain of both male and female *G. bimaculatus* during the 30 days rearing period. At the onset of the experiment, it was difficult to differentiate between sexes; hence, the weight of males and females was not determined at this age. After 15 days of rearing, a steady increase in the body weight in both feed conditions was observed, with weights ranging 0.76 – 0.78 g (male) and 0.86 – 0.89 g (female), but no significant difference was observed (Table 5.2).

At the end of the rearing period, the body weight of female crickets was significantly higher than that of male crickets in both feed conditions (Table 5.2). Normally, at this age the female crickets are adults, have developed ovipositors, and are feeding voraciously to meet the protein demand for egg development, which could be associated with the observed increase in weight gain, as compared to their male counterparts (Caparros Megido et al., 2015).

Table 5.2: Body weight and weight gain of male and female *G. bimaculatus* fed Starter chicken feed only (SO) and Starter chicken feed + Wandering Jew weeds (S+W) for 30 days

	Body weight (g)			
Duration (days)	SO		S+W	
	Male	Female	Male	Female
15	0.77±0.1[a]	0.87±0.1[a]	0.78±0.1[a]	0.86±0.1[a]
30	1.12±0.1[a]	1.42±0.1[b]	1.14±0.2[a]	1.44±0.1[b]
	Weight gain (g)			
15-30	0.37	0.55	0.36	0.58

Data are the means ± standard deviations

[a,b,] Means with the same superscript within the same row do not differ significantly ($P > 0.05$).

At the end of rearing trial, the mortality averaged 17.50% and 15.00% for SO and S+W, respectively, and no significant difference was observed between feed conditions (Table 5.1). Researchers have demonstrated that the type and quantity of feed provided to the crickets profusely influenced the mortality. For instance, when *T. testaceus* were fed young cashew leaf flour only, or a mixture of young cashew leaf flour and brown rice flour, the mortality ranged between 81.11 – 94.44%. However, when young cassava leaf flour only or native broiler finisher only or a mixture of young cassava leaf flour and brown rice flour were provided as feeds to *T. testaceus*, the mortality rate was less than 13.13% (Caparros Megido et al., 2015). In a separate study, when *A. domesticus* were fed with either agricultural side streams or farm weeds, less than 2.00% mortality was observed (Kinyuru & Kipkoech, 2018).

In the above findings (Caparros Megido et al., 2015; Kinyuru and Kipkoech, 2018), the mortality was mostly associated with unpalatable feeds, cannibalism, and predation. However, in the current study, a number of whole crickets were found dead and the cause of their death could not be stated with certainty. In this regard, further research is proposed to determine the cause(s) of mortality, such as assessing the parasitoids associated with the crickets' life cycle.

5.4.2 Classical microbiological analyses of *G. bimaculatus*

Microbial counts of farmed adult *G. bimaculatus* are presented in Table 5.3. In food products, plate counts are used as indicators of good hygienic conditions or good handling practices (Stoops et al., 2016). On average, microbial counts were generally high, as shown in Table 5.3. The total aerobic counts averaged 7.4 and 7.5 log cfu/g for SO and S+W, respectively, and did not differ significantly $t(14) = 0.738$, $P = 0.473$. The observed numbers were above 6.7 log cfu/g, the recommended hygiene criterion for raw minced meat (Grabowski and Klein, 2017a). In the literature, Caparros Megido et al. (2017) reported slightly lower counts (8 log cfu/g) on *A. domesticus* bought from rearing company while Vandeweyer et al. (2018) found counts ranging 8.2 – 8.4 log cfu/g for reared tropical house crickets *Gryllodes sigillatus*. However, the total aerobic counts in this study compare closely to numbers found for *A. domesticus* purchased from a rearing company (Klunder et al.,

2012), as well as to samples of *A. domesticus* and *G. bimaculutus* that were not meant for human consumption (Grabowski & Klein, 2017a).

Similarly, no significant differences were observed between crickets from both feed conditions for counts of *Enterobacteriaceae* t (14) = 0.599, *P* = 0.558, and yeasts and moulds t (14) = 1.483, *P* = 0.160, although they were above recommended limits of raw minced meat (*Enterobacteriaceae* <2.0 and yeasts and moulds <4.0 log cfu/g) (Grabowski and Klein, 2017a). In other studies, Grabowski and Klein (2017a) reported high counts of *Enterobacteriaceae* (7 log cfu/g) for both *A. domesticus* and *G. bimaculutus*, while Vandeweyer et al. (2018) found counts between 7.2 – 7.5 log cfu/g for farmed *G. sigillatus*. In contrast, Klunder et al. (2012) reported significantly lower value (4 log cfu/g) for *A. domesticus* purchased from a local rearing company. It could be that for some of the rearing companies, from where samples were taken, the insects were not intended for human consumption, possibly explaining the huge variation. However, the counts for yeasts and moulds in this study are in line with the ones reported in literature for *A. domesticus* (Caparros Megido et al., 2017) and for *G. bimaculutus* (Grabowski & Klein, 2017a).

Table 5.3: Microbial counts (log cfu/g) of *G. bimaculatus* fed Starter chicken feed only (SO) and Starter chicken feed + Wandering Jew weeds (S+W) for 30 days

	Type of feeds	
Microbial counts	SO	S+W
Total aerobic viable counts	7.4±0.1[a]	7.5±0.3[a]
Enterobacteriaceae	6.4±0.4[a]	6.2±0.8[a]
Lactic acid bacteria	6.8±0.3[a]	6.1±0.1[b]
Bacterial endospores	3.9±0.1[a]	2.7±0.2[b]
Yeasts and moulds	5.2±0.2[a]	4.8±0.6[a]

Data are the means ± standard deviations

[a,b,] Means with the same superscript within the same row do not differ significantly (*P* > 0.05)

On the other hand, a significant difference was observed for lactic acid bacteria t (14) = 5.697, *P* = 0.001, and bacterial endospores t (14) = 4.429, *P* = 0.021, between the two feed conditions. The bacterial endospores averaged at 3.9 log cfu/g and 2.7 log cfu/g for feeds SO and S+W, respectively. Klunder et al. (2012) reported bacterial endospores (3.6 log cfu/g) of *A. domesticus*, which is comparable to crickets fed with SO, but slightly higher than those fed with S+W. This finding may suggest that by partially replacing the commercially available chicken feeds with Wandering Jew, there is a possibility of reducing the risks posed by bacterial endospores. Previously, Wandering Jew leave extracts have indeed shown promising antibacterial activities against common bacterial pathogens, such as *Bacillus cereus, Bacillus subtilis* and *Staphylococcus* (Dash et al., 2017), although this cannot be stated with certainty as the main reason for low endospores counts in crickets fed with S+W. Moreover, bacterial endospores could be acquired when insects come into contact with the soil or through feed (Klunder et al., 2012). Although the bacterial endospores in both feeds were not determined in this study, high ratios of commercial chicken feeds, such as SO, usually consist of grains that are normally spread-dried on the floor and that could act as points of contamination. Therefore, during the processing of feeds such SO, proper application of good manufacturing practices is important.

5.4.3 Metagenetics analyses

The bacterial community composition of the *G. bimaculatus* was characterized using high-throughput 16S rRNA. A total of 1976 Operational Taxonomic Units (OTUs) were obtained (Appendix 1). The *G. bimaculatus* that were reared using SO were dominated by three bacterial phyla ascribed to Proteobacteria (55.56 - 96.90%; *Coxiellaceae*), Firmicutes (0.25 - 26.15%; *Lactobacillus*), Tenericutes (0.02 - 8.12%; *Spiroplasma*), and also, those reared using S+W; Proteobacteria (53.56 - 98.20%; *Coxiellaceae*), Firmicutes (0.15 - 26.41%; *Lactobacillus*) and Tenericutes (0.03 - 8.97%; *Spiroplasma*) were also the dominant phyla (Figure 5.1). Similarly, *A. domesticus* and powdered crickets were dominated by Proteobacteria (42.6% and 28.4%, respectively) and Firmicutes (34% and 54%, respectively), while Tenericutes (0.7% and 1.6%, respectively) was detected in low abundance (Garofalo et al., 2017).

However, comparing these results to those obtained by Vandeweyer et al. (2017c), on average, *A. domesticus* and *G. sigillatus* were dominated by Bacteroidetes (43%) and Firmicutes (35%) while Proteobacteria was low in abundance. Similarly, during rearing and processing of *G. sigillatus*, the most abundant phyla were Bacteroidetes ranging from 35.8 to 71.2%, Firmicutes (5.4 - 56.6%) and Proteobacteria ranging from 4.9 to 16.0% (Vandeweyer et al., 2018). In other similar studies, Firmicutes and Proteobacteria were also the dominant phyla in *T. molitor* larvae (Wynants et al., 2018) and *L. migratoria* (Stoops et al., 2017), while Proteobacteria, Firmicutes, Actinobacteria, Bacteroidetes and Fusobacteria were dominant phyla in *R. differens* (Ssepuuya et al., 2019).

When the bacterial community of *T. molitor* larvae from three rearing cycles within the same company was characterized, one cycle was dominated by the phyla *Spiroplasma* (80%) (Vandeweyer et al., 2017c). In this study, however, the bacterial family *Coxiellaceae* (OTUs 7) dominated, representing 70.58% of the total sequences (SO) and 79.98% of the total sequences (S+W), and the bacterial pattern seems to be similar between the two experimental feeds (Figure 5.1).

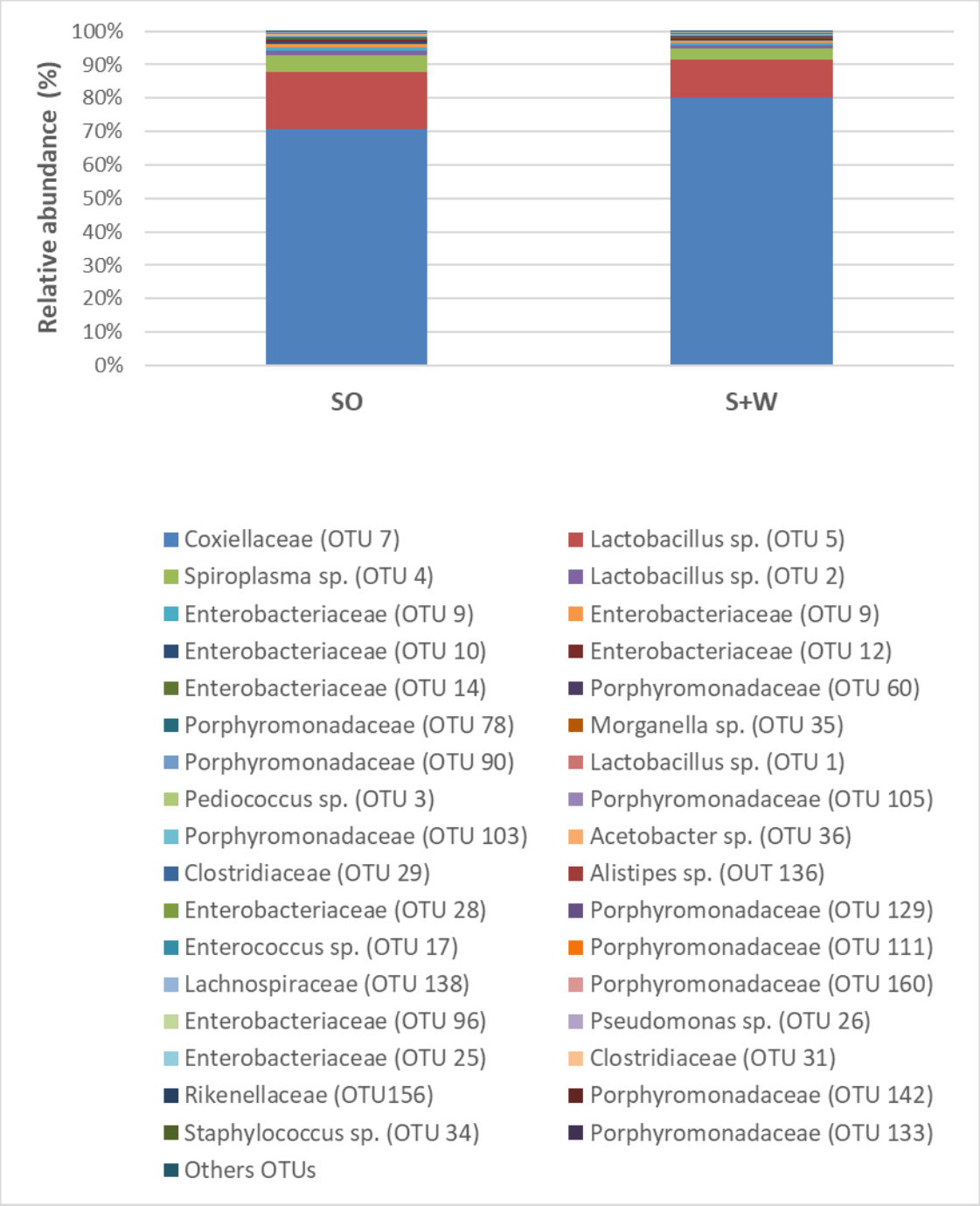


Figure 5.1: Relative abundance (%) of Operational Taxonomic Units (OTUs) present in samples of raw G. *bimaculatus* fed Starter chicken feed only (S0) and Starter chicken feed + Wandering Jew weeds (S+W) for 30 days

The dominance of the phyla Proteobacteria, Firmicutes and Bacteroidetes in this study, and what is reported in the literature demonstrate that different edible insect species can be colonized by similar bacterial communities, although with significant variations. Normally, these phyla represent a group of bacteria that are gut-associated with insects and highly influenced by the diets and the hosts' intestinal bacterial community composition (Colman et al., 2012).

The diversity indices were averaged for replicate samples per experimental feed condition and presented in Table 5.4. The total coverage, based on Chao1 calculation was 74.42 ± 1.60% and 73.81 ± 1.30% for SO and S+W, respectively, indicating that most abundant bacterial communities in *G. bimaculatus* were recovered. However, these values are slightly lower compared with those reported in literature for *G. sigillatus* (Vandeweyer et al., 2018) and *A. domesticus* (Vandeweyer et al., 2017c). The observed richness, Chao1, Shannon-Wiener, and evenness diversity indices did not differ significantly between the two experimental feed conditions (Table 5.4). Although it maybe hypothesized that the addition of the Wandering Jew weeds in the commercial starter chicken feed may increase the bacterial diversity, the Shannon-Wiener diversity index, which denotes the diversity based on both richness and abundance, did not differ significantly, but it was slightly higher for S+W than that of SO (Table 5.4).

Table 5.4: Microbial community diversity indices of the *G. bimaculatus* fed Starter chicken feed only (S0) and Starter chicken feed + Wandering Jew weeds (S+W) for 30 days

Type of feed	**Alpha diversity index**				
	OTUs richness	Chao1	Coverage	Shannon-Wiener	Evenness
SO	32±5.20[a]	43±7.80[a]	74.42±1.61[a]	1.32±0.07[a]	0.12±0.01[a]
S+W	33±1.73[a]	44±7.13[a]	73.81±1.30[a]	1.43±0.05[a]	0.14±0.01[a]

Sequences were grouped into Operational Taxonomic Units (OTUs) defined by 97% sequence identity at the 16S rRNA gene (V4 region, 250 bp). Values are the mean ±

standard deviation of analyses performed on two replicate samples, with two technical replicates per sample (n= 2 × 2), Richness = the number of observed OTUs; Chao1 = the species richness estimator (Delong, 2013); Coverage = observed richness/Chao1 estimate × 100 (Vandeweyer et al., 2017c); Shannon-Wiener index = an indication of species diversity, a combined measure of the number of species in a community (richness) and the number of individuals per species (Di Bitetti, 2017); Evenness = the relative frequency of species in a community (Di Bitetti, 2017).

Generally, except the *Coxiellaceae* (OTU 7), *Lactobacillus* (OTU 5), and *Spiroplasma* (OTU 4), other OTUs were found at relative abundance lower than 1% (Appendix 1). The occurrence of *Coxiellaceae* (OTU 7) and *Spiroplasma* (OTU 4) in relatively high abundance plus other OTUs with less than 1% abundance that included *Staphylococcus*, *Porphyromonadaceae*, *Acetobacter*, *Pseudomonas*, *Rikenellaceae*, and *Morganella* in both experimental feed conditions could have contributed to high total viable counts (Table 5.3). On average, 17.4% of all bacterial sequences (SO) and 11.5% of all bacterial sequences (S+W) belonged to members of the genus *Lactobacillus* (OTUs 5 and 1; Figure 5.1), corresponding to high counts of lactic acid bacteria (SO: 6.8 log cfu/g and S+W: 6.1 log cfu/g). These counts differed significantly between the two feeds (Table 5.3). In addition, *Pediococcus* and *Enterococcus* were also detected in both experimental feed conditions. Although for some species of the genera *Lactobacillus, Pediococcus* and *Enterococcus* probiotic properties have been claimed when ingested in right amount (Fijan, 2014), it is also worth noting that some species of the genera *Lactobacillus, Leuconostoc*, and *Lactococcus* also act as food spoilage organisms particularly in refrigerated meat products (Nieminen et al., 2011). In this regard, it is possible that lactic acid bacteria cause spoilage in edible insects when poorly preserved since they contain high protein content just like conventional meats or meat products.

Although the occurrence of pathogenic bacteria, such as *Escherichia coli*, *Listeria monocytogenes* and *Salmonella* spp., was not detected after metagenetic analysis, these pathogens can't be completely ruled out. *Escherichia coli* and *Salmonella* can still be within the family *Enterobacteriaceae* which occurred at relatively low abundance (<1%; Figure 5.1) between the two experimental feed conditions.

Enterobacteriaceae are normally associated with human and animal feaces and are frequently used as indicators of good/ bad handling or good/ bad processing practices (Stoops et al., 2016). In a similar study, after pyrosequencing, powdered crickets contained a low abundance (below 2%) of *Listeria* spp., while other major pathogens were not detected (Garofalo et al., 2017). Furthermore, the occurrence of the family *Clostridiaceae* and the genus *Staphylococcus* in very low abundance (<1%; Figure 5.1) in crickets from both feeding conditions could represent the presence of pathogenic species as well. Recently, *Clostridium* spp. were characterized from *A. domesticus* and powdered crickets (Garofalo et al., 2017). Normally, these classes of bacterial endospores have a potential to cause foodborne diseases, and represent a challenge in the production and processing of edible insects. An important source of contamination is when insects get into contact with the soil during rearing, via the feed containing spores or by unhygienic handling during production (Klunder et al., 2012). As a result, good sourcing and preparation of feeds, as well as good handling and processing practices, are crucial during the entire production process of insects intended for human consumption.

5.5 Conclusion

This study provides a summary of the body weight, mortality, microbial numbers and bacterial communities' composition of farmed crickets. The study has demonstrated that the commercial starter chicken feed, which is usually expensive for local farmers, can be partially replaced with farm weed (Wandering Jew) and this results in crickets with higher sellable weight. Although most microbial counts observed exceeded the recommended process hygiene criteria of comparable food matrices, such as minced meat, the bacterial endospore counts in crickets fed with S+W were significantly lower and thus promising, and could reduce the risks associated with ready-to-eat insects. However, studies on antimicrobial properties of Wandering Jew weed and reason (s) for higher preference are recommended.

Metagenetics analyses have demonstrated that the crickets in two experimental groups can harbour similar bacterial communities with some minor variations. With regard to food safety, a few possible risks were identified such as OTUs related to

the genus *Staphylococcus*, and the families *Enterobacteriaceae* and *Clostridiaceae*. These may contain pathogenic species, and involve the risk of foodborne diseases. However, culture-dependent assays, such as presence-absence tests or specific counts of pathogens are needed for a full evaluation of the microbiological safety of the crickets reared on the alternative substrates.

CHAPTER SIX

EFFECT OF DIETARY SUPPLEMENTATION WITH POWDER DERIVED FROM DRUMSTICK TREE (*MORINGA OLEIFERA*) AND NEEM TREE (*AZADIRACHTA INDICA*) LEAVES ON GROWTH AND MICROBIAL LOAD OF EDIBLE CRICKETS

6.1 Abstract

Cricket farming has shown its potential to address food and nutrition insecurity in many parts of the world. However, one of the remaining challenges limiting mass production of these edible insects is the affordability of feeds. One possible way to address this issue would be to explore the use of traditional plants such as *Moringa oleifera* leaf (MOL) and *Azadirachta indica* leaf (AIL) that are locally available, are protein-rich and possesses some antibacterial properties in insect feed formulation. In this regard, a study was conducted to evaluate the effect of supplementation of MOL (5% or 10%) and AIL (5% or 10%) powder in the commercially used starter chicken feed on growth and microbial load of house cricket (*Acheta domesticus*) and field cricket (*Gryllus bimaculatus*).

After feeding for four weeks, the supplemented feeds with 10% MOL or 10% AIL in *A. domesticus* and *G. bimaculatus* significantly decreased ($P < 0.05$) the body weights compared to the control feed. Also, supplementation with MOL (5% or 10%) and AIL (5% or 10%) increased the mortality in both cricket species. Although high microbial counts of total aerobic, *Enterobacteriaceae*, lactic acid bacteria, bacterial endospores, yeasts and moulds were observed, significantly low counts ($P < 0.05$) of lactic acid bacteria and bacterial endospores were observed in experimental feed treatments containing 10% MOL or 10% AIL in both cricket species.

After processing (toasting and boiling), significant reductions ($P < 0.001$) of all microbial counts were observed, but log reductions in bacterial endospores were small. *Salmonella* and *Escherichia coli* were not detected in processed crickets' samples. In conclusion, supplementation with 5% MOL or 5% AIL powder can produce crickets with similar body weight as control feed. Also, some microbial

counts were significantly reduced when feeds were supplemented with either 10% MOL or 10% AIL in both cricket species.

6.2 Introduction

The potential of edible insects to address food and nutrition insecurity is receiving an increasing amount of attention (Halloran et al., 2016). In Africa, however most of the communities still rely heavily on artisanal, non-sustainable collection of edible insects from the wild and, thus, their utilization is hampered by seasonal availability and food safety risks (Murefu et al., 2019; Ssepuuya et al., 2019). In this context, sustainable insect farming and harvesting has been advocated for better nutrition, improved food safety, as well as for a steady household income generation (Durst & Hanboonsong, 2015; Kinyuru & Kipkoech, 2018).

In low and middle income countries, cricket farming has shown the potential to address food and nutrition insecurity (Halloran et al., 2018), as well as having a positive impact on the rural development and rural economy (Caparros Megido et al., 2015; Durst & Hanboonsong, 2015; Halloran et al., 2016). Crickets have received a greater attention because they are one of a handful of insects with high feed conversion efficiency and a short generation time, tolerate high densities, do not undergo diapauses and are generalist feeders (Caparros Megido et al., 2015; Kinyuru and Kipkoech, 2018). In addition, insects like crickets have been associated with a low ecological foot print because fewer resources are required compared to conventional food sources e.g livestock for their mass production (Oonincx et al., 2010).

Although cricket farming is making progress, some of the challenges hindering cricket mass production is lack of an understanding on the effect of various feeds on the nutrition and safety of insects intended for human consumption. As one way of addressing this, researchers are testing locally available agricultural by-products and agricultural farm weeds (Caparros Megido et al., 2015; Kinyuru & Kipkoech, 2018; Orinda et al., 2017), but with varying degree of success. Despite this, recent studies have shown that the microbial quality of farmed crickets does not meet the proposed criterion for raw edible insects (Caparros Megido et al., 2017; Grabowski & Klein,

2017a; Murefu et al., 2019; Vandeweyer et al., 2017a; Vandeweyer et al., 2018). This demonstrates that there is incomplete knowledge on the effects of various available feeds on the growth performance and safety of crickets intended for human consumption.

In this perspective, cost-effective ways need to be exploited for rearing edible crickets using feeds that are readily available, affordable, accessible and safe. One possible way is to explore the use of traditional plant resources such as drumstick tree (*Moringa oleifera* Lam) and neem tree (*Azadirachta indica*) leaves that are locally available to be tapped in insect feed formulations. Previous studies have shown that *Moringa oleifera* leaf (MOL) powder is nutritionally rich and contains crude protein ranging between 23.1 and 29.5% (Gadzirayi et al., 2012; Gupta et al., 1989; Lu et al., 2016; Oduro et al., 2008), while the amount of crude protein in *Azadirachta indica* leaf (AIL) powder ranged between 11.5 and 20.9% (Adjorlolo et al., 2016; Bhowmik et al., 2008; Obikaonu, 2012).

Due to this high protein content in MOL and AIL powder, studies have shown that animal nutrition and performance can be improved by dietary supplementation of MOL and AIL powder. For instance, when poultry feeds were supplemented with MOL, an improvement in egg production, weight gain, and feed conversion efficiency was observed in hens (Mohammed et al., 2012). Similarly, when feeds were supplemented with AIL, egg weight and egg yolk pigmentation increased (Obikaonu and Udedible, 2015), while buck rabbits tolerated up to 15% AIL dietary inclusion of meal without effects on body weight (Ogbuewu et al., 2010).

Furthermore, the leaf extracts of MOL and AIL have shown potential antibacterial activity against some common human pathogens. For instance, *in vitro* studies have demonstrated that MOL powder extracts exhibited antibacterial activity against some Gram negative bacteria such as *Pseudomonas aeruginosa*, *Shigella sonnei* and Gram positive bacteria including *Staphylococcus aureus*, *Bacillus cereus*, *Bacillus subtilis*, *Sarcina lutea* and *Bacillus megaterium* (Rahman et al., 2009; Singh and Tafida, 2014). In a similar study, AIL extracts showed higher antimicrobial activity against Gram positive bacteria including *Bacillus cereus*, *Listeria monocytogenes* and

Staphylococcus aureus (Hoque et al., 2007). In a separate study, dietary inclusion of a mixed powder of herbal plant leave extracts was found to enhance the immune function of broiler chicken (Hoque et al., 2007).

Despite the promising results on animal nutrition enhancement and antibacterial activities as described above, use of MOL and AIL powder in formulating insects' feed remains largely untested. Therefore, it was hypothesized that the inclusion MOL and AIL powders in crickets' feed could improve the quality of feed and consequently crickets' nutrition and safety. From this perspective, a study was designed to assess whether supplementation of MOL and AIL powder in commercial starter chicken feed could have an impact on the feed intake, feed conversion ratio, body weight, mortality and microbial load of farmed house cricket (*A. domesticus*) and field cricket (*G. bimaculatus*).

6.3 Materials and Methods

6.3.1 Study site and cricket colonies initiation

The rearing of *A. domesticus* and *G. bimaculatus* was carried out at the Jomo Kenyatta University of Agriculture and Technology (JKUAT) insects' farm (1°08′S, 37°02′E, 1519 m.a.s.l). In order to obtain nymphs of both *A. domesticus* and *G. bimaculatus*, eggs were collected from the stock colonies already in existence in JKUAT insects' farm using moist cotton balls. The cotton balls containing the eggs were thinly spread-out and placed in sterilized 2 Litres transparent rectangular plastic containers (21×14×15 cm) (Kenpoly Manufacturers Ltd., Nairobi, Kenya), and incubated in controlled environmental chamber maintained at 30°C, 70% relative humidity, and a photoperiod of 12 hours: 12 hours light: dark until hatched. The emerging nymphs were kept in room without climate control and fed until the age of 4 weeks.

6.3.2 MOL and AIL powder preparation, and crude protein determination

Five kilograms of each MOL and AIL were harvested in JKUAT's botanical garden and sun-dried immediately for 48 hours. The dried leaves were subsequently ground

using a two-speed warring laboratory blender, (Camlab, Over, UK), sieved through a mesh screen (0.5 mm), mixed in different proportions into airtight plastic bags and stored at ambient temperature (23°C – 27°C). Crude protein content of commercial starter chicken feed, MOL and AIL powder was determined by the Kjeldahl method (Chang, 2010), using a steam distillation apparatus (Vapodest 20, Gerhardt, Königswinter, Germany). A nitrogen-to-protein conversion factor of 4.43 was applied to determine protein content (Yeoh and Wee, 1994).

6.3.3 Experimental setup

The experiment was carried out in the same room without climate control where the nymphs were produced. For each cricket species, the experiment was carried out in 50 Litres plastic containers with a top diameter of 56.4 cm, a bottom diameter of 48.5 cm and a height of 33.4 cm (Kenpoly Manufacturers Ltd., Nairobi, Kenya). Cardboard egg cartons (29 × 29 cm) were placed horizontally inside the plastic containers to increase the insect living areas and to provide shelter. A completely randomized design was applied during the experiment for each cricket species. The plastic containers containing cardboard egg cartons were arranged in the rearing room, and 40 nymphs, 30 days old were added. For each cricket species, the experimental treatments consisted of: commercial starter chicken feed (control), and the control supplemented with 5% or 10% of either MOL or AIL powder (5 treatments performed in triplicates). The starter chicken feed was considered as control because previously its potential usage has been shown during cricket farming (Caparros Megido et al., 2015; Ng'ang'a et al., 2020).

The crickets' feeds were administered as follows: 20 g/ rearing container/ 2 days. Wet cotton wools, which were changed after every 2 days were placed on 16 cm diameter saucers and placed in each rearing container to provide drinking water. The rearing containers were cleaned after every 2 days to remove the residual substrates, feaces and dead crickets. The rearing containers were covered by a 2 mm net to contain the insects inside and keep off predators. Three EL-USB-2 data loggers (Lascar electronics Inc., Pennsylvania, USA), programmed to record data every one hour, were placed in strategic locations within the rearing room to record the

temperature and relative humidity. The experiment was carried out for four weeks during the month of April 2019.

6.3.4 Determination of body weight and feed conversion efficiency

The body weight (BW) was determined at the onset of the experiment, as well as after 2 and 4 weeks for each cricket species. At each sampling period, two rearing containers per experimental treatment were randomly selected, and cardboard egg cartons were lifted and shaken in a clean container. Then, 20 crickets per experimental treatment were weighed for BW determination. The individual nymphs/ adults crickets were weighed in pre-weighed small containers. The weighed crickets were only put back into the rearing containers at week two sampling period as the feeding ended in fourth week.

During each feeding period, feed intake (FI) was calculated as the difference between the amount of feed offered and substrate residue. Crickets feaces dropped in the substrate were carefully removed before weighing the substrate residue. The total amount of feed consumed for 4 weeks during rearing was recorded. This data was then used to determine feed conversion ratio (FCR), calculated as the proportion of dry feeds consumed to fresh body weight of harvested crickets (Lundy and Parrella, 2015).

6.3.5 Determination of mortality

Mortality was assessed at second and fourth week of rearing and determined as percentage of dead crickets. At each sampling period, after samples of BW determination were obtained as described above, then, all cardboard egg cartons were removed, and live and dead crickets recorded for each experimental treatment for mortality determination. At the end of 4 weeks of rearing, the dead crickets were carefully removed. Then, the remaining crickets were harvested (per experimental treatment) and refrigerated immediately for 24 hours to sedate the crickets. Thereafter, samples for microbial counts and pathogens detection were withdrawn and analyzed.

6.3.6 Culture dependent microbiological analysis

Microbiological analyses were done at the end of 4 weeks of rearing. The microbial counts (Total aerobic viable counts, *Enterobacteriaceae*, lactic acid bacteria, bacterial endospores, and yeasts and moulds) were determined as described in section 4.3.2, and counts expressed as log cfu/g.

6.3.7 Heat processing of harvested crickets

The microbial counts of processed crickets in the experimental treatment starter chicken feed only were analysed for both insect species. The processing involved (1) Toasting: about 50 g of crickets were placed into a hot air oven for approximately 24 hours at 60°C, (2) Boiling: about 50 g of crickets were transferred into 2 litres of boiling water for 1 minute. The microbial counts were determined as described in section 4.3.2, and counts expressed as log cfu/g.

6.3.8 Foodborne pathogens detection

After heat processing as described above, samples of both cricket species were withdrawn and the presence of two foodborne pathogens was assessed. The presence of *Salmonella* and *Escherichia coli* was assessed as described in section 4.3.3.

6.3.9 Statistical analysis

Statistical analyses were performed using Stata SE version 12 (StataCorp LP, TX). Data are presented as means with their standard deviations. Microbial counts were expressed as log cfu/g. ANOVA tests were performed to determine significant differences in individual microbial counts (Total aerobic counts, *Enterobacteriaceae*, lactic acid bacteria, bacterial endospores, yeasts and moulds), and body growth parameters (body weight and feed conversion ratio), and mortality. Mean separation was obtained by the Bonferroni's option of the ANOVA test. For all tests, a significance level of ≤ 0.05 was considered.

6.4 Results and Discussion

6.4.1 Feed crude protein content

The crude protein contents of the control feed, MOL, AIL and supplemented feeds are shown in Table 6.1. The crude protein content of MOL observed was low compared to what is reported in other studies. In the literature, a higher crude protein content for MOL powder is reported ranging between 23.10 and 29.50% (Gadzirayi et al., 2012; Lu et al., 2016; Oduro et al., 2008). The crude protein content in AIL powder ranged from 11.50 to 20.90% and was in line with what has been reported previously (Bhowmik et al., 2008; Obikaonu, 2012). Variations in protein contents in MOL and AIL powder compared to what is reported in the literature could be attributed to environmental factors, such as temperature, water availability, light, soil nutrition, and even diseases and pests which are strongly associated with plant health and growth (Baldwin, 1975). The protein content of formulated feeds ranged between 20.19 and 21.01g similar to the control (Table 6.1).

Table 6.1: Crude protein (g/100g) of commercial starter chicken feed only, MOL, AIL and formulated feeds on dry weight basis

Diets	% Crude protein
Starter feed only	21.17±0.89
MOL	18.15±1.72
AIL	14.42±0.48
5% MOL + 95% Starter feed	21.01±0.32
5% AIL + 95% Starter feed	20.83±1.09
10% MOL + 90% Starter feed	20.86±0.81
10% AIL + 90% Starter feed	20.19±0.92

Data are the means ± standard deviations of three replicates

6.4.2 Temperature and relative humidity in the rearing room

Figure 6.1 shows the mean temperature and relative humidity prevailing in the rearing room of *A. domesticus* and *G. bimaculatus* over the four weeks of the study. The mean atmospheric temperature and relative humidity were 26.6 ± 4.6°C and 53.5 ± 12.4%, respectively. These patterns were characterized by wide ranges of between 17.5 – 36.8°C (temperature), and 25.5 - 80.5% (relative humidity). In a similar trial carried out within JKUAT insects' farm in four cycles of the year 2016, in the month of April the temperature ranged between 20.0°C – 22.0°C and relative humidity ranging 65.0 – 75.0% although that year was characterized by low temperatures (Kinyuru and Kipkoech, 2018). Other studies (Lundy and Parrella, 2015; Nakagaki and DeFoliart, 1991), have shown that high temperature (29.0 - 35.0°C) and humidity more than 50.0% are associated with fast cricket growth and development and this weather conditions compares closely with the findings of the current study.

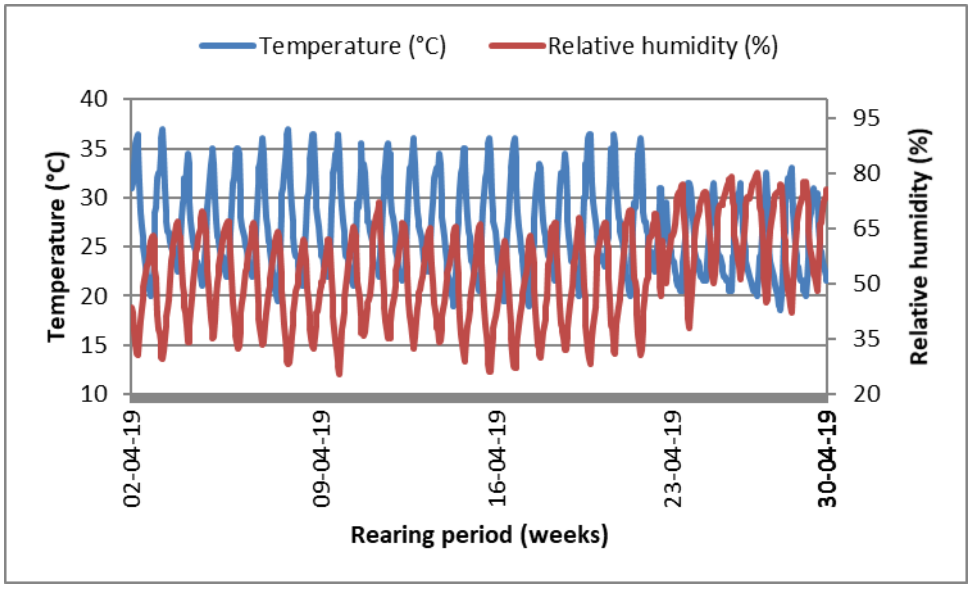


Figure 6.1: Temporal variations in temperature and relative humidity in the rearing room of *A. domesticus* and *G. bimaculatus* during the four weeks feeding period

6.4.3 Cricket body weight

Table 6.2 and Table 6.3 summarizes the body weight (BW), feed intake (FI), average weight gain (AG), feed conversion ratio (FCR) and mortality of *A. domesticus* and *G. bimaculatus* respectively within the four weeks of rearing. By the time of initiating the experiment, the nymphs of both insect species were already 4 weeks old. Normally, the average weight gain of newly hatched nymphs is small during early stages of growth, thus, delaying the initiation of the experiment until 4 weeks after hatching ensured that cricket nymphs had equivalent metabolic capacity during their growth phase (Lyn et al., 2011).

At the onset of the experiment, the nymphs BW per experimental feed treatment of both *A. domesticus* and *G. bimaculatus* did not differ significantly (Table 6.2 and Table 6.3 respectively). In the literature, Nakagaki and DeFoliart (1991) reported average body weight of 0.44 g for 28 – 30 days old *A. domesticus* when reared at 33.0°C – 35.0°C and fed chick starter diet.

Table 6.2: Effect of dietary supplementation of MOL and AIL powder on body weight, feed conversion ratio and mortality of *A. domesticus*

	Experimental treatments				
Duration (weeks)	**Starter feed only**	**5% MOL**	**10% MOL**	**5% AIL**	**10% AIL**
		BW (g)			
0	0.37±0.10^{a}	0.35±0.10^{a}	0.39±0.10^{a}	0.37±0.10^{a}	0.36±0.10^{a}
2	0.83±0.10^{a}	0.74±0.13^{a}	0.72±0.13^{a}	0.71±0.11^{a}	0.70±0.10^{a}
4	1.25±0.10^{a}	1.14±0.12^{a}	0.89±0.10^{b}	1.03±0.15^{a}	0.86±0.13^{b}
		FI (g/insect)			
0-4	1.33±0.12^{a}	1.03±0.10^{b}	0.71±0.02^{c}	1.21±0.13^{a}	0.72±0.00^{c}
		AG (g)			
0-4	0.88	0.81	0.50	0.66	0.52
		FCR			
0-4	1.31±0.21^{ab}	1.27±0.03^{a}	1.42±0.11^{b}	1.83±0.13^{c}	1.64±0.17^{c}
		Mortality (%)			
0-2	5.0±1.63^{a}	5.0±1.90^{a}	30.0±2.33^{b}	22.5±1.48^{c}	27.5±1.81^{b}
0-4	12.5±2.16^{a}	25.0±2.06^{b}	47.5±2.91^{c}	27.5±2.27^{b}	52.5±3.24^{c}

Data are the means ± standard deviations

^{a,b,c} Means with the same superscript within the same row do not differ significantly ($P > 0.05$)

BW: body weight; FI: feed intake; AG: average weight gain; FCR: feed conversion ratio

Within the two weeks of feeding, a steady increase in the weight gain of both *A. domesticus* (Table 6.2) and *G. bimaculatus* (Table 6.3) was observed, although without significant differences among experimental feed treatments. The final body weight of adult *A. domesticus* ranged between 0.86 g and 1.25 g and differed significantly among the experimental feed treatments as shown in Table 6.2. The experimental feed treatments 10% MOL and 10% AIL produced the lowest body weights and this corresponded with lowest feed intake during the entire feeding period. The body weight of adult *G. bimaculatus* ranged from 0.91 g to 1.46 g and differed significantly among the experimental feed treatments as shown in Table 6.3.

Table 6.3: Effect of dietary supplementation of MOL and AIL powder on body weight, feed conversion efficiency and mortality of *G. bimaculatus*

	Experimental treatments				
Duration (weeks)	**Starter feed only**	**5% MOL**	**10% MOL**	**5% AIL**	**10% AIL**
		BW (g)			
0	0.36±0.11^{a}	0.37±0.10^{a}	0.39±0.10^{a}	0.37±0.11^{a}	0.36±0.10^{a}
2	0.82±0.12^{a}	0.85±0.14^{a}	0.70±0.10^{a}	0.73±0.12^{a}	0.69±0.11^{a}
4	1.46±0.11^{a}	1.39±0.11^{a}	0.94±0.10^{b}	1.01±0.13^{b}	0.91±0.12^{b}
		FI (g/insect)			
0-4	1.59±0.40^{ac}	1.61±0.23^{a}	0.80±0.01^{b}	1.15±0.14^{c}	0.78±0.02^{b}
		AG (g)			
0-4	1.10	1.02	0.55	0.70	0.55
		FCR			
0-4	1.46±0.03^{a}	1.56±0.10b^{c}	1.55±0.10b^{c}	1.64±0.07b^{c}	1.51±0.01^{b}
		Mortality (%)			
0-2	10.0±1.91^{a}	7.6±2.30^{a}	22.5±2.08^{b}	12.5±2.63^{a}	30.0±1.77^{c}
0-4	17.5±2.52^{a}	20.0±1.74^{a}	35.0±2.70^{b}	30.0±2.68^{b}	33.5±2.91^{b}

Data are the means ± standard deviations

^{a,b,c} Means with the same superscript within the same row do not differ significantly ($P > 0.05$)

BW: body weight; FI: feed intake; AG: average weight gain; FCR: feed conversion ratio

In the literature, when *G. bimaculatus* were fed either starter chicken feed or starter chicken feed supplemented with fresh wandering Jew weeds for four weeks, the body weights averaged at 1.11 and 1.39 g respectively (Ng'ang'a et al., 2020). On the other hand (Orinda et al., 2017) reported average body weights of 0.98 g and 0.61 g of *G. bimaculatus* and *A. domesticus* respectively when fed growers chick mash. In the current study, the control feed was the most preferred by both cricket species. Normally, when the temperature conditions are favourable (29.0 - 35.0°C), the crickets do not hide in the cardboard egg cartons and feed veraciously if the feed is palatable, and this could contribute to increased body weight (Lundy & Parrella, 2015).

In general, the control feed was the most preferred by both cricket species and produced the highest body weights. All the same, the body weight of *A. domesticus* in the experimental feed treatments 5% MOL and 5% AIL, and body weight of *G. bimaculatus* in the experimental feed treatments 5% MOL did not differ from the control feed. On the other hand, the experimental feed treatments 10% MOL and 10% AIL were least preferred feeds and produced the lowest body weights in both cricket species. These findings have shown that the commonly used starter chicken feed can be supplemented by 5% MOL or 5% AIL powder during the rearing of *A. domesticus* and by 5% MOL in the rearing of *G. bimaculatus* and produces crickets with the similar body weights.

6.4.4 Feed conversion efficiency

Animals that have a low FCR are considered efficient users of feed. The overall FCR of *A. domesticus* and *G. bimaculatus* differed significantly among the experimental feed treatments (Table 6.2 and Table 6.3 respectively). In both cricket species, the experimental feed treatment control had the lowest FCRs while supplemented feeds produced the highest FCRs values. In the literature, FCRs values of 1.47 and 1.80 when *A. domesticus* was fed poultry feed and food waste respectively were reported (Lundy and Parrella, 2015). When comparing the above FCRs values, they are higher than the values (range: 0.92 – 1.08) reported for *A. domesticus* when fed four type of feeds, one of which was starter chicken feed (Nakagaki and DeFoliart, 1991). In

contrast, Oonincx et al. (2015) reported much higher values (range 2.3 – 10.0) when *A. domesticus* were fed four type of feeds (beet molasses; potato steam peelings; spent grains and beer yeast; bread remains and cookie remains) formulated from by-products of food manufacturing companies.

The diet composition is the main factor determining the feed conversion efficiency depending on insect species (Oonincx et al., 2015). Therefore, the type of feed provided to crickets depends on how efficiently it is converted to body mass during rearing. The FCRs values obtained in current study were much lower to those reported for conventional production animals, *i.e.,* 2.3 for poultry meat, 4.0 for pork, and 8.8 for cereal beef (Wilkinson, 2011). This demonstrates that the *A. domesticus* and *G. bimaculatus* considered suitable for human consumption were more efficient in converting their feeds to food for humans.

6.4.5 Cricket mortality

At the end of rearing, the mortality differed significantly for *A. domesticus* (P = 0.001; Table 6.2) and *G. bimaculatus* (P = 0.021; Table 6.3). For *A. domesticus*, the lowest mortality was recorded in the control feed (12.5%), while mortality in experimental feed treatments 10% MOL (47.5%) and 10% AIL (52.5%) were the highest. Similar trends were observed in *G. bimaculatus* where control feed recorded the lowest mortality (17.5%), while experimental feed treatment 10% MOL (35.0%), mortality rate was highest. These high mortalities in both insect species could be attributed to low diet intake, hence, poor nutrition; and high levels of inclusion of these powders making the feeds less palatable.

Previous studies have shown that the type and quantity of feed provided to the cricket profusely influences the survival rate (Caparros Megido et al., 2015). For example, when *G. bimaculatus* were fed either starter chicken feed or starter chicken feed supplemented with fresh wandering Jew weeds for four weeks, the mortality averaged at 15.0% and 17.5% respectively (Ng'ang'a et al., 2020). On the other hand, when *A. domesticus* were fed with agricultural side streams (maize stalks and leaves, kale and spinach leaves, sorghum stalks and leaves, mango fruit peels, carrot pulp) for 8 weeks, less than 2.0% mortality was reported (Kinyuru and Kipkoech,

2018). In a different study, when *Teleogryllus testaceus* were fed either native broiler finisher only or young cassava leaf flour, the mortality averaged at 8.9% and 13.3% respectively (Caparros Megido et al., 2015). On the contrary, when *T. testaceus* were fed young cashew leaf flour only or a mixture of young cashew leaf flour and brown rice flour, the mortality averaged at 94.4% and 81.1% respectively (Caparros Megido *et al*., 2015). High mortalities have profound effect on the final quantities of insects available for harvesting. In addition, dead insects if not regularly sorted out, can lead to spread of diseases and subsequent colony collapse (Vega and Kaya, 2012).

6.4.6 Microbial load of raw *A. domesticus* and *G. bimaculatus*

Microbial counts of raw *A. domesticus* and *G. bimaculatus* are presented in Table 6.4. Due to high mortality experienced in supplemented feeds compared to the control, only two replications were considered for this analysis. The total aerobic counts for *A. domesticus* ranged between 7.9 and 8.6 log cfu/g and differed significantly (P = 0.031), while that of *G. bimaculatus* ranged from 7.6 to 8.4 log cfu/g (P = 0.042), among the experimental feed treatments (Table 6.4). In both cricket species, the experimental feed treatments 5% AIL and 10% AIL resulted in significantly high aerobic counts compared to the control. Although the microbial counts in the substrates used were not determined, previously, substrates and farming environments have been the major sources of bacterial infections in edible insects (Klunder et al., 2012).

Nevertheless, the observed aerobic counts are in agreement with other studies in the literature. Vandeweyer et al. (2018) reported total aerobic counts (8.2 – 8.4 log cfu/g) for reared tropical house cricket *Gryllodes sigillatus*. On the other hand, Grabowski and Klein (2017a) reported aerobic counts ranging 7.3 - 7.5 log cfu/g for both *A. domesticus* and *G. bimaculatus* samples bought from pet shops. A slightly lower aerobic counts of 7.2 log cfu/g for *A. domesticus* purchased from a rearing company was observed (Klunder et al., 2012).

These high aerobic counts could be associated with rapid spoilage of insect and insect-based products if inadequately processed and during storage (Vandeweyer et al., 2017a).

Table 6.4: Effect of dietary supplementation of MOL and AIL powder on microbial counts (log cfu/g) of raw *A. domesticus* and *G. bimaculatus*

		Microbial counts (log cfu/g)				
Insect species	**Experimental treatments**	**Total viable counts**	***Enterobacteriaceae***	**Lactic acid bacteria**	**Bacterial endospores**	**Yeasts and moulds**
A. omesticus	Starter feed only	7.9±0.1^{a}	6.9±0.1^{a}	7.3± 0.1^{a}	3.7±0.1^{a}	5.0±0.1^{a}
	5% MOL	8.2±0.1^{a}	6.8±0.1^{a}	6.6±0.2^{bc}	4.0±0.1^{b}	5.3±0.2^{a}
	10% MOL	8.0±0.2^{a}	6.6±0.2^{a}	6.2±0.2^{b}	3.2±0.1^{c}	4.9±0.2^{a}
	5% AIL	8.6±0.1^{b}	7.2±0.1^{b}	7.1±0.2^{a}	3.0±0.1^{c}	5.0±0.2^{a}
	10% AIL	8.3± 0.2^{ab}	6.5±0.2^{a}	6.8±0.1^{c}	3.1±0.1^{c}	4.3±0.1^{b}
G. bimaculatus	Starter feed only	7.8±0.1^{a}	6.9±0.1^{a}	7.0±0.1^{a}	3.9±0.1^{a}	5.3±0.1^{a}
	5% MOL	8.1±0.3^{ab}	7.3±0.1^{a}	6.6±0.1^{bc}	4.3±0.2^{c}	5.9±0.1^{b}
	10% MOL	7.6±0.2^{a}	6.5±0.2^{b}	6.4±0.1^{c}	3.3±0.1^{b}	4.9±0.1^{a}
	5% AIL	8.4±0.3^{b}	7.1±0.1^{a}	6.7±0.2^{abc}	3.8±0.1^{a}	5.2±0.3^{a}
	10% AIL	8.1±0.1^{b}	6.3±0.1^{b}	6.8±0.2^{ab}	3.2±0.1^{b}	5.0±0.1^{a}

Data are the means ± standard deviations of three replicates

^{a,b,c} Means with the same superscript within the same column for each insect species do not differ significantly ($P > 0.05$)

Significant difference was observed in *Enterobacteriaceae* for both *A. domesticus* (*P* = 0.041), and *G. bimaculatus* (*P* = 0.022) among the experimental feed treatments (Table 6.4). For *A. domesticus*, the experimental feed treatment 5% AIL had significantly higher counts (7.2 log cfu/g) compared to the control while for *G. bimaculatus* significantly lower counts were observed in the experimental feed treatments 10% AIL (6.3 log cfu/g) and 10% MOL (6.3 log cfu/g) compared to the control. Although *in vitro* study has shown that MOL powder extracts exhibits antibacterial activity against common pathogens in the genera *Escherichia, Salmonella* and *Shigellai* within the family *Enterobacteriaceae* (Rahman et al., 2009), it can only be hypothesised this was the cause for the low counts in this study because *Enterobacteriaceae* are normally concentrated in the gastrointestinal tract of the insect.

In the literature, huge variations in *Enterobacteriaceae* counts have been reported. For instance, Vandeweyer et al. (2018) found high counts ranging 7.2 – 7.5 log cfu/g for reared *G. sigillatus* and likewise, Grabowski and Klein (2017a) reported counts ranging between 7.0 - 7.2 log cfu/g for both *A. domesticus* and *G. bimaculatus*. In contrast, when *A. domesticus* samples from a rearing company were analyzed, lower counts (4.2 log cfu/g) were observed (Klunder et al., 2012). The bacteria within the family *Enterobacteriaceae* are frequently used to assess enteric contamination in foods and are used as indicators of good handling or good processing practices. In addition, some species within the family are known to be food pathogens (Stoops et al., 2016).

Statistically significant difference was observed in lactic acid bacteria for both *A. domesticus* (*P* = 0.029) and *G. bimaculatus* (*P* = 0.035) among the experimental feed treatments (Table 6.4). In *A. domesticus*, the experimental feed treatments 5% MOL and 10% MOL resulted in significantly low counts compared to the control while in *G. bimaculatus* significant low counts in the experimental feed treatments 5% MOL, 10% MOL and 10% AIL were observed compared to the control. Previously, MOL extracts have been shown to exhibit antimicrobial activity against some strain of lactic acid bacteria including *Lactobacillus plantarum* in presence of acetic acid and propionic acid (Wang et al., 2018), although this was *in vitro* study, and probably

was the cause of low lactic acid bacteria in these treatments. In a separate study, high counts ranging 6.7 – 7.8 log cfu/g were observed for reared *G. sigillatus* (Vandeweyer et al., 2018). Normally, both lactic acid bacteria and *Enterobacteriaceae* have been reported to emanate mostly from the substrates supplied to the insects (Klunder et al., 2012), and are usually concentrated in the gastrointestinal tract, and after insect pulverization, the micro-organisms are generated from insect matrix, and thus high counts observed in all treatments (Vandeweyer et al., 2017a). Some lactic acid bacteria have been associated with food spoilage in the past. For example, some species of the genera *Lactobacillus, Leuconostoc*, and *Lactococcus* act as food spoilage organisms particularly in refrigerated meat products (Nieminen et al., 2011), and could cause spoilage of insects, as well as insect-derived products.

Whereas the bacterial endospore counts were statistically different for both *A. domesticus* and *G. bimaculatus* among the experimental feed treatments (Table 6.4), numerical differences were small (<1.0 log cfu/g). In both cricket species, significantly lower counts in the experimental feed treatments 10% MOL and 10% AIL were observed while significantly higher counts in experimental feed treatment 5% MOL were recorded. *In vitro* studies have shown that MOL and AIL powder extracts could inhibit the growth of pathogenic bacteria within the genus *Bacillus* including *Bacillus cereus*, *Bacillus subtilis* and *Bacillus megaterium* that largely constitute bacterial endospores (Rahman et al., 2009; Singh and Tafida, 2014), and probably inclusion of 10% MOL and 10% AIL contributed to low counts. It could be argued that probably 5% MOL concentration was not enough to cause inhibitory effect. Normally, high ratios of commercial starter chicken feed usually consist of grains that are normally spread-dried on the floor and that could act as points of contamination, and that could explain also high endospore counts in this study.

Other studies have reported similar counts ranging 3.2 – 4.4 log cfu/g for *G. sigillatus* (Vandeweyer et al., 2018), while bacterial endospore counts (3.6 log cfu/g) of *A. domesticus* were observed (Klunder et al., 2012). One major source of contamination is when insects get into contact with the soil during rearing, or via the feed containing spores (Klunder et al., 2012), but it cannot be stated with certainty

that this was the case for the high counts observed in this study. Bacterial endospores within the genera *e.g.*, *Bacillus* and *Clostridium*, have potential to cause food borne diseases, and represent a challenge in the production and processing of insects (Garofalo et al., 2017).

The yeast and mould counts had small variations for each cricket species among the experimental feed treatments. The counts ranged between 4.3 and 5.3 log cfu/g for *A. domesticus* while *G. bimaculatus* counts were ranging 4.9 – 5.9 log cfu/g (Table 6.4), and were slightly above the recommended limits of raw edible insects of less than 4.0 log cfu/g (Grabowski and Klein, 2017a). Similar counts (5.4 – 5.6 log cfu/g) were observed for *G. bimaculatus* and *G. sigillatus* (Grabowski and Klein, 2017a; Vandeweyer et al., 2018 respectively), while Caparros Megido et al. (2017) reported counts of 4.8 log cfu/g for *A. domesticus*. When conditions (temperature and relative humidity) are conducive, some fungi can germinate and eventually produce secondary metabolites such as mycotoxins that are toxic to humans. For example, the accumulation of aflatoxin B1 in edible stink bugs (*Encosternum delegorguei* Spinola) that were wild harvested and stored in gunny bags has been reported (Musundire et al., 2016).

Generally, in all experimental feed treatments high microbial counts were observed for both cricket species. However, the dietary supplementation with MOL (5% or 10%) powder in *A. domesticus* and, MOL (5% or 10%) and 10% AIL powder in *G. bimaculatus* resulted in significantly low counts of lactic acid bacteria compared to the control. In addition, inclusion of AIL (5% or 10%) and 10% MOL in *A. domesticus* and, 10% MOL and 10% AIL in *G. bimaculatus*, significant low counts of bacterial endospores were recorded. Also, inclusion of 10% MOL and 10% AIL in *G. bimaculatus* significantly reduced the *Enterobacteriaceae* counts.

6.4.7 Microbial load of processed *A. domesticus* and *G. bimaculatus*

Table 6.5 summarizes and compares the microbiological quality of raw and processed (toasted and boiled) crickets. Owing to high mortality experienced in other experimental treatments during rearing, only the control feed produced enough sample sizes for this analysis in both cricket species.

A statistically significant reduction of all microbial counts was observed, but log reductions in bacterial endospores were small (Table 6.5), for both cricket species. For total aerobic counts, and yeasts and moulds, reductions of about 5.5 and 2.5 log cfu/g respectively were observed after processing using either of the method. Similarly, Vandeweyer et al. (2018) found a reduction of about 5.8 and 4.0 log cfu/g for total aerobic counts, and yeasts and moulds respectively when *G. sigillatus* were boiled.

Table 6.5: Effect of heat processing on microbial counts (log cfu/g) of *A. domesticus* and *G. bimaculatus* fed Starter chicken feed only

		Microbial counts (log cfu/g)				
Insect species		Total viable counts	*Enterobacteriaceae*	Lactic acid bacteria	Bacterial endospores	Yeasts and moulds
A. domesticus	Raw crickets	7.9±0.1[a]	6.9±0.1[a]	7.3± 0.1[a]	3.7±0.1[a]	5.0±0.1[a]
	Toasted crickets	2.2±0.1[b]	<1.0±0.0[b]	<1.0±0.0[b]	2.5±0.1[b]	2.5±0.1[b]
	Boiled crickets	2.1±0.1[b]	<1.0±0.0[b]	<1.0±0.0[b]	2.2±0.1[b]	2.3±0.1[b]
G. bimaculatus	Raw crickets	7.8±0.1[a]	6.9±0.1[a]	7.0±0.1[a]	3.9±0.1[a]	5.3±0.1[a]
	Toasted crickets	2.6±0.2[b]	<1.0±0.0[b]	<1.0±0.0[b]	2.1±0.1[b]	<2.0±0.1[b]
	Boiled crickets	2.3±0.1[b]	<1.0±0.0[b]	<1.0±0.0[b]	2.0±0.1[b]	2.4±0.1[c]

Data are the means ± standard deviations of three replicates

[a,b,c] Means with the same superscript within the same column for each insect species do not differ significantly (P > 0.05)

In a different study, Klunder et al. (2012) found log reductions of 4.1 cfu/g of total aerobic counts when fresh *A. domesticus* were boiled for one minute. Elsewhere, low aerobic counts were observed when yellow mealworm larvae (*Tenebrio molitor*) was blanched for 40 seconds (Vandeweyer et al., 2017b). Some authors (Klunder et al., 2012; Vandeweyer et al., 2018) have argued that total aerobic counts may constitute some heat resistant bacteria and could not be eliminated completely using common processing techniques.

Enterobacteriaceae and lactic acid bacteria were completely eliminated or reduced to a level below the detection limit when *A. domesticus* and *G. bimaculatus* were processed using any of the two methods. Similar findings were reported when *A. domesticus* were boiled for one minute (Klunder et al., 2012), and *G. sigillatus* were brought to a boil in a kettle with water (Vandeweyer et al., 2018). Other heat treatment methods, i.e toasted, deep-fried and smoked *Ruspolia differens* sampled from the markets (Ng'ang'a et al., 2018), and blanched *T. molitor* for 40 seconds (Vandeweyer et al., 2017b) produced similar findings. These groups of bacteria are usually heat sensitive (Klunder et al., 2012), and that fact is supported by this study, where any of the two methods under study involved heating.

Although a significant reduction in bacterial endospores counts of about 1 log cfu/g was observed for two insects under study, the two processing methods could not eliminate them completely. In the literature, a reduction of about 1.6 log cfu/g was reported when fresh *A. domesticus* were boiled for one minute (Klunder *et al.*, 2012), and log reduction of 1.4 cfu/g for boiled *G. sigillatus* (Vandeweyer et al., 2018). Other studies have found similar findings for toasted, deep-fried and smoked *R. differens* (Ng'ang'a et al., 2018) and *T. molitor* blanched for 40 seconds (Vandeweyer et al., 2017b). The spore-forming bacterial species such as *Bacillus* spp., are often introduced from soil and the gastrointestinal tract of insects, and are not entirely inactivated by simple heat processing method such as boiling, and this may imply food safety risks associated with the consumption of edible insects (Klunder et al., 2012). As complete inactivation is often impossible without negatively altering food quality, aim to find agents capable of stopping growth such as acidifying (use of weak acids) and drying has given promising results (Ter Beek &

Brul, 2010). In addition, whatever insect species is considered, spore-forming bacteria and their survival should be given special attention in order to minimize them during the production, *e.g.*, by optimizing a combination of factors such as the substrates used, the farming environments, and good agricultural and handling practices.

6.4.8 Major foodborne pathogens

None of the food-borne pathogens, *i.e.*, *Salmonella* and *E. coli*, were detected in *A. domesticus* and *G. bimaculatus* processed samples. *Salmonella* is one of the most frequent causes of food poisoning, and its detection in foods before they are consumed is vital for safeguarding public health (Duguid and North, 1991), while *E. coli* is used an indicator organism for the presence of faecal coliforms. The most significant group based on severity of illness is *E. coli* O157:H7 (Diez-gonzalez and Russell, 1997). Normally, it is important to determine the presence/ absence of food pathogens in insects intended for human consumption. In a rearing cycle of *G. sigillatus*; *Salmonella* spp. and *Listeria monocytogenes* were not detected, while *Bacillus cereus* and coagulase-positive staphylococcal counts were below detection limit (Vandeweyer et al., 2018). In other studies, however, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Bacillus cereus* were reported in rhinoceros beetles *Oryctes Monoceros* (Banjo et al., 2006), while dried and powdered insects contained *B. cereus*, coliforms, and *Listeria ivanovii* (Grabowski and Klein, 2017b). The food-borne pathogens under study are normally heat sensitive, and were either absent in raw samples (not determined) or eliminated during processing using either of the heating method.

6.5 Conclusion

This study provides an overview of the effect of dietary supplementation with MOL and AIL powder on body weight, feed conversion efficiency, mortality and microbial load of farmed *A. domesticus* and *G. bimaculatus* intended for human consumption. In general, our findings have shown that the commonly used starter chicken feed can be supplemented by 5% MOL or 5% AIL powder during the rearing of *A. domesticus*; and by 5% MOL in the rearing of *G. bimaculatus* and produce crickets

with similar body weights. However, the inclusion of MOL or AIL above 5% increased the mortality in both cricket species compared to the control feed. The FCRs values obtained in this study were less than 2 and much lower than those reported for conventional production animals, indicating that *A. domesticus* and *G. bimaculatus* were more efficient in converting their feed into food for human consumption.

The dietary supplementation with MOL (5% or 10%) powder in *A. domesticus* and MOL (5% or 10%) and 10% AIL powder in *G. bimaculatus* resulted in significantly lower counts of lactic acid bacteria compared to the control feed. The inclusion of AIL (5% or 10%) and 10% MOL in *A. domesticus* and, 10% MOL and 10% AIL in *G. bimaculatus*, significant low counts of bacterial endospores were recorded. Also, inclusion of 10% MOL and 10% AIL in *G. bimaculatus* significantly reduced the *Enterobacteriaceae* counts compared to the control.

The applied processing methods resulted in killing vegetative cells (to levels deemed safe for human consumption) but bacterial endospores were not completely eliminated. Thus, during production and processing of insects and insect-derived products, bacterial endospores would require special attention *e.g.*, additional processing step, but this may have an impact on nutritional quality and may prompt further research. If the prevailing environmental conditions are conducive (temperature and relative humidity), sporulating bacteria may germinate and spoilage/ reduce the shelf life of the insects and insect-derived products after processing and during the storage.

CHAPTER SEVEN

DETERMINATION OF HEAVY METAL CONTAMINATION IN WILD HARVESTED *RUSPOLIA DIFFERENS*, AND FARMED *ACHETA DOMESTICUS* AND *GRYLLUS BIMACULATUS*

7.1 Abstract

Due to the rapid increase in world population, the use of alternative food sources is strongly promoted. In this respect, edible insects may act as a valuable alternative to the more common food sources due to their comparable nutritional content. However, edible insects may also act as source of food hazards and thus perceived as an unappealing food source. In this study, the levels of non-essential metals (Pb and Cd) and essential metals (Cu, Cr, Mn and Zn) were investigated in farmed crickets (*Acheta domesticus* and *Gryllus bimaculatus*), and wild harvested edible grasshoppers (*Ruspolia differens*). Metal levels were determined according to AOAC method 1995.01. In cricket samples, Cu and Zn were consistently the most abundant metals, with mass fractions ranging from 16.21 to 19.13 mg/kg and from 56.98 to 64.06 mg/kg, respectively. Similarly, in grasshopper samples, Cu and Zn were also the most abundant metals, with mass fractions ranging between 7.00 and 11.08 mg/kg and from 53.03 to 60.28 mg/kg, respectively. The mass fractions of Mn and Cr in all edible insects samples occurred in levels <6.71 mg/kg and <0.82 mg/kg, respectively. In all edible insect samples, Pb and Cd were not detected. These results indicates that farmed crickets and wild harvested edible grasshoppers can act as source of essential microminerals and they may not pose a food safety health risk in relation to heavy metal contamination.

7.2 Introduction

The demand for food to the world population is increasing tremendously. In this perspective, feeding the future population would involve the employment of alternative sources of proteins such as edible insects (FAO, 2013). The benefits associated with consumption of edible insects were broadly explored in a document by FAO (2013), and insects are considered suitable alternatives to common animal

sources of food. Although the use of insects as a food source brings important environmental, economic, and food security benefits (Mlcek et la., 2014; Oonincx et al., 2010), consumer acceptance remains one of the largest barriers to the adoption of insects as viable sources of nutrients (Mlcek et al., 2014; Orsi et al., 2019). In particular, in western societies, *e.g.*, in Europe, consumption of edible insects and insect-derived products is still limited, and food safety concerns, both chemical and microbiological have contributed immensely to their rejection (Mancini et al., 2019; Orsi et al., 2019).

Although insect farming is evolving in some parts of the world, up to date however, the majority of consumed insects particularly in Africa are collected from the wild. Therefore, depending on the environment they inhabit, these edible insects could be associated with food safety issues, such as heavy metal contamination (Imathiu, 2020; Murefu et al., 2019). Generally, contamination of foods by heavy metals is known to cause adverse health effects, both acute and chronic, in humans and animals (D'Souza & Peretiatko, 2000). Heavy metal contaminants may be present in edible insects (when consumed as food directly), and also in the feed (originally derived from insects), and they occur as a result of the presence of the particular contaminant in the environment and /or the substrate (van der Fels-Klerx et al., 2018). The main chemical exposure route is through the substrate on which the insects are reared and, therefore, the heavy metal(s) of concern will vary accordingly (van der Fels-Klerx et al., 2018). Accumulation of heavy metals in edible insects may depend on many factors including insect species, growth phase, feed substrate, and metal element (Diener et al., 2015; van der Fels-Klerx et al., 2018).

Currently, there is limited information on the safety of edible insects in regard to heavy metal contamination. Studies by van der Fels-Klerx et al. (2018) and Vijver et al. (2003) observed that the metabolism of essential metals, such as copper, zinc and manganese, is efficiently controlled in the insect body and no correlation has been observed between substrate concentration and the insect internal concentration. On the other hand, non-essential heavy metals, such as cadmium, lead, mercury and arsenic, have been shown to accumulate in insects, and a positive correlation between the substrate concentration and the insect internal concentration has been

observed in several studies (Diener et al., 2015; Lindqvist and Block, 1995; Poma et al., 2017; van der Fels-Klerx et al., 2018).

The few available studies of wild harvested or market collected edible insects (Devkota & Schmidt, 1999; Handley et al., 2007; Kasozi et al., 2019; Köhler et al., 2019), and farmed insects and insect-derived products (Poma et al., 2017; Vijver et al., 2003) suggest the possibility that edible insects bioaccumulate heavy metals. Although concentrations of all tested heavy metals (cadmium, arsenic, chromium, lead and tin) in farmed insects and insect-derived products were within the acceptable levels for human consumption (Poma et al., 2017), there is a likelihood for edible insects and insect-derived products, particularly for the case of wild harvested ones, to contribute to unsafe food in future, and this merits further research. Therefore, this study aimed at assessing the residual levels of the metals, *i.e.*, lead, cadmium, copper, chromium, manganese, and zinc in three edible insects, *i.e.*, wild harvested long-horned grasshoppers (*Ruspolia differens*), and farmed crickets (*Acheta domesticus* and *Gryllus bimaculatus*) intended for human consumption.

7.3 Materials and Methods

7.3.1 Sample collection and preparation

Samples of *R. differens* were collected when swarms were observed during the months of March - May in 2017 in the Kagera region, Tanzania (1°45′S and 32°40′E), and in the Mbarara district in Uganda (0°36' S and 30°39'E), as well as in the Nandi Hills, Kenya (0°06'N and 35°10'E) during the months of March - May in 2018. From each sampling site, samples weighing approximately 1.0 kg were collected, pooled together, packed in cool boxes and transported to the Food biochemistry laboratory, JKUAT for analysis.

The farmed crickets (*A. domesticus* and *G. bimaculatus*) used in this analysis were withdrawn from the crickets harvested at the end of feeding experiment described in chapter six. The crickets analyzed involved those that were fed starter chicken feed only (for other treatments, due to high mortality, the sample sizes obtained were

small for this analysis). Approximately 20 g of each insect species was placed into a hot air oven for about 24 hours at 60°C to dry. Then, for each insect species, individuals were pooled, homogenized and ground with a two-speed warring laboratory blender, (Camlab, Over, UK) to produce fine powder. In addition, starter chicken feed that was used in cricket rearing (section 6.3.3) was also analyzed for metals. The ground samples were stored at -4°C awaiting analysis.

7.3.2 Heavy metal analysis

Heavy metal determination was conducted according to AOAC method 1995.01 (AOAC, 1995). To determine levels of lead (Pb), cadmium (Cd), chromium (Cr), zinc (Zn) and manganese (Mn), approximately 3 g of insect powder was weighed and 5 ml of a mixture of nitric acid, sulphuric acid and hypochloric acid in the ratio of 6:3:1 added. The mixture was then allowed to stand for 5 minutes after shaking thoroughly. The mixture was digested on a hot plate starting at 70 °C through to 120 °C until the volume reduced to approximately 1 ml, and the white fumes of surfur trioxide were evolved. The samples were then allowed to cool to room temperature, after which approximately 20 ml of 5% hydrochloric acid solution was added. The samples were heated on a hot plate at 75 °C for 15 minutes and then cooled. They were filtered through Whatman® Grade 42 filter paper into a 100 ml volumetric flask, and filled to the mark using 5% hydrochloric acid. A blank sample was also prepared (Osborn & Voogt, 1978).

The Pb, Cd, Cr, Zn and Mn levels in the samples were determined using a Shimadzu Atomic Absorption Spectrophotometer, Model AA-6200 flame (Shimadzu, Tokyo, Japan) according to Osborn and Voogt (1978). For each metal, analysis were conducted in triplicates and expressed as mg/kg. Commercial samples of Pb, Cd, Cr, Zn and Mn standards were used as reference compounds (Wako Pure Chemical Industries Ltd., Japan). Thereafter, standard curves were generated and used to determine the concentration of each metal in each insect sample.

7.3.3 Statistical analysis

Statistical analyses were performed using Stata SE version 12 (StataCorp LP, TX). Data are presented as means with their standard deviations. Metal levels were expressed as mg/kg ww. One-way ANOVA tests were performed to determine significant differences in individual metal levels in wild harvested grasshoppers and farmed crickets. Mean separation was obtained by the Bonferroni's option of the ANOVA test. For all tests, a significance level of ≤ 0.05 was considered.

7.4 Results and Discussion

Table 7.1 summarizes the results of metals quantified in farmed crickets and wild harvested grasshoppers. In both cricket species, although Cu and Zn were the most abundant metals, with mass fractions ranging from 16.21 to 19.13 mg/kg and from 56.98 to 64.06 mg/kg respectively, their bioaccumulation was not statistically different. On the other hand, the amount of Mn quantified in *G. bimaculatus* was significantly higher than in *A. domesticus*. Normally, the *G. bimaculatus* are considered voracious feeder and tend to gain more weight than *A. domesticus* (Kinyuru and Kipkoech, 2018), and with significantly higher level of Mn quantified in starter chicken feed (Table 7.1), this probably could explain higher bioaccumulation of Mn in *G. bimaculatus* compared to *A. domesticus*. In a different study, when several species of edible insects, *i.e.*, larvae of the greater wax moth (*Galleria mellonella*), adults of the migratory locust (*Locusta migratoria*), larvae of the mealworm beetle (*Tenebrio molitor*), and larvae of the buffalo worm (*Alphitobius diaperinus*) were analyzed, measured levels of Zn and Cr were similar to those of farmed crickets in this study (Poma et al., 2017).

Table 7.1: Quantification of heavy metals in farmed and wild harvested edible insects (mg/kg ww)

	Metals					
Insect/ feed samples	Pb	Cd	Cu	Cr	Mn	Zn
Farmed crickets						
A. domesticus	ND	ND	16.21±3.54[a]	0.82±0.37[a]	1.86±0.22[a]	64.06±7.48[a]
G. bimaculatus	ND	ND	19.13±1.79[a]	0.68± 0.28[a]	4.29±1.19[b]	56.98±9.65[a]
Starter chicken feed	ND	ND	22.29±4.54[a]	0.24±0.12[b]	20.37±4.64[c]	37.19± 5.81[b]
Wild harvested grasshoppers						
R. differens - Tanzania	ND	ND	11.08±0.99[a]	0.59±0.31[a]	4.95±1.91[a]	60.28±5.71[a]
R. differens - Uganda	ND	ND	9.58±1.38[ab]	0.71±0.20[a]	4.27± 2.08[a]	53.03±7.54[a]
R. differens - Kenya	ND	ND	7.00±2.02[b]	0.66±0.21[a]	6.71±2.11[a]	58.17±11.92[a]

Data are the means ± standard deviations of three replicates

[a,b,c] Means with the same superscript letters within the same column do not differ significantly ($P > 0.05$)

ND: Not Detected

Detection limit of Cd and Pb was 0.01 and 0.005 mg/kg, respectively

In starter chicken feed, Cu, Mn, and Zn were the most abundant metals with significantly higher level of Mn observed in starter chicken feed than in both cricket species. Studies by van der Fels-Klerx et al. (2018) and Vijver et al. (2003) observed that the metabolism of essential metals such as Cu, Mn, and Zn, is efficiently controlled in the insect body and no correlation was found between their concentrations in the substrate and in the insect. This may justify the reason why higher levels of Cu and Mn were observed in starter chicken feed compared to that of farmed crickets.

It is worth noting that mineral supplements including Cu, Fe, Mn and Zn are part of broiler feed formulations (Bao and Choct, 2009), and this may explain why higher levels of Cu, Mn and Zn were quantified in starter chicken feed. In the literature, the importance of inclusion of Cu, Mn and Zn in broiler chicken feeds has been reported, with dietary deficiency of Zn strongly depressing the feed intake, and hence slower growth of broiler chickens (Bao & Choct, 2009). Therefore, feeds containing mineral supplements could result to insects with enhanced mineral levels as observed in this study, *e.g.*, Zn levels in both cricket species.

For the case of wild harvested grasshoppers, it was difficult to establish the diets the grasshoppers were feeding on in the fields or bushes, hence only the grasshoppers were analyzed. In grasshopper samples, Cu and Zn were also the most abundant metals, with mass fractions ranging between 7.00 and 11.08 mg/kg, and from 53.03 to 60.28 mg/kg, respectively (Table 7.1). However, grasshoppers from the three sampling sites did not differ significantly in their Zn, Mn and Cr level (Table 7.1). The obtained values of Zn, Cu and Mn in grasshoppers in this study are similar to those found in *R. differens* harvested from Uganda by Fombong et al. (2017), confirming that edible grasshoppers are likely to provide these essential metals if they are readily bioavailable when consumed.

Nevertheless, high standard deviations were obtained for Zn in grasshoppers collected in three countries, indicating high variability, but similar findings have been reported by other researchers working on *Ruspolia* grasshoppers (Fombong et al., 2017; Kasozi et al., 2019). On the other hand, the Cu level quantified in grasshoppers from Tanzania was significantly higher than those harvested in Kenya. Similar variations were observed by Fombong et al. (2017) in grasshoppers harvested in different locations in Uganda, and attributed it to grasshoppers feeding on different foliage materials while swarming from one region to another. Normally, the recommended daily intake of Zn for adults is between 4.9 and 7.0 mg (moderate bioavailability), that of Cu is between 1.0 to 1.4 mg while that of Mn is from 1.8 to 2.6 mg (FAO, 2007). As such, consumption of either crickets or grasshoppers in this study would provide rich source of these trace metals but these will depend on the frequency of consumption and quantity consumed.

In all edible insect samples and in the feed, Pb and Cd were consistently below the limit of detection. In other studies in the literature, the levels of Cd and Pb measured in edible grasshoppers from Korea averaged at 0.02 and 0.73 mg/kg ww, respectively, and were considered safe for human consumption (Hyun et al., 2012). Poma et al. (2017) reported mean levels of Cd (<0.04 mg/kg ww) and Pb (<0.03 mg/kg ww) in four edible insect species namely; *G. mellonella, L. migratoria, T. molitor* and *A. diaperinus,* as well as low levels of Pb and Cd (all <0.03 mg/kg ww) in insect-derived foods. Similarly, low levels of Pb, Cd and As were detected in four edible insects (mulberry silkworm, scarab beetle, house cricket and bombay locust) consumed in Thailand and deemed safe for human consumption (Köhler et al., 2019). On the contrary, Handley et al. (2007) found a high Pb content in chapulines (dried grasshopper) from Oaxaca (Mexico) and associated it to grasshoppers feeding in highly polluted mine areas.

In general, the levels of essential and non-essential heavy metals in all insect samples analyzed in this study were lower than the maximum acceptable levels of metals in similar types of foods set by the European Commission (Commission Regulation 1881/2006), including meat of bovine animals, sheep, pig, poultry, and muscle meat of fish (Pb <0.4 mg/kg and Cd <0.3 mg/kg). These results indicate that the risk of exposure to heavy metals emanating from the consumption of edible insects is relatively low.

7.5 Conclusion

This study suggests that these edible insects have the potential to provide essential metals such as Cu, Mn and Zn in human diet. On the other hand, the level of non-essential metals including Pb and Cd that are known to bioaccumulate in insect body was below the detection limit. These results indicates that farmed crickets and wild harvested edible grasshoppers may not pose a food safety health risk in relation to heavy metal contamination when used as food or feed.

CHAPTER EIGHT

GENERAL CONCLUSION AND RECOMMENDATIONS

8.1 Conclusion

In East Africa, the consumption of long-horned grasshoppers is based on wild collections, and food safety risks are usually associated with this practice. On the other hand, cricket farming is making progress in many parts of the world, however, the efficacy and safety of various locally available materials used as feed is being tested because of possibility of introducing food safety risks such as microbial hazards and toxic metals in crickets intended for human consumption. Findings of this study showed that the long-horned grasshoppers that exists in E. Africa is *Ruspolia differens* (Orthoptera: Tettigonidae) based on 18S rDNA partial sequences. Although a recent study has identified *R. differens* using COI gene region, it was noted that there is very limited genomic information on the long-horned grasshoppers occurring from E. Africa, such that other genetic markers (28S and COII) were incapable of identifying *R. differens* from other related tettigoniids due to the absence of the corresponding gene sequences from *R. differens* in the GenBank. Thus, in order to identify the (sub) species that occur in E. Africa, a deep sequencing method, such as restriction site associated DNA markers (RAD-Seq) is suggested.

As insects are living organisms, they harbour a complex microbiota that are not just localized in the gut but also on various anatomical parts. This microbiota is comprised of microbes that are either intrinsically part of an insect's life cycle or are introduced during rearing and processing. The microbial analysis of raw wild harvested *R. differens* samples collected at harvesting points revealed high microbial counts including total viable count (TVC), *Enterobacteriaceae*, lactic acid bacteria (LAB), bacterial endospores, and yeasts and moulds, exceeding the recommended process hygiene criteria. Further, handling of *R. differens* samples along the food value chain (transportation and plucking) increased the contamination levels. These numbers indicate that raw long-horned grasshoppers offer a suitable matrix for growth of variety of microorganisms, including possible spoilage microorganisms or foodborne pathogens. Thus, since most insects are usually consumed in their entirety,

as removal of the gut is not always possible, attention should be paid to the high numbers of these microbial groups when edible insects are harvested, processed and stored.

During the rearing of *A. domesticus*, when S+W was supplied as the main feed, this resulted in crickets with higher sellable weight compared to the control feed (SO). This indicates that the Wandering Jew weeds were palatable and highly accepted by the crickets, and thus, it should be tapped in insect feed formulation. On the other hand, high microbial counts that included TVC, *Enterobacteriaceae*, LAB, and yeasts and moulds exceeding the recommended process hygiene criteria of comparable food matrices were observed in raw *A. domesticus*. However, the bacterial endospore counts in crickets fed with S+W were significantly lower than those SO fed ones. Bacterial sporeformers, such as *Clostridium botulinum* and *Bacillus cereus* are foodborne pathogens that can produce toxins, causing illness to consumers. Thus, this low count of bacterial endospores in crickets fed with S+W, could reduce the risks associated with sporeformers because they are hardly eliminated by commonly applied processing methods. When these crickets were subjected to metagenetics analyses, a total of 1976 Operational Taxonomic Units (OTUs) were yielded. However, only a few OTUs with potential food pathogens that included the genus *Staphylococcus*, and the families *Clostridiaceae* and *Enterobacteriaceae* were obtained. Thus, culture-dependent assays, such as presence-absence tests or specific counts of pathogens are needed for a full evaluation of the microbiological safety of these crickets.

When feed was supplemented with 5% MOL or 5% AIL in feeding of *A. domesticus*; and 5% MOL in feeding of *G. bimaculatus*, the body weights were similar to control feed, and thus, at these levels of supplementation they can be tapped in insect feed formulation. In contrast, the inclusion of MOL (5% or 10%) and AIL (5% or 10%) increased the mortality in both cricket species. The feed conversion ratio (FCR) values obtained in this study were less than 2 and much lower than those reported for production animals such as poultry, pork, and beef, indicating that *A. domesticus* and *G. bimaculatus* were more efficient in converting the supplied feeds into biomass for human consumption. Although high counts of TVC, *Enterobacteriaceae*, LAB,

bacterial endospores, yeasts and moulds exceeding the recommended process hygiene criteria were recorded, significantly lower counts of LAB and bacterial endospores were observed in treatments containing MOL (5% or 10%) and 10% AIL in both cricket species. Thus, the risks associated with bacterial endospores and LAB in these farmed edible crickets is low. In this regard, the microbial risks of farmed edible insects can be greatly controlled by following good hygienic practices during rearing, handling, harvesting, processing, storing and transporting.

Heat treatments in this study were shown to completely eliminate *Enterobacteriaceae* and LAB, while TVC and, yeasts and moulds were reduced to levels deemed safe for human consumption in both wild harvested long-horned grasshoppers and in farmed crickets. On the contrary, bacterial endospores were never eliminated. The presence of endospore-forming bacteria in edible insects is major food safety concern as the spores, being heat-resistant, may withstand the common processing methods like boiling, toasting, smoking and deep-frying adopted in this study. Therefore, the development of techniques to reduce spore populations while still maintaining a high-quality product, as well as preventing germination of spores and growth of vegetative cells during processing and storage is advocated. Valuable alternatives to traditional boiling and/or blanching such as high-pressure decontamination combined with heating or (gamma) irradiation are suggested.

This study showed that farmed crickets and wild harvested grasshoppers have potential to provide trace elements such as Cu, Mn and Zn. On the other hand, three heavy metals with no dietary requirements *i.e,* Cr the levels measured were relatively low in all insect samples analyzed, while Pb and Cd were not detected or below limit of detection. Although more research is advocated particularly in Africa where insect consumption is mainly based on wild collections, the findings in this study have shown that farmed crickets and wild harvested long-horned grasshoppers may not pose a food safety health risk in relation to heavy metal contamination when used as food or feed.

8.2 Recommendations

The recommendations that have been identified and suggested from this study include:

1. In order to identify (sub)species of long-horned grasshoppers (of the genus *Ruspolia*) that exist(s) in E. Africa, it is recommended that the extant populations be analyzed in more detail via molecular genetic methods based on deep sequencing methods, such as restriction site associated DNA markers (RAD-Seq).
2. Wandering Jew weed that was highly accepted and resulted in crickets with higher sellable weight should be tapped in insect feed formulation. On the other hand, commercial starter chicken feed can be supplemented with 5% MOL or 5% AIL and results in crickets with higher body weight, while supplementation with 10% MOL or 10% AIL and improved microbial quality.
3. High microbial loads were recorded particularly on crickets fed on commercial chicken feeds only (SO) and this could be attributed to the quality of the feeds, and therefore, during the processing of feeds such SO, proper application of good manufacturing practices is important.
4. Techniques on how to completely eliminate or inactivate bacterial endospores should be explored further. Development of new techniques to reduce spore populations and preventing germination of spores while still maintaining nutritional quality and texture of edible insects during processing, distribution and storage.
5. High fungal counts in this study provides evidence for the possible presence of mycotoxins in insects under study. Thus, a more in-depth characterization of the fungal community and assessment of the prevalence of mycotoxins in edible insects is recommended.
6. Mechanism behind the inhibitory effect against some bacteria group when starter chicken feed was supplemented with Wandering Jew weed, and *Moringa oleifera* and *Azadirachta indica* leaves powder need to be explored.

7. Characterization of the bacterial community in farmed crickets using metagenetics could only classify group of bacteria up to genus level, hence, more specific techniques such as whole genome sequencing for bacteria or sequencing of the internal transcribed spacer region (ITS) of the nuclear DNA for fungi, to identify foodborne pathogens associated with edible insects is recommended.

REFERENCES

AOAC (1995). *Official Methods of Analysis*. (15th Edition), Arlington, VA, USA: Association of the Official Analytical Chemists.

Adjorlolo, L. K., Timpong-jones, E., & Adogla-bessa, T. (2016). Potential contribution of neem (Azadirachta indica) leaves to dry season feeding of ruminants in West Africa. *Livestock Research for Rural Development*, *28*.

Agea, J. G., Biryomumaisho, D., Buyinza, M., & Nabanoga, G. N. (2008). Commercialization of Ruspolia nitidula (nsenene grasshoppers) in central Uganda. *African Journal of Food Agriculture Nutrition and Development*, *8*, 291–303.

Anand, H., Ganguly, A., & Haldar, P. (2008). Potential value of acridids as high protein supplement for poultry feed. *International Journal of Poultry Science*, *7*, 722–725.

Ayieko, M. A., Kinyuru, J. N., Ndong, M. F., Kenji, G. M., & Box, P. O. (2012). Nutritional Value and Consumption of Black Ants (Carebara vidua Smith) from the Lake Victoria Region in Kenya. *Advance Journal of Food Science and Technology*, *4*(1), 39–45.

Ayieko, M. A., Obonyo, G. O., Odhiambo, J. A., Ogweno, P. L., Achacha, J., & Anyango, J. (2011). Constructing and using a light trap harvester: Rural technology for mass mollection of Agoro termites (Macrotermes subhylanus). *Research Journal of Applied Sciences, Engineering and Technology*, *3*(2), 105–109.

Ayieko, M., Joseph Magala, H., & Ogola, O. (2016). Introducing rearing crickets (gryllids) at household levels: Adoption, processing and nutritional values. *Journal of Insects as Food and Feed*, *2*, 203–211.

Bailey, W. J., & McCrae, A. W. R. (1978). The general biology and phenology of swarming in the east african tettigoniid Ruspolia differens (Serville) (Orthoptera). *Journal of Natural History*, *12*(3), 259–288.

Bailey, W.J. (1975). A review of the African species of the genus Ruspolia Schulthess (Orthoptera, Tettigonioidea). *The Bulletin of the Fundamental Institute of Black Africa*, (37), 171–226.

Baldwin, J. P. (1975). A quantitative analysis of the factors affecting plant nutrient uptake from some soils. *Journal of Soil Science*, *26*(3), 195–296.

Banjo, A. D., Lawal, O. A., & Adeyemi, A. I. (2006). The microbial fauna associated with the larvae of Oryctes monocerus. *Journal of Applied Sciences Research*, *2*, 837–843.

Bao, Y., & Choct, M. (2009). Trace mineral nutrition for broiler chickens and prospects of application of organically complexed trace minerals: A review. *Animal Production Science*, *49*, 269–282.

Barennes, H., Phimmasane, M., & Rajaonarivo, C. (2015). Insect consumption to address undernutrition, a national survey on the prevalence of insect consumption among adults and vendors in Laos. *PloS one*, *10*(8), e0136458.

Belluco, S., Losasso, C., Maggioletti, M., Alonzi, C. C., Paoletti, M. G., & Ricci, A. (2013). Edible insects in a food safety and nutritional perspective: A critical review. *Comprehensive Reviews in Food Science and Food Safety*, *12*, 296–313.

Bhowmik, S., Chowdhury, S. D., Kabir, M. H., & Ali, M. A. (2008). Chemical composition of some medicinal plant products of indigenous origin. *The Bangladesh Veterinarian*, *25*, 32–39.

Brits, J. A., & Thorton, C. H. (1981). ON the biology of Ruspolia differens (serville) (Orthoptera: Tettigoniidae) in South Africa. *Phytophylactica*, *173*(13), 169–173.

Brown, S. P., Veach, A. M., Rigdon-huss, A. R., Lickteig, S. K., Lothamer, K., Oliver, A. K., …& Davey, M. L. (2015). Scraping the bottom of the barrel : are rare high throughput sequences artifacts ? *Fungal Ecology*, *13*, 221–225.

Caparros Megido, R., Alabi, T., Nieus, C., Blecker, C., Danthine, S., Bogaert, J., & Francis, F. (2015). Optimisation of a cheap and residential small-scale production of edible crickets with local by-products as an alternative protein-rich human food source in Ratanakiri Province , Cambodia. *Journal of the Science of Food and Agriculture*, *96*, 627–632.

Caparros Megido, R., Desmedt, S., Blecker, C., Béra, F., Haubruge, É., Alabi, T., & Francis, F. (2017). Microbiological load of edible insects found in Belgium. *Insects*, *8*, 1–8.

Caparros Megido, R., Poelaert, C., Ernens, M., Liotta, M., Blecker, C., Danthine, S., Francis, F. (2018). Effect of household cooking techniques on the microbiological load and the nutritional quality of mealworms (Tenebrio molitor L. 1758). *Food Research International*, *106*, 503–508.

Caparros Megido, R., Sablon, L., Geuens, M., Brostaux, Y., Alabi, T., Blecker, C., Francis, F. (2014). Edible insects acceptance by belgian consumers : promising attitude for entomophagy development. *Journal of Sensory Studies*, *29*, 14–20.

Caporaso, J. G., Lauber, C. L., Walters, W. A., Berg-lyons, D., Lozupone, C. A., Turnbaugh, P. J., … Knight, R. (2011). Global patterns of 16S rRNA diversity at a depth of millions of sequences per sample. *Proceedings of the National Academy of Sciences of the United States of America*, *108*, 4516–4522.

Chang, S. K. . (2010). *Protein Analysis. In Food Analysis* (4th edition) *Nielsen, S.S., Ed.;* New York: Springer.

Charlton, A., Dickinson, M., Wakefield, M., Fitches, E., Kenis, M., Han, R., … & Smith, R. (2015). Exploring the chemical safety of fly larvae as a source of protein for animal feed. *Journal of Insects as Food and Feed*, *1*(1), 7–16.

Choo, B. Y. R., Yan, A. T., Loh, R. H. J., Chin, Z. Y., Jo, C. S., Lee, K. E. M., & Tan, H. T. W. (2017). Can waste vegetables and weeds be used to farm crickets and grasshoppers in Singapore ? *Utar agriculture science journaL*, *3*(November), 18–22.

Christensen, D. L., Orech, F. O., Michael, N., Larsen, T., & Friis, H. (2006). Entomophagy among the Luo of Kenya: a potential mineral source? *International Journal of Food Sciences and Nutrition*, *57*(June), 198–203.

Colman, D. R., Toolson, E. C., & Takacs-vesbach, C. D. (2012). Do diet and taxonomy influence insect gut bacterial communities ? *Molecular Ecology*, *21*, 5124–5137.

D'Souza, C., & Peretiatko, R. (2000). The nexus between industrialization and environment: A case study of Indian enterprises. *Environmental Management and Health*, *13*, 80–97.

Danley, P. D., Mullen, S. P., Liu, F., Nene, V., Quackenbush, J., & Shaw, K. L. (2007). A cricket Gene Index: a genomic resource for studying neurobiology, speciation, and molecular evolution. *BMC Genomics*, *16*, 1–16.

Dash, G. K., Swe, M., & Mathews, A. (2017). Tradescantia Zebrina : A Promising Medicinal Plant. *Indo american journal of pharmaceutical sciences*, *4*, 3498–3502.

De Smet, J., Lenaerts, S., Borremans, A., Scholliers, J., Van Der Borght, M., & Van Campenhout, L. (2019). Stability assessment and laboratory scale fermentation of pastes produced on a pilot scale from mealworms (Tenebrio molitor). *LWT - Food Science and Technology*, *102*, 113–121.

Defoliart, G. R. (1999). Insects as food : Why the Western attitude is important. *Annual Review of Entomology*, (80), 21–50.

de Gier, S., & Verhoeckx, K. (2018). Insect (food) allergy and allergens. *Molecular immunology*, *100*, 82-106.

Delong, E. (2013). Methods in enzymology*: Microbial Metagenomics, Metatranscriptomics, and Metaproteomics*, *531*, 2-598.

Devkota, B., & Schmidt, G. H. (1999). Accumulation of heavy metals in food plants and grasshoppers from. *Agriculture Ecosystems & Environment*, *78*, 85–91.

Dhanasekaran, D., Shanmugapriya, S., & Thajuddin N. P. A. (2011). Aflatoxins and aflatoxicosis in human and animals. In: Guevara-González RG, editor. Aflatoxins - *Biochemistry and Molecular Biology.*, Croatia: Rijeka.

Di Bitetti, M. (2017). The distribution of grooming among female primates : testing hypotheses with the Shannon-Wiener diversity index. *Beyond Behaviour*, *127*, 1517–1540.

Dian-feng, L., Zi-mei, D., Da-yu, Z., Yan-ze, G., Pei-jun, G., Rui-hua, H., & Guo-fang, J. (2008). Molecular phylogeny of the higher category of acrididae (Orthoptera: Acridoidea). *Zoological Research*, *6*, 585−591.

Diener, S., Zurbrügg, C., & Tockner, K. (2015). Bioaccumulation of heavy metals in the black soldier fly, *Hermetia illucens* and effects on its life cycle. *Journal of Insects as Food and Feed*, *1*(4), 261–270.

Diez-gonzalez, F., & Russell, J. B. (1997). The ability of *Escherichia coli* 0157: H7 to decrease its intracellular pH and resist the toxicity of acetic acid. *Microbiology*, (1 997), 1175–1180.

Dijk, R., Beumer, R. R., Hummelen, T., Brinkman, E., Debevere, J., van Dijk, J. C., Dijkstra, A. F. & Stegeman, H. (2007). *Microbiologie van voedingsmiddelen, methoden, principes en criteria* (4th ed.). Houten: Noordervliet Media

Dillon, R. J., & Dillon, V. M. (2004). The gut bacteria of insects: Nonpathogenic Interactions. *Annual Review of Entomology*, *49*, 71–92.

Duguid, J. P., & North, R. A. E. (1991). Eggs and Salmonella food-poisoning: an evaluation. *Journal of Medical Microbiology*, *34*, 65–72.

Durst, P. B., & Hanboonsong, Y. (2015). Small-scale production of edible insects for enhanced food security and rural livelihoods : Experience from Thailand and Lao People ' s Democratic Republic. *Journal of Insects as Food and Feed*, *1*, 25–31.

Edgar, R. C. (2013). UPARSE : highly accurate OTU sequences from microbial amplicon reads. *Nature Methods*, *10*, 996–998.

Edgar, R. C. (2016). SINTAX : a simple non-Bayesian taxonomy classifier for 16S and ITS sequences. *BioRxiv*, *74161*.

EFSA. (2015). Risk profile related to production and consumption of insects as food and feed. *EFSA Journal*, *13*(10), 4257.

European Commission. (2006). Commission Regulation (EC) No 1881/2006 of 19 December 2006 setting maximum levels for certain contaminants in foodstuffs. *Off. J. Eur. Union*, *364*, 5-24.

European Commission (2017). Commission regulation (EU) 2017/893 of 24 May 2017 amending annexes I and IV to regulation (EC) No 999/2001 of the

European Parliament and of the council and annexes X, XIV and XV to commission regulation (EU) No 142/2011 as regards the provisions on processed animal protein. *Official Journal of the European Union*, *138*, 92-116.

FAO/ United Nations (2013). World Population Prospects: The 2012 Revision, Highlights and Advance Tables (Working Paper No. ESA/P/WP. 228). Retrieved from http://esa.un.org/unpd/wpp/ExcelData/population.htm.

FAO (2007). Protein and amino acid requirements in human nutrition. Report of a Joint WHO/FAO/UNU Expert Consultation. World Health Organ. *Tech. Rep. Series 2007*, *935*, 1–265.

Fijan, S. (2014). Microorganisms with Claimed Probiotic Properties : An Overview of Recent Literature. *International Journal of Environmental Research and Public Health*, *11*, 4745–4767.

Flook, P. K., Klee, S., & Rowell, C. H. F. (1999). Combined molecular phylogenetic analysis of the Orthoptera (Arthropoda, Insecta) and implications for their higher systematics. *Systematic Biology*, *48*(2), 233–253.

Flook, P. K., & Rowell, C. H. F. (1998). Inferences about orthopteroid phylogeny and molecular evolution from small subunit nuclear ribosomal DNA sequences. *Insect Molecular Biology*, *7*, 163–178.

Fombong, F. T., Van Der Borght, M., & Vanden Broeck, J. (2017). Influence of freeze-drying and oven-drying post blanching on the nutrient composition of the edible insect *Ruspolia differens*. *Insects*, *8*, 102.

Francis, F., Doyen, V., Debaugnies, F., Mazzucchelli, G., Caparros, R., Alabi, T., & Corazza, F. (2019). Limited cross reactivity among arginine kinase allergens from mealworm and cricket edible insects. *Food chemistry*, *276*, 714-718.

Gadzirayi, C. T., Masamha, B., Mupangwa, J. F., & Washaya, S. (2012). Performance of broiler chickens fed on mature *Moringa oleifera* leaf meal as a protein supplement to soyabean meal. *International Journal of Poultry Science*, *11*(1), 5–10.

Garofalo, C., Milanović, V., Cardinali, F., Aquilanti, L., Clementi, F., & Osimani, A. (2019). Current knowledge on the microbiota of edible insects intended for human consumption : A state-of-the-art review. *Food Research International*, *125*, 108527.

Garofalo, C., Osimani, A., Milanovi, V., Taccari, M., Cardinali, F., Aquilanti, L., & Clementi, F. (2017). The microbiota of marketed processed edible insects as revealed by high-throughput sequencing. *Food Microbiology*, *62*, 15–22.

Grabowski, N., & Klein, G. (2017b). Microbiology of processed edible insect products – Results of a preliminary survey. *International Journal of Food Microbiology*, *243*, 103–107.

Grabowski, N. T., & Klein, G. (2017a). Microbiological analysis of raw edible insects. *Journal of Insects as Food and Feed*, *3*, 7–14.

Grabowski, N.T., Tchibozo, S., Abdulmawjood, A., Acheuk, F., M'Saad Guerfali, M., Sayed, W.A.A. & Plötz, M. 2020. Edible insects in Africa in terms of food, wildlife resource, and pest management legislation. *Foods*, *9*, 502.

Gupta, K., Barat, G. K., Wagle, D. S., & Chawla, H. K. L. (1989). Nutrient content and nntinutrional factors in conventional and non-conventional leafy vegetables. *Food Chemistry*, *31*, 105–116.

Gurevich, A., Saveliev, V., Vyahhi, N., & Tesler, G. (2013). Bioinformatics applications note Genome analysis QUAST : quality assessment tool for genome assemblies. *Bioinformatics*, *29*, 1072–1075.

Halloran, A., Caparros Megido, R., Oloo, J., Weigel, T., Nsevelo, P., & Francis, F. (2018). Comparative aspects of cricket farming in Thailand, Cambodia, Lao People's Democratic Republic, Democratic Republic of the Congo and Kenya. *Journal of Insects as Food and Feed*, *1*, 1–14.

Halloran, A., Roos, N., Flore, R., & Hanboonsong, Y. (2016). The development of the edible cricket industry in Thailand. *Journal of Insects as Food and Feed*, *2*(2), 91–100.

Halloran, A., Vantomme, P., Hanboonsong, Y., & Ekesi, S. (2015). Regulating edible insects: the challenge of addressing food security, nature conservation, and the erosion of traditional food culture. *Food Security*, *7*(3), 739–746.

Hanboonsong, Y., Jamjanya, T., & Durst, P. B. (2013). Six-legged livestock: edible insect farming, collection and marketing in Thailand. *RAP publication*, *3*, 8-21.

Handley, M. A., Hall, C., Sanford, E., Diaz, E., Gonzalez-mendez, E., Drace, K., & Croughan, M. (2007). Globalization, binational communities, and imported food risks: Results of an outbreak investigation of lead poisoning in Monterey County, California. *American Journal of Public Health*, *97*(5), 900–906.

Hemp, C., Heller, K., Kehl, S., Warchałowska-Sliwa, E., Wagele, J. W., & Hemp, A. (2010). The Phlesirtes complex (Orthoptera, Tettigoniidae, Conocephalinae, Conocephalini) reviewed: integrating morphological, molecular, chromosomal and bioacoustic data. *Systematic Entomology*, (35), 554–580.

Hoque, M. D. M., Bari, M. L., Inatsu, Y., Juneja, V. K., & Kawamoto, S. (2007). Antibacterial activity of guava (*Psidium guajava* L.) and Neem (*Azadirachta indica* A. Juss.) extracts against foodborne pathogens and spoilage bacteria. *Foodborne Pathogens and Disease*, *4*, 481–488.

Houbraken, M., Spranghers, T., De Clercq, P., Cooreman-Algoed, M., Couchement, T., De Clercq, G., … & Spanoghe, P. (2016). Pesticide contamination of *Tenebrio molitor* (Coleoptera: Tenebrionidae) for human consumption. *Food Chemistry*, *201*, 264–269.

Huffman, R. D. (2002). Current and future technologies for the decontamination of carcasses and fresh meat. *Meat Science*, *62*, 285–294.

Hyun, S.-H., Kwon, K. H., Park, K.-H., Hjeong, H. C., Kwon, O., Tindwa, H., & Han, Y. S. (2012). Evaluation of nutritional status of an edible grasshopper, *Oxya chinensis* Formosana. *Entomological Research*, *42*, 284–290.

Imathiu, S. (2020). Benefits and food safety concerns associated with consumption of edible insects. *NFS Journal*, *18*, 1–11.

ISO 11290-2:2004 (2004). *Microbiology of food and animal feeding stuffs - horizontal method for the detection and enumeration of Listeria monocytogenes with modification of medium*. Geneva, Switzerland: International Organization for Standardization.

ISO 6579:2007 (2007). Microbiology of food and animal feeding stuffs - horizontal method for the detection and enumeration of *Salmonella* species.

ISO 7251: 2005 (2005). Microbiology of food and animal feeding stuffs - horizontal method for the detection and enumeration of presumptive *Escherichia coli.*

Ji, K., Chen, J., Li, M., Liu, Z., Wang, C., Zhan, Z., & Xia, Q. (2009). Anaphylactic shock and lethal anaphylaxis caused by food consumption in China. *Trends in food science & technology*, *20*(5), 227-231.

Ji, K. M., Zhan, Z. K., Chen, J. J., & Liu, Z. G. (2008). Anaphylactic shock caused by silkworm pupa consumption in China. *Allergy*, *63*(10), 1407-1408.

Kachapulula, P. W., Akello, J., Bandyopadhyay, R., & Cotty, P. (2018). Aflatoxin contamination of dried insects and fish in Zambia. *Journal of Food Protection*, *81*(9), 1508–1518.

Kasozi, K. I., Namazi, C., Basemera, E., Atuheire, C., Odwee, A., Majalija, S., & Kateregga, J. N. (2019). Inorganic pollutants in edible grasshoppers (*Ruspolia nitidula*) of Uganda and their major public health implications. *African Health Sciences*, *19*(3), 2679–2691.

Kearse, M., Moir, R., Wilson, A., Stones-havas, S., Sturrock, S., Buxton, S., & Drummond, A. (2012). Geneious Basic: An integrated and extendable desktop software platform for the organization and analysis of sequence data. *Bioinformatics*, *28*(12), 1647–1649.

Kelemu, S., Niassy, S., Torto, B., Fiaboe, K., Affognon, H., Tonnang, H., & Ekesi, S. (2015). African edible insects for food and feed: inventory, diversity, commonalities and contribution to food security. *Journal of Insects as Food and Feed*, *1*(2), 103–119.

Kenya Bureau of Standards KS 2921:2020 (2020). *Standard guide on the primary production and handling of insects for food and feed.* Nairobi: KBS.

Kinyuru, J., Kenji, G., Muhoho, S., & Ayieko, M. (2010). Nutritional potential of long-horned grasshopper (*Ruspolia differens*) consumed in Siaya district, Kenya. *Journal of Agriculture Science and Technology*, *12*, 32–46.

Kinyuru, J N, & Kipkoech, C. (2018). Production and growth parameters of edible crickets: experiences from a farm in a high altitude, cooler region of Kenya. *Journal of Insects as Food and Feed*, *1*(1), 1–5.

Kinyuru, J.N, Konyole, S. O., Roos, N., Onyango, C. A., Owino, V. O., Owuor, B. O., & Kenji, G. M. (2013). Nutrient composition of four species of winged termites consumed in western Kenya. *Journal of Food Composition and Analysis*, *30*(2), 120–124.

Klunder, H. C., Wolkers-Rooijackers, J., Korpela, J. M., & Nout, M. J. R. (2012). Microbiological aspects of processing and storage of edible insects. *Food Control*, *26*, 628–631.

Köhler, R., Kariuki, L., Lambert, C., & Biesalski, H. K. (2019). Protein, amino acid and mineral composition of some edible insects from Thailand. *Journal of Asia-Pacific Entomology*, *22*, 372–378.

Kozich, J. J., Westcott, S. L., Baxter, N. T., Highlander, S. K., & Schloss, P. D. (2013). Development of a dual-Index sequencing strategy and curation pipeline for analyzing amplicon sequence data on the MiSeq. *Applied Environmental Microbiology*, *79*, 5112–5120.

Kumar, S., Nei, M., Dudley, J., & Tamura, K. (2008). MEGA: A biologist-centric software for evolutionary analysis of DNA and protein sequences. *Briefings in Bioinformatics*, *9*(4), 299–306.

Legendre, F., Robillard, T., Song, H., Whiting, M., & Desutter-Grandcolas, L. (2010). One hundred years of instability in ensiferan relationships. *Systematic Entomology*, *35*, 475–488.

Leonard, A., Khamis, F. M., James, P. E., Kyamanywa, S., Ekesi, S., Tanga, C. M., & Subramanian, S. (2020). Identification of edible short- and long-horned grasshoppers and their host plants in East Africa. *Journal of Economic Entomology*, *113*, 2150–2162.

Lindqvist, L., & Block, M. (1995). Excretion of cadmium during moulting and metamorphosis in *Tenebrio molitor* (Coleoptera; Tenebrionidae). *Comparative Biochemistry and Physiology. Part C*, *111*, 325–328.

Lu, W., Wang, J., Zhang, H. J., Wu, S. G., Qi, G. H., Lu, W., & Qi, G. H. (2016). Evaluation of *Moringa oleifera* leaf in laying hens : effects on laying performance, egg quality, plasma biochemistry and organ histopathological indices. *Italian Journal of Animal Science*. *15*(4), 658-665.

Lundy, M. E., & Parrella, M. P. (2015). Crickets are not a free lunch : Protein capture from scalable organic side-streams via high-density populations of *Acheta domesticus*. *PLoS ONE*, *10*, 1–12.

Lyn, J. C., Naikkhwah, W., Aksenov, V., & Rollo, C. D. (2011). Influence of two methods of dietary restriction on life history features and aging of the cricket *Acheta domesticus*. *Age*, 509–522.

Magara, H. J. O., Tanga, C. M., Ayieko, M. A., Hugel, S., Mohamed, S. A., Khamis, F. M., & Roos, N. (2019). Ecology and behavior performance of newly described native edible cricket *Scapsipedus icipe* (Orthoptera : Gryllidae) on various diets of relevance for farming. *Journal of Economic Entomology*, *112*, 653–664.

Makkar, H. P. S., Tran, G., Heuzé, V., & Ankers, P. (2014). State-of-the-art on use of insects as animal feed. *Animal Feed Science and Technology*, *197*, 1–33.

Malaisse, F., & Latham, P. (2014). Human consumption of lepidoptera in Africa: An updated chronological list of references (370 quoted !) with their ethnozoological. *Geo-Eco-Trop*, *2*, 339–372.

Mancini, S., Moruzzo, R., Riccioli, F., & Paci, G. (2019). European consumers' readiness to adopt insects as food . A review. *Food Research International*, *122*, 661–678.

Matojo, N. D. (2017). A review work on how to differentiate the long-horned grasshoppers *Ruspolia differens* and *Ruspolia nitidula* (Orthoptera: Tettigoniidae). *Journal of Applied Life Sciences International*, *15*(2), 1–4.

Matojo, N. D., & Hosea, K. M. (2013a). Phylogenetic relationship of the long-horned grasshopper *Ruspolia differens* Serville (Orthoptera: Tettigoniidae) from Northwest Tanzania based on 18S Ribosomal nuclear sequences. *Journal of Insects*, 1–5.

Matojo, N. D., & Yarro, J. G. (2013b). Anatomic Morphometrics of the“ Senene” Tettigoniid *Ruspolia differens* Serville (Orthoptera: Conocephalidae) from North-West Tanzania. *ISRN Entomology*, *2013*, 12.

Matojo, N. D., & Njau, M. A. (2010b). Plasticity and biosystematics of swarming of the conehead *Ruspolia differens* Serville (Orthoptera: Conocephalidae). *Internatinal Journal of of Integrated Biology*, *9*(2), 97–103.

Matojo, N. D., & Yarro, J. G. (2010a). Variability in polymorphism and sex ratio of the conehead *Ruspolia differens* Serville (Orthoptera: Conocephalidae) in north-west Tanzania. *Internatinal Journal of of Integrated Biology*, *9*(3), 131–136.

Mbata, K. J., & Chidumayo, E. N. (2003). Traditional calues of caterpillars (Insecta: Lepidoptera) among the Bisa people of Zambia. *International Journal of Tropical Insect Science*, *23*(4), 341–354.

McCrae, N. (1982). Characteristics of swarming in the African edible bush-cricket *Ruspolia differens* (Serville) (Orthoptera; Tettigonioidea). *Journal of the East Africa Natural History Society and National Museum*.

Miech, P., Berggren, Å., Lindberg, J. E., Chhay, T., Khieu, B., & Jansson, A. (2016). Growth and survival of reared Cambodian field crickets (*Teleogryllus testaceus*) fed weeds , agricultural and food industry by-products Abstract. *Journal of Insects as Food and Feed*, *2*(4), 285–292.

Mlcek, J., Rop, O., Borkovcova, M., & Bednarova, M. (2014). A comprehensive look at the possibilities of edible insects as food in Europe - A Review. *Polish Journal of Food and Nutrition Sciences*, *64*, 147–157.

Mmari, M. W., Kinyuru, J. N., Laswai, H. S., & Okoth, J. K. (2017). Traditions, beliefs and indigenous technologies in connection with the edible long-horned grasshopper *Ruspolia differens* (Serville 1838) in Tanzania. *Journal of Ethnobiology and Ethnomedicine*, *13*(1), 1–11.

Mohammed, K. A. F., Sarmiento-franco, L., Santos-ricalde, R., & Solorio-sanchez, J. F. (2012). The nutritional effect of *Moringa oleifera* fresh leaves as feed supplement on Rhode Island Red hen egg production and quality. *Tropical Health Animal Production*, *44*, 1035–1040.

Mpuchane, S., Gashe, B. A., Allotey, J., Siame, B., Teferra, G., & Ditlhogo, M. (2000). Quality deterioration of phane, the edible caterpillar of an emperor moth *Imbrasia belina*. *Food Control*, *11*, 453–458.

Murefu, T. R., Macheka, L., Musundire, R., & Manditsera, F. A. (2019). Safety of wild harvested and reared edible insects : A review. *Food Control*, *101*, 209–224.

Musundire, R., Osuga, I. M., Cheseto, X., Irungu, J., & Torto, B. (2016). Aflatoxin contamination detected in nutrient and anti-oxidant rich edible stink bug stored in recycled grain containers. *PLoS ONE*, *11*, 1–16.

Nakagaki, B. J., & DeFoliart, G. R. (1991). Comparison of diets for mass-rearing *Acheta domesticus* (Orthoptera : Gryllidae) as a novelty food and comparison of food conversion efficiency with values reported for livestock. *Journal of Economic Entomology*, *84*, 891–896.

Ng'ang'a, J., Imathiu, S., Fombong, F., Ayieko, M., Vanden Broeck, J., & Kinyuru, J. (2018). Microbial quality of edible grasshoppers *Ruspolia differens* (Orthoptera: Tettigoniidae): From wild harvesting to fork in the Kagera Region, Tanzania. *Journal of Food Safety*, *12549*, 1–6.

Ng'ang'a, J., Imathiu, S., Fombong, F., Borremans, A., Van Campenhout, L., Vanden Broeck, J., & Kinyuru, J. (2020). Can farm weeds improve the growth and microbiological quality of farmed crickets (*Gryllus bimaculatus*)? *Journal of Insects as Food and Feed*, *6*(2), 199–209.

Nieminen, T. T., Vihavainen, E., Paloranta, A., Lehto, J., Paulin, L., Auvinen, P., & Björkroth, K. J. (2011). Characterization of psychrotrophic bacterial communities in modified atmosphere-packed meat with terminal

restriction fragment length polymorphism. *International Journal of Food Microbiology*, *144*, 360–366.

Nowak, V., Persijn, D., Rittenschober, D., & Charrondiere, U. R. (2016). Review of food composition data for edible insects. *Food Chemistry*, *193*, 39–46.

Nwaru, B. I., Hickstein, L., Panesar, S. S., Roberts, G., Muraro, A., Sheikh, A., and EAACI Food Allergy and Anaphylaxis Guidelines Group. (2014). Prevalence of common food allergies in Europe: a systematic review and meta-analysis. *Allergy*, *69*(8), 992-1007.

Nyangena, D. N., Mutungi, C., Imathiu, S., Kinyuru, J., Affognon, H., Ekesi, S., & Fiaboe, K. K. M. (2020). Effects of traditional processing techniques on the nutritional and microbiological quality of four edible insect species used for food and feed in East Africa. *Foods*, (9), 1–16.

Obikaonu, H. O. (2012). Evaluation of the nutritinal value of neem (*Azadirachta indica*) leaf meal on the performance of finisher broilers. *Iternational Journal of Agiculture and Rural Development*, *15*(3), 1235–1239.

Obikaonu, H. O., & Udedible, A. B. I. (2015). Evaluation of neem (*Azadirachta indica*) leaf meal in the diets of black leghorn laying hens for protein sustaininability and national development. *International Journal of Agricultural Technology*, *11*, 1089–1095.

Obopile, M., & Seeletso, T. G. (2013). Eat or not eat: an analysis of the status of entomophagy in Botswana. *Food Security*, *5*, 817–824.

Oduro, I., Ellis, W. O., & Owusu, D. (2008). Nutritional potential of two leafy vegetables : *Moringa oleifera* and *Ipomoea batatas* leaves. *Scientific Research and Essay*, *3*, 57–60.

Ogbuewu, I. P., Uchegbu, M. C., Okoli, I. C., & Iloeje, M. U. (2010). Assessment of blood chemistry, weight gain and linear body measurements of pre-

puberal buck rabbits fed different levels of neem (*Azadirachta indica*) leaf meals. *Chilean Journal of Agricultural Research, 70*, 515–520.

Olembo, R. J. (1970). Polymorphism in the African edible grasshopper *Homorocoryphus nitidulus* (Tettigoniidae: Conocephalidea) revisited. *Acta Tropica, 27*, 387-390.

Oonincx, D. G. A. B., Broekhoven, S. Van, Huis, A. Van, & Loon, J. J. A. Van. (2015). Feed conversion, survival and development, and composition of four insect species on diets composed of food by-products. *PLoS ONE, 10*, 12.

Oonincx, D. G. A. B., Van Itterbeeck, J., Heetkamp, M. J. W., Van Den Brand, H., Van Loon, J. J. A., & Van Huis, A. (2010). An exploration on greenhouse gas and ammonia production by insect species suitable for animal or human consumption. *PLoS ONE, 5*(12), 1–7.

Orinda, M. A., Mosi, R. O., & Ayieko, M. A. (2017). Growth performance of common house cricket (*Acheta domesticus*) and field cricket (*Gryllus bimaculatus*) fed on agro-byproducts. *Journal of Entomology and Zoology Studies, 5*, 1664–1668.

Orsi, L., Voege, L. L., & Stranieri, S. (2019). Eating edible insects as sustainable food? Exploring the determinants of consumer acceptance in Germany. *Food Research International, 125*, 108573.

Osimani, A., Garofalo, C., Milanovic, V., Aquilanti, L., Pasquini, M., Mozzon, M., & Clementi, F. (2017). Insight into the proximate composition and microbial diversity of edible insects marketed in the European Union. *European Food Research and Technology, 243*, 1157–1171.

Piromrat, K., Chinratanapisit, S., & Trathong, S. (2008). Anaphylaxis in an emergency department: a 2-year study in a tertiary-care hospital. *Asian Pacific Journal of Allergy and Immunology, 26*(2-3), 121.

Poma, G., Cuykx, M., Amato, E., Calaprice, C., Focant, J. F., & Covaci, A. (2017). Evaluation of hazardous chemicals in edible insects and insect-based food intended for human consumption. *Food and Chemical Toxicology*, *100*, 70–79.

Pratt, R. C., Morgan-richards, M., & Trewick, S. A. (2008). Diversification of New Zealand weta (Orthoptera: Ensifera: Anostostomatidae) and their relationships in Australasia. *Philosophical Transactions of the Royal Society*, *363*, 3427–3437.

Rahman, M. M., Sheikh, M. M. I., Sharmin, S. A., Islam, M. S., Rahman, M. A., Rahman, M. M., & Alam, M. F. (2009). Antibacterial activity of leaf juice and extracts of *Moringa oleifera* Lam against some human pathogenic bacteria. *Chiang Mai University Journal of Natural Sciences*, *8*, 219–228.

Ravindran, V., & Blair, R. (1993). Feed resources for poultry production in Asia and the Pacific. III. Animal protein sources. *World's Poultry Science Journal*, *49*, 219–235.

Rimmer, C. C., Miller, E. K., & Faccio, S. D. (2010). Mercury bioaccumulation and trophic transfer in the terrestrial food web of a montane forest. *Ecotoxicology*, (19), 697–709.

Rumpold, B. A., & Schlüter, O. K. (2013a). Nutritional composition and safety aspects of edible insects. *Molecular Nutrition and Food Research*, *57*(5), 802–823.

Rumpold, B. A., & Schlüter, O. K. (2013b). Potential and challenges of insects as an innovative source for food and feed production. *Innovative Food Science and Emerging Technologies*, *17*, 1–11.

Saeed, T., Abu Dagga, F., & Saraf, M. (1993). Analysis of residual pesticides present in edible locusts captured in Kuwait. *Arab Gulf Journal of Scientific Research*, *11*, 1–5.

Saitou, N., & Nei, M. (1987). The neighbor-joining method: A new method for reconstructing phylogenetic Trees. *Molecular Biology and Evolution*, *4*(4), 406–425.

Schabel, H. G. (2008). Forest insects as food: A global review. In Edible Forest Insect*: Human Bite Back, Proceedings of a Workshop on Asia-Pacific Resources and Their Potential for Development, Chiang Mai, Thailand, 19–21 February 2008*.

SHC and FASFC. (2014). *Food safety aspects of insects intended for human consumption* (SciCom dossier 2014/04; SHC dossier n° 9160)., (9160), 1–22.

Sheng, Z., Dong, Z. *Æ*., Zheng, M., & Lv, Q. C. (2008). Bioaccumulation of total and methyl mercury in three earthworm species (*Drawida* sp ., *Alolobophora* sp ., and *Limnodrilus* sp). *Bulletin of Environmental Contamination and Toxicology*, 937–942.

Singh, K., & Tafida, G. M. (2014). Antibacterial activity of *Moringa oleifera* (Lam) leaves extracts against some selected bacteria. *International Journal of Pharmacy and Pharmaceutical Sciences*, *6*, 52–54.

Ssepuuya, G., Mukisa, I. M., & Nakimbugwe, D. (2016). Nutritional composition, quality, and shelf stability of processed *Ruspolia nitidula* (edible grasshoppers). *Food Science and Nutrition*, *5*, 103–112.

Ssepuuya, G., Wynants, E., Verreth, C., Crauwels, S., Lievens, B., Claes, J., & Van Campenhout, L. (2019). Microbial characterisation of the edible grasshopper *Ruspolia differens* in raw condition after wild-harvesting in Uganda. *Food Microbiology*, *77*, 106–117.

Stoops, J., Crauwels, S., Waud, M., Claes, J., Lievens, B., & Van Campenhout, L. (2016). Microbial community assessment of mealworm larvae (*Tenebrio molitor*) and grasshoppers (*Locusta migratoria migratorioides*) sold for human consumption. *Food Microbiology*, *53*, 122–127.

Stoops, J., Vandeweyer, D., Crauwels, S., Verreth, C., Boeckx, H., Van Der Borght, M., & Van Campenhout, L. (2017). Minced meat-like products from mealworm larvae (*Tenebrio molitor* and *Alphitobius diaperinus*): microbial dynamics during production and storage. *Innovative Food Science and Emerging Technologies*, *41*, 1–9.

Svenson, G. J., & Whiting, M. F. (2004). Phylogeny of Mantodea based on molecular data: evolution of a charismatic predator. *Systematic Entomology*, *29*, 359–370.

Takeja, J. (1990). The dietary repertory of the Ngandu people of the tropical rain forest: an ecological and anthropological study of the subsistence activities and food procurement technology of a slash-and burn agriculturist in the Zaire river basin. *African Study Monographs Supplementary*, *11*, 1–75.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., & Kumar, S. (2011). MEGA5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum marsimony methods. *Molecular Biology and Evolution*, *28*(10), 2731–2739.

Tanzania Bureau of Standards TZS 789:2003 (2003). *The standard requirements for both drinking waters and bottled drinking waters*. Tanzania: TZS.

Ter Beek, A., & Brul, S. (2010). To kill or not to kill Bacilli: opportunities for food biotechnology. *Current Opinion in Biotechnology*, *21*(2), 168–174.

Unicef, & World Health Organization (2019). State of food security and nutrition in the World: safeguarding against economic slowdowns and downturns. Retrieved from https://www.wfp.org/publications/2019-state-food-security-and-nutrition-world-sofi-safeguarding-against-economic

Van der Fels-Klerx, H. J., Camenzuli, L., Belluco, S., Meijer, N., & Ricci, A. (2018). Food safety issues related to uses of insects for feeds and foods.

Comprehensive Reviews in Food Science and Food Safety, *17*(5), 1172–1183.

Van Huis, A, Van Itterbeeck, J., Klunder, H., Mertens, E., Halloran, A., Muir, G., & Vantomme, P. (2013a). *Edible insects. Future prospects for food and feed security. FAO Forestry Paper 171.*, Rome, Italy: FAO.

Van Huis, Arnold. (2013b). Potential of insects as food and feed in assuring food Security. *Annual Review of Entomology*, *58*(1), 563–583. https://doi.org/10.1146/annurev-ento-120811-153704

Van Huis, Arnold. (2017). Cultural significance of termites in sub-Saharan Africa. *Journal of Ethnobiology and Ethnomedicine*, *8*, 1–12.

Vandeweyer, D., Crauwels, S., Lievens, B., & Van Campenhout, L. (2017c). Metagenetic analysis of the bacterial communities of edible insects from diverse production cycles at industrial rearing companies. *International Journal of Food Microbiology*, *261*, 11–18.

Vandeweyer, D., Crauwels, S., Lievens, B., & Van Campenhout, L. (2017b). Microbial counts of mealworm larvae (*Tenebrio molitor*) and crickets (*Acheta domesticus* and *Gryllodes sigillatus*) from different rearing companies and different production batches. *International Journal of Food Microbiology*, *242*, 13–18.

Vandeweyer, D., Lenaerts, S., Callens, A., & Van Campenhout, L. (2017a). Effect of blanching followed by refrigerated storage or industrial microwave drying on the microbial load of yellow mealworm larvae (*Tenebrio molitor*). *Food Control*, *71*, 311–314.

Vandeweyer, Dries, Wynants, E., Crauwels, S., Verreth, C., Viaene, N., Claes, J., & Van Campenhout, L. (2018). Microbial dynamics during industrial rearing, processing, and storage of tropical house crickets (*Gryllodes sigillatus*) for human consumption. *Applied Environmental Microbiology*, *84*, 1–12.

Vantomme, P., Göhler, D., & Deckere-ziangba, F. N. (2004). Contribution of forest insects to food security and forest conservation: The example of caterpillars in Central Africa. *In: ODI Wildlife Policy Briefing*, *3*(4), 345 -352.

Vega, F. E., & Kaya, H. K. (2012). *Insect pathology*. Amsterdam: Academic Press.

Vijver, M., Jager, T., Posthuma, L., & Peijnenburg, W. (2003). Metal uptake from soils and soil-sediment mixtures by larvae of *Tenebrio molitor* (L.) (Coleoptera). *Ecotoxicology and Environmental Safety*, *54*(3), 277–289.

Virgilio, M., Backeljau, T., Nevado, B., & De Meyer M. (2010). Comparative performances of DNA barcoding across insect orders. *BMC Bioinformatics 11: 206.*

Wang, Y., Wang, C., Zhou, W., Yang, F., Chen, X., Zhang, Q., & Chen, X. (2018). Effects of wilting and *Lactobacillus plantarum* addition on the fermentation quality and microbial community of *Moringa oleifera* leaf silage. *Frontiers in Microbiology*, *9*, 1–8.

Waud, M., Busschaert, P., Ruyters, S., Jacquemyn, H., & Lievens, B. (2014). Impact of primer choice on characterization of orchid mycorrhizal communities using 454 pyrosequencing. *Molecular Ecology Resources*, *14*, 679–699.

Whiting, M. F. (2002). Mecoptera is paraphyletic: multiple genes and phylogeny of Mecoptera and Siphonaptera. *Zoologica Scripta*, *1*, 93–104.

Wilkinson, J. M. (2011). Re-defining efficiency of feed use by livestock. *Animal*, *5*, 1014–1022.

Wynants, E., Crauwels, S., Lievens, B., Luca, S., Claes, J., Borremans, A., … Van Campenhout, L. (2017). Effect of post-harvest starvation and rinsing on the microbial numbers and the bacterial community composition of mealworm larvae (*Tenebrio molitor*). *Innovative Food Science and Emerging Technologies*, *42*, 8–15.

Wynants, E., Crauwels, S., Verreth, C., Gianotten, N., Lievens, B., Claes, J., & Van Campenhout, L. (2018). Microbial dynamics during production of lesser mealworms (*Alphitobius diaperinus*) for human consumption at industrial scale. *Food Microbiology*, *70*, 181–191.

Yeoh, H., & Wee, Y. (1994). Leaf protein contents and nitrogen-to-protein conversion factors for 90 plant species. *Food Chemistry*, *49*, 245–250.

APPENDICES

Appendix I: Bacterial community composition and relative abundance of the G. bimaculatus fed S0 and S+W for 30 days

		S0	S+W
OTUs	**Bacterial community**	**Relative abundance (%)**	**Relative abundance (%)**
7	Coxiellaceae	69.71593949	79.11877
5	Lactobacillus sp.	16.93583515	11.15115
4	Spiroplasma sp.	4.926488755	3.439687
2	Lactobacillus sp.	1.409242161	0.977117
9	Enterobacteriaceae	0.946330578	0.618206
11	Enterobacteriaceae	0.859899854	0.556678
10	Enterobacteriaceae	0.782260982	0.52738
12	Enterobacteriaceae	0.572779671	0.38821
14	Enterobacteriaceae	0.309097238	0.191907
60	Porphyromonadaceae	0.240242043	0.325203
78	Porphyromonadaceae	0.169926796	0.098149
35	Morganella sp.	0.128914934	0.007324
90	Porphyromonadaceae	0.137699125	0.067384
1	Lactobacillus sp.	0.169929822	0.127449
3	Pediococcus sp.	0.177254284	0.124519
105	Porphyromonadaceae	0.112795851	0.045411
103	Porphyromonadaceae	0.093752418	0.062992
36	Acetobacter sp.	0.136236241	0.080572
29	Clostridiaceae	0.096683657	0.074712
136	Alistipes sp.	0.020508442	0.084966
28	Enterobacteriaceae	0.067385938	0.065922
129	Porphyromonadaceae	0.07031441	0.027833
17	Enterococcus sp.	0.111332324	0.109869
111	Porphyromonadaceae	0.036622503	0.118656
138	Lachnospiraceae	0.064454763	0.020509
160	Porphyromonadaceae	0.054200606	0.021973
96	Enterobacteriaceae	0.060060446	0.082033
26	Pseudomonas sp.	0.061526742	0.016114
25	Enterobacteriaceae	0.060061541	0.051273
31	Clostridiaceae	0.067386131	0.032229
156	Rikenellaceae	0.048341217	0.023438
142	Porphyromonadaceae	0.014649053	0.051272
34	Staphylococcus sp.	0.041016884	0.019044
133	Porphyromonadaceae	0.033692293	0.067384
	Others	0.012671376	0.012247

Appendix II: List of publications

1. **Ng'ang'a J**, Imathiu S, Fombong F, Ayieko M, Vanden Broeck J and Kinyuru J (2018). Microbial quality of edible grasshoppers *Ruspolia differens* (Orthoptera: Tettigoniidae): From wild harvesting to fork in the Kagera Region, Tanzania. *Journal of Food Safety* 12549: 1–6. https://doi.org/10.1111/jfs.12549.
2. **Ng'ang'a J**, Imathiu, S., Fombong, F., Borremans, A., Van Campenhout, L., Vanden Broeck, J. and Kinyuru, J. (2020). Can farm weeds improve the growth and microbiological quality of farmed crickets (Gryllus bimaculatus)? *Journal of Insects as Food and Feed*, *6*(2), 199–209.
3. **Ng'ang'a J**, S. Imathiu, F. Fombong, J. Vanden Broeck and J. Kinyuru (2021). Effect of dietary supplementation with powder derived from *Moringa oleifera* and *Azadirachta indica* leaves on growth and microbial load of edible crickets. *Journal of Insects as Food and Feed*. https://doi.org/10.3920/JIFF2020.0056.
4. **Ng'ang'a J**, F. Fombong, S. Kiiru, C. Kipkoech and J. Kinyuru (2021). Food safety concerns in edible grasshoppers: A review of microbiological and heavy metal hazards. *International Journal of Tropical Insect Science*: https://doi.org/10.1007/s42690-020-00372-9.

LIST OF PROCEEDINGS

Ng'ang'a J, Imathiu S, Fombong F, Ayieko M, Vanden Broeck J and Kinyuru J (2020). Microbial aspects of delicacy *Ruspolia* grasshoppers found in East Africa. 1st International *Ruspolia* grasshoppers Symposium, 11 December 2020, SAJOREC Conference Center, JKUAT, Nairobi, Kenya.

J. Ng'ang'a, S. Imathiu, F. Fombong, J. Vanden Broeck and J. Kinyuru (2021). Effect of dietary inclusion of Wandering Jew (*Commelina sinensis*) weed on growth and bacterial community composition of farmed edible cricket (*Gryllus bimaculatus*). The 15th JKUAT Scientific, Technological, & Industrialization Conference; 25th & 26th March, 2021, JKUAT, Nairobi, Kenya.