ANALYSIS OF MICROBIAL LOAD AND DIVERSITY IN CRICKETS (*GRYLLUS BIMACULATUS* AND *SCAPSIPEDUS ICIPE*) USED AS A SOURCE OF PROTEIN FOR FOOD

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DECLARATION

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

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DEDICATION

This work is dedicated to the Almighty God for giving me life. To my family especially my husband Nicholas Kinyua, my sons Quincy Murimi, Adrian Gatheru and Edison Nyagah for the love, patience and understanding they showed me during the entire study without which this work could not have seen the light of the day.

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ABBREVIATIONS AND ACRONYMS

ANOVA	Analysis of Variance
DNA	Deoxyribonucleic acid
FAO	Food and Agricultural organization
GLM	General Linear Model
HSD	Honest Significant Difference
OTUs	Operational Taxonomic Units
PBS	Phosphate-buffered Saline
PCR	Polymerase Chain Reaction
PDA	Potato Dextrose Agar
RNA	Ribonucleic acid
SDA	Sabouraud Dextrose Agar
SSU	Small Sub Unit
MWs	Mopane worms

ABSTRACT

Crickets are drawing interest as sustainable source of protein for food and feed worldwide. However, there is little information on microbial quality of edible crickets thus the need for a profound insight into their safety. The objective of the study was to determine the microbial load of two edible cricket species *Scapsipedus icipe* and *Gryllus bimaculatus* and to evaluate the efficiency of different processing methods (boiling, sun-drying, freeze-drying, snap freezing and deep frying) in reducing microbial counts. The wild-caught crickets were obtained from Nguruman (Kajiado County) and Mbita (Homa Bay County) while the farmed crickets were reared at Animal Rearing and Containment Unit at the International Centre of Insect Physiology and Ecology (*icipe*). The cricket samples were screened for microbiota using culture-dependent method. Fifteen adult crickets were pooled together and homogenized in Phosphate-buffered Saline (PBS). The solution was used for mycological and bacterial isolation and analysis. Bacterial microbiota was isolated using Nutrient agar (NA) and MacConkey agar (MCA). Fungal microbiota was isolated using Sabouraud Dextrose Agar (SDA) and Potato Dextrose Agar (PDA). The isolates were characterized morphologically and through sequencing of bacterial 16S SSU rRNA genes and fungal internal transcribed spacer (ITS) rRNA gene. Most of the bacterial isolates (70 %) on NA had characteristics typical of members of *Bacillus* spp. while on MCA, most bacteria (90 %) had characteristics suggestive of members of *E. coli*. Majority (80 %) of the fungal isolates on PDA had characteristics similar to those of *Aspergillus* spp. while most isolates (80 %) on SDA were members of *Trichoderma* spp. Microbial counts of fresh cricket samples were generally high, with bacterial population ranging from 2.5×10^4 - 3.8×10^4 CFU /g fresh weight. The fungal populations ranged from $2.0 \times 10^4 – 3.0 \times 10^4$ CFU /g fresh weight. Upon processing the microbial counts reduced considerably with bacterial counts ranging from $0.1 \times 10^2 – 0.8 \times 10^2$ CFU /g dry weight (boiled) and $0.7 \times 10^3 – 2.2 \times 10^3$ CFU /g dry weight (sun dried). The fungal counts ranged from 0.7×10^2 - 0.9×10^2 CFU /g dry weight (boiled) and 0.9×10^3- 1.7×10^3 CFU /g dry weight (sun-dried). Freeze-dried and deep fried samples had no microbial counts. The diversity of bacteria and fungi species in wild-caught crickets was considerably high compared to the lab-reared crickets with most isolates belonging to species pathogenic to humans. Phylogenetic analysis revealed that most bacterial isolates from the wild-caught crickets related to members of *Bacillus* spp. (57 %), *Staphylococcus* spp. (43 %) and *E. coli* (14 %). Fungal isolates related to *Aspergillus* spp. (57 %) and *Penicillium roseopurpureum* (29 %). From the farmed crickets, most of the bacterial isolates related to *E. coli* (60 %), *Enterobacter* (40 %) and *Lactococcus garvieae* (20 %). The fungal isolates related to *Trichoderma asperellum* (75 %), *Aspergillus* spp. (25 %) and *Tetrapisispora fleetii* (12.5 %). From the cricket samples processed by boiling and sun drying, the microbial diversity was very low with only two bacterial isolates related to *Rickettsiella grylli* (42 %) and *Wolbachia* spp. (29 %), while the two fungal isolates related to members of *Aspergillus* spp. (67 %) and *Trichoderma asperellum* (33 %). Thirteen potentially novel bacterial and fungal isolates from wild and reared crickets had no close matches from gene bank and need further investigation. This study shows that crickets harbor diverse microbial communities some of which are potentially pathogenic. Deep-frying, freeze drying and snap freezing completely eliminated bacterial and fungal contaminants thus minimizing microbial risks in crickets meant for food.

CHAPTER ONE

INTRODUCTION

1.1 Background

Feeding on insects, which is also known as entomophagy, has a long history as part of the human diet (Klunder *et al*., 2012). A great number of insect species are being served as a source of food in many parts of the world. Approximately 2.5 billion people, mainly from Africa, Latin America and Asia feed on insects as part of their main diet in the same manner they eat fish or meat (Hanboonsong *et al*., 2013). Insects constitute high quality human and animal food and recently, there has been an increasing global interest to utilize them as a food resource, to compliment the diet of the ever-increasing human population (EFSA, 2015).

The world population is estimated to surpass 9 billion people by 2050, an addition of more than 2 billion people to an already crowded globe (Mpuchane *et al*., 2000). Food and Agricultural Organization (FAO) estimates that the current food production will need to expand by about 70 percent to meet global food necessities come 2050 (van Huis *et al*., 2015). To meet this massive additional demand for food, there should be concerted focus to increase production and consumption of currently under-appreciated and under-utilized foods (Belluco *et al*., 2013). Edible insects encompass one such category of under-utilized foods that would contribute to meeting future universal demands (Makkar *et al.,* 2014).

Insects grow and reproduce easily, have high feed conversion efficiency and can feed on waste biomass and transform it to a high value feed resource. Additionally, insects require far much less breeding space and their greenhouse gas emission is minimal

compared to other livestock (Jansson and Berggren, 2015). Apart from their environmental benefits, insects are comparable to the usual livestock meat in terms of their nutritional content. The crude protein content of insects ranges from 40 – 75 % on dry weight basis, mostly depending on the species and the stage of development (Klunder *et al*., 2012).

It is estimated that about 842 million people, which amounts to 12 % of the world population could not meet their dietary requirement from 2010 through 2013 (Kelemu *et al.,* 2015). Majority of these people live in developing nations where undernourishment was at 15 % in 2011 through 2013 (van Huis *et al.,* 2015). Africa remains the region with high levels of undernourishment despite increasing economic growth and continuing decline in poverty rates in the recent years (Mpuchane *et al.,* 2000). Majority of the people are unable to access quality food because the region is vulnerable to recurrent famines and food crisis triggered by unlikely events such as conflicts, floods, pests as well as drought. Furthermore, hunger is expected to worsen unless radical measures are taken to overturn the state food insecurity (Jansson and Berggren, 2015).

As the number of people continue to rise, there is need to reconsider other measures to enhance food and nutrition security to the rising population. It is in this regard that FAO has recommended the application of sustainable diets to improve food and nutritional security (EFSA, 2015). Sustainable diets are foods with minimal environmental effects, respectful and protectful of ecosystems and biodiversity, accessible, culturally accepted, affordable, safe, nutritionally adequate and healthy. Utilization of insects for food and feed perfectly fits within the context of sustainable diets (Hanboonsong *et al.,* 2013).

Insects are the most abundant organisms on earth constituting more than 70 % of all the organisms (Mpuchane *et al.,* 2000). They are among the most diverse group of organisms, which have been in existence for at least 400 million years, making them some of the earliest animals on earth (Rumpold and Schlüter, 2013). It is believed that about 2000 species of insects are consumed worldwide by approximately 2 billion people, mainly from the developing countries (Mlcek *et al.,* 2014). Mujuru *et al.* (2014) noted that, apart from insects being used as food, they provide incomes, which can in turn be used to purchase foods and farm inputs. A case in point is where *Gonimbrasia belina* also referred to as Mopane worms are an important source of income and nutrition for lower income households in Zimbabwe. The caterpillar stage of the worm is an excellent source of minerals and proteins, with a crude protein value of about 50 % (Mujuru *et al.,* 2014).

Owing to their high nutritional profiles, insects provide a favourable environment for microbes to grow and survive (Klunder *et al*., 2012). However, survival and growth of microbes could also be influenced by processing and storage conditions applied to them along the value chain. In most cases, traditional processing methods are used which include, sun drying, roasting and boiling. This is mostly done to improve on palatability, storability and taste with an assumption of providing safe food products (Belluco *et al*., 2013).

It is of fundamental importance that insect’s value chain be in such a manner that ensures the production of safe and good quality products (van Huis *et al.,* 2015). In many cases processing, storage and packaging of insects is poorly done, leading to spoilage by microorganisms and pests. Traditionally, insects are degutted by hand followed by

drying, by either sun or hot sand, as it is the case with mopane worms (Allotey and Mpuchane, 2003). The drying process can be effective in reducing microbial contamination and spoilage in dry environments. However, in humid environments the growth of microbes could be stimulated. Additionally, contamination could also occur through soil, air and packaging material used, resulting in unsafe and poor quality products unfit for human consumption (Hanboonsong *et al.,* 2013).

As noted by Mujuru *et al.* (2014), edible insects can be contaminated by fungi, bacteria or even pesticide residues. Thus, regulatory guidelines should be formulated based on scientific data to protect the consumers. The data should be used to develop suitable legislative framework for insects to be considered as food at different stages of the value chain (Belluco *et al*., 2013).

There are three major ways to consume edible insects, first as a whole recognizable form. Secondly, they can be turned into a non-recognizable form, where whole insects are dried, ground into powder and used to enrich low nutrient food and feed (Kelemu *et al*., 2015). Additionally, insect's protein can be extracted and used to substitute meat and soy proteins in food. Most edible insects are still gathered from the wild where their accessibility is seasonal and limited to certain localities. However recently, there has been an emergence of commercial insect farming in some countries (Charlton *et al*., 2015).

Since man uses insects as food, this study is aimed at documenting the microflora associated with fresh wild and lab reared *Scapsipedus icipe* and *Gryllus bimaculatus*, with the aim of advising on any health implication. The reared species were subjected to

various processing methods to establish the best method to be used by consumers to minimize the risks that could arise from consumption of these insects.

1.2 Statement of the problem

The rising world population combined with increasing economic growth and urbanization has led to rising demand for high-value protein. More food has to be produced to cater for the increasing population, which is negatively affecting the environment considering the dwindling resources, carbon emissions and public health risks (Kelemu *et al*., 2015).

The increasing human population is exerting more pressure on the earth's dwindling resources resulting to Climate change, diminishing fresh water resources, and overfishing, less productive agricultural lands as well as pollution from pesticides and fertilizers. A clear indication of how the human population is placing a disproportionate burden on the earth's ecosphere, thus something has to be done (Jansson and Berggren, 2015).

The continuous undernourishment manifest in most developing nations is the other problem triggering the search for alternative sources of food. For example, recent statistics show that 40 % of children below the age of 5 years in Lao Peoples Democratic Republic (PDR) are constantly malnourished or exhibit stunted development (Klunder *et al*., 2012).

1.3 Justification

Edible insects are a valued source of proteins across the world. It is estimated that approximately 2000 species of insects are used as food globally (Charlton *et al*., 2015). Insects can be compared to the conventional livestock meat based on nutritional content.

The crude protein content of insects ranges from 40 – 75 %, depending on the stage in the life cycle and the species (Belluco *et al*., 2013). Additionally, insects have high contents of zinc and iron as well as high levels of polyunsaturated fatty acids (Rumpold and Schlüter, 2013).

Rearing insects require less space and minimal feed to generate the same amount of nutrients produced by other larger animals. It also reduces the amount of water and land used to grow animal feed as well as the use of pesticides (Allotey and Mpuchane, 2003). In addition to this, their greenhouse gas emission is minimal compared to that of larger animals. Insects are rich in nutrients, and they can act as a sole source of major nutrients, especially in the developing world, which is frequently famine stricken (van Huis *et al*., 2013).

Both farmed and wild caught insects may harbor pathogenic microorganisms, including bacteria and fungi that can influence their safety as food. Potential pathogens known to be associated with edible insects should be assessed to reduce the risks for consumers by identifying the appropriate processing methods e.g. drying, boiling, frying etc. Specific processing steps with antimicrobial effects should be adopted to minimize the risks involved with the consumption of the edible insects (Makkar *et al*., 2014).

1.4 Hypotheses

i. Microbial load of reared edible insects is significantly different from that of wild edible insects.
ii. The diversity of microbial isolates of reared edible insects is significantly different from that of wild edible insects.

iii. There is a significant shift in microbial load of edible insects when raw and under different processing methods.

1.5 Objectives

1.5.1 General objective

To analyze microbial load and diversity in crickets (*Scapsipedus icipe* and *Gryllus bimaculatus)* used as a source of protein for food.

1.5.2 Specific objectives

i. To determine microbial load of reared and wild crickets.
ii. To isolate and characterize microorganisms in reared and wild edible insects
iii. To evaluate shifts in microbial load of edible crickets when raw and under different processing methods.

1.6 Significance of the study

The recognition of edible insects as an alternative source of protein is increasing. Nonetheless, strong insight into the microbial safety of edible insects and their products is lacking. Therefore, this study helped in characterizing the microbiota of fresh and processed crickets in a qualitative and quantitative way. Based on the research findings, information on the best processing methods was suggested and passed on to the consumers to reduce the risks associated with the consumption of crickets and their products. Papers for disseminating research findings through workshops and journals were published as well as a research thesis.

CHAPTER TWO

LITERATURE REVIEW

2.1 Depending on edible insects to improve livelihoods

In most developing nations, the problem of protein malnutrition, the increasing demand and the resulting high cost of usual proteins from plants, animals and aquatic sources have led to the need for alternative sources of proteins, which include edible insects (van Huis *et al*., 2013). Insects appear to be a promising solution to the problem of food insecurity and protein malnutrition. Nevertheless, as many other foodstuffs, attention should be focused on the microbiological hazards associated with consumption of edible insects (Makkar *et al*., 2014).

As noted by Klunder *et al.* (2012), insects are rich in nutrients and moisture, therefore providing a favourable environment for growth and survival of microbes. Some cases of botulism plus other food borne diseases connected to the consumption of poorly stored insects has been reported in some parts of Africa (Mujuru *et al.,* 2014). Furthermore, consumption of whole insects with the digestive system intact has been reported to considerably increase microbial risks (Amadi and Kiin-Kabari, 2016). Therefore, consumption of whole insects should be avoided and a sterilization, pasteurization or blanching step is recommended (Mlcek *et al.,* 2014).

Very few studies have focused on microbiological risks resulting from insect consumption (Megido *et al.,* 2017). The first study was carried out on a mopane worm (*Gonimbrasia belina*) which was subjected to 24 hours fasting period followed by 15-30 min blanching and 1-3 days of sundrying before consumption (Mujuru *et al.,* 2014).

Several species from 7 genera of bacteria and 5 genera of fungi were identified from the processed mopane caterpillars. Despite the reduction in bacterial load after a suplementary boiling step, recontamination of the insects occurred during the sun drying period due to contact with soil as well as from poor storage conditions.

Klunder *et al.* (2012) in their study on reared house crickets (*Acheta domesticus*), reared mealworms (*Tenebrio molitor*) and harvested large crickets (*Brachytrupes* sp.) showed that fresh insects have high microbial load composed mainly of *enterobacteriacea* and bacterial spores. The study demonstrated that 10 to 15 min blanching step at 90 °C considerably reduced the total aerobic count (TAC) while roasting decreases the bacterial spores (Klunder *et al.,* 2012). The studies emphasized on proper processing, packaging and storage conditions in reducing bacterial loads, particularly bacterial spores in insects meant for food.

2.2 Nutritional value of edible insects

Edible insects are known to have diverse nutritional value mainly because of their large number and variability of the species (Akullo *et al.,* 2018). Generalising the nutritional value of edible insects can be difficult as it varries with diet, developmental stage and the environment. Equally, the nutritional value keeps on changing based on the processing methods used before consumption i.e boiling, frying, drying (Gere *et al.,* 2017).

According to Kouřimská and Adámková, (2016) the nutrional score value of mealworm, crickets, and palm weevil larvae is considerably higher than in beef and chicken. Majority of the edible insects provide adequate energy and protein intake in human diet. Insects are rich in mono and polyunsaturated fatty acids as well as trace elemet such as

iron, copper phosporous, magnesium, selenium, manganese and zinc. They are also rich in vitamins like pantothenic acid, riboflavin and biotin (Akullo *et al.,* 2018).

Based on dry matter, insects protein content ranges between 7-91 % with most species containing approximately 60 % protein (Payne *et al.,* 2016). Rumpold and Schlüter, (2013) reported on 236 nutrient composition found in edible insects, clearly indicating that insects generally meet the requirements of WHO for amino acids especially species from the order orthoptera which includes grasshoppers, crickets and locusts. The protein content of orthoptera is considerably high in both adults and nymphs and it ranges from 23-65 % in dry matter (Rumpold and Schlüter, 2013).

Fats comprise the second largest portion of nutrients in edible insects. According to Nowak *et al.* (2016) the lipids content ranges from 13 % for orthoptera (crickets, grasshoppers and locust) to 33 % in coleoptera (grubs and beetles). Fats content in cricket species ranges from 13-34 % in dry matter. In general, the unsaturated fatty acids content of insects can be compared to those found in fish and chicken (van Huis, 2016). Insects contain little calcium because they are invertebrates and thus do not have a mineralised skeleton. Kouřimská and Adámková, (2016) shows that several species of insects including palm weevils, caterpillar, termites and crickets are rich in micronutrients such as iron and zinc. Majority of the insects have a high iron content ranging from 10-77 mg per 100 g dry matter, with members of orthoptera having iron content ranging from 8-20 mg per 100 g dry matter (Kouřimská and Adámková, 2016).

Edible insects also contain a variety of water soluble vitamins for example thiamine with content ranging from 0.1 to 4 mg per 100 g of dry matter (Akullo *et al.,* 2018). Riboflavin

is also present with its content ranging from 0.11-8.9 mg in 100 g. (Nowak *et al.,* 2016) Vitamin B12 is present in abundance especially in the yellow meal worm beetle *Tenebrio molitor* larvae (0.47 µg per 100 g) as well as in the house cricket *Achetus domesticus* where it ranges from 5.4 µg per 100 g in adults and 8.7 µg per 100 g in nymphs (Gere *et al.,* 2017).

2.3 Insect farming for food

Although insects have been used by man for different purposes for many years, rearing insects for direct human consumption began only recently (Rumpold and Schlüter, 2013). Farming of crickets for food is now being practiced in Vietnam, Cambodia, Thailand, USA, Netherlands and Africa (Megido *et al.,* 2017). In western countries, insect farming is mainly family-run with the main focus of feeding pets and zoos, with some farms producing small amounts for human consumption (Jansson and Berggren, 2015).

Most farmed species include; locusts (*Locusta migratoria*), wax moths *(Galleria mellonella),* housefly maggots (*Musca domestica),* crickets (*Gryllodes sigillatus, Gryllus bimaculatus, Acheta domesticus),* cockroaches (*Blaptica dubia*), mealworms (*Zophobas morio, Alphitobius diaperinus, Tenebrio molitor)* and subeetles (*Pachnoda marginata peregrina)* (Charlton *et al*., 2015).

For insects to become a profitable dietary constituent for humans, big quantities of insects will have to be produced and on a continuous basis. Both farming and processing will need to be automated as well as developing efficient and safe mass farming systems (Belluco *et al*., 2013). Mass rearing systems development is already underway in some countries for example, the Netherlands. To make mass production of insects attractive, it

is necessary to come up with rearing, harvesting and processing technologies as well as quality and safety monitoring to reduce production costs and guarantee food and feed safety (Mlcek *et al.*, 2014).

For an insect species to qualify as a good candidate for food and feed, it should have high egg production, short larval stage duration, high egg hatchability, high feed conversion rate, low production cost, low vulnerability to diseases, have high quality protein and should be able to live in high densities (Rumpold and Schlüter, 2013).

2.4 Major groups of edible insects

Insects are a major group of animals on earth. They constitute an enormous biodiversity and make up a huge amount of biomass. The actual number of insect species is not known, but it has been estimated to be about 3-4 hundred million species (Megido *et al*., 2017). Insects are found everywhere since they can colonise any type of habitat from aquatic to terrestrial environment where they play a vital role in the ecology. Because of their ubiquitous nature, insects have formed part of the human diet for a long time all over the universe (Makkar *et al*., 2014). Many different species of insects are consumed in different countries, as at now, there are more than 2000 registered species of edible insects eaten by people in the five continents (Jansson and Berggren, 2015).

The most commonly consumed insects include beetles (Coleoptera); locusts, crickets, grasshoppers (Orthoptera) and termites (Isoptera). Cicadas, true bugs, planthoppers, leafhoppers (Hemiptera); dragonflies (Odonata) and flies (Diptera) are also consumed in some parts of the world (EFSA, 2015). Crickets are a major group of edible insects consumed in different parts of the world. Species used for food include *Gryllus*

bimaculatus, *Teleogryllus occipitalis, Acheta domesticus, Brachytrupes portentosus* and *Tarbinskiellus portentosus* (van Huis, 2013). Despite the extensive practice of insect farming, only two species (*Acheta domesticus* and *Gryllus bimaculatus*) are reared economically. The house cricket (*Acheta domesticus*) is usually preferred by farmers over other species because of its soft body. Other species for example, *Tarbinskiellus portentosus* are harvested in the wild and are not preferred for farming because of their long life cycles (EFSA, 2015).

2.5 Insects consumption patterns

People of all ages and races eat insects. They are consumed by individuals of all social status, but mostly by those who reside in rural areas. Consumption of insects forms part of a people's cultural heritage where knowledge on how to locate, gather, cook, and conserve them is handed down through oral communication from one generation to the other (Amadi and Kiin-Kabari, 2016). Most insects are captured casually, but in some cases, they are lured. Insects are consumed in their various developmental stages including eggs, larvae, pupae and adults, but mostly they are eaten in the immature stages when soft and with reduced exoskeleton (Charlton *et al*., 2015).

Insects are taken as food in about 80 % of the world’s nations mainly from Africa Asia and larger parts of South America (van Huis, 2015). Termites, grasshoppers, crickets, bee and the larvae of the sago palm weevil form part of the diet of most Asian communities while the leafcutter ant (*Atta laevigata*), beetles (*Platycoelia lutescens*), larvae of the South American palm weevil (*Rhynchophorus palmarum*) and bearded weevil (*Rhinostomus barbirostris*) are consumed in parts of South America (Manditsera *et al.,*

2018). In Southern Africa, the large caterpillar moth *Gonimbrasia belina* commonly known as the mopane worm is a common protein source.

In Australia, the witchetty grub is a common diet of the indigenous people while Termites, grasshoppers and crickets are widely accepted in most parts of Eastern and central Africa. Eating of insects is not yet a widespread practice in the West. However, there is an emerging trend towards accepting insects as a source of protein (Deroy *et al.,* 2015). Crickets are consumed as whole insects in most parts of Africa and Asia but in the west, they are often integrated into food whereby they are dried and ground into a powder. The cricket powder is then incorporated in protein bars, cakes and pasta (Manditsera *et al.,* 2018).

2.6 Microbes associated with edible insects

Two types of microbes are considered as possible hazards in insects used for food and feed (van Huis *et al*., 2015). Those intrinsically related to insects as part of their life (gut microbes) and those introduced during rearing and processing (Belluco *et al.*, 2013). When processing insects for food, the gut content is usually not removed. Even if they are degutted, traces of gut microbiota will remain which should be viewed as contamination. Insects also carry microbiota on their tentacles and body surface, which can result in contamination (Mujuru *et al.,* 2014).

2.6.1 Bacterial hazards

The insect gut is inhabited by both commensal and opportunistic microbiota usually shaped by a combination of endogenous (host genetics and gut environment) and exogenous (habitat and diet) factors (Engel and Moran, 2013). In edible insects, the

microbiota can also be influenced by rearing conditions, handling and processing procedures as well as storage conditions applied to them along the value chain process (Mpuchane *et al.*, 2000).

Generally, the microbiota of insects comprises of various bacterial genera, including; *Bacillus, Staphylococcus, Proteus, Streptococcus, Micrococcus, Acinetobacter, Lactobacillus, Escherichia*, *Pseudomonas, Wolbachia* and *Bacteroidetes* (Braide and Nwaoguikpe, 2011). A study carried out by Hwee *et al.* (2018) on wild caught and farmed crickets indicated that the most dominant phyla associated with the gut of all the examined crickets were *Bacteroidetes, Firmicute* and *Proteobacteria.* However, the wild crickets had higher microbial diversity and greater *Firmicutes* to *Bacteroidetes* ratios in contrast with the farmed crickets.

Previous studies investigating microbial contamination of edible insects found *Klebsiella aerogenes* and *Escherichia coli* in freshly harvested *Rhynchophorus phoenicis* (palm grubs) in Nigeria. In addition, *Staphylococcus* sp. was isolated in heat-treated palm grabs in the same studies, which was thought to be as a result of improper handling and processing (Rumpold and Schlüter, 2013).

Several microorganisms have been isolated from the gut and the body surface of *Musca domestica* (domestic housefly), including the pathogenic *Staphylococcus aureus, Pseudomonas aeruginosa and Bacillus cereus* and non-pathogenic *Streptococcus faecalis* and *Bacillus subtilis* (Banjo *et al.,* 2005). Similar observations were reported on microbiota isolated from the body surface and the gut of African rhinoceros beetle (*Oryctes monocerus*) larvae in Nigeria. In this study pathogenic organism such as

Bacillus cereus, Pseudomonas aeruginosa and *Staphylococcus aureus* and non-pathogenic organisms such as *Bacillus firmis* and *Bacillus subtilis* were found (Amadi and Kiin-Kabari, 2016).

Klunder, Wolkers-Rooijackers, Korpela, and Nout (2012) investigating the microbial aspect of fresh, processed and stored house crickets (*Acheta domesticus*) and farmed mealworm larvae (*Tenebrio molitor*) found high levels of microbes, spore forming bacteria and enterobacteriacea typical of fresh food that has been harvested from the soil. Boiling the insects for 5-10 minutes, averted enzymatic processes for example black discolouration of the house crickets. Boiling also resulted in a decrease in the microbial load as well as elimination of enterobacteriacea in all the insects. Roasting for about 10 minutes did not eliminate all the enterobacteriacea. The two processing methods (boiling and roasting) did not completely eliminate the bacterial spores hence spore forming bacteria pose a possible risk in entomophagy. Therefore, to eliminate the spore forming bacteria there is a need for thorough decontamination techniques for example conventional sterilization at 110 – 150 °C, high pressure thermal sterilization followed by proper storage conditions (Rumpold and Schlüter, 2013).

A study carried out in Lao Peoples Democratic Republic on fresh-farmed crickets (*Achetus domesticus, Brachytrupes)* and mealworm larvae *(Tenebrio molitor)* demonstrated the presence of enterobacteriaceae and spore forming bacteria (Klunder *et al*., 2012). Furthermore, in preliminary studies carried out on fresh mealworms (*T. molitor*), locusts (*Locusta migratoria*) and morio worms (*Zophobus atratus*) in Belgium reported high counts of enterobacteriaceae, aerobic and anaerobic bacteria were found

(EFSA, 2015). The risk of transmitting these bacteria can be mitigated through efficient processing of the insects.

2.6.2 Fungal hazards

Generally, the major fungal genera associated with insects include *Aspergillus* sp., *Fusarium* sp., *Alternaria* sp., *Rhizopus* sp., *Penicillum* sp., *Mucor* sp., *Phycomycetes, Cladosporium* (Mpuchane *et al*., 2000) and four genera of entomopathogenic fungi including; *Beauveria bassiana, Metarhizium anisopliae, Purpureocillium lilacinum* and *Lecanicillium attenuatum* (EFSA, 2015). Some genera of fungi have been found to be in association with crickets including; *Trichoderma*, *Penicillium*, *Hyphopichia*, *Cladosporium, Fusarium*, and *Aspergillus*. Most of these fungi are usually found in air and soil, as well as on the surface of plants or even on the bodies of other dead insects where they are collected by the insects.

Entomopathogenic fungi produce toxins that are insect specific, resulting in high mortality in insects. However, immuno-compromised individuals can suffer from diseases associated with these fungi (EFSA, 2015). Insects may be carriers of fungi as well as yeasts that are hazardous to humans and animals. Fungal risks associated with consumption of insects can be reduced by ensuring proper hygiene in the entire production system (Mpuchane *et al*., 2000).

The quality of mopane caterpillar (*Imbrasia belina*) has been studied widely due to its regular consumption in most African countries. A study carried out in Botswana showed deterioration in quality (inner flesh disintegration and colour change due to mould growth and formation of cavities in the chitinous exoskeleton) of sun dried mopane caterpillar

commonly known as phane (Mujuru *et al.,* 2014). Fungal species isolated from the phane included *Aspergillus, Fusarium, Penicillium, Phycomycetes* and *Cladosporium.* Strains of *Fusarium*, *Penicillium* and *Aspergillus* are associated with the production of mycotoxins. The level of aflatoxins in the study varied from 0-50 µg per kg of product, thus within the maximum safe level set by FAO which is 20 µg per kg. Regular consumption of such foods over a long period would pose a health risk. The study recommended quick and even drying of the caterpillars after harvesting and storing them in a cool and dry place (Mujuru *et al.,* 2014).

2.7 Identification of microbial contaminants of insects

Food borne disease incidences have increased over the years resulting to major public health problems worldwide. Food borne pathogens are common in various foods and thus it is necessary to detect them in order to provide safe food and prevent food borne illnesses (Law *et al.,* 2015). The conventional methods; that is, culture-dependent methods used to detect food borne pathogens are laborious and time consuming. Hence, a number of methods that are labor-saving, time-efficient, sensitive and specific have been established for rapid detection of food borne pathogens.

Rapid detection methods are categorized into nucleic acid-based, immunological-based and biosensor-based methods (Alum *et al.,* 2016). The methods include simple polymerase chain reaction (PCR), multiplex PCR, real-time PCR, nucleic acid sequence-based amplification (NASBA), loop-mediated isothermal amplification (LAMP) and oligonucleotide DNA microarray. Others include enzyme-linked immunosorbent assay (ELISA) and lateral flow immunoassay. Rapid detection methods are critical in preventing and treatment of foodborne diseases (Ceuppens *et al.,* 2014).

2.7.1 Fungal identification

Molecular methods relying on DNA analysis have the potential to provide a new perspective on fungal community composition in edible insects. In addition, rapid molecular fingerprinting methods; for example, terminal restriction fragment length polymorphism (T-RFLP) analysis can be used to track dynamics in fungal communities over time compared to culture and microscopy reliant techniques (Alwakeel, 2016). The internal transcribed spacer (ITS) regions are DNA stretches between 18S, and 28S rRNA genes and are used as appropriate targets for molecular analysis of fungal communities. These ITS genes have high sequence variability compared to the bordering rRNA genes, making them valuable for genus and species level identification (Alwakeel, 2016).

2.7.2 Bacterial identification

Traditional identification of bacteria based on phenotypic characteristics in some cases is not as accurate as using the genotypic characteristics (Sujatha *et al.,* 2012). Comparison of the 16S rRNA bacterial gene sequence has been fronted as the preferred genetic technique (Wei *et al.,* 2013). The 16S rRNA gene sequence has been widely used to approximate relationships among bacteria. More recently, it has become important in identification of unknown bacteria up to the genus or species level. The 16S rRNA gene sequence is the most commonly used gene marker because it is present in almost all the bacteria, the gene has not changed over time and it is large enough for bioinformatics purposes (Gusmo *et al.,* 2007). The analysis based on rRNA is not only used to investigate microbial diversity, but it is also used to identify new strains.

2.8 Accepted microbial levels in raw and processed meat

The microbial level in ready to eat meat depends on the type and the duration of processing as well as the handling and the storage procedures. The accepted microbial levels in raw, processed and packaged meat should be below 10^5 colony-forming units per 1 gram of the meat (Koutsoumanis and Angelidis, 2007). If the level goes beyond 10^7 cfu/gram then the meat is not fit for human consumption (Centre for Food Safety, 2014).

Presence of hygiene indicator organisms in cooked meat is an indication of inadequate cooking or post processing contamination. If the indicator organisms surpass 10^2 cfu/g the meat should not be consumed (Bradeeba and Sivakumaar, 2013). Some microorganisms for example *Shigella, E. coli* and *Salmonella* should not be detected in 25 g of meat such that if they are detected then the meat is not safe for consumption (Mead, 2004). The level of aflatoxins should be within the maximum safe level set by FAO, which is 20 µg per kg of meat (Mujuru *et al.,* 2014).

2.9 Processing procedures

Processing procedures greatly influence survival and growth of microbes, thus proper processing procedures should be applied to have safe high quality products. Generally, insects are processed using a sequence of steps that will include: -

2.9.1 Freezing

Freezing insects at -20 °C stops the microbial growth but does not kill the organisms (Jansson and Berggren, 2015). The insects should be heated immediately after thawing to prevent the microbes from swinging back to action.

2.9.2 Heating

Heating the insects will kill all the microbes (depending on the temperature). Most microbes will die at a temperature of 100 ℃. However, some bacterial spores will survive this and need temperatures around 130 ℃ to kill them (Mujuru *et al*., 2014).

2.9.3 Drying

Generally, insects have moisture content levels in the range of 55-65 %, which is too high to guarantee an extended shelf life. Therefore, a drying process that will reduce the moisture content to less than 10 % is required to stop microbial growth (Mpuchane *et al*., 2000).

2.10 Storage of edible insects

In some places, insects are a staple food. The peasants collect them during periods of abundance, preserve and store them for consumption at a later date. For example, in Africa caterpillars such as *Anaphe* spp. *Gonimbrasia bellina*, *Bunaea* spp. *Lobobunaea* spp. as well as some termites and grasshoppers of *Schistocerca* spp., *Locusta* spp., *Nomadacris* spp. and *Zonocerus* spp are collected during the abundance period, preserved and stored for later use (Amadi and Kiin-Kabari, 2016). Preservation of the insects is usually done by drying the insects in the sun, on hot ashes, or even in an earth oven. For boiled insects, preservation is usually done using salt (Mujuru *et al.,* 2014).

Studies carried out by Klunder *et al*. (2012) on Mealworm larvae (*Tenebrio molitor*), small cricket (*Acheta domesticus*) and large crickets (*Brachytrupus* sp.) showed quality deterioration of fresh insects stored in the refrigerator (4 ℃) and at room temperature (28-30 ℃). The studies recommended the application of a heating step, e.g. boiling

before storage. Bacteria levels on boiled *Acheta domesticus* remained stable for more than two weeks of storage in the refrigerator. In the same studies, drying as well as acidifying the insects with vinegar to a pH of 4.5 were considered as the best methods of processing if the insects are to be stored at room temperature without the use of a refrigerator (Klunder *et al.,* 2012).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study sites

Wild-caught populations of *G. bimaculatus* and *S. icipe* were obtained through light-pit fall traps placed in Mbita (0°25'49.2"S 34°12'24.8"E), Homabay County and Nguruman (36°100.31"S 1°84'75.56"E), Kajiado County, Kenya (Fig. 3.1). The study was part of a bigger project that targeted edible crickets from these two regions.

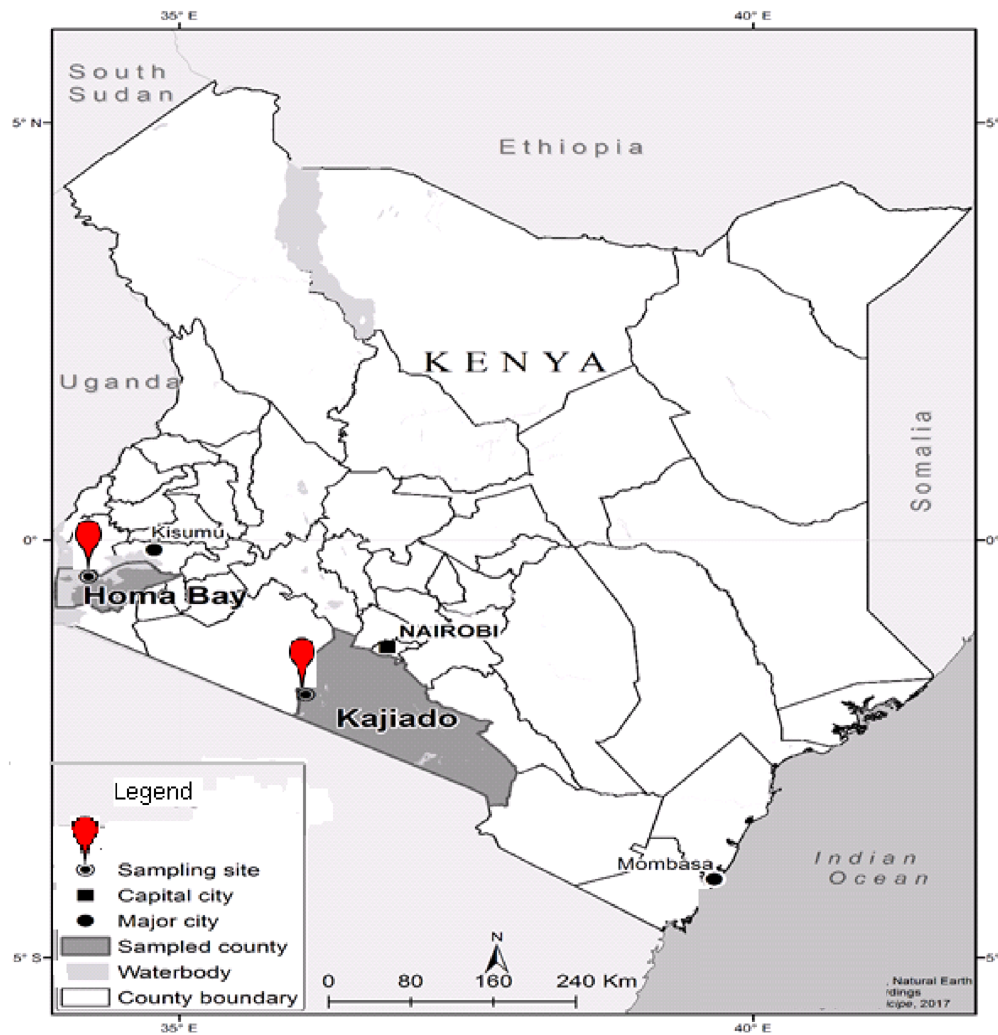

Figure 3.1: A map of Kenya showing Homabay and Kajiado Counties where wild crickets were collected (Geo-Information unit, icipe, 2017).

3.2 Traps

The pitfall trap used were similar to those described by Barbara (2005) with slight modifications. The traps were installed directly into the soil and the lid of each container was screened with a wire mesh to prevent the capture of small mammals and amphibians (Plate. 3.1). The top of each trap was covered at all times to prevent excessive exposure to sunlight and precipitation. The daily servicing of the traps included removal of catches, cleaning of the devices and careful leveling of the immediate surroundings of the trap-rims.

Plate 3.1: A simple cricket trap made of a tin and a raised cover to keep away predators.

3.3 Sample rearing

The *G. bimaculatus* and *S. icipe* for analyses were reared at Animal Rearing and Containment Unit at the International Centre of Insect Physiology and Ecology (*icipe*). Eggs were collected from the stock colony using oviposition devices (70 % moist cotton balls), which were replaced after every 2 days. Each cotton ball containing the eggs was

thinly spread-out and placed in a 2-litre transparent rectangular plastic container (21 x 14 x 15 cm) (Kenpoly Manufacturer Ltd., Nairobi, Kenya) and incubated in controlled environmental chamber maintained at a temperature of 28 ± 2 °C, relative humidity of 65 ± 5 % and photoperiod of 12L: 12D until hatched. The pinheads were transferred into transparent Perspex cages (60 x 60 x 60 cm).

The sides and top surface of the cages were designed with rectangular opening (14 cm length x 20 cm width), screened with wire mesh to provide aeration. The cages were provided with twelve cardboard egg trays to serve as refuge sites for the crickets (Plate 3.2). The newly hatched nymphs were fed on millet flour or finger millet flour or soybean flour or sorghum flour or wheat bran or sweet potato leaves or kales or a combination of 3 – 4 of the products *ad libitum*. Water was provided in shallow bowls with pumice granules to prevent drowning, which was always replaced whenever necessary (that is; every 2-days). The rearing cultures were monitored daily to record and remove dead insects. The stock colony was refreshed every six months with wild-caught crickets to maintain the genetic vigour of the colonies and prevent inbreeding depression.

Crickets undergo an incomplete metamorphosis with three distinct stages namely egg, nymph and adult. The nymph resembles the adult only that they do not have fully developed wings. Egg to adult development takes about three months. Eggs take three weeks to hatch and in about one month, the crickets are large enough to use (Plate 3.3). Because they are cold-blooded, their development rate can be sped up or even slowed down to some extent by adjusting the rearing temperatures.

Plate 3.2**:** Cages used for rearing the crickets. A: clear cage where rearing was done and B: egg cartons acting as hiding places for the crickets.

Plate 3.3: Reared crickets; A: male *S. icipe*, B: female *S. icipe*, C: male *G. bimaculatus* and D: female *G. bimaculatus.*

3.4 Sample preparation

The crickets were divided into 2 batches; the first batch was analyzed as fresh raw edible insects and the second batch was processed by either boiling for 5 min at 94 °C, sun drying for 7 days, deep-frying until brown and crunchy, snap freezing (using liquid nitrogen) and finally deep-freezing at -20 °C for 7 days. One gram of the raw and processed crickets was ground with a sterile mortar and pistol and homogenized

individually in 9 ml phosphate-buffered saline (PBS) in a test tube to form the stock solution. One ml of the stock solution was transferred using a sterile pipette to another 9 ml PBS in the test tube; this dilution was done until the sixth test tube when an appropriate concentration of cells was obtained. The sixth dilution was used for mycological and bacterial isolation and analysis. The bacterial and fungal counts were expressed as colony forming units per gram (CFU/g).

3.5 Isolation of bacteria and fungi

Fungal isolation was done by aseptically inoculating 1 ml of the homogenate from the 6th dilution onto freshly prepared Sabouraud Dextrose Agar (SDA) and Potato Dextrose Agar (PDA) plates supplemented with 0.05 g/L chloramphenicol to inhibit growth of bacteria (Zarrin *et al*., 2007). The plates were incubated at 25 ± 2 °C for up to three weeks and checked every day for any fungal growth (Mar *et al*., 2012). The experiments were done in triplicates for each medium.

Bacterial isolation was carried out by inoculating 1 ml homogenate from the 6th dilution onto freshly prepared Nutrient Agar and MacConkey Agar and incubating the plates at 37 °C for 2 to 3 days (Omoya and Akinyosoye, 2013). In both cases, the inoculums were spread evenly over the surface of the medium using an L-shaped glass rod sterile spreader. The experiments were done in triplicates for each medium.

3.6 Determination of microbial load of crickets

On establishment of growth, the number of bacteria colonies formed from each plate was counted using the Gallenkamp electronic colony counter. The fungal counts were obtained using the dissecting microscope. The mean from the triplicate plates was

computed, and then multiplied by the dilution factor to get the total viable cells per unit weight of the sample, expressed as colony forming units per gram (CFU/g). Sub-culturing was made from plates with mixed colonies where single colonies were picked using a sterile loop and inoculated onto fresh plates. This was done repeatedly until pure colonies were obtained.

3.7 Morphological characterization

Preliminary identification of the bacteria species was done by describing the morphology of a single colony based on colony characteristic; that is; shape, size, texture, form, color edge and degree of opacity (van Teeseling *et al.,* 2017). Colonies that appeared different especially when grown on the same medium were assumed to belong to different bacterial species. However, since numerous species have the same colony morphology, the contrary (that similar colonies belong to the same species) is not always true.

Fungal cultures were identified using macro and micro-morphology and surface coulouration of the colonies. Microscopic observations were done by making microscopic mounts in distilled water from both PDA and SDA colonies. Species characteristics; that is, the colour of the colony, conidial heads structure and conidia shapes were observed (Kim *et al.,* 2013).

3.8 Harvesting of cells for molecular characterization

The pure bacterial isolates were aseptically inoculated into 7 ml luria broth (LB), in sterile 50 ml falcon tubes and placed into an Innova 44 (Eppendorf) shaker set at 180 rpm and 37 °C for 18 hrs. The cells were harvested by centrifugation at 2000 rpm for 3 minutes in 1.5 ml Eppendorf tubes, four times and discarding the supernatant.

The fungal colonies were harvested by scrapping the mycelial network or spores into 1.5 ml Eppendorf tubes each containing 3 sterile beads into which 400-µl lysis buffer (Plant DNA extraction kit, Bioline (UK) Ltd) then vortexed to break the cells in readiness for genomic DNA extraction.

3.9 Molecular characterization

3.9.1 Bacterial DNA extraction

The bacterial DNA was extracted using the Isolate II Genomic DNA Kit (Bioline (UK) Ltd) following the manufacturer's protocol. Extracted DNA was quantified using Nanodrop 2000/2000c spectrophotometer (Thermo Scientific) and samples stored at -20 °C (Yu and Morrison, 2004).

3.9.2 Fungal DNA extraction

Fungal DNA extraction was carried out using the Isolate II Plant DNA extraction kit (Bioline (UK) Ltd) following the manufacturer's instructions. The resultant extracted DNA quality and quantity checks were done using the Nanodrop 2000/2000c spectrophotometer (Thermo Scientific) and samples stored at -20 °C (Mahuku, 2004).

3.9.3 Polymerase Chain Reaction (PCR)

PCR amplification for bacteria was carried out using universal primers that target bacterial small sub unit (SSU) rRNA genes from the V1 to the V2 hyper variable region (Rinttila *et al*., 2004). These were 27F (5-' AGAGTTTGATCMTGGCTCAG3') and 1492R (5-' TACCTTGTTACGACTT3'). All PCR reactions were carried out in a final 20 µL volume containing 5x MyTaq reaction buffer (Bioline, UK) (5mM dNTPs, 15 mM $MgCl_2$, stabilizers and enhancers), 0.5 pm`ol μl^{-1} of each primer, 0.25 mM $MgCl_2$, 0.0625

U μl^{-1} MyTaq DNA polymerase (Bioline, UK) and 15 ng μl^{-1} of DNA template. PCR reactions were set up in a Mastercycler Nexus thermal cycler (Eppendorf, Germany) and the cycling conditions were initial denaturation for 2 min at 95 °C, then 35 cycles of denaturation for 30 s at 95 °C, annealing for 40 s at 52 °C and extension for 1 min at 72°C, followed by a final elongation step of 10 min at 72 °C. The expected product size was approximately 1500 bp (Bakke *et al.,* 2011).

PCR amplification of the fungal ITS regions was carried out using the universal fungal ITS primers; ITS5, (5'- TCCTCCGCTTATTGATATGC -3') and ITS4 (5'-GGAAGTAAAAGTCGTAACAAGG-3') (White *et al*., 1990). The PCR reactions set up was as described above with the exception of the annealing temperature at 54 °C for 1 min. The expected product size ranged between 450 – 600 bp (Martin and Rygiewicz, 2005).

3.9.4 Gel electrophoresis

This was done in order to confirm whether the target regions were successfully amplified before sequencing was done. The amplified PCR products were resolved through a 1.2 % agarose gel stained with ethidium bromide (10 mg/mL) and subjected to electrophoresis at 100 volts for 1 hour. DNA bands on the gel were visualized and documented under ultraviolet (U.V.) trans-illuminator using the KETA GL imaging system trans-illuminator (Wealtec Corp, Meadowvale Way Sparks, Nevada, USA) (Johansson, 2009).

3.9.5 Purification of PCR products

Successfully amplified products were excised and purified using Isolate II PCR and Gel Kit (Bioline, London, UK) as per the manufacturer's instructions (Werle *et al.,* 1994).

3.9.6 Sequencing

The amplicons were first taken through sequence PCR. The sequence PCR was carried out in a 25 µl reaction mixture that contained; 3 µl template DNA, 1.5 µl 5X buffer, 1.5 µl forward primer, 1.5 µl reverse primer, 2 µl Big Dye and 10.5 µl distilled water. The PCR amplification was run for 25 cycles under the following conditions; initial denaturation at 96 °C for 5 minutes, denaturation at 96 °C for 10 seconds, annealing at 62 °C for 45 seconds and extenstion at 60 °C for 4 minutes. The PCR products were then purified using Qiaquick reagents (Qiagen) following the manufacturer's instructions. The PCR products were then cycle sequenced using Big Dye V3.1 reagents following the manufacturers protocol (Bayley, 2006). The sequencing products were then purified using the CleanSeq Sequence Purification System and automated sequencing done by capillary electrophoresis on an ABI3700. The sequences were finally aligned and then examined by use of visual inspection of the electropherogram by using the Sequencher soft.

3.10 Data Analysis

Percentage occurrence data of different fungi/bacteria species between reared and wild insects were compared using the Chi-square test. Because the cfu/g data were not normally distributed, the non-parametric Kruskal–Wallis multiple-range test with Bonferroni p-values adjustment using agricolae package (De Mendiburu, 2015) in R 3.1.3 was used to compare cfu/g among processing methods at $\alpha < 0.05$ significance level.

Bacterial 16S rRNA gene-based sequence data, that is, the forward and reverse sequences were cleaned and edited using Bio Edit Sequence Alignment Editor Version 7.2.5

software (Hall, 1999), and multiple alignment done in Clustal X (version 2.1) (Thompson *et al*., 1997). Blasting of the bacterial and fungal isolate sequences was done using NCBI BLAST site (https://blast.ncbi.nlm.nih .gov/Blast.cgi). The reference strains having the highest max score, total score, query cover, and identity were considered as the closest matches and were selected alongside their accession numbers.

Fasta formats of the bacterial and fungal isolate sequences and the reference strain sequences were aligned using MEGA7 software using the Clustal W option. The phylogenetic trees were constructed using MEGA7 software with the following algorithms: Bootstrap method for test of phylogeny with 1,000 bootstrap replications: Nucleotide substitution type; UPGMA; Homogeneous pattern among lineages; Complete deletion of gaps and a Neighbour-joining statistical method. The phylogenies were inferred by comparing the bacterial and fungal sequences to the main lines of descent within the bacterial and fungal phyla of more than 98 % threshold to assign sequences to the same operational taxonomic units (OTUs). The bacterial and fungal isolates were categorized into either species or genus based on the 98 % threshold match (Smith *et al.,* 2009).

CHAPTER FOUR

RESULTS

4.1 Morphological characteristics of fungal Isolates

From the study, 26 pure fungal isolates were obtained from *S. icipe* and *Gryllus bimaculatus* both reared and wild. Most of the isolates from the reared cricket species had dark greenish mass with no clear aerial mycelia, characteristics suggestive of members of *Trichoderma* spp. (Plate 4.1). Few isolates had a yellow luxuriant growth with dust like conidia, which suggested that they could belong to members of *Aspergillus niger* (Plate 4.1). Only one isolate had white colonies, which appeared like dots even after being incubated for a long time, a characteristic suggestive of members of *Beauveria* spp. (Plate 4.1). The majority of the isolates from the wild species (*G. bimaculatus* and *S. icipe*) had dark brownish colonies, with mycelia that had visible tiny spores on SDA suggesting that they belonged to *Aspergillus s*pp. (Plate 4.2). Few isolates had light greenish rough surfaced colonies with spores, which were not clearly visible (Table 4.1) suggesting that they belonged to *Penicillium* spp (Plate 4.2).

Table 4.1: Description of fungal isolates from *S. icipe* and *G. bimaculatus* both reared and wild

Isolate	morphological characteristics	Suspected organism
FGRF1	Dark greenish mass with no aerial mycelia visible, conidiophores are septated and loosely packed.	*Trichoderma* spp.
FGWF2	Light greenish rough surfaced mass, with spores that are not clearly visible.	*Penicillium* spp.
FGWF2	Dark brownish colonies, the mycelia have visible, tiny, colourless, rough spores. The spores are colourless and rough.	*Aspergillus* spp.
FSWF1	Colonies are white like dots; the colonies remain white even after long incubation. Spores appear white and round like balls.	*Beauveria* spp.
FSRPB2	Yellow luxuriant growth which later bears black dust-like conidia. The spores appear long and globose at the tip.	*Aspergillus niger*

Key: FGRF: Fungi *Gryllus* Reared Fresh, FGWF: Fungi *Gryllus* Wild Fresh, FSWF: Fungi *Scapsipedus* Wild Fresh, FSRPB: Fungi *Scapsipedus* Reared Processed Boiled.

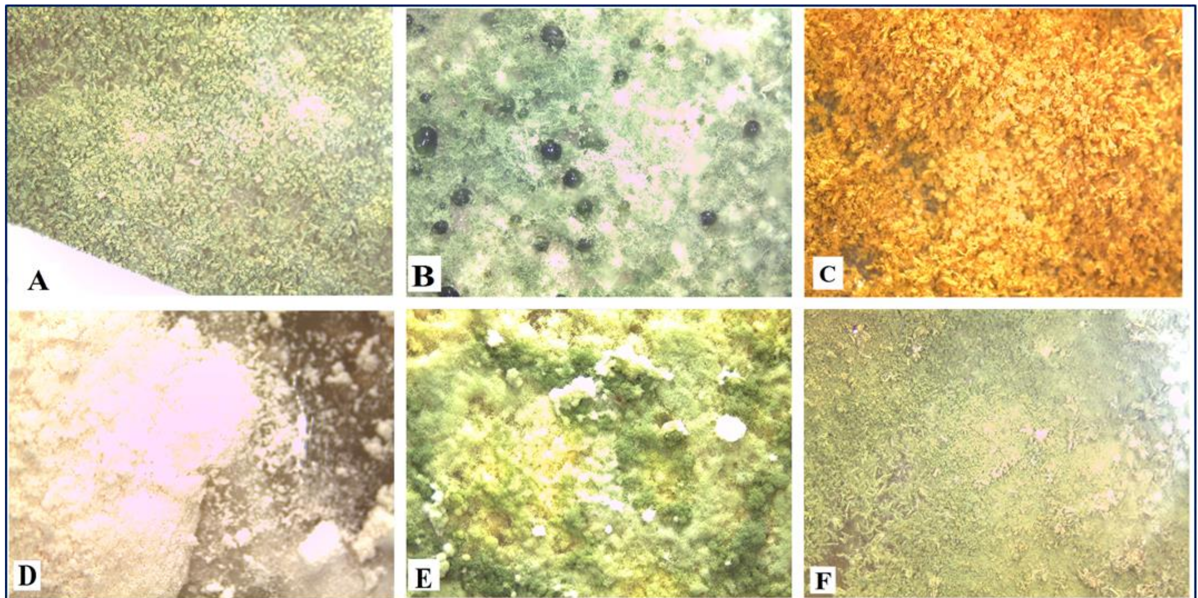

Plate 4.1: Fungal isolates from the wild and the reared *S. icipe* and *G. bimaculatus* grown on Potato Dextrose Agar showing A and F: Dark greenish colonies, B and E: Light greenish rough surfaced colonies, C: Yellow luxuriant growth, D: white colonies that appear like dots.

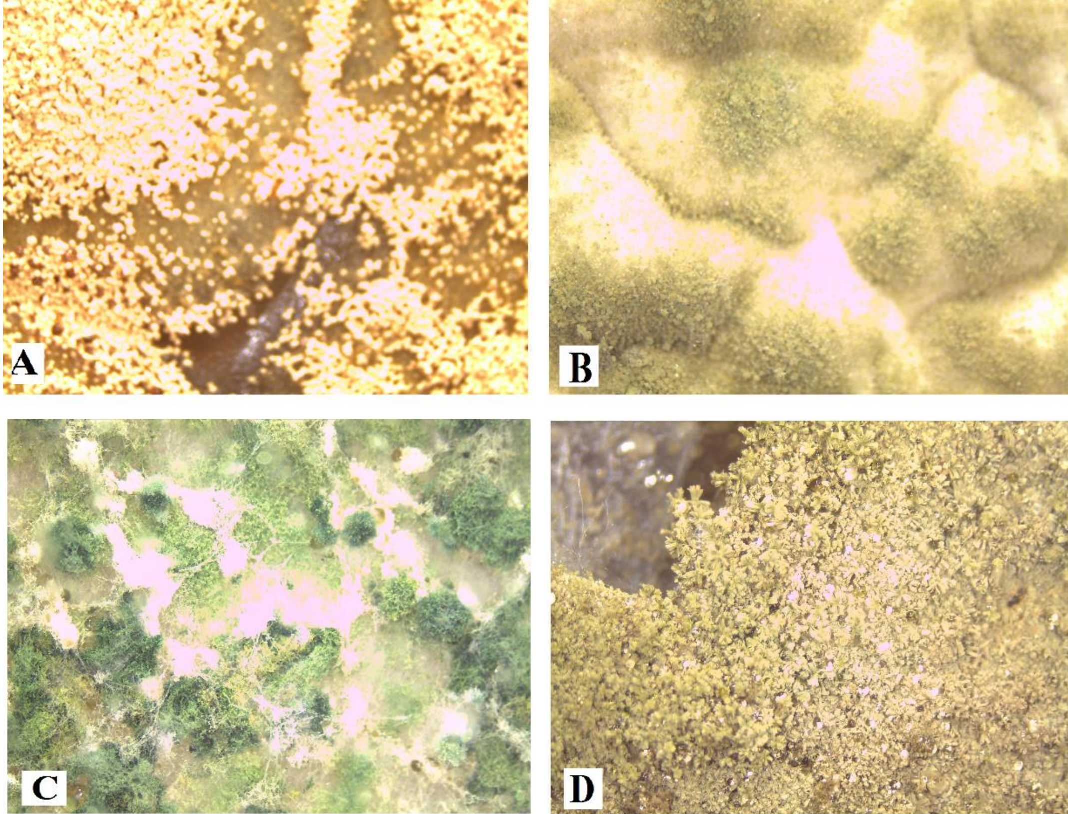

Plate 4.2: Fungal Isolates from wild and reared *S. icipe* and *G. bimaculatus* grown on Sabouraud Dextrose Agar (SDA). A and D: Dark brownish colonies, B: Dark greenish colonies, C: Light greenish rough surfaced colonies.

4.2 Morphological characteristics of bacterial isolates

A total of 40 pure bacterial isolates were obtained from *G. bimaculatus* and *S. icipe* both wild and reared. On MacConkey agar 90 % of the colonies were pink in colour, had smooth margins with a raised elevation and convex in shape characteristics typical to *Escherichia coli* (Plate 4.3). About 5 % of the colonies had dark red, rough margined colonies with a firm gummy typical of members of *Enterobactor* spp. The remaining colonies were light pink in colour, raised in elevation, smooth margined and had a soft texture typical of members of *Klebsiella* spp. (plate 4.3).

Most of the isolates (70 %) on nutrient agar had clear, convex shaped colonies with smooth margins (Plate 4.4). The colonies were also translucent, circular in shape and raised in elevation (Table 4.2) typical of members of *Lactococcus* spp. About 20 % of the colonies were milky white in appearance, opaque with rough margins suggesting they belong to *Bacillus* spp. (Plate 4.4). The remaining 10 % of the colonies were creamy white in colour, opaque, raised in elevation with smooth margins, which is typical of members of *Staphylococcus* spp. (Plate 4.4).

Table 4.2: Colony morphological characteristics of bacterial isolates on nutrient agar (NA) and MacConkey agar (MCA).

Isolate Characteristic	**I**	**II**	**III**	**IV**	**V**	**VI**
Medium used	NA	NA	NA	MC	MC	MC
Margin	Sc	R	S	Sc	R	Sc
Colour	Cl	Mw	Cw	P	Dr	P
Elevation	Rs	Rs	Cvx	Rs	Dmd	Cvx
Transparency	T	O	O	O	O	O
Colony shape	C	C	C	C	C	C
Texture	St	St	Ft	St	Ft	St
Suspected micro-organism	*Lactococcus* spp.	*Bacillus* spp.	*Staphylococcus* spp.	*E. coli*	*Enterobactor* spp.	*Klebsiella* spp.

Key: NA, Nutrient agar; MC, MacConkey agar; S, smooth; Sc, smooth clear; R, rough; Cw, creamy white; Mw, milky white; Ww, watery white; W, white; Cl, Clear; Rs, raised; Cvx, convex; Dmd, domed; O, opaque; T, translucent; C, circular; St, soft texture; Ft, firm texture.

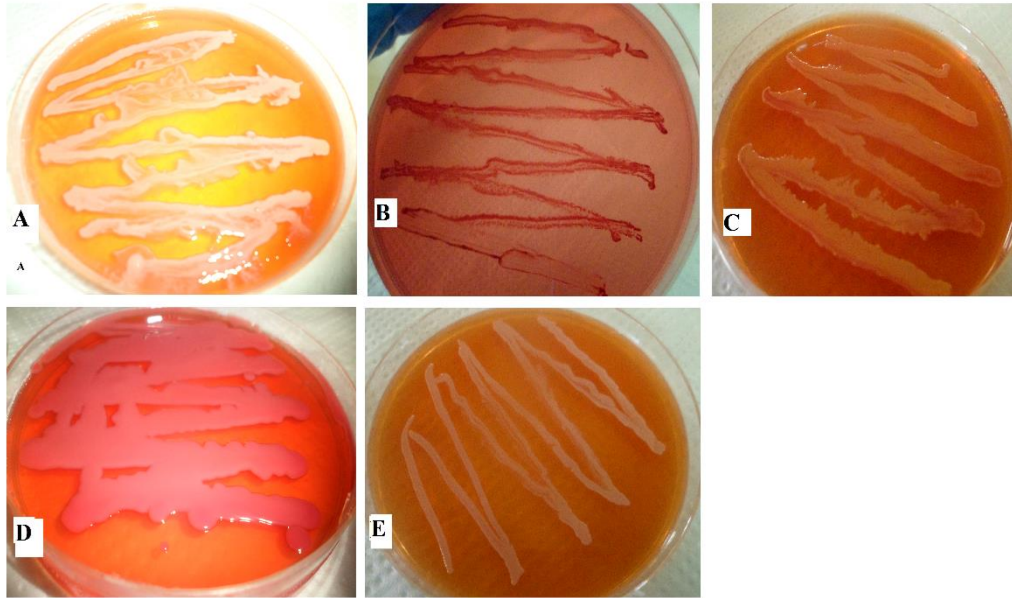

Plate 4.3: Pure isolates of bacteria from wild and reared *S. icipe* and *G. bimaculatu*s grown on MacConkey Agar (MCA). A and D represents isolates from the reared *S. icipe* while B, C and E are isolates from the wild *G. bimaculatus*.

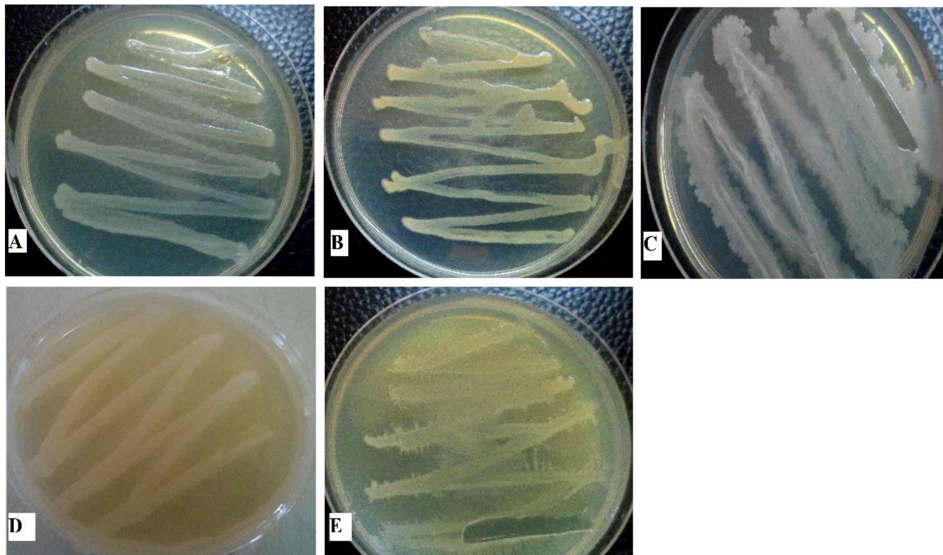

Plate 4.4: Pure bacterial isolates from both wild and reared *G. bimaculatus* and *S. icipe* grown on Nutrient Agar (NA). A is from reared fresh *G. bimaculatus*, B and C are isolates from sun-dried samples of S. *icipe* while D and E are from fresh wild species of *S. icipe*.

4.3 Relative abundance of fungal and bacterial species found in association with both wild and reared crickets

4.3.1 Relative abundance of fungi associated with *S. icipe*

The fungal isolates recovered from wild species mainly belonged to *Aspergillus* spp, *Cyberlindnera jadinii* and *Tetrapisispora fleetii* among others. *Aspergillus* spp. (86.7 % of the fungal isolates in *S. icipe*) was the most predominant in the wild species, followed by *Cyberlindnera jadnii* (40.0 %), *Tetrapisispora fleetii* (33.3 %) and *Beauveria bassiana* (6.7 %) (Table 4.3). The reared *S. icipe* was dominated by *Trichoderma asperellum* (46.7 %), and *Tetrapisispora fleetii (*40.0 %). There was no significance difference in prevalence of fungi isolated from the wild and the reared species (Table 4.3).

Table 4.3: Relative abundance of fungal isolates found in association with reared and wild *S. icipe.*

Wild/ Reared	*B. bassiana*	*C. jadnii*	*T. fleetii*	*Aspergillus spp.*	*T. asperellum*	*A. niger*	others	Total
Reared (n=15)	0 a	0 b	40.0 a	0 b	46.7 a	40.0 a	0 b	48.3a
Wild (n=15)	6.7 a	40.0 a	33.3 a	86.7 a	0 b	0 b	33.3 a	51.7a
$\chi 2$	1	6	0.091	13	7	6	5	0.034
P value	0.317	0.014	0.763	0.0003	0.008	0.014	0.025	0.85

Values are means of triplicate samples; means within a row followed by different letters show significant difference, means followed with the same letter show no significance difference (P<0.05). (Key: *B*: *Beauveria, C: Cyberlindnera*, *T: Tetrapisispora*, *T: Trichoderma*, *A*: *Aspergillus*)

4.3.2 Relative abundance of fungi associated with *G. bimaculatus*

Aspergillus spp. (80.0 % of the fungal isolates in *G. bimaculatus*) was the most abundant fungal isolate from the wild *Gryllus bimaculatus,* followed by *Penicillium roseopurpureum* (46.7 %) and *Hyphopichia burtonii* (40.0 %) (Table 4.4). The reared species was dominated by *Trichoderma asperellum* (73.3 %) followed by *Aspergillus flavus* (53.3 %). There was no significance difference between the number of fungi isolated from both the wild and the reared species (Table 4.4).

Table 4.4: Relative abundance of fungi found in association with reared and wild *G. bimaculatus.*

Wild/Reared	*Aspergillu* sp.	*H. burtonii*	*P. roseopurpureum*	*T. asperellum*	*A. flavus*	Total
Reared (n=15)	0 b	0 b	0 b	73.3 a	53.3 a	44.4a
Wild (n=15)	80.0 a	40.0 a	46.7 a	0 b	0 b	55.6a
$\chi 2$	12	6	7	11	8	0.33
P value	0.001	0.014	0.008	0.0009	0.005	0.56

Values are means of triplicate samples; means within a row followed by different letters show significant difference, means followed with the same letter show no significance difference ($P<0.05$). (*H: Hyphopichia, P: Penicillium,T: Trichoderma, A: Aspergillus*).

4.3.3 Relative abundance of bacteria in association with *Scapsipedus icipe*

In addition to fungi, bacteria were also isolated from the wild and reared *S. icipe* in high numbers. *Bacillus nealsonii, Chryseobacterium gleum, Lactococcus garvieae* and *Erwinia* sp. were among the bacteria isolated from the wild species while, *Enterobacter* sp, *E. coli* and *Lactococcus garvieae* were from the reared species (Table 4.5). From the wild species, the most abundant bacteria was *Bacillus nealsonii* (66.7 % of the total

number of bacterial isolates in *S. icipe*) followed by *Lactococcus garvieae* (53.3 %) and *Chryseobacterium gleum* (40 %) respectively. *Erwinia* sp. (33.3 %) was the least isolated (Table 4.5). The reared species was predominated by *Escherichia coli* (73.3 %) followed by *Enterobacter* sp. (53.3 %) and *Lactococcus garvieae* (33.3 %). There was no significance difference between the number of bacteria isolated from the wild and the reared cricket species.

Table 4.5: Relative abundance of bacterial isolates associated with reared and wild *S. icipe.*

Wild /Reared	*B. nealsonii*	*L. garvieae*	*Erwinia sp*	*C. gleum*	*Enterob acter sp.*	*E.coli*	Others	Total
Reared (n=15)	0 b	33.3 b	0 b	0 b	53.3 a	73.3 a	0 b	51.7a
Wild (n=15)	66.7 a	53.3 a	33.3 a	40 a	0 b	0 b	33.3 a	48.3a
$\chi 2$	10	0.69	5	6	8	11	5	0.034
P value	0.002	0.405	0.025	0.014	0.005	0.0009	0.025	0.85

Values are means of triplicate samples; means within a row followed by different letters show significant difference, means followed with the same letter show no significance difference (P<0.05). (Key: *B: Bacillus, L: Lactococcus, C: Chryseobacterium, E: Escherichia).*

4.3.4 Relative abundance of bacteria in association with *G. bimaculatus*

The wild *G. bimaculatus* had a wide diversity of bacteria, including; *Streptococcus* sp, *Lactococcus garvieae, Staphylococcus sciuri*, *Lactobacillus brevis* and *Klebsiella oxytoca,* while the reared species had low diversity with only *Aeromonas media* and *Rickettsiella grylli* recovered (Table 4.6). *S. sciuri and L. garvieae* (53.3 %) were the most predominant bacteria in the wild species, followed by *Streptococcus sp* (46.7 %) and *L. brevis* (40.0 %). *K. oxytoca* was less frequently isolated (26.7 %). In the reared

species, *A. media* (66.7 %) was the most frequently isolated bacteria followed closely by *R. grylli (*53.3 %) (Table 4.6). There was no significance difference in percentage occurrence of bacteria between the reared species and the wild species.

Table 4.6: Relative abundance of bacterial isolates associated with reared and wild *G. bimaculatus*

Wild/Reared	*Streptococcus* sp	*L. brevis*	*L. garvieae*	*S. sciuri*	*K. oxytoca*	*A. media*	*R. grylli*	Others	Total
Reared (n=15)	0 b	0 b	0 b	0 b	0 b	66.7 a	53.3 a	26.7 a	46.4a
Wild (n=15)	46.7 a	40.0 a	53.3 a	53.3 a	26.7 a	0 b	0 b	0 b	53.6a
$\chi2$	7	6	8	8	4	10	8	4	0.14
P value	0.008	0.014	0.005	0.005	0.046	0.002	0.005	0.046	0.71

Values are means of triplicate samples; means within a row followed by different letters show significant difference, means followed with the same letter show no significance difference (P<0.05). *(*Key: *L: Lactobacillus, L: Lactococcus, S: Staphylococcus, K: Klebsiella, A: Aeromonas, R: Rickettsiella).*

4.4 Microbial loads in reared fresh and processed crickets

Culture plates from the non-processed insects had an overgrowth of microbial colonies that greatly reduced after a processing step, which included boiling and sun drying (Plate 4.5). Freeze-dried, snap dried and deep fried samples gave clear plates meaning that neither bacteria nor fungi were cultured from them (Plate 4.5).

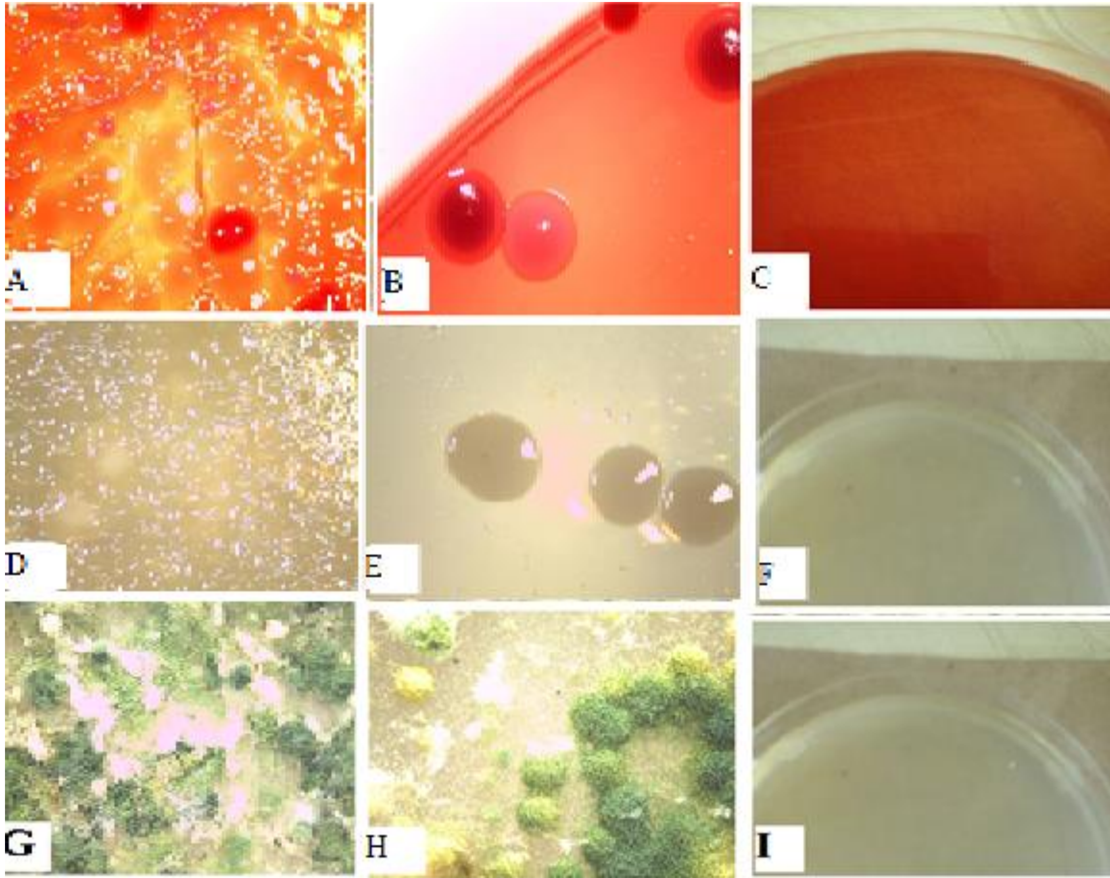

Plate 4.5: A and D show overgrowth of bacteria, while G shows an overgrowth of fungi cultured from non-processed *S. icipe*. B and E show significant reduction in number of bacteria while H shows a decrease in the number of fungi in processed *S. icipe.* C, F and I show clear plates from samples processed through deep-frying, snap drying and deep freezing.

4.4.1 Microbial loads in reared fresh and processed *Scapsipedus icipe*

Table 4.7 shows the microbial levels in reared *S. icipe* whereby, the fresh samples recorded highest bacterial and fungal populations compared to the samples processed through various ways. The bacteria levels in the fresh samples ranged from 2.1 ± 0.7 colony forming units (CFU) g^{-1} fresh weight to 4.31 ± 0.7 $CFUg^{-1}$ fresh weight. The sun dried samples recorded the second highest bacterial populations ranging from 2.07 ± 0.45 to 2.62 ± 0.12 $CFUg^{-1}$ dry weight (Table 4.7). The boiled samples had lower counts of

bacteria, ranging from 0.32 ± 0.21 to 0.73 ± 0.26 $CFUg^{-1}$ dry weight. Freeze-dried, snap dried and deep fried samples recorded zero levels of bacterial infestation.

The fungal populations were also notably high in the fresh samples with populations ranging from 2.83 ± 0.62 to 4.48 ± 0.04 $CFUg^{-1}$ fresh weight (Table 4.7). Sun-dried samples came in second with fungal populations ranging from 2.94 ± 0.33 to 3.71 ± 0.13 $CFUg^{-1}$ dry weight. The boiled samples recorded lower fungal counts with levels ranging from 0.64 ± 0.2 to 0.87 ± 0.36 $CFUg^{-1}$ dry weights. The samples processed though freeze-drying, snap drying and deep-frying had no fungal infestation.

Table 4.7: Effect of different processing methods on bacterial and fungal densities in reared *Scapsipedus icipe*

Processing Method	Bacteria log (cfu/g)					Fungi log (cfu/g)		
	P. anthropi	*Wolbachia*	*R. grylli*	*L. garvieae*	Others	*T. asperellum*	*A. niger*	Others
Fresh	0±0a	0±0b	2.1 ±0.7a	2.57 ±0.7a	4.31 ±0.07a	4.23 ±0.06a	2.83 ±0.62a	4.48 ±0.04a
Boiled	0.32±0.21a	0±0b	0±0b	0±0b	0.73 ±0.26c	0.64±0.2c	0±0b	0.87 ±0.36c
Sun dried	0±0a	2.07 ±0.45a	1.34 ±0.45a	0±0b	2.62 ±0.12b	2.94 ±0.33b	0±0b	3.71 ±0.13b
Freeze dried	0±0a	0±0b	0±0b	0±0b	0±0d	0±0d	0±0b	0±0d
Snap dried	0±0a	0±0b	0±0b	0±0b	0±0d	0±0d	0±0b	0±0d
Deep fried	0±0a	0±0b	0±0b	0±ob	0±0d	0±0d	0±0b	0±0d

Values are means of $CFUg^{-1}$ fresh/dry weight of triplicate experiments ± standard deviation. In the same row, means with different letters are significantly different while means followed with the same letter show no significance difference ($P < 0.05$)

4.4.2 Microbial loads in reared fresh and processed *Gryllus bimaculatus*

Fresh samples of *G. bimaculatus* had the highest populations of bacteria and fungi. The level of bacterial infestation in the fresh samples ranged from 4.35 ± 0.08 to 4.77 ± 0.06 $CFUg^{-1}$ fresh weight which was way higher than the sun-dried samples which had populations ranging from 2.53 ± 0.56 to 3.65 ± 0.09 $CFUg^{-1}$ dry weight (Table 4.8). The bacterial densities greatly reduced in the boiled samples with populations ranging from 0.57 ± 0.24 to 0.51 ± 0.21 $CFUg^{-1}$ dry weight. The fungal counts were also high in the fresh samples with populations ranging from 2.34 ± 0.64 to 4.39 ± 0.06 $CFUg^{-1}$ fresh weight. The sun-dried samples recorded lower counts of fungi 2.87 ± 0.32 to 3.25 ± 0.08 $CFUg^{-1}$ dry weight, while the boiled samples recorded the least number of fungal populations ranging from 0 ± 0 to 0.84 ± 0.44 $CFUg^{-1}$ dry weight (Table 4.8). Samples processed through snap drying, freeze-drying and deep-frying had no fungal infestation.

Table 4.8: Effects of different processing methods on bacterial and fungal loads in reared *Gryllus bimaculatus*

Processing Method	Bacteria log (cfu/g)				Fungi log (cfu/g)			
	Wolbachia	*R. grylli*	*A. media*	others	*A. tamarii*	*A. flavus*	*T. asperellum*	others
Fresh	0±0*b*	4.35 ±0.08*a*	4.08 ±0.3*a*	4.77 ±0.06*a*	0±0*b*	2.34 ±0.64*a*	3.4±0.57*a*	4.39 ±0.06*a*
Boiled	0.57 ±0.24*a*	0±0*b*	0±0*b*	0.51 ±0.21*c*	0±0*b*	0±0*b*	0±0*b*	0.84 ±0.44*c*
Sun dried	0±0*b*	2.53 ±0.56*c*	0±0*b*	3.65 ±0.09*b*	2.87 ±0.32*a*	0±0*b*	0±0*b*	3.25 ±0.08*b*
Freeze dried	0±0*b*	0±0*c*	0±0*b*	0±0*d*	0±0*b*	0±0*b*	0±0*b*	0±0*c*
Snap dried	0±0*b*	0±0*c*	0±0*b*	0±0*d*	0±0*b*	0±0*b*	0±0*b*	0±0*c*
Deep fried	0±0*b*	0±0*c*	0±0*b*	0±0*d*	0±0*b*	0±0*b*	0±0*b*	0±0*c*

Values are means of $CFUg^{-1}$ fresh/dry weight of triplicate experiments ± standard deviation. In the same row, means with different letters are significantly different while means followed with the same letter show no significance difference ($P < 0.05$).

4.5 Molecular characterization

4.5.1 PCR amplification of the ITS region

PCR amplifications of 25 fresh and processed isolates from the reared crickets (Plate 4.6) as well as PCR amplifications of 15 samples from the fresh wild crickets (Plate 4.7) produced 600 base pairs using ITS4 (5’- GGAAGTAAAAGTCGTAACAAGG-3’) and ITS5 (5’- TCCTCCGCTTATTGATATGC -3’) primers.

Plate 4.6: PCR amplification products of the ITS region of reared fresh and processed *G. bimaculatus* and *S. icipe* on 1.2 % agarose gel. Lane M, 1kb DNA ladder used as a molecular marker; lane 1-25 had cricket isolates. (1, FGRP; 2, FGRF; 3, FSRP; 4, FSRP; 5, FGRP; 6, FSRP; 7, FGRF; 8, FGRF; 9, FGRF; 10, FGRF; 11, FSRF; 12, FSRP; 13, FGRF; 14, FSRF; 15, FGRP; 16, FGRP; 17, FGRF; 18, FGRP; 19, FSRF; 20, FSRF; 21, FGRP; 22, FGRF; 23, FGRF; 24, FSRF, 25, FSRF)

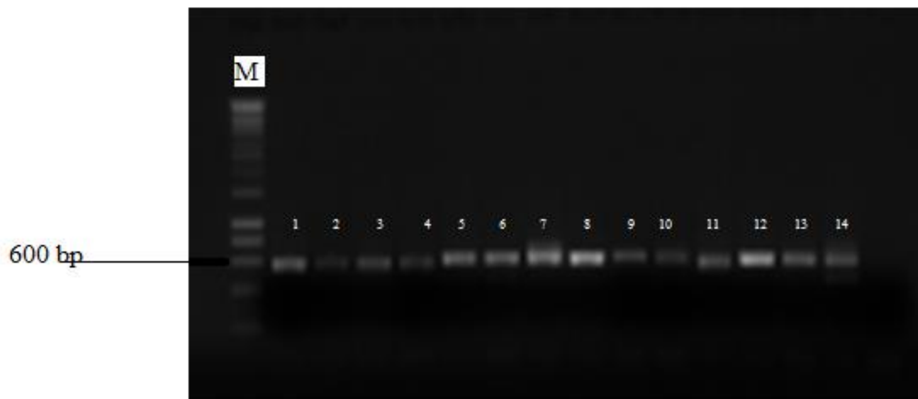

Plate 4.7: PCR amplification products of the ITS region of the fresh wild *G. bimaculatus* and *S. icipe* samples using 1.2 % agarose gel. Lane M, 1kb DNA ladder used as a molecular marker; lane 1-14 had the wild samples (1, FSWF; 2, FSWF; 3, FSWF; 4, FGWF; 5, FGWF; 6, FSWF; 7, FSWF; 8, FSWF; 9, FSWF; 10, FSWF; 11, FGWF; 12, FGWF; 13, FGWF; 14, FGWF; 15,FGWF).

4.5.2 Phylogenetic analysis of fungal isolates

The fungal isolates clustered into seven distinct clades (Figure 4.1). The 1st cluster consisted of isolates; FSRPB1 obtained from boiled *S. icipe*, FGWF4 from wild fresh *G.*

bimaculatus and FGRF2 from fresh reared *G. bimaculatus.* These isolates had *Beauvaria bassiana,* Accession number JQ320366 as their closest reference match (99 %). Other isolates in this cluster included isolates FSRPB2 (boiled *S. icipe)*, FGRPS1 (sun-dried *G. bimaculatus*), FSWF3 (wild *S. icipe*), FGRF4 (reared fresh *G. bimaculatus*), FGWF2 (wild *G. bimaculatus*) and FSWF5 (wild *S. icipe*). This isolates closely related (99 %) to *Aspergillus* sp. (AM901694). *Beauvaria bassiana* and *Aspergillus* sp. are fungal species mostly found in soil and dust (Raja *et al,* 2013).

The 2nd cluster was composed of isolates FGRF3 (reared fresh *G. bimaculatus*) and FSRF4 (reared fresh *S. icipe*) which clustered closely (98 %) with *Trochoderma asperellum* (KC898193) a fungus commonly associated with rhizosphere of endophytes (Chow *et al.,* 2017). The third cluster consisted of lab isolates FGWF3 (wild *G. bimaculatus*) and FSWF2 (wild *S. icipe*). These two isolates did not have any close references to match with in the NCBI database.

The fourth cluster had isolate FSRPB1 (boiled *S. icipe*) clustering closely (95 %) with *Aspergillus oyrzae* (EU301638) a fungus commonly associated with forest soils (Mukunda *et al.,* 2012). The fifth and the sixth clusters consisted of isolates FGRF1, FGRF5 and FGRF6 (reared fresh *G. bimaculatus*); FSWF1, FSWF6 and FSWF4 (wild *S. icipe*); FGWF5 and FGWF1 (wild *G. bimaculatus*); FGRPB1 (boiled *G. bimaculatus*); FSRF2 and FSRF1 (reared fresh *S. icipe*). These isolates did not have any close reference matches in the NCBI database suggesting they could be potential novel isolates. The seventh cluster consisted of isolates FSRF3 and FSRF5 (reared fresh *S. icipe*) which clustered together (99 %) with *Aspegillus flavus* (KU978916) a biodegrading endophyte commonly found in air (Hedayati *et al.,* 2007).

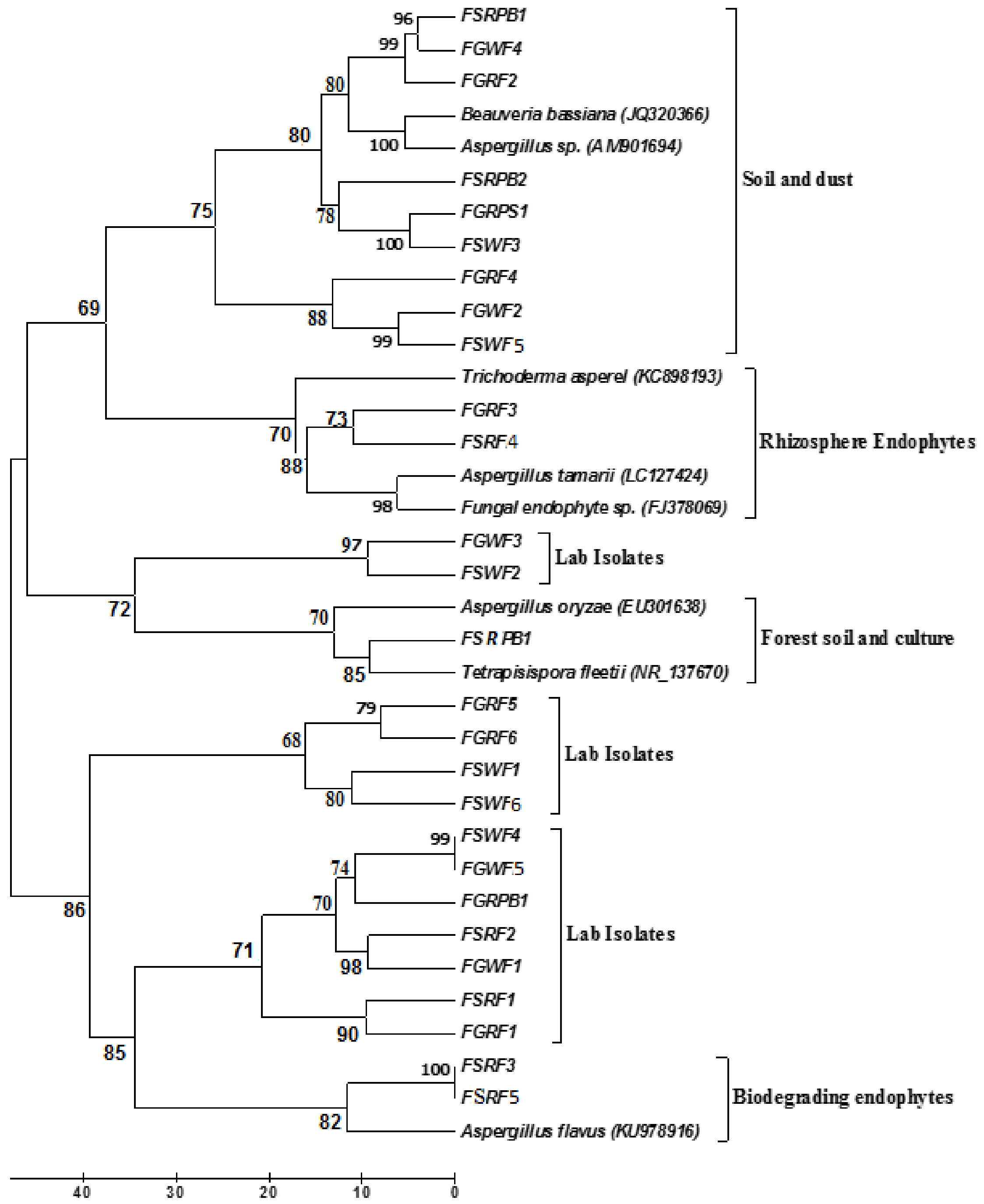

Figure 4.1: Phylogenetic tree inferred using the maximum likelihood method for fungal (F) isolates from both wild (W) and reared (R) crickets (fresh -F and processed -P) samples from *G. bimaculatus* (G) and *S. icipe* (S) with their reference isolates. Bootstrap values for the associated taxa that clustered together in the bootstrap test (1,000 replicates) is indicated at the nodes. The scale bar shows the rate of substitution per nucleotide position. Evolutionary analyses were conducted using MEGA7.

4.5.3 PCR amplification of 16S rRNA

PCR amplification of 16S rRNA gene of 42 bacterial isolates produced a single band of 1500 base pair in size using 27f (5-'AGAGTTTGATCMTGGCTCAG3') and 1492r (5-' TACCTTGTTACGACTT3') primers (Plate 4.8 and plate 4.9).

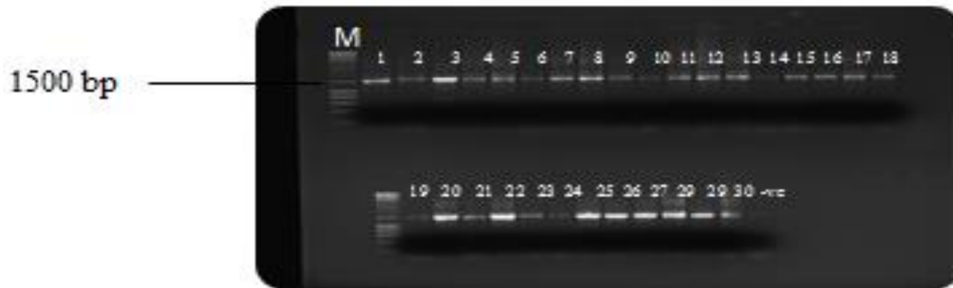

Plate 4.8: PCR amplification products of the 16S rRNA gene for representative bacterial isolates of reared fresh and processed *S. icipe* and *G. bimaculatus* on 1.2 % agarose gel. Lane M, 1kb DNA ladder used as a molecular marker; lane 1-30 had bacterial isolates (1, BGRF; 2,BSRF; 3, BSRP; 4, BGRP; 5, BSRP; 6, BGRF; 7, BSRF; 8, BSRP; 9, BSRF; 10, BSRF; 11, BSRF; 12, BGRF; 13, BGRP; 14, BGRP; 15, BSRF; 16, BSRF; 17, BSRP; 19, BGRF; 20, BGRF; 21, BSRF; 22, BGRF; 23, BSRP; 24, BSRP; 25, BSRF; 26, BSRF; 27, BSRF; 28, BGRF; 29, BSRF; 30, BGRF).

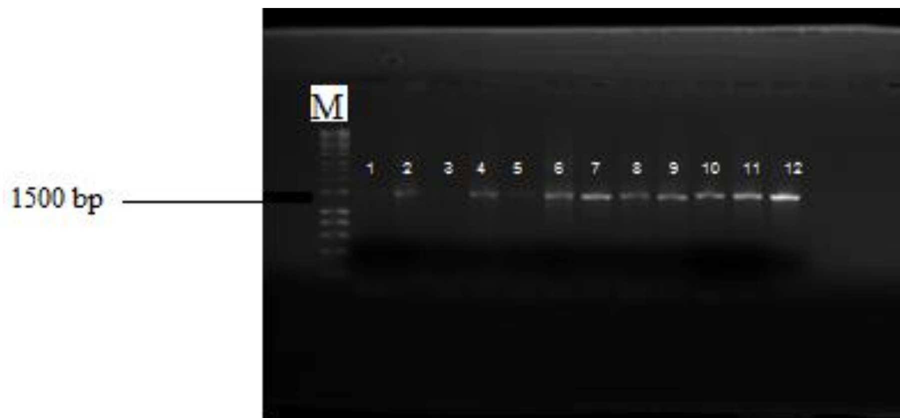

Plate 4.9: PCR amplification products of the 16S rRNA gene for representative bacterial isolates of some of the processed *G. bimaculatus* and *S. icipe* using 1.2 % agarose gel. Lane M, 1kb DNA ladder used as a molecular marker; lane 1-12 had bacterial Isolates; (1, BGWF; 2, BGWF; 3, BSWF; 4, BGWF; 5, BGWF; 6, BGWF; 7, BSWF; 8, BSWF; 9, BGWF; 10, BSWF; 11, BSWF; 12, BSWF).

4.5.4 Phylogenetic analysis of bacterial isolates

Phylogenetic analysis showed that the bacterial isolates were clustered into sixteen distinct clades (Figure 4.2). In the 1st cluster, isolate BGRF9 isolated from fresh reared *G. bimaculatus* and BGWF2 from wild *G. bimaculatus* clustered closely (90 %) with *Bacillus* sp. (LC146400) commonly isolated from soil sediments (Amin *et al.,* 2015). In the second cluster, isolate BGWF1 from *wild G. bimaculatu*s clustered closely (97 %) with *Klebsiella oxytoca* (JN102560), bacteria previously isolated from mesenteric lymph nodes of vertebrates (Darby *et al*., 2014). The third cluster consisted of only isolate BSRF1 isolated from reared fresh *S. icipe*. This isolate had no closest reference strains to match with in NCBI database.

The fourth cluster also comprised of one isolate; BSWF1 from wild *S. icipe* matching closely (97 %) with *Erwinia sp.* (KM878581) a bacterium commonly associated with insects (Anand *et al.,* 2009). In the fifth cluster, isolates BSRPB3 and BSRPB1 both from boiled *S. icipe* clustered closely to *Pseudocitrobacter faecalis* (NR-125690) previously isolated from fecal samples (Kämpfer *et al.,* 2014). Isolates BGRF6 (reared fresh *G. bimaculatus*) in cluster six, BSRF3 (reared fresh *S. icipe*) in cluster seven, BGRF1 (reared fresh *G. bimaculatus*) and BSWF3 (wild *S. icipe*) in cluster eight had no close reference strains to match with in NCBI database suggesting they could be potential novel isolates.

The nineth cluster consisted of only one isolate BGRF5 (fresh reared *G. bimaculatus*) clustering closely (83 %) with *Pseudomonas aeruginosa* (KP119458), a bacterial species previously isolated from hydrogen carbon contaminated soil (Ebadi *et al.,* 2017). Isolates BSRPS1 (sun-dried *S. icipe*), BGRPS1 (sun-dried *G. bimaculatus*), BGRPB1; BGRPB2

(boiled *G. bimaculatus*), and BSRPB2 (boiled *S. icipe*) grouped together in cluster ten and their closest relative (97 %) was *Wolbachia endosymbiont* (HE583204). This bacterial species was previously isolated from bugs (*Macrolophus pygmaeus*) (Miller, 2013).

Isolates BGRF4 (reared fresh *G. bimaculatus*), BSRPB4 (boiled *S. icipe)*, BSRPS4 and BSRPS2 (sun-dried *S. icipe*), BGRPS2 and BGRPS3 (sun dried *G. bimaculatus*) grouped together in cluster eleven with *Rickettsiella grylli* (U97547) previously isolated from ticks, as their closest match (99 %) (Jauset *et al*., 2015). Cluster twelve comprised of lab isolates BSWF4, BSWF3 (both from wild *S. icipe*) and BGRF8 (reared fresh *G. bimaculatus*). These isolates had no close reference strains in the NCBI database suggesting they may be potential novel isolates. The thirteenth cluster had isolates BGRF2 and BGRF3 from fresh reared G. *bimaculatus*, BSRF5 and BSRF2 from fresh reared *S. icipe* clustering closely (96 %) to *Lactococcus garvieae* (MF348235), previously isolated from humans (Wang *et al.,* 2007). Cluster fourteen had lab isolates BGRF7 (Fresh reared *G. bimaculatus*) and BSRF6 (fresh reared *S. icipe*). The two isolates had no close matches in the NCBI database.

BSWF2 (wild *S. icipe*) in the fifteenth cluster matched closely (99 %) with *Chryseobacterium gleum* (KX579964), suggesting it is a member of this species. Members of this species had been previously isolated from activated sludge soil (Szoboszlay *et al.,* 2008). Isolates BSRF4 (reared fresh *S. icipe*) and BSRPS3 (sun-dried *S. icipe*) grouped together in cluster sixteen with *Bacteroides* sp. (KR822443) as their closest match. *Bacteroides* have previously been isolated from the hindgut of the cockroach *Periplaneta americana* (Schauer *et al.,* 2012).

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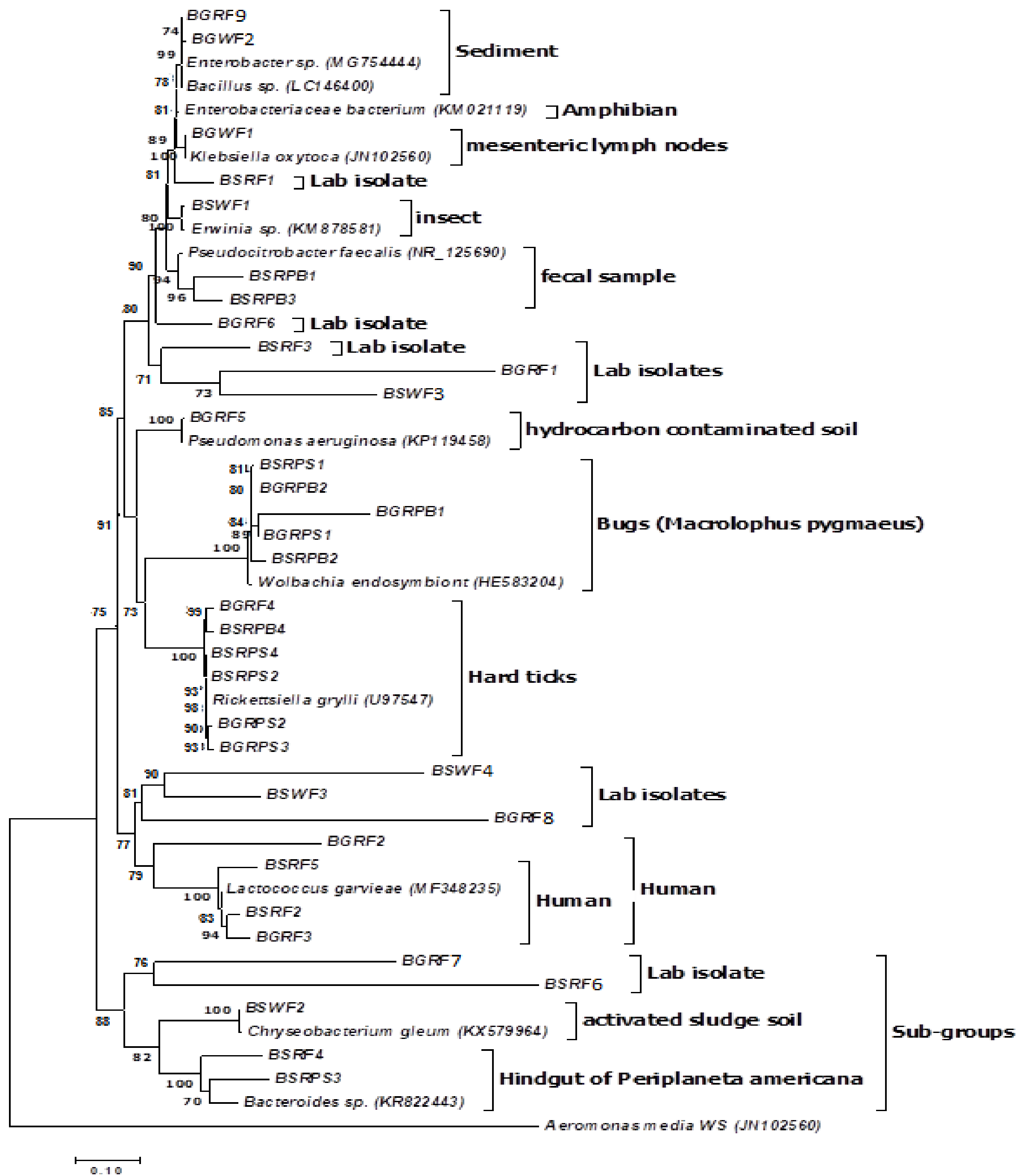

Figure 4.2: Phylogenetic tree inferred using the maximum likelihood method for bacterial (B) isolates from both wild (W) and reared (R) crickets (Fresh-F and Processed-P) samples from *G. bimaculatus* (G) and *S. icipe* (S) with their closest reference isolates. Bootstrap values for the associated taxa that clustered together in the bootstrap test (1,000 replicates) are indicated at the nodes. The scale bar shows the rate of substitution per nucleotide position. Evolutionary analyses were conducted using MEGA7.

CHAPTER FIVE

DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

5.1 Discussion

5.1.1 Morphological characteristics of bacterial and fungal isolates from crickets

The majority of the fungal isolates from the wild species had dark brownish colonies, with mycelia that had visible tiny spores, characteristics attributed to members of *Aspergillus* spp. This is in tandem with the work of Braide and Nwaoguikpe (2011) where colonies with similar characteristics were isolated while assessing the microbial quality of processed wild edible weevil caterpillar (*Rhynchophorus phoenicis*) in Port Harcourt, southern Nigeria.

A good number of the isolates had light greenish rough surfaced colonies with spores that were not clearly visible, characteristics associated with members of *Penicillium* spp. Colonies with similar characteristics had previously been isolated by Braide *et al.* (2011) while assessing the microbiological quality of wild edible caterpillar of an emperor moth *Bunaea alcinoe*. Most of the fungal isolates from the reared cricket species had dark greenish mass with highly branched and loosely packed conidiophores characteristics associated with members of *Trichoderma* sp. Similar results had been observed by Omoya and Kelly (2017) while investigating the entomopathogenic organisms associated with the wild grasshopper (*Zonocerus variegatus)*. Only one isolate had white colonies, which appeared like dots even after being incubated for a long time, characteristics belonging to members of *Beauveria* spp. (Braide *et al.,* 2011).

The bacterial diversity in the wild crickets was higher than in the reared crickets. Most of the bacterial isolates in both reared and wild crickets had milky white colonies with rough margins on nutrient agar. The colonies were also opaque, circular in shape and raised in elevation. The results tallied with the work of Hernández-Flores *et al*. (2015) while investigating the bacteria present in reared *Comadia redtenbacheri* larvae, where most of the colonies had similar characteristic commoly associated with menbers of the *Bacillus* spp.

On nutrient agar, some of the colonies were creamy white in colour, opaque, raised in elevation with smooth margins, characteristics associated with members of *Staphylococcus* spp. Similar observations had been made by Braide *et al.* (2011), while investigating the microbiological quality of an edible caterpillar of an emperor moth *Bunaea alcinoe* in Nigeria. On MacConkey agar, 90 % of the colonies from the reared *S. icipe* and *G. bimaculatus* were pink in colour, had smooth margins with a raised elevation and convex in shape suggesting that they belonged to members of *E. coli*. The results tallied with the work of Banjo, Lawal and Adeyemi (2006) while investigating the microbial fauna associated with edible beetle larvae of *Oryctes Monocerus* in South Western Nigeria.

About 5 % of the remaining bacterial colonies, mostly from the wild species of *S. icipe* and *G. bimaculatus* were dark red, rough margined and viscous. Colonies with similar characteristics believed to belong to members of *Enterobacter* spp. had been previously observed by Omoyo and Kelly, (2017) while investigating entomopathogenic organisms in association with *Zonocerus variegatus*. The remaining 5 % of the colonies were light

pink in colour, raised in elevation, smooth margined and mucoid in texture (Hernández-Flores *et al*., 2015) characteristics typical to members of *Klebsiella oxytoca.*

5.1.2 Molecular characterization of fungal and bacterial isolates

Fungal isolates FSRPB1, FGWF4, FGRF2 in cluster 1 closely matched with *Beauveria bassiana,* while isolates FSRPB2, FGRPS1, FSWF3, FGRF4, FGWF2 and FSWF5 also from cluster 1 closely matched with *Aspergillus* sp. *Beauveria bassiana* and *Aspergillus* sp. are fungal species mostly found in soil and dust (Raja *et al.,* 2013) where they were easily picked by the crickets. *B. bassiana* is rarely known to be pathogenic to humans and due to its pathogenicity to insect species, it is integrated into several pesticides (Hu *et al.*, 2016). Some species of *Aspergillus* are pathogenic to humans causing an assortment of allergic reactions and life threatening infections (Paulussen *et al.,* 2017).

Isolates FGRF3 and FSRF4 in the 2nd cluster closely matched *Trochoderma asperellum* a fungus commonly associated with rhizosphere of endophytes as well as in most soils (Chow *et al.*, 2017) where it could have been picked by the crickets during their foraging activities. *Trochoderma asperellum* is not known to be pathogenic to humans except in severely immunocompromised individuals (Rani *et al.,* 2009). However, the fungus is a universally recognized biocontrol agent owing to its effective broad-spectrum antimicrobial activity, where it displays hostile effects against at least 29 species of pathogenic fungi, and a good number of pathogenic bacteria (Mbarg *et al.*, 2012).

The third cluster consisted of lab isolates FGWF3 and FSWF2. These two isolates did not have any close references to match with in the NCBI database suggesting they potentially form novel isolates associated with crickets. Their identity should be investigated to

establish whether they are human pathogens and the role they play in insects. The fourth cluster had isolate FSRPB1 clustering closely with *Aspergillus oyrzae* a fungus commonly associated with forest soils (Mukunda *et al.*, 2012). *Aspergillus oyrzae* is not pathogenic to humans but has been in use for many years in the food fermentation industry and no strain of the fungus is associated with aflatoxin production (Payne *et al.,* 2006).

The fifth and the sixth clusters consisted of isolates FGRF1, FGRF5, FGRF6 FSWF1, FSWF6, FSWF4, FGWF5, FGWF1, FGRPB1, FSRF2 and FSRF1. All these isolates did not have any close reference matches in the NCBI database suggesting they could be potential novel isolates whose identities need to be investigated to establish whether they are human pathogens.

The seventh cluster consisted of isolates FSRF3 and FSRF5 clustering closely with *Aspegillus flavus* a biodegrading endophyte (Hedayati *et al*., 2007). *A. flavus* is a human pathogen known to cause human invasive aspergillosis together with *A. fumigatus* (Payne *et al.,* 2006). Additionally, it is the main *Aspergillus* species known to infect insects, as well as cause diseases in economically important crops for example, peanuts and maize producing strong mycotoxins (Hedayati *et al.,* 2007).

Phylogenetic analysis of bacterial isolates (fig.4.2) revealed that in the 1st cluster, isolates BGRF9 and BGWF2 clustered closely with *Bacillus* spp. commonly found in soil sediments (Amin *et al.,* 2015). *Bacillus* spp. are ubiquitous spore-forming bacteria typically occurring as saprophytes in water and soil as indicated by Blackburn and McClure, (2009) and it is possible that these communities may have been picked from the

environment during the foraging activities of the insects. Some species of *Bacillus*, for example, *B. cereus*, are known to cause food poisoning (Blackburn and McClure, 2009). Other species known to be pathogenic to humans include*; B. brevis, B. subtilis, B. macerans, B. licheniformis, B. circulans, B. alvei, B. coagulans, B. pumilus,* and *B. sphaericus* (Blackburn and McClure, 2009).

In the second cluster, isolate BGWF1 clustered closely with *Klebsiella oxytoca*, a bacterium previously isolated from mesenteric lymph nodes of vertebrates (Darby *et al.,* 2014). *Klebsiella oxytoca* is an opportunistic pathogen associated with numerous clinical infections in humans and animals (Wang *et al.,* 2014) and has been isolated from diverse environments. Most of *Klebsiella* spp. infections are associated with hospitalization, with the urinary tract being the most commonly affected site. According to Podschun and Ullmann, (1998), *Klebsiella* spp. can colonize different areas of the human body e.g. the colon, skin, urine and sterile wounds. *K. oxytoca* has also been identified as one of the many bacteria colonizing the gut system of insects thus inhibiting the formation and multiplication of pathogenic bacteria (Darby *et al.,* 2014)

Isolate BSRF1 in the third cluster had no closest reference strains to match with in NCBI database. Its identity need to be investigated to establish whether it is pathogenic to humans and the role it plays in insects. The fourth cluster also comprised of one isolate; BSWF1 matching closely with *Erwinia sp*. a bacterium commonly associated with insects (Anand *et al.*, 2009). *Erwinia* spp. are plant pathogens, which are not known to be associated with any human disease (Shin *et al.,* 2008). The crickets most probably acquired the bacteria through feeding.

In the fifth cluster isolates BSRPB3 and BSRPB1 clustered closely to *Pseudocitrobacter faecalis* previously isolated from fecal samples (Kämpfer *et al.,* 2014). *Pseudocitrobacter faecalis* is considered an opportunistic pathogen capable of causing infections in immunocompromised individuals (Al-Kharousi *et al.,* 2016). Isolates BGRF6 in cluster six, BSRF3 in cluster seven, BGRF1 and BSWF3 in cluster eight had no closest reference strains to match with in NCBI database suggesting they could be potential novel isolates.

The nineth cluster consisted of only one isolate BGRF5 clustering closely with *Pseudomonas aeruginosa* (KP119458), a bacterial species that was previously isolated from hydrogencarbon contaminated soil (Ebadi *et al.,* 2017). Gellatly and Hancock, (2013) describes *Pseudomonas aeruginosa* as an opportunistic pathogen causing severe infections in humans. Some of the infections resulting from the bacteria include pneumonia, infections of the central nervous system, urinary tract, wounds, ears, eyes, skin, and musculoskeletal system. *Pseudomonas* species normally inhabit soil, water, and vegetation (Ebadi *et al.,* 2017) where they could have easily been picked by the insects.

Isolates BSRPS1, BGRPS1, BGRPB1, BGRPB2 and BSRPB2 grouped together in the tenth cluster with their closest relative being *Wolbachia endosymbiont* previously isolated from bugs (*Macrolophus pygmaeus*) (Miller, 2013). *Wolbachia* is not known to be pathogenic to humans but has a mutualistic relationship with arthropods (Wei *et al.*, 2013) and probably forms symbiotic association with crickets. Isolates BGRF4, BSRPB4, BSRPS4, BGRPS2 and BGRPS3 grouped together in cluster eleven with *Rickettsiella grylli* previously isolated from ticks as their closest match (Jauset *et al.,* 2015). The bacterium is also not known to be pathogenic to humans. It infects arthropods, where the relationship is more mutualistic than parasitic (Wei *et al.*, 2013).

Cluster twelve comprised of lab isolates BSWF4, BSWF3 and BGRF8. These isolates had no close reference strains in the NCBI database suggesting they may be potential novel isolates whose identity need to be established. The thirteenth cluster had isolates BGRF2, BGRF3, BSRF5 and BSRF2 clustering closely to *Lactococcus garvieae*, previously isolated from humans (Wang *et al.,* 2007). *Lactococcus garvieae* is a Gram-positive bacterium traditionally known to be a low virulence organism, but recently it is being considered as an emerging opportunistic human pathogen causing skin and urinary tract infections (Aguado-Urda *et al.,* 2011). Cluster fourteen had lab isolates BGRF7 and BSRF6. The two isolates had no close matches in the NCBI database.

Isolate BSWF2 in the fifteenth cluster closely related to *Chryseobacterium gleum*, suggesting it is a member of this species. Members of this species had previously been isolated from activated sludge soil (Szoboszlay *et al.,* 2008). *Chryseobacterium* species are uncommon human pathogens, known to cause nosocomial infections only in immunosuppressed individuals (Arouna *et al.,* 2017). The bacterium is typically found in plants, soil, and water (Szoboszlay *et al.,* 2008) where it could have easily been picked by insects.

Isolates BSRF4 and BSRPS3 grouped together in cluster sixteen with *Bacteroides sp*. as their closest match. *Bacteroides* species are among the most predominant bacteria in the gut of cockroach *Periplaneta americana* (Schauer *et al.,* 2012) where they have a mutualistic relationship with the host. Some *Bacteroides* species for example, *B. fragilis* are known to induce abscess formation, which if not treated can result to intestinal obstruction, erosion of blood vessels in the affected area, and eventually fistula formation

(Wexler, 2007). *Bacteroides* species also composes the gut microbiota of crickets (Domingo *et al.,* 1998).

5.1.3 Comparison between wild and reared crickets

From the study, 26 pure fungal isolates were obtained from both reared and wild crickets. *Trichoderma* sp. was the most predominant fungi isolated from the reared crickets, while *Aspergillus* sp. was the predominant fungi in crickets collected from the wild. *Trichoderma* spp. has previously been isolated in high numbers in studies carried out by Mpuchane *et al.* (2006) on domestic cockroaches. The occurrence of *Aspergillus* sp. as the main fungal isolate in wild crickets is in agreement with the work of Banjo *et al.* (2006) who observed similar results from wild edible beetle *Oryctes monocerus* larvae as well as Mpuchane *et al.* (2000) on wild ready to eat mopane caterpillar (*Imbrasia belina*) obtained from the market. Certain strains of *Aspergillus* (*A. niger* and *A. flavus*) and *Penicillium* species are known to produce mycotoxins which would have toxic effects on consumers (Mujuru *et al.,* 2014). The relatively high presence of moulds in the crickets samples could be attributed to spore deposition from the air as they are ubiquitously distributed in soil and air.

The bacterial flora of the reared and wild crickets had a mixed population of both Gram negative and Gram-positive bacteria. The gram-positive bacteria included *Bacillus* sp., *Lactobacillus* sp., *Staphylococcus* sp., *Streptococcus* sp. while the gram negatives included *Klebsiella* sp*, Erwinia* sp., *Enterobacter* sp. and *Escherichia coli.* All these microorganisms have previously been isolated from insects (Wei *et al.*, 2013; Grabowski and Klein, 2017). However, not all the species of bacteria isolated from the crickets under study cause food spoilage during storage, thus they do not pose health risks to consumers.

The bacterial diversity in field-collected crickets was more than the lab-reared crickets. *Bacillus nealsonii* (66.7 %) was the most abundant bacterial isolate in the wild *S. icipe* (Table 4.5) while the reared species was predominated by *E. coli* (73.3 %). *Staphylococcus sciuri* and *Lactococcus garvieae* (53.3 %) dominated the wild *G. bimaculatus* while the reared species was dominated by *Aeromonas media* (66.7 %) (Table 4.6).

The wild crickets could have had a higher diversity of microorganisms because of the exposure to the microbial flora of the environment, whereas the lab-reared ones are in a more controlled environment. Rani *et al.* (2009) while comparing between lab reared and wild mosquitoes reported a similar observation. In terms of microbe numbers, there was no significance difference between the lab reared crickets and the ones collected in the field as their relative abundances ranged from 46.4-53.6 % (Tables 4.5 and 4.6). This is because despite being in a controlled environment, the insects are fed with food directly from the market without sterilization. Furthermore, most of the vegetables are rotten by the time the insects eat them, resulting in high levels of microorganisms.

Food spoilage and disease causing coliforms including E*nterobactor*, *Erwinia* sp. and *Staphylococcus* sp. were present in both wild and reared crickets. Coliforms have previously been isolated by Mpuchane *et al.* (2006) from domestic cockroaches. Lab-reared crickets might have obtained them from feed usually obtained from the market, while the wild ones just picked them from the environment (Rani *et al.,* 2009).

Lactobacillus sp. and *Staphylococcus* sp. were isolated from the edible crickets which resonates with studies by Braide and Nwaoguikpe, (2011) on the edible weevil caterpillar

(*Rhynchophorus phoenicis*). These bacterial species are effective food spoilage organisms and usually give rise to food borne illnesses. Presence of spore forming bacteria e.g. *Bacillus* sp. in the wild *S. icipe* could be due to the fact that the crickets are always in direct contact with the soil since *Bacillus* is known to be a soil inhabitant (Banjo *et al.,* 2006). *Bacillus* spp. Spores are not completely inactivated even after application of a heating step, thus could cause food spoilage or health risks when favourable conditions return (Klunder *et al.,* 2012).

5.1.4 Effects of different processing methods

In this study, boiling emerged as the most affordable and efficient processing method that do not add cost to insects as a raw material for food (table 4.8). Moreover, no microorganisms were isolated from deep-fried, freeze-dried and snap dried samples thus these methods could be considered as effective processing techniques. However, these practices are expensive adding the cost of edible insects as a raw material for food companies thus the need to look for more sustainable processing methods (Vandeweyer *et al.,* 2017). Nevertheless, it has been observed (Megido *et al.,* 2017) that freeze drying and snap freezing techniques only inactivate microorganisms rather than killing them, thus when the food substance is rehydrated many could be revitalized back to the vegetative state that could prove harmful. Consequently, we recommend the application of a heating step to freeze dried insects before consumption.

Sun drying the insects did not eliminate all the microbiota from the insect’s gut. That is why *Escherichia coli* (a gut microbe) was still detected from the sun dried insects. It is in this regard that blanching the insects in hot water for a few minutes before drying to assist in eliminating *E. coli* and other enteric bacteria is suggested. This is in line with

studies carried out by Klunder *et al.* (2012) on *Tenebrio molitor* and *Achetus domesticus.* The studies suggested blanching the insects in hot water for a few minutes before roasting to eliminate most of enterobacteriaceae, *Bacteroides,* which are gut bacteria were not eliminated by sun drying though they were effectively eliminated in boiling.

Most fungi were eliminated during boiling apart from *Trichoderma asperellum*, which was still isolated from samples, processed in this manner, thus emerging as the most resistant fungus to sun drying and boiling. However, the fungus is not known to cause any human infection except in individuals with severely compromised immune system (Rani *et al.,* 2009). Most samples processed through sun drying had *Aspergillus* spp. This is because *Aspergillus* spp. are ubiquitous and have the ability to grow at low water activity making them important processed food contaminants (Mpuchane *et al.,* 2006).

Boiling the insects for 5 minutes killed most of the enteric bacteria including *E. coli* for both species. Increasing the boiling time to 15 and 20 minutes resulted to the water having an appearance of a turbid broth suggesting an additional loss of fats and other nutrients. Additionally, boiling distorted the appearance of the insects, making them look less appealing and unpalatable. Consequently, 5 minutes boiling was considered sufficient because boiling is already known to reduce protein and fat content in raw insects as noted by Grabowski and Klein (2017). Furthermore, increasing the boiling time from 5 to 10 min did not have an influence on the level of the remaining microorganisms.

Pseudocitrobacter anthropi was not eliminated in insects boiled for 5 minutes. However, the bacterium is not known to be pathogenic to humans, although it can cause infections in immune suppressed individuals (Rani *et al.,* 2009). *Rickettsiella grylli* and *Wolbachia*

spp. were also found in the boiled samples, thus emerging as the most resistant bacteria in this experiment. These two bacteria are known to infect arthropods with some of them having a more mutualistic relationship than parasitic (Wei *et al.,* 2013). The presence of these two bacteria could have resulted from contamination during processing.

5.2 Conclusions

In terms of microbial load, there was no significance difference in the number of microbes isolated from the reared and the wild crickets. In the reared crickets, suspected contamination mostly occurred from the feed and the handling of the insects during rearing, whereby the feed is obtained from the market and directly fed to the insects without sterilization. Moreover, some of the feed is not very fresh during the time of feeding, for example, the green vegetables are usually already turning yellow in colour and rotten by the time the insects feed on them. In addition, unhygienic handling of crickets for example changing them from one cage to the other was the other factor that contributed to high contamination of the reared crickets.

From the study, the wild crickets had higher diversity of microorganisms than the reared crickets. This is because the reared crickets are in a more controlled environment than the wild crickets, which have direct exposure to the microbial flora of the environment.

The fresh insect had high microbial loads, which reduced significantly after application of a processing step. Boiling emerged as the most efficient and affordable processing method which do not add cost of edible insects as a raw material for food. Deep-frying, deep-freezing and snap freezing could also be considered as effective processing techniques since neither fungi nor bacteria was isolated from the cricket samples

processed in this manner. However, these practices are expensive adding the cost of edible insects as a raw material for food companies thus the need to look for more sustainable processing methods. Most of the contamination from the dried and the boiled samples could be due to improper handling, processing and exposure to air. With proper handling and processing of the insects, microbial risks could be minimized.

5.3 Recommendations

i. Application of a heating step is recommended to both wild and reared crickets to minimize the risks involved with consumption of these edible insects.

ii. Apart from microbial hazards tested in this study, other hazards, e.g. parasitic and chemical hazards need to be investigated for insects to be fully accepted as a safe source of food.

iii. Culture-independent approaches should be applied in screening the crickets for potential hazards to avoid the biasness associated with culture-based approaches.

5.4 Recommendation for further studies

i. Investigating other hazards for example, parasitic and chemical hazards that could pose a risk to consumers hindering the acceptance of insects as a safe source of food.

ii. Screening the insects using culture-independent methods to capture all the potential microbes.

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APPENDICES

Appendix 1:Guidance for the interpretation of results on the levels of indicator organisms and specific food borne pathogens in meat according to Centre for Food Safety, 2014)

Meat and meat products	Result (colony-forming unit (cfu) /g)		
	Satisfactory	**Borderline**	**Unsatisfactory**
Raw, Processed and packaged meat	$<10^5$	10^5-$<10^7$	$\geq 10^7$
Hygiene indicator organisms	20	20 - $<10^2$	$> 10^2$
Campylobacter spp, E coli, Salmonella spp, V. cholera, Shigella spp.	Not detected in 25g	N/A	Detected in 25g
Listeria monocytogene, Vibrio parahaemolyticus, Staphylococcus aureus and other Coagulase-positive *staphylococci, Clostridium perfringens, Bacillus cereus*	< 10	10 - < 100	> 100

Appendix II: Bacterial and fungal codes with their meaning.

Bacterial codes	Meaning
FGRF	Fungi *Gryllus* reared fresh
FSRF	Fungi *scapsipedus* reared fresh
FGWF	Fungi *Gryllus* wild fresh
FSWF	Fungi *Scapsipedus* wild fresh
FSRPB	Fungi *Scapsipedus* reared processed boiled
FGRPB	Fungi *Gryllus* reared processed boiled
FSRPS	Fungi *Scapsipedus* reared processed sun-dried
FGRPS	Fungi *Gryllus* reared processed sun-dried

Fungal Codes	Meaning
BGRF	Bacteria *Gryllus* reared fresh
BSRF	Bacteria *Scapsipedus* reared fresh
BGWF	Bacteria *Gryllus* wild fresh
BSWF	Bacteria *Scapsipedus* wild fresh
BSRPB	Bacteria *Scapsipedus* reared processed boiled
BGRPB	Bacteria *Gryllus* reared processed boiled
BSRPS	Bacteria *Scapsipedus* reared processed sun-dried
BGRPS	Bacteria *Gryllus* reared processed sun-dried

Appendix III: DNA extraction and quantification of fungal samples

Genomic DNA quantification gave good DNA concentrations of the fungal samples. The samples with DNA concentrations of less than or close to 10 ng/µl were discarded.

Sample name	Nucleic Acid Concentration	Unit
1F	34.5	ng/µl
1F	14.3	ng/µl
3F	33.1	ng/µl
4F	50.7	ng/µl
5F	47.2	ng/µl
6F	47.7	ng/µl
7F	39.7	ng/µl
8F	57.7	ng/µl
9F	11	ng/µl
10F	27.1	ng/µl
11F	54.5	ng/µl
12F	33.4	ng/µl
13F	51.2	ng/µl
14F	52.3	ng/µl
15F	44.4	ng/µl
16F	68.6	ng/µl
17F	51.6	ng/µl
18F	58.2	ng/µl
19F	78.5	ng/µl
20F	78.8	ng/µl
21F	69.9	ng/µl
22F	41.4	ng/µl
23F	10.8	ng/µl
24F	31.9	ng/µl
25F	22.8	ng/µl
26F	34.7	ng/µl
27F	7.9	ng/µl
28F	6.7	ng/µl
27F	7.4	ng/µl
29F	28.2	ng/µl
30F	9.8	ng/µl
31F	15.6	ng/µl
32F	6.4	ng/µl
35F	23.3	ng/µl
36F	27.1	ng/µl
37F	6.1	ng/µl
38F	10.8	ng/µl

Appendix IV: DNA extraction and quantification of bacterial samples.

The samples with DNA concentrations above 100 ng/µl were diluted using PCR water (at a ratio of 1 parts samples and 9 parts of PCR water).

Sample Name	Nucleic Acid Concentration	Unit
1B	159.6	ng/µl
2B	1480.8	ng/µl
3B	571.4	ng/µl
4B	2100	ng/µl
5B	2024.8	ng/µl
6B	1644.7	ng/µl
7B	280.8	ng/µl
8B	954	ng/µl
9B	699.2	ng/µl
10B	184.4	ng/µl
11B	255.8	ng/µl
12B	539.3	ng/µl
13B	488.6	ng/µl
14B	145.5	ng/µl
15B	1950.4	ng/µl
16B	962	ng/µl
17B	-1.7	ng/µl
18B	422.9	ng/µl
19B	484.9	ng/µl
20B	1515.1	ng/µl
21B	370.3	ng/µl
22B	677.6	ng/µl
23B	225.1	ng/µl
24B	468.8	ng/µl
25B	616.3	ng/µl
26B	379.5	ng/µl
27B	180.3	ng/µl
28B	176	ng/µl
29B	306.1	ng/µl
30B	261.6	ng/µl
31B	1381.1	ng/µl
32B	607.1	ng/µl
33B	401.1	ng/µl
34B	121.9	ng/µl
35B	233.6	ng/µl