EVALUATING THE EFFICIENCY OF OVEN AND SUN-DRYING TRADITIONAL PROCESSING METHODS AT BACTERIAL QUALITY IMPROVEMENT FOR BLACK SOLDIER FLY (*HERMETIA ILLUCENS*) LARVAE

BY

PRUDENCE MAPIKI

A DISSERTATION

SUBMITTED IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE MASTER OF SCIENCE DEGREE IN FOOD SAFETY AND RISK ANALYSIS

SCHOOL OF VETERINARY MEDICINE
UNIVERSITY OF ZAMBIA

COPY RIGHT

DECLARATION

I, Prudence Mapiki, do hereby declare that the dissertation entitled evaluating the efficiency of oven and sun-drying traditional processing methods at bacterial quality improvement for black soldier fly (*hermetia illucens*) larvae has been done by me for the award of a Master of Science degree in Food Safety and Risky Analysis.

I also declare that this dissertation has not been submitted for the award of any degree in the University.

Date: ………………………………Signature: ……………………………

.

APPROVAL

The University of Zambia approves this dissertation of Prudence Mapiki as fulfilling the requirements for the award of the degree of Master of Science in Food Safety and Risk Analysis.

Supervisor	**Signature**	**Date**
Examiner (1)	**Signature**	**Date**
Examiner (2)	**Signature**	**Date**
Examiner 3	**Signature**	**Date**
Chairperson **(Board of Examiner)**	**Signature**	**Date**

DEDICATION

Every challenging work needs self-efforts as well as guidance from family members especially those who are very close to our heart. My humble effort I dedicate to my sweet and loving husband, my mother, my children and all relatives, whose affection, love, encouragement and prays of day and night made me able to get this success and honour.

ACKNOWLEDGMENTS

I would like to thank my supervisors Professor Hang'ombe Bernard and Doctor Mufungwe Joyce for their invaluable assistance and insights leading to the writing of this paper. My sincere thanks also go to them for their patience and understanding during the period of effort that went into the production of this thesis.

I also express my sincere thanks to all the laboratory technicians at the University of Zambia, School of Veterinary Medicine, and Microbiology Laboratory under the leadership of Professor Hang'ombe B. for the help rendered to me while I was working in the laboratory.

Not forgetting the Hokudai Centre for Zoonosis Control in Zambia through Dr. Shawa Misheck's guidance regarding molecular characterization in the laboratory.

I would also like to sincerely appreciate Professor Yngvild Wasteson and Professor Ann Katrin from Norwegian University of Life Sciences through Professor Eystein Skjerve for the invaluable assistance through corrections and guidance they rendered to me towards the writing of the thesis during my stay in Norway.

This work was funded by the National Science Technology Council of Zambia (NSTC), which is encouraging research in this field to identify a sustainable and safe alternative source of proteins for animals and humans in conjunction with the African Centre of Excellence in Infectious Diseases of Humans and Animals (ACEIDHA) which provided tuitions fees for the writer. I would like to also appreciate FORTCASE Project which provided funds for me to travel to Norway for more learning in research under the Norwegian University of Life Sciences.

ABSTRACT

Black soldier fly (*Hermetia illucens*) larvae (BSFL) are a promising, sustainable source of nutrients for animals and humans. However, they could harbour potentially pathogenic bacteria such as *Enterobacteriaceae* and *Staphylococcus aureus* which could be carriers of antimicrobial resistance (AMR) genes due to the environment they are reared in. This is coupled with the limited knowledge regarding processing techniques to ensure food and feed safety on the utilization of BSFL. This study compared the efficiency of sun- and oven-drying methods which are the two commonly used traditional processing methods. The larvae processed under the two methods were assessed for bacterial load and isolation. Furthermore, antibiotic susceptibility testing was done using disk diffusion, on the identified *Escherichia coli* and *Staphylococcus* isolates. PCR was used to identify ESBL and *mecA* genes in *Escherichia coli* and *Staphylococcus* isolates, respectively. Bacterial load for the two processing methods were compared using Jamovi version 2.3.21.0 software by using a Mann Whitney U test and the results indicated a significant difference ($p < 0.001$) in the efficiency of both methods. The oven-drying method reduced the Estimated Aerobic Total Plate Count by 86.4% compared to the sun-drying method. Specifically, the observed cell counts were 3×10^3 CFU/mL and 2.6×10^4 CFU/mL for oven and sun-drying, respectively. The results of the total bacterial load for both methods were below the acceptable limit for insects of animal feed which is 10^9 CFU/mL for an insect material to be considered safe for use in feed. One AMR gene (*bla*$_{\text{CTX-M}}$) was detected from an *E. coli* strain isolated from sun-dried BSFL, indicating the need to use more effective processing methods and the need for more diligence in the selection of suitable substrate for BSFL rearing. Altogether, the results suggest that oven-drying is a better method for processing BSFL for feed but, if one cannot afford, sun-drying can as well be used as it can result in a safe end product as long as source of the substrate is considered before use.

Table of Contents

LIST OF FIGURES

LIST OF TABLES

LIST OF ABREVIATION

NSTC	National Science and Technology Council
ACEIDHA	Centre of Excellence in Infectious Diseases of Humans and Animals
BSFL	Black Soldier Fly Larvae.
AMR	Antimicrobial Resistance
PCR	Polymerases Chain Reaction
ESBL	Extended-Spectrum β-lactamase
MecA	Methicillin Resistant gene
CFU	Colony Forming Unit
HACCP	Hazard Critical Control Point
MRSA	Methicillin Resistance *Staphylococcus aureus*
EFSA	European Food Safety Authority
PBP	Penicillin Binding Protein
ETPC	Expected Total Plate Count
EMB	Eosin Methylene Blue
CTX	Cefotaxime
TET	Tetracycline
GEN	Gentamicin
SXT	Co-trimoxale
CHL	Chloramphenicol
CIP	Ciprofloxacin
AMP	Ampicillin
CLSI	Clinical Laboratory Institute

EUCAST	European Committee on Antimicrobial Susceptibility Testing
ECOFF	Epidemiology Cut-off value
MDR	Multi-drug Resistance
DNA	Deoxyribonucleic acid
BP	Base pair
UNZAREC	University of Zambia Biomedical Research Committee
APC	Aerobic Plate Count
AST	Antimicrobial Susceptibility Testing

CHAPTER ONE

INTRODUCTION

1.1 Background to Black Soldier Fly Larvae utilization and safety in agriculture

In several regions, increased consumption of animal-based foods requiring protein to feed livestock has been linked to global population growth (Hussein et al., 2017). The increase in demand for protein sources has resulted in the sources being expensive (van-Huis, 2013; Makkar et al., 2014). To meet the demand for sources of proteins, insects are now considered alternative protein sources for animal feed due to their high nutrient content, short lifecycle, and less competition for their consumption with humans. The Black Soldier Fly Larvae *Hermetia illucens* (BSFL) is one of the insects used as such. This is due to the many advantages it has, such as its short lifecycle, the ability to feed on a wide range of biological wastes (Nyakeri et al., 2019), and its high nutritional content. Figure 1.1 shows the nutritional and chemical composition of the BSFL.

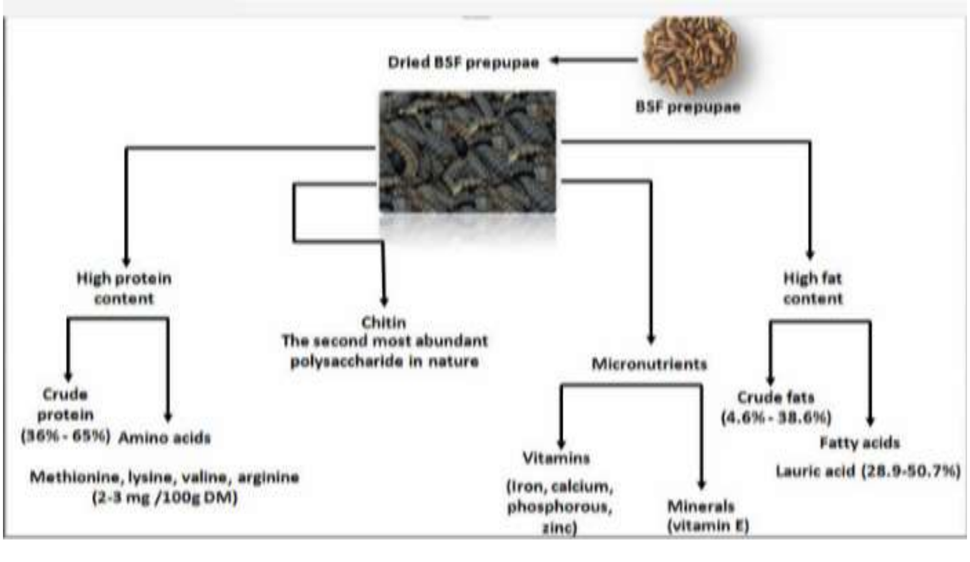


Figure 1.1.1 Nutritional and chemical composition of black soldier fly (Shehata, 2020)

Humans and animals have been consuming insects in many parts of the world for many years and were suggested as a source of food that that could be used to ease global food shortages. In East Africa, insect farming initiatives have taken off, and some countries have developed regulatory mechanisms to mainstream their production and use, for example, in animal feeds (Kebs, 2017).

To utilize edible insects on a commercial scale, producing large quantities of biomass is necessary, either through mass rearing or sustainable harvesting. There is need for suitable postharvest techniques to accumulate reasonable amounts of high quality and safety. Before considering commercialization, it is critical to consider the insect's safety in food and feed (Nyangena et al., 2020). This is because insects contain a variety of microorganisms that can be pathogenic to humans and animals. Insect contamination with human and animal pathogens can occur as a result of the substrate used for rearing and improper processing methods (Nyangena et al., 2020).

Many traditional processing methods, such as boiling, roasting, sun-drying, oven-drying, toasting, frying, smoking, drying, or a combination of these, have been used to process insects in Africa (Nyangena et al., 2020). There is still little information available on their effectiveness when the insects are found in the wild. There is also a scarcity of literature on assessing the presence of antimicrobial resistance (AMR) in processed larvae while taking the environment into account. The majority of the processing literature was done on insects from controlled environments, with little literature on AMR (Rumpold & Schlüter, 2013b).

Li et al., (2016) highlighted the need to consider sources of insects when considering safety, as sources may pose different food safety concerns. Therefore, the difference in the habitat the insects are harvested from can contribute to differences in their safety (Murefu et al., 2019). This is very important to consider in localities such as Zambia, where the mass rearing of BSFL is under consideration. There was need to determine whether the commonly used processing methods will improve the hygienic quality of the wild larvae to avoid contaminating the controlled environment and assess the presence of AMR in the bacteria associated with the processed larvae.

Contamination of feed with pathogenic bacteria originating from BSFL can result in animal diseases that can spread to humans through direct contact where animals act as vehicles to transfer pathogens to humans (Erickson et al., 2004; Wang & Shelomi, 2017). At the same time, these potential pathogens from the contaminated BSFL may be carriers of AMR genes, complicating matters further by making it difficult to treat diseases caused by such pathogens. AMR poses a significant public and animal health risk (Ortega-Paredes. et al., 2020). Infections caused by drug-resistant bacteria are associated with increased morbidity and mortality, as well as a significant increase in healthcare costs). In this study, oven- and sun-drying methods were evaluated for their efficiency to ensure safety in BSFL for feed. This was after considering the fact that sun drying is easy to be used by most people while oven-drying is easily adapted to continuous industrial operations worldwide.

1.2. Problem Statement

BSFL feed on a wide variety of biological wastes (Nyakeri et al., 2019), such as garden waste and animal excrement, which could contain a wide variety of zoonotic pathogens and Antimicrobial Resistant gene carrying bacteria. To ensure the safety of BSFL in the feed chain, the BSFL must be subjected to processing. Although many traditional processing methods have been used in Africa, such as steaming, boiling, roasting, toasting, sun-drying, frying, smoking, and drying, or a combination of these (Nyangena et al., 2020), the effectiveness of these processing methods to reduce bacterial load and amounts of AMR bacteria is not thoroughly established, especially for BSFL grown in the natural environment (Mutungi et al., 2019). Most of the processing evaluations has been on insects grown in controlled environments without considering where the starter larvae (wild) are coming from and the presence of AMR. (Rumpold & Schlüter, 2013b).

With the possibility of mass rearing of BSFL for feed in Zambia, there was an urgent need to evaluate common traditional methods for bacterial quality improvement and check for the presence of AMR to ensure safety of the feed that will be produced. The present study, therefore, aims to evaluate the effectiveness of sun- and oven-drying processing methods and assess the AMR in *E. coli* and *Staphylococcus* from the processed larvae.

1.3 Justification

Unsafe food and feed threaten public health, prosperity, and development, especially in developing countries (Gustafson, 2018). Livestock or poultry may be asymptomatic to certain bacterial diseases, while humans may not be. As a result, food contaminated with pathogenic bacteria can cause illness and even death in the person who encounters it through poor hygienic conditions and breeches in biosecurity. In addition, the chances of potentially pathogenic bacteria, including AMR conveying bacteria, are high in larvae or pre-pupae reared on the animal waste that contains infectious bacteria (Gwenzi et al., 2021). Processing is critical because it provides a vital line of defence against potential hazards. Processing also stops spoilage processes, which improves product safety and reduces product losses. As a result, there is a need to standardize and monitor the efficiency of processing methods in order to ensure the safety and nutritional value of insect-based food/feeds (Mutungi et al., 2019).

Common traditional processing methods require evaluation and validation in order to build on traditional knowledge, and it would be an excellent starting point for developing and implementing a successful food/feed safety mechanism, particularly in Zambia where insect rearing and collection can have the greatest impact on securing livelihoods (Nyangena et al., 2020). Furthermore, to commercialize the use of BSFL, product safety must be continuously monitored. This reduces human health problems arising from being in contact with animals carrying such bacteria or eating meat contaminated with pathogenic bacteria that may be carrying AMR genes. Thus, the present study aimed to evaluate the effectiveness of two common processing methods on bacterial quality improvement and assess the presence of AMR to ensure the end product's safety.

1.4. Importance of this research

The knowledge will guide technological improvements in hazard control plans targeting small and medium-scale actors involved in insect rearing and preliminary processing activities to ensure hygienic quality of the BSFL in Zambia.

1.5. Research questions

1. Do oven-drying and sun-drying processing methods have the same efficiency at improving hygienic quality of BSFL?
2. Do processed BSFL carry AMR bacteria?

1.5. Objectives of the study

1.5.1. General objective

To evaluate the effectiveness of the sun-drying and oven-drying processing methods at bacterial quality improvement and assess the presence of AMR on hygiene indicator bacteria from processed BSFL.

1.5.2. Specific objects

1. To compare the bacterial load of the BSFL under two commonly used processing methods (oven- and sun-drying).
2. To isolate *E. coli* and *Staphylococcus* as fecal and hygienic indicator bacteria.
3. To determine the AMR patterns of *E. coli* and *Staphylococcus* isolated from the processed BSFL.

CHAPTER TWO

LITERATURE REVIEW

2.1. Introduction to insects as an alternative protein source in animal feed

Insect production is now accepted as mini livestock farming worldwide (Durbin & Senate, 2000). Depending on the species, insects can be used as an alternative protein source for human and animal nutrition. Insects also provide other components, such as lipids for biodiesel production and bio-chemicals in cosmetics, can also be applied in waste management to support the circular economy (Sogari et al., 2019). Currently, the most commonly farmed insect species is the Black Soldier Fly (*Hermetia illucens*) and it is the larval stage that is primarily used in animal feed (Arru et al., 2019). The BSF (Diptera): Stratiomyidae) has many advantages as a protein alternative over other cultivated edible insects (Wang & Shelomi, (2017). They have ability to convert various types of waste into high-quality proteins, fats, and minerals and demonstrate the potential to be scalable and efficient as an alternative source of protein for animal feed (EFSA, 2015). The different types of wastes used to rear BSFL affect microbial quality of the larvae resulting in the BSFL being contaminated with potential pathogenic bacteria and AMR gene conveying bacteria. This could lead to diseases with animals which feeds on the contaminated feed and eventually human being through many pathways.

2.2. Colonization or infection of food animals due to contaminated animal feed.

According to Crump, et al. (2002), contaminated food causes more than 5,000 deaths and 76 million illnesses. Humans might not be asymptomatic carriers of some pathogens, but cattle or fowl might be. As a result, food contaminated with pathogenic bacteria and possibly carrying AMR genes can make people sick and even kill them if they come into touch with sick animals. AMR is a major threat to human and animal health (Graham et al., 2019). The spread of AMR bacteria and genes between systems can occur through many agricultural and non-agricultural pathways, including sewage, soil, manure application, direct human-animal exchange, and food contact. In addition, AMR genes for different drug classes are often located on the same mobile genetic elements (plasmids, insertion sequences, transposons, integrons, etc.), meaning that selection for one type of resistance can lead to the transmission of various kinds of resistance (Verraes et al., 2013). In turn, enteric antibiotic resistant bacteria in faeces can spread to other livestock and agricultural workers, re-entering the environment through sewage and manure and exposing other animals. Finally, commensal bacteria such as *Staphylococcus aureus* can infect food animals and people who work on farms or live near livestock. Indeed, recent evidence (Vaney, 2020) from the Netherlands indicates that the proximity of livestock, such as farm workers, compared to their family members, significantly increases their carriage of MRSA; 38% versus 16% for nasal and throat swabs, respectively (Graham, et al., 2019).

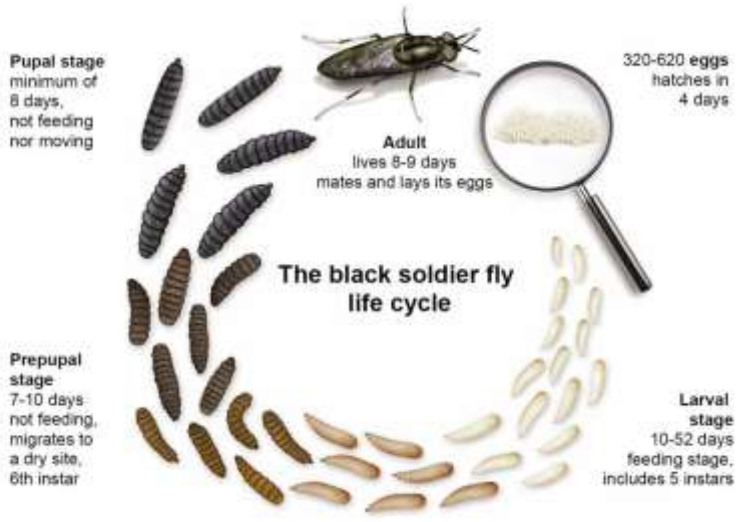
2.3. β-lactam Antibiotics

An antibiotic may either kill or inhibit the growth of bacteria. Antibiotics are from different classes such as penicillins (e.g., penicillin), tetracyclines (e.g., tetracycline), cephalosporins (e.g., Cefotaxime), fluroquinolones (e.g., ciprofloxacin), carbapenems (e.g., *Cilastatin*) and aminoglycosides (e.g., gentamicin). The β-lactam antibiotics have a β-lactam ring in their molecular structure. They include penicillin, cephalosporins, monobactams, and carbapenems. They are bactericidal antibiotics that bind covalently to and inhibit penicillin-binding proteins (PBPs). Resistance to β-lactam antibiotics is now widespread and results primarily from β-lactamase production. It can also result from the production of altered PBPs (such as PBP2a) and, among gram-negative bacteria, the exclusion of drugs that generally diffuse through porins to their site of action. Gram-negative β-lactamases are strategically located just beneath the outer lipopolysaccharide layer, which acts as the barrier to drug penetration, while Gram-positive bacteria secrete β-lactamases into their immediate surroundings. Many different βeta-lactamase enzymes vary in their specificity for β-lactam drugs.

With proper treatment, the BSFL can be used as stable feedstock. Therefore, post-harvest processing is critical as it significantly impacts the chemical and microbiological properties of all food and feed ingredients. In the case of BSFL, given the substrates on which they grow, serious consideration must be given to avoid microbial contamination of the final product, as this can lead to animal and, ultimately, human disease. BSFL can be reared from many organic side streams such as kitchen waste, brewer’s waste, and animal manure or from the wild (Van-Huis, 2013).

2.4. Life cycle of the Black Soldier Fly

The BSF, a tropical and temperate dipteran insect, is adapted to large-scale production considering its short life cycle, the great size of its immature stage, the high number of eggs, and bioconversion efficiency. It takes 10 to 52 days for the larvae to reach the prepupal stage, which is the right size for use in animal feed. Figure: 2.1 gives a summary of the life cycle of the BSFL.



(Adapted from: http://uniquebiotechnology.com/).

Figure 2.4.1. Life cycle of the Black Soldier Fly Larvae.

2.5. Insect Processing.

Processing has a significant impact on final product quality and can lessen microbial burden while enhancing color and nutritional value (Bogusz et al., 2022). According to Kooh et al., (2019), it is necessary to undertake several hygienic procedures when using insects as a source of food or feed in order to lower microbial populations. Several hygienic precautions that can be taken to ensure that the finished product is free from insects are highlighted in Figure 2.5.1. In Africa, a variety of traditional processing techniques, including steaming, boiling, roasting, toasting, frying, smoking, and drying, as well as combinations of these, have been used.(Nyangena et al., 2020) as a way of decontaminating and increasing the shelf life of insects. The effectiveness of the above stated commonly used processing methods is not thoroughly established (Mutungi et al., 2021). The present study evaluated the effectiveness of oven- and sun-drying at hygiene quality improvement and assess the presence of AMR in the isolated bacteria. This is in line with Medigo et al., (2017), who indicated the need for any processing method to reduce microbial load while maintaining the nutritional value of the food.

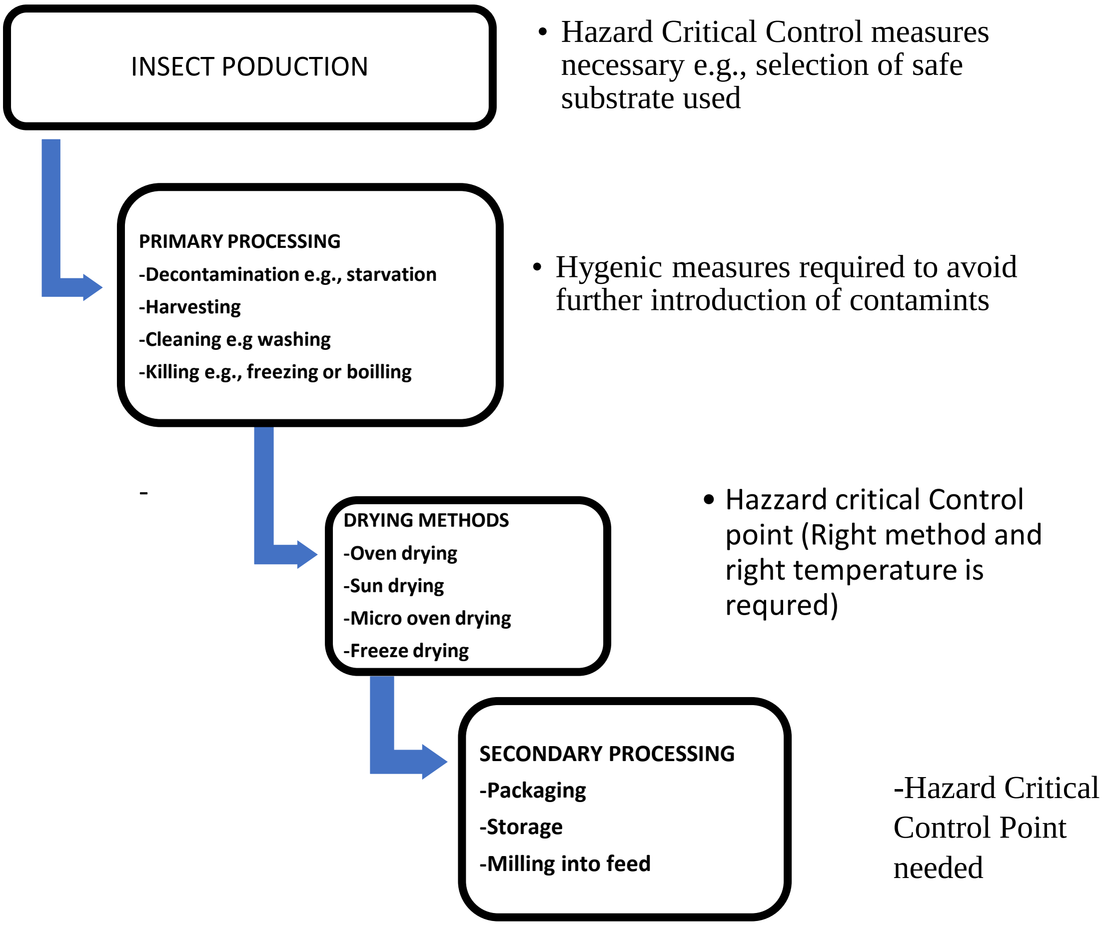


Figure 2.5.1. Schematic drawing of necessary Hazards Critical Control Points (HACCP) during insect processing.

2.5.1 Oven and Sun-drying

To extend the shelf life and safety of edible insects, drying (such as roasting, frying, sun-drying, freeze-drying, and microwave-assisted drying) is frequently utilized (Melgar-Lalanne et al., 2019). In many insect species, including termites, grasshoppers, and caterpillars, sun-drying is a time honoured, low-cost approach that has been effective especially where ambient temperatures are high to about 38°C while where temperatures are low the method is considered to be less effective due to being labour intensive and time consuming as it takes long to dry. However, the favoured techniques for drying whole insects are oven and freeze drying (Melgar-Lalanne et al., 2019) as they are fast and adapted for commercial production of insects for food and feed.

In order to evaluate the effectiveness of the traditional methods, Mujuru et al., (2014) in Zimbabwe conducted a study on the microbiological quality of *G. belina* processed using different traditional methods: boiling in salted water (5% w/w salt; 30 min) followed by solar drying and boiling in salted water followed by open-pan roasting; drum roasting or hot-ash roasting, all after degutting of the insects. Hot-ash roasting was least effective and retained the highest levels of coliforms, *E. coli* and *S. aureus* whereas these organisms were not detected in the boiled and open-pan roasted samples. Sun-drying of the boiled samples encouraged recontamination by molds. The results revealed the importance of sanitation and hygienic handling during processing and that different processing methods show different decontamination effects.

Nyangena et al., (2020) also compared the effect of toasting (5 min, 150°C), boiling (5 min, 96°C), solar-drying (2 days, 50-60°C, 15-25% RH) and oven-drying (2 days, 60°C) on *Acheta domesticus, H. illucens* and *Spodoptera littoralis* observed that in all the species, an important reduction was detected for TVC and *Staphylococcus aureus*, yeasts-moulds and *Salmonella* spp. when toasting or boiling were used. On the contrary, solar-drying and oven-drying could not guarantee the same results, if not preceded by toasting or boiling (Nyangena et al., 2020).

In Nigeria, Braide et al., (2011) reported high bacterial and fungal populations in processed (degutted, washed, spiced, roasted and sun-dried) *Bunaea acinoe* larvae, and isolated *Pseudomonas* and *Proteus* spp. in addition to the toxigenic *S. aureus, B. cereus* and *E. coli* which shows inadequate processing or post-processing contamination. In a separate study, the quality characteristics of *Rhynchophorus phoenicis* collected in the tropical rainforest zone of Nigeria were reported (Opara et al., 2012). *E. coli* and *Klebsiella aerogenes* were identified in the freshly harvested, whereas *Staphylococcus* spp. was isolated in heat-processed samples collected from hawkers. The contamination of heat-processed *R. phoenicis* was attributed to insufficient heat processing and unhygienic handling by healthy carriers of *Staphylococcus* spp.

Nyangena et al., (2020); Braide et al., (2011) reported processing methods with different combinations without referring to an established standard for animal feed. Most of the studies were done on edible insects. There is no laid down procedure to achieve a safe product that can systematically be followed. Moreover, these studies were done in different countries with different habitants on farmed insects, meanwhile Ngwenzi et al., (2021), emphasizes the importance of considering the source of the larvae. Difference substrates carry different bacteria. With reports of AMR in poultry in Zambia (Mukonka et al., 2022), there is need to assess specific methods that will be used in the country to ensure feed safety. As a result, there is a need to standardize and monitor the efficiency of the processing methods in order to ensure the safety and nutritional value of insect-based food/feeds (Mutungi et al., 2019).

In general, oven drying is most commonly used since it is the least expensive among the more efficient drying processes. In addition, it can be easily adapted to continuous industrial operations (Saucier et al., 2020). Temperatures between 50°C and 120°C are generally applied from an hour to a few days, but lower temperature is favourable in order to maintain protein solubility and to reduce Maillard reaction, shrinkage and tissue collapsing (Larouche et al., 2019). Despite this advantage, traditional and commonly used methods for rural and medium-income people have to be evaluated for their efficiency and extent they offer safety in insects in Africa (Mutungi, et al., 2019) and assess the presence of AMR. Therefore, the present study evaluated the two traditional methods for bacterial quality improvement and evaluated the presence of AMR as a way of ensuring safety in BSFL for food/feed

CHAPTER THREE

MATERIALS AND METHODS

3.1. Study area

Samples were collected from the dumpsite for a commercial poultry farm rearing over 100,000 layers. The farm is in Kitwe, Copperbelt Province of Zambia. The farm uses an intensive rearing method where chickens are housed and kept in cages, and water, heating, and feed are automated. In this case, the dump site is where poultry carcasses, feed wastes, excrements, process wastewater, and other wastes associated with poultry confinement from a poultry feeding operation are disposed off

3.2. Study design

An experimental study design was used in the study where the BSFL was exposed to two different processing conditions according to the two drying processing methods under consideration (oven- and sun-drying)

3.3. Sampling method

BSFL samples were collected using the purposive sampling, based on the fact that Copper University would be getting their BSF starter Larvae for use in their laboratory for mass rearing. The larvae were collected at the pre-pupae stage. Seventeen BSFL samples, each weighing 200 grams were collected. Each sample was divided into two (100 grams) groups according to the two drying methods. The number of samples collected depended on the number of heaps with larvae found on the site.

The larvae were collected using gloved hands using forceps for picking the larvae and were then put in small sterile buckets to avoid scorching by the sun's rays. After collection, the larvae were placed in Ziploc plastic bags and in cool boxes with ice packs to prevent the larvae from deteriorating. Upon reaching the Copperbelt University Microbiology laboratory, the samples were washed with tap water to remove debris. After washing, samples were packed in Ziploc bags, frozen at -20°C for storage according to(CDC, 2014) transportation to the microbiology laboratory at the University of Zambia, Lusaka, where laboratory analysis was conducted.

3.4. Processing

Upon arrival at the laboratory, each sample was given a unique identification number before drying, isolating, and quantifying the bacteria and their indexes. Samples were then dried according to the study's selected methods before being cultured for bacterial load count and identification. Processing involved two stages: firstly, the samples were thawed at room temperature, then processed according to the selected methods.

3.4.1 Oven-drying

Freshly thawed BSFL weighing 100g was put on kitchen paper on trays with holes for air circulation in the oven (Figure 3.4.1). Then the larvae were dried at 65°C in a one-night phase of 16 hours followed by a two-day phase of each 4 hours where trays were reshuffled in between phases accordingly. The process was completed when the larvae were hard with a dry texture and shrunken shape, weighing 25-35% of the original weighed fresh larvae (about 25 to 35g).

Figure 3.4.1 Oven-drying of BSFL (Picture: Prudence).

3.4.2 Sun-drying

Gloves were worn whenever handling with the BSFL. The raw insects (100 g) were placed in the sun in individual plastic plates as indicated in figure 3.4.2, first disinfected with 70% ethanol before use. Then, the larvae were dried according to the condition of the day temperatures. Samples were considered dry when the yield was within the 25-35% acceptable range. The products were removed from the sun and microbiological analysis was done.

Figure 3.4.2. Sun-dried samples of raw BSFL in plastic plates.

3.5. Isolation and Identification of Bacteria

The figure 3.5.1 summarizes the procedures for bacterial isolation and AMR gene identification that were carried out during the study.

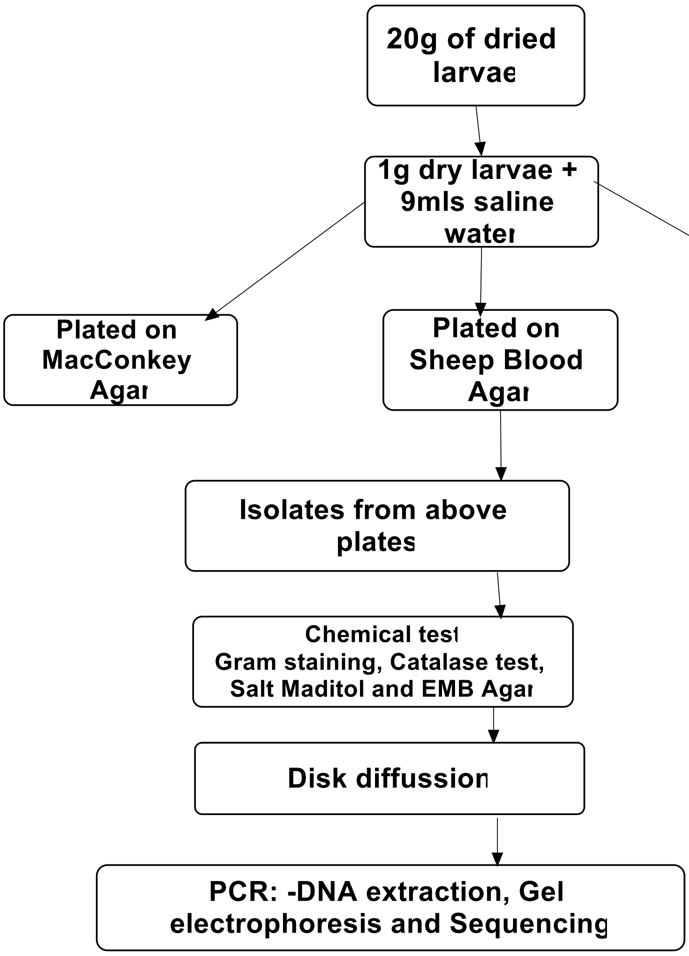


Figure 3.5.1. Schematic representation of the bacterial isolation and AMR gene identification

3.6 Enumeration of bacterial load

Twenty gram of each dried BSFL sample was weighed and shredded using a hand mortar pre-rinsed with 70% ethanol and sterilized by autoclaving. This was done to homogenize the BSFL samples. Then 1g of the homogenized dried BSFL sample was transferred into a test tube containing 9mL of normal saline, and the mixture was vortexed for 1 min. Then 1 mL of the mixture was serially diluted up to 10^3 fold in 9 mL of the diluent. Estimated Total Plate Counts (ETPC) were determined by plating 100 µL of diluted solution on each nutrient agar plate and spreading using a spreader around the agar plate. This was in duplicate on Nutrient agar (Hi Media M001) and were then incubated at 37°C for 24 hours according to Gutierrez et al., (2018). The ETPC estimated the total viable aerobic microbial population present in the dried BSFL samples. Colonies were counted from all the plates, and the average colony number was then expressed as Colony-Forming Units per mL (CFU/mL) for each dried BSFL sample. One Nutrient Agar plate was plated with saline water as a negative control.

3.7 Characterization of hygienic indicator bacteria (*E. coli* and *Staphylococcus*)

Selective media was used to assess the presence of *E. coli* and *Staphylococcus* as fecal and hygiene indicator bacteria representing the Gram-negative and Gram-positive bacteria respectively. MacConkey Agar and Sheep Blood Agar were used to grow the two species. The culture was inoculated on the agar surface by picking single colonies from previously prepared MacConkey and Sheep Blood Agar plates for both bacteria and streaking on the respective media. Characteristics features of the colonies on solid agar media were used to identify the bacteria in this case. MacConkey Agar (HiMedia M001) was used to determine *E. coli* following 24 hours of incubation of the cultured plates at 37°C. Typical colonies were distinguished by their pink-red color as a general property for all the *Enterobacteriaceae*, while Sheep Blood Agar was used to grow *Staphylococcus* spp. bacteria, and differentiation was based on their haemolytic properties and circular, smooth, convex appearance.

Further, Gram staining was used to identify Gram-positive and Gram-negative bacteria. Gram staining technique was done as described by Tripathi & Sapra, (2022). The pure colonies produced was Gram-stained to determine Gram-positive or Gram-negative nature and microscopic morphological appearance. Each individual pure colony was first emulsified in sterile normal saline on a well labelled, clean, dry glass slide. This was then air-dried and fixed under a Bunsen burner. Thereafter, the slides were stained with Crystal Violet solution for 45 seconds, and then washed under gently running tap water. The slides were then flooded with Iodine solution for 45 seconds and then gently washed under gently running tap water. The slides were then be decolorized with 70 percent alcohol solution, followed by gentle washing under gently running tap water. Finally, the slides were counter-stained with safranin solution for 45 seconds, followed by gentle washing under gently running tap water. The slides were air dried and viewed at X100 magnification under oil immersion. The microscopic morphology characteristics and Gram-stain was viewed and noted. The purpose of staining was to confirm if our samples are really Gram-negative or positive as literature say.

Finally confirmatory tests, such as the catalase test on *Staphylococcus* spp., were done to distinguish it from *Streptococcus*. Catalase is an enzyme that converts hydrogen peroxide to water and oxygen gas. To this end, catalase test was performed according to the American Society for Microbiology, (2016). A small number of bacteria isolated using a sterile disposable inoculating loop was got from plate agar where it was grown for at least 24 hours and put on a sterile glass slide. Then one drop of hydrogen peroxide was added using a dropper and bubbles appeared (due to the production of oxygen gas), then the bacteria were said to be catalase positive. Mannitol Salt Agar was also used to identify *Staphylococcus*, and a yellow colour appearance was used as an indicator for the bacteria. Eosin methylene blue (EMB) media was further used to distinguish *E. coli* from other *Enterobacteriaceae*. This resulted in growth with green-metallic sheen colonies.

3.8. Antibiotic sensitivity test on isolated bacteria

Isolates identified as *E. coli* and *Staphylococcus* were subjected to AST against the following antibiotic drugs: cefotaxime 30 µg (CTX), tetracycline 30 µg (TET), gentamicin 10 µg (GEN), ciprofloxacin 5 µg (CIP), ampicillin 5 µg (AMP), co-trimoxazole 25 µg (SXT) and chloramphenicol 30 µg (CHL) on Muller-Hinton Agar. The diameter of inhibition surrounding each antibiotic disk was measured, and the results for sensitivity were noted after 24 hours at 37°C by measuring the zone of inhibition with a standard scale and interpreting the results according to International Clinical Laboratory Institute (CLSI 2020) and European Committee on Antimicrobial Susceptibility Testing (EUCAST/ECOFFS). Isolates displaying resistance to three or more classes of antimicrobials were defined as multidrug-resistant (MDR), as proposed by Magiorakos et al. (2012).

3.9. Quantification of resistant bacteria

The quantification was done by swabbing the inoculum on the surface of Mueller Hinton Agar completely. After swabbing, the plates were allowed to dry for five minutes before placing the antibiotic disks on the agar. Then, using a pair of sterile forceps, antibiotic discs were removed from the dispenser and placed on the swabbed agar. The plates were then incubated upside down at 37 °C. This was done in duplicates, and the average disk diameters were used to estimate the antibiotic sensitivity.

3.10. Polymerase chain reaction (PCR) for determination of ESBL-encoding genes

To further identify ESBL genes among the isolated bacteria associated with the processed BSFL, the PCR method allowed prompt identification of the genes. Genomic DNA was extracted using QIAamp DNA mini kit (QIAGEN). The extracted DNA was then stored at 20°C. PCR was done as summarized in the tables below. This was done to amplify and detect the genes that confer resistance to broad-spectrum cephalosporins, such as CTX; these genes include *bla*$_{\text{CTX-M}}$, *bla*$_{\text{TEM}}$, *bla*$_{\text{OXA}}$, and *bla*$_{\text{SHV}}$. Table: 3. 10.1 summarizes the conditions for the PCR used.

Table: 3. 10.1 Conditions for PCR

Steps	Temperature	Period	Cycles
Initial denaturation	94°C	1 min	-
Denaturation	94°C	10 sec	35
Annealing	59.5°C	15 sec	35
Extension	68°C	30 sec	35
Hold	4°C	∞	

The PCR products were run through electrophoresis on ethidium bromide-stained 1.5% agarose gel and a UV trans-illuminator was used to visualize the amplified DNA bands with band size 544 bp for *bla*$_{\text{CTX-M}}$. To run the PCR, Takara Enzyme was used under the conditions indicated in Table: 3. 10.2.

Table 3.10. 2 PCR Master Mix for blaCTX-M, blaTEM, blaSHV, and blaOXA

Reagents	Volume (1 µL)
Nuclease-free water	9.5
Forward primer	0.5
Reverse primer	0.5
DNA template	1.5
Takara enzyme	12.5
Total	24.00

The PCR product was then purified using the Wizard ® SV gel and PCR Clean-Up System kit (Promega) according to the manufacturer's instructions, as attached in the appendix. Sequencing for confirmation was done using Big Dye, 5X sequencing buffer, primers, and nuclease-free water as indicated in the table: 3.10.3

Table: 3. 10.3 Requirements for sequencing using the Big Dye method

Reagent	Volume (µL)
Big dye	1.00
Primers	0.32
DNA template	0.32
5x sequencing buffer	5.00
Nuclease free water	3.80
Total	9.88

The amplified DNA fragments of the isolate were sequenced using forward primer and reverse primers in Table 3.10.5. The sequences were assembled using SnapGene and the obtained consensus sequences were subjected to a BLAST search on the National Centre for Biotechnology Information platform. PCR products were sequenced under the condition shown in Table 3.10. 4. The sequencing was based on Sanger method of sequencing.

Table 3.10. 4. Conditions for the Big Dye Kit

NO-		Steps	Temperature	Period	Cycles
1		Initial denaturation	96°C	1 min	-
2		Denaturation	96°C	10 sec	35
3		Annealing	50°C	5 sec	35
4		Extension	60°C	4 min	35

Table 3.10.5 indicates all the primers used to determine ESBL and *MecA* genes of resistance. The primer sequences, target, amplification product sizes (bp) and references are included in the table

Table: 3.10.5 List of primers used

Name	Sequence	Size (bp)	Target	Reference
CTX-MA1	*SCSATGTGCAG≠YACCAGTAA	544	*bla*$_{\text{CTX-M}}$	(Pokhrel et al., 2014)
CTX-MA2	CCGC¥RATATGRTTGGTGGTG			
yaiO-F	TGATTTCCGTGCGTCTGAATG	115	*E. coli*	(Molina et al., 2015)
yaiO-R	ATGCTGCCGTAGCGTGTTTC			
SHV-F2	AGGATTGACTGCCTTTTTG	392	*bla*$_{\text{SHV}}$	(Colom et al., 2003)
SHV-R2	ATTTGCTGATTTCGCTCG			
TEM-C	ATCAGCAATAAACCAGC	516	*bla*$_{\text{TEM}}$	(Mabilat & Courvalin, 1990)
TEM-H	CCCCGAAGAACGTTTTC			
OXA-F	ATATCTCTACTGTTGCATCTCC	619	*bla*$_{\text{OXA}}$	(Colom et al., 2003)
OXA-R	AAACCCTTCAAACCATCC			
SAU327	GGA CGA CAT TAG ACG AAT CA	1250	*S. aureus*	(Rocchetti et al., 2018)
SAU1645	CGG GCA CCT ATT TTC TAT CT			
MRSA$_1$	AAAATCGATGGTAAAGGTTGGC	533	*mec*A	(Rocchetti et al., 2018)
MRSA$_2$	GTTCTGCAGTACCGGATTTGC			

Note; *S = G or C, ≠Y = C or T, ¥R = A or T.

3.11. Determination of MRSA

Luria Bertani (LB) agar supplemented with 64 µg/mL of cloxacillin was inoculated with *Staphylococcus* and incubated at 37°C for 24 hours. Then a single colony was transferred to LB broth media containing 64 µg/mL of cloxacillin and incubated for 18 hours at 37 °C. The broth was then subjected to DNA extraction using the Quick – DNA Miniprep plus Kit (Zymo) following the manufacturer’s instructions. DNA concentration was measured using the Nano Drop 1000 equipment, and PCR was carried out using KOD enzyme by using forward and reverse primers for the gene amplicon to identify the *mecA* gene of resistance.

3.12 Data Processing and Analysis

Data on bacterial load was firstly cleaned using the pivot table in Excel, the imported to Jamovi 2.3.21.0 version for analysis using a Mann Whitney U test in order to check for the significance of the two processing methods used. AST was analyzed for visualizations using Excel 2013 version (Silicon Valley, USA).

3.13 Ethical consideration

Ethical approval was sought from the University of Zambia Biomedical Research Ethics Committee (UNZAREC) with approval number 2227-2022.

CHAPTER FOUR

RESULTS

4.1 Microbial load

This study compared the Aerobic Plate Count (APC) and CFU/mL for oven- and sun-drying methods. The results revealed that the Average Plate Count (APC) ranged from 100 CFU/mL to 1.1 x 10^4 CFU/mL and 3 to 3.6 x 10^4 CFU/mL for oven- and sun-drying methods, respectively. Meanwhile, the Estimated Aerobic Total Plate Count (EATPC) was 3.0 x 10^3 CFU/mL and 2.2 x 10^4 CFU/mL for oven-drying and sun-drying methods, respectively. Colonies per sample are as indicated in the Table 4.1 and are shown side by side for both oven- and sun-drying methods for easy comparison.

Table 4.1.1: Colony Forming Unit for both oven-dried and sun-dried BSFL per sample

SAMPLE NO.	Sun Drying		Differences between methods CFU/mL/sample	Oven Drying	
	Colonies/mL	CFU/ mL/Sample		Colonies /mL	CFU/mL/ Sample
1	26000	2.6 x 10^4	2.4 x 10^4	2000	2.0 x 10^3
2	33000	3.3 x 10^4	3.3 x 10^4	1000	1.0 x 10^3
3	3000	3.0 x 10^3	2.55 x 10^3	450	4.5 x 10^2
4	10000	1.0 x 10^4	9.73 x 10^3	270	2.7 x 10^2
5	18000	1.8 x 10^4	1.5 x 10^4	3000	3.0 x 10^3
6	26000	2.6 x 10^4	2.55 x 10^4	450	4.5 x 10^2
7	28000	2.8 x 10^4	2.7 x10^4	1000	1.0 x 10^3
8	36000	3.6 x 10^4	3.4 x 10^4	2000	2.0 x 10^3
9	27000	2.7 x 10^4	1.6 x 10^4	11000	1.1 x 10^4
10	28000	2.8 x 10^4	2.1 x 10^4	7000	7.0 x 10^3
11	16000	1.6 x 10^4	6.0 x 10^3	10000	1.0 x 10^4
12	25000	2.5 x 10^4	1.9 x 10^4	6000	6 .0 x 10^3
13	31000	3.1 x 10^4	2.3 x 10^4	8000	8.0 x 10^3
14	9000	9.0 x 10^3	8.73 x 10^3	270	2.7 x 10^2
15	9000	9.0 x 10^3	8.55 x 10^3	450	4.5 x 10^2
16	21000	2.1 x 10^4	1.9 x 10^4	2000	2.0 x 10^3
17	36000	3.6 x 10^4	3.6 x 10^4	000	<100
TAPC	382000	N/A	N/A	54890	N/A
Average	22000	2.2 x 10^4	1.9 x 10^4	3000	3.0 x 10^3

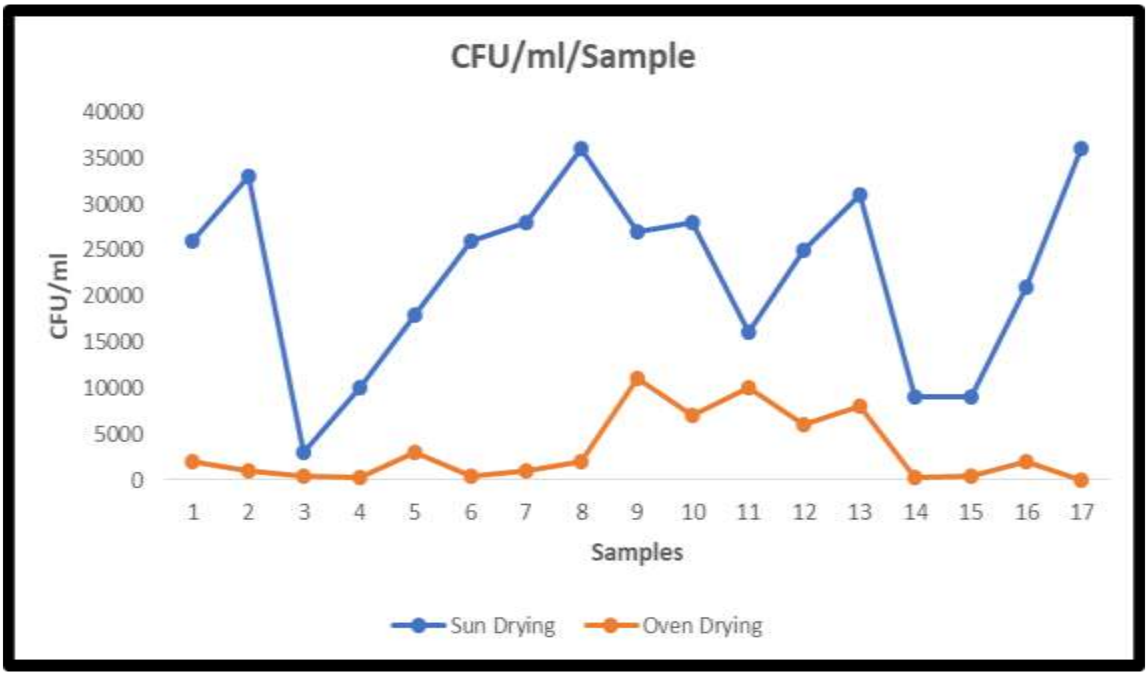


Figure: 4.1.1 Line graph for colony trends for each sample per method

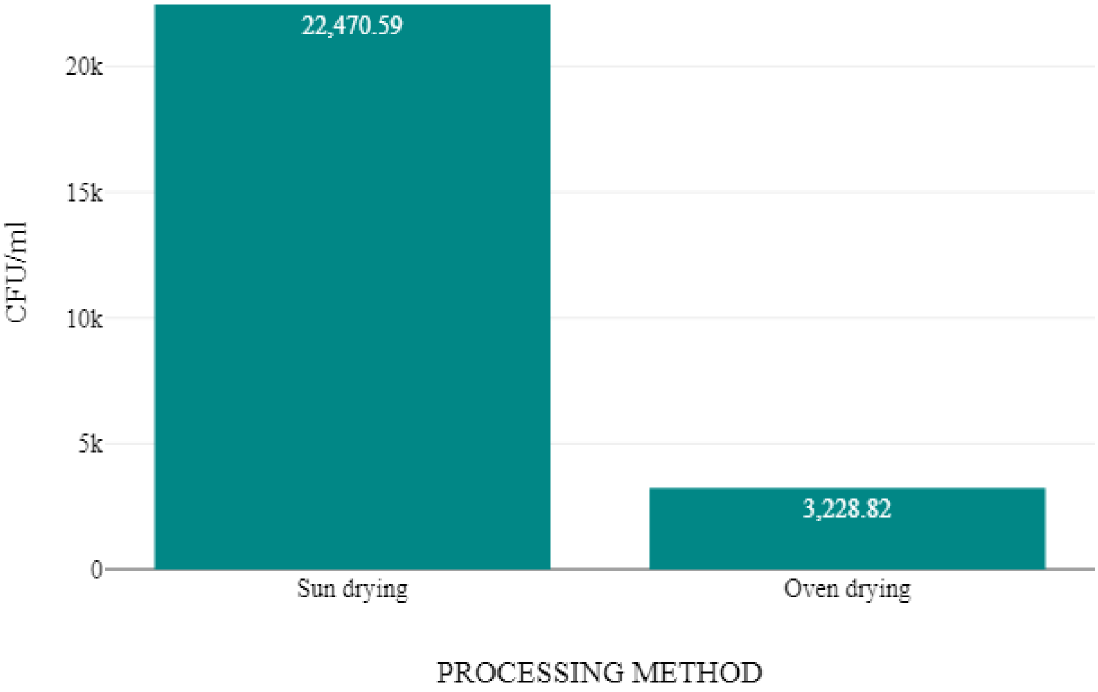


Figure: 4.1.2. Bar chart showing mean CFU/mL for oven- and sun-drying methods

Table 4.1.1 and figure 4.1.1 and 4.1.2 shows the difference in the ETPC. Figure 4.1.1 shows the colony trends per sample per method and figure 4.1.2 shows the different means for each method. The results generally in the figures above shows the differences in the two processing methods. Statistical analysis using a Mann Whitney U Test showed a significant difference ($p<0.001$) in the median of the ETPC obtained from samples dried by both oven- and sun-drying.

4.2 Isolation of hygienic indicator bacteria

Thirty-three (33) bacterial isolates were obtained from both oven- and sun-dried BSFL samples as indicated in Table 4.2.1 by using selective culture media (figure 4.2.1) and biochemical tests accomplished by gross colony morphology examination and colour changes (figure 4.2.2) The study limited itself to *E. coli* and *Staphylococcus* as they represent fecal and hygienic indicators. They also have the ability to acquire genes coding for extended-spectrum β-lactamases (conferring resistance to broad-spectrum cephalosporins) and Methicillin Resistant *Staphylococcus Aureus* (MRSA) which contain *mecA* gene coding for a variant penicillin binding protein (PBP2A) and cause resistance to a penicillin class (Poiret, et al., 2018).

Figure 4.2.1 Left; MSA plate with *Staphylococcus*. Right; EMB Agar plate with *E. coli* isolates respectively.

Figure 4.2.2.: Microscope images from Samples PS8 and PS16. Left; *E. coli* and Right; *Staphylococcus*.

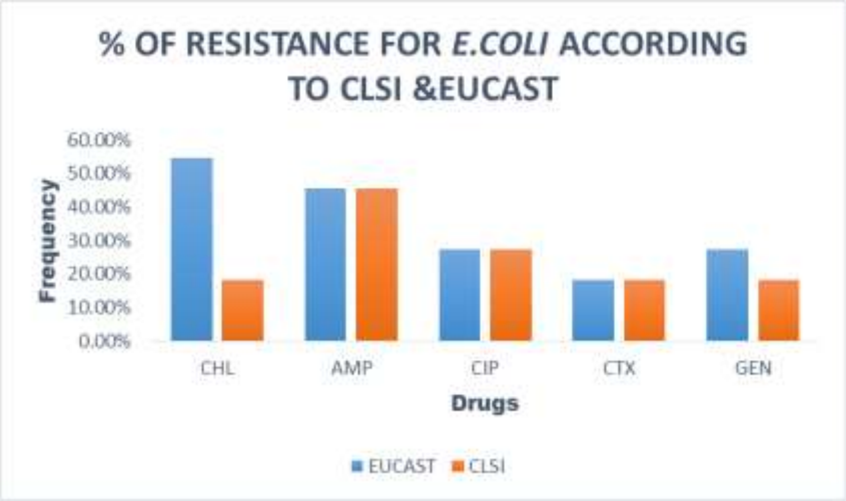
Table 4. 2.1. Total Bacterial isolates from oven- and sun-drying.

	Oven-drying	Sun-drying	Bacteria	Sun-drying	Oven-drying	Bacteria	Isolates positive for bla_{CTX}-M Gene	Isolates positive for *meCA* Gene
1	PS1	PO3	*Staphylococcus*	PS3	PO13	*E. coli*	nil	nil
2	PS2	PO6	*Staphylococcus*	PS3	PO14	*E. coli*	“	“
3	PS2	P07	*Staphylococcus*	PS9		*E. coli*	“	“
4	PS2	PO10	*Staphylococcus*	PS6		*E. coli*	“	“
5	PS3	PO14	*Staphylococcus*	PS7		*E. coli*	“	“
6	PS4	-	*Staphylococcus*	PS8		*E. coli*	**PS8**	“
7	PS5	-	*Staphylococcus*	PS14		*E. coli*	nil	“
8	PS6	-	*Staphylococcus*	PS16		*E. coli*	“	“ ‘
9	PS8	-	*Staphylococcus*	PS17		-	-	“
10	PS7	-	*Staphylococcus*	-		-	-	“
11	PS10	-	*Staphylococcus*	-		-	-	“
12	PS11	-	*Staphylococcus*	-		-	-	“
13	PS12	-	*Staphylococcus*	-		-	-	“
14	PS14	-	*Staphylococcus*	-		-	-	“
15	PS15	-	*Staphylococcus*	-		-	-	“
16	PS5	-	*Staphylococcus*	-		-	-	“
17	PS16	-						
Total	17	05		09	02			

4.3 Antimicrobial susceptibility tests

AST was carried out on 33 characterized fecal and hygienic indicator bacteria isolates, 33.3% (11/33) *E. coli* and 66.7% (22/33) *Staphylococcus*. These bacteria represented the Gram-negative and Gram-positive species, respectively. Two standards testing bodies were used to interpret the results of the AST (CLSI and EUCAST/ECOFFs). EUCAST/ECOFF was used since the BSFL was got from the wild environment where ECAST/ECOFFs guidelines are used. The CLSI was also used considering the illnesses the bacteria characterized may present may affect animals and eventually human beings where CLSI guidelines for the clinical routine screenings is used.

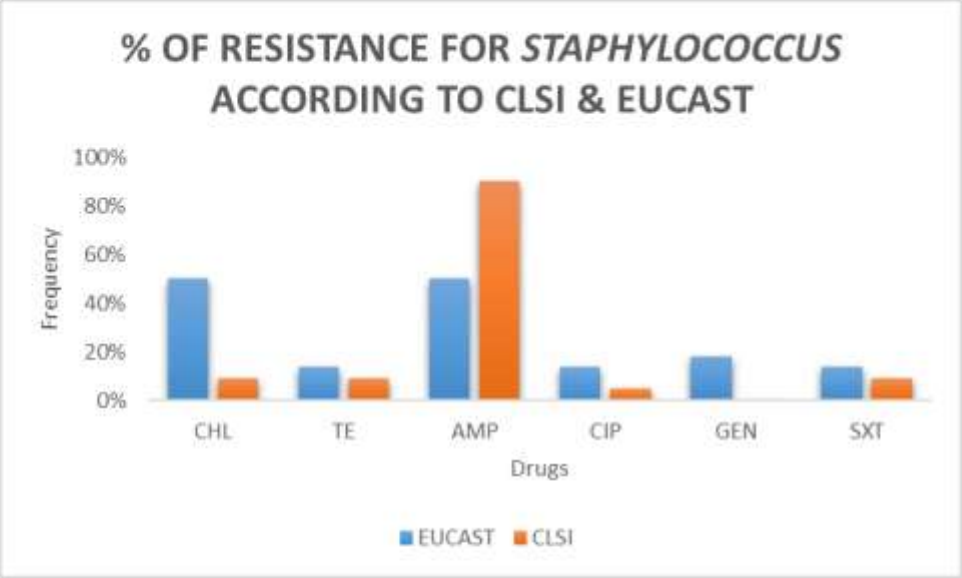
AST for *E. coli* based on the CLSI (2020) and EUCAST/ECOFFs guidelines showed only minor differences between the two standards. The proportion of resistance to AMP, CIP, and CTX were the same regardless of the approach used. However, EUCAST guidelines showed a much higher resistance rate to CHL compared to CLSI standard. This is summarized in the Figure: 4.3.1 Furthermore, while the proportion of GEN resistance was higher when EUCAST guidelines were applied compared to CLSI, this difference was minimal. Altogether, resistance was highest to CHL (54.4%) followed by AMP (45.5%), CIP 27.3%), GEN (27.3%), and CTX (18.2%) when EUCAST guideline were applied. On the other hand, when the CLSI were applied, AMP (31.2%) showed the highest resistance rate, followed by CIP (27.3%, CHL (18.2%), CTX (18.2%), and GEN (18.2%).



Key: CHL= chloramphenicol, CTX = cefotaxime, AMP = ampicillin, CIP= ciprofloxacin, Gen = gentamicin.

Figure: 4.3.1. Percentage of Resistance for *E. coli*. According to CLSI and EUCAST.

Despite the general less resistance AST results observed for *Staphylococcus* according to both CLSI and EUCAST/ECOFFs, AMP had high resistance (90% and 54.5% according to CLSI and EUCAST/ECOFFs respectively. Figure 4.3.2 gives a full summary of the resistance pattern according to both methods.



Key: CH = chloramphenicol, TE = tetracycline, AMP = ampicillin, CIP = ciprofloxacin, GEN = gentamicin, SXT = co-trimoxazole

Figure: 4.3.2. Percentage of Resistance for *Staphylococcus* according to CLSI and EUCAST

4.4. Multidrug resistance

To check for MDR, isolates were subjected to the disk diffusion test using seven different antibiotic classes. The results showed that, 24.2% (8/33) of the total isolates were resistant to at least three or more drug classes. *E. coli* was resistant to three or more drugs classes by 9.1% (3/33) while *Staphylococcus* was resistant by 15.2% (5/33).

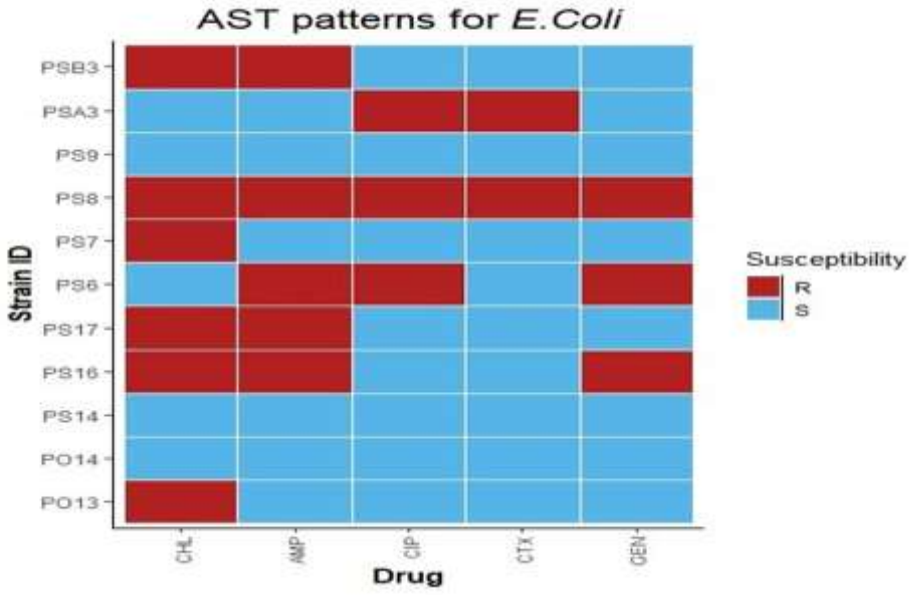


Figure: 4.4.1. MDR pattern for *E. coli.*

R = resistance, S = susceptibility, FEP = cefepime, AMP = ampicillin, CIP = ciprofloxacin, GEN = gentamicin.

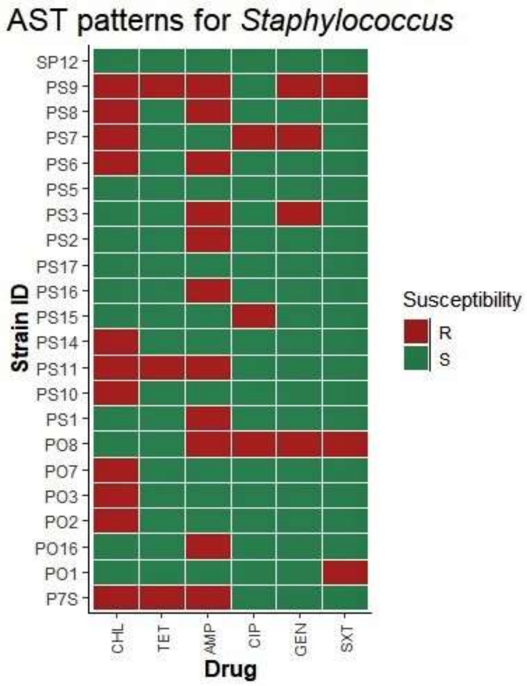


Figure: 4.4.2. MDR Pattern for *Staphylococcus*.

R = resistant, S = susceptible, CHL = chloramphenicol, TE = tetracycline, AMP = ampicillin, CIP = ciprofloxacin, GEN = gentamicin, SXT = cotrimoxazole

Figure 4.4.1 and 4.4.2 above shows the MDR pattern for *E. coli* and *Staphylococcus*, respectively. Figure 4.4.1 shows that, *E. coli* isolates were 27.3% (3/11) resistance to at least three or more drug classes, while Figure 4.4.2 shows that *Staphylococcus* isolates were 22.7% (5/22) to at least three or more drug classes

4.5. Detection of AMR genes

Based on the phenotypic profiles (i.e., resistance to penicillins and cephalosporins), the *E. coli* isolates were screened for ESBL genes. Of the 11 CTX-resistant strains, one tested positive for the *bla*$_{\text{CTX-M}}$ gene, which yielded a 544 bp band on electrophoresis. Figure 4.10, shows the image of the gel electrophoresis for *bla*$_{\text{CTX-M}}$ gene. However, none of the strains tested positive for *bla*$_{\text{TEM}}$, *bla*$_{\text{SHV}}$, or *bla*$_{\text{OXA}}$. Meanwhile, the *mecA* gene in *Staphylococcus* is associated with resistance to anti-Staphylococcal penicillins such as methicillin and cloxacillin. When the

Staphylococcus strains in this study were streaked on cloxacillin-supplemented plates, 66.7% (22/33) exhibited resistance. However, despite the high rate of cloxacillin resistance, none of the *Staphylococcus* isolates tested positive for the *mecA* gene.

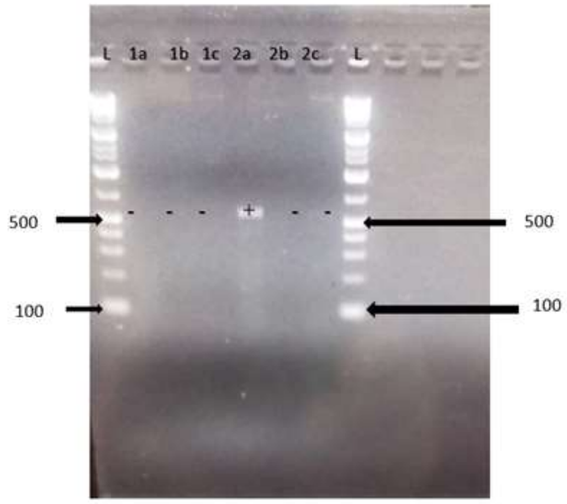


Figure 4.5.1.: DNA maker (L), gel image without bands Z. 2a positive band *bla*$_{\text{CTX-M}}$ (544 bp) gene

CHAPTER FIVE

DISCUSSION

5.1. General discussion on the BSFL

This study evaluated oven- and sun-drying commonly used traditional processing methods at hygienic quality improvement in BSFL. The results showed that the oven-drying method was 86.4% more effective at improving the hygienic quality of BSFL products compared to the sun-drying method. While the two processing methods reduced the bacterial load below (10^9 CFU/mL) the threshold for animal feed materials, a few bacterial species were still isolated from the processed BSFL. When two AST standards (CLSI and EUCAST) were applied to these isolates, the results were similar, although the EUCAST indicated higher CHL resistance in both species and AMP resistance in *Staphylococcus*. Regardless of the standard used, AMP resistance was high in both species, while CIP resistance was only significant in *E. coli*. Importantly, MDR was detected in both species, including the *bla*$_{\text{CTX-M}}$ gene in one (9.10%) *E. coli* strain. However, while cloxacillin resistance was prevalent in *Staphylococcus*, none of the strains tested positive for the *mecA* gene.

The results of the bacterial load for the present study revealed that both oven- and sun-drying suppressed the bacterial load to levels below the acceptable limit (10^9 CFU/mL) (KEBS, 2017) for insects to be safely used in animal feed. While the result highlights that both methods effectively ensure food safety, the effect of oven-drying was significantly more. This was probably due to the higher temperature (65°C) associated with oven-heating compared to sun-drying whose temperature depended on the weather of the day. Sun-drying was done in May when the temperatures are usually low in Zambia with mean average temperature of 19.9°C (Mt. Makulu Research Centre Meteorological Station, May 2022). Moreover, a previous study by Nyangena et al., (2020), reported a similar effect at 60°C, although their CFU decrease was smaller than the present study found. The difference in the present study and the previous study from Nyangena, et al., (2020) suggests that a higher temperature is more effective at reducing bacterial load. However, the temperature should not be raised above a certain threshold if the nutritional value of the larvae is to be maintained (Larouche et al., 2019). Therefore, an optimum temperature should be identified to balance between safety and maintenance of nutrients. While the methods used in this study achieved the desired outcome, there was still some bacterial growth even under oven-drying.

The growth was dominated by Gram-positive bacteria where bacilli belong to. This is probably because most *Bacillus* strains (e.g., *B. cereus*) form spores that can withstand harsh environmental conditions, including high temperature (Bogusz et al., 2022). The presence of *B. cereus* is important to consider because of their ability to produce enterotoxins and the presence of spores which makes it difficult to kill. (Braide et al., 2011) This spore-forming organisms may require a severe heat treatment process such as canning (Klunder et al., 2012) and a temperature range which must be investigated.

AST used two standard guideline methods to interpret the results (EUCAST and CLSI). The two methods showed a big difference in CHL resistance, 54.5% and 18.2% resistance against *E. coli* and 50% and 9.1% against *Staphylococcus* according to EUCAST and CLSI were observed respectively. Notably, CHL is an important drug that is used to treat serious diseases like meningitis and Salmonella infection in the hospitals (Modal & Sumanta, 2017). The implication of the differences in the results in AMR surveillance or in the hospitals would be in two folds. Firstly, if EUCAST is used, we might misclassify susceptible isolates as resistant, depriving patients of a potent drug they could benefit from. On the other hand, if we used CLSI, we might wrongly classify resistant isolates as susceptible, resulting in treatment failure.

Highest resistance was observed on ampicillin (90.1%) according to CLSI by *Staphylococcus*. According to the Ministry of Fisheries and Livestock, penicillins are among the most imported antibiotics for animal use in Zambia (Mukonka, 2020). Therefore, the high resistance shown by ampicillin could be due to overuse of the drug in livestock and passing on the resistance to other animals around including bacteria that are found in insects as they feed from the wastes from animals. Therefore, this could be true and the reason for the high resistance observed with ampicillin against *E. coli* and *Staphylococcus* species as the BSFL were collected from a dump site for a poultry farm. The BSFL could have got the resistance from antibiotic residues in the droppings from the chickens which were used to treat diseases.

Thirty-three (33) bacterial isolates from the processed BSFL which were collected from the dumpsite for a layer's poultry farm were found in the study. Insects acquire AMR via the substrates they are raised from as claimed by Gwenzi et al., (2021). This may be the case even with the isolates got in the study, given the location of their collection. Less MDR was also observed in both *E. coli* and *Staphylococcus*, 9.1% (3/33) and 15.2% (5/33) respectively, compared to earlier reports of high MDR *E. coli* in the fresh BSFL in Zambia by un published data by Laiser 2022 who assessed the presence of AMR resistance in fresh BSFL and found out that *E. coli* was 100% MDR to the same antibiotic drugs used in this study. This suggests that processing could have killed most of the bacteria including those that were MDR to the antibiotic drugs used. This indicates that there is very little chance of spreading MDR strains that are difficult to treat through processed BSFL. Moreover, just one isolate (9.1%) tested positive for the *bla*$_{\text{CTX-M}}$ gene, the most prevalent ESBL gene.

5.2 Public health implications on the isolated positive ***Bla***$_{CTX}$**-M gene.**

Antimicrobial resistance (AMR) is a significant threat to both human and animal health (Pomba et al., 2017.) The presence of AMR is of great concern since the occurrence of ESBL producers and other AMR bacteria may not only entail treatment failure in diseased animals, but also poses a potential health hazard to humans, either through direct transmission of AMR bacteria from animals to humans or indirectly through transmission of resistance genes (Nilsson, 2015). Currently, the most prevalent ESBLs belong to the CTX-M type, with CTX-M-15 globally the most common among human clinical isolates (Pomba et al., 2017). The spread of AMR bacteria and genes across systems can occur through many pathways, both related and unrelated to agriculture, including via wastewater, soils, manure applications, direct exchange between humans and animals, and food exposure (Thanner et al., 2016; Marshal, et al., 2011). “Indirect” antibiotic resistance bacteria (ARB) selection also occurs when ARGs conferring different types of resistance are closely located on a chromosome or plasmid, which means the selection for one type of resistance might confer other types of resistance. In turn, enteric ARB shed in faeces can spread to other livestock and farm workers, re-entering the environment and exposing other animals through drainage water and manure (Marshal et al., 2011).

Finally, commensal bacteria such as *Staphylococcus aureus* and *E. coli* can be transmitted to and from food animals and humans who work on farms or live in close proximity to livestock (Voss, et al., 2005). In fact, recent evidence from the Netherlands suggests that immediate proximity to livestock, such as being a farm worker versus a member of their family, significantly increases their carriage of methicillin-resistant *S. aureus* (MRSA); 38% versus 16%, respectively, in nasal and throat swabs (much higher than wider Dutch populations) (Graveland et al., 2011).

5.3. The isolated bacteria of *E. coli* and *Staphylococcus* and its implications in humans and livestock

Bachir & Raho, (2015), indicates that *Staphylococcus aureus* and *E. coli* are a major cause of various humans and animals' infections. The first causes skin and soft tissues infections, surgical site infections, and bone and joint infections. *Staphylococcus aureus* is a common cause of hospital-acquired bacteremia and it is associated with hospital-acquired respiratory tract infections (Abulreesh et al., 2011). *Escherichia coli* are an important zoonotic pathogen that can cause severe human disease, and can be transmitted from animals into humans through consumption of foods made from these animals or contact with foods contaminated from animal wastes (Fegan et al., 2012). *E. coli* is the most common cause of urinary tract infections (UTIs) in humans (Foxman, 2010), and is a leading cause of enteric infections and systemic infections (Kaper et al., 2004). *E. coli* is also leading cause of neonatal meningitis (Kim, 2012). Now, many nosocomial and community-acquired *E. coli* are now resistant to the several important antimicrobial classes (Pitout et al., 2012).

Antibiotic resistant staphylococci are major public health concern since the bacteria can be easily circulated in the environment. Infections due to methicillin-resistant *Staphylococcus aureus* (MRSA) have increased world-wide during the past twenty years (Dersinki et al., 2005; Ippolito et al., 2010). Multiple drug-resistant *S. aureus* have been frequently recovered from foodstuffs, water and biofilm formation (Lancellotti et al., 2012) nasal mucosa of humans (Acco et al., 2003) clinical cases (Stefani et al., 2010) and livestock (Wulf et al., 2008).

CHAPTER SIX

CONCLUSION AND RECOMMENDATIONS

6.1. Conclusion

1. The oven-drying process was much more efficient at improving the bacterial quality of the BSFL, the results for the bacterial load for both methods were below the threshold (10^9 CFU/mL) for insects used as animal feed.

2. The BSFL's bacterial quality can be improved by both methods, moreover, both Gram positive and Gram-negative bacteria were isolated from both methods.

6.2. Recommendation

1. Oven drying is the better method to use between the two methods, but, if one cannot afford, sun-drying can as well be used as it can result in a safe product.

2. If one cannot afford the oven-drying, more diligence is needed in selection of suitable substrate for BSFL rearing.

6.3. Further research and actions:

1. Importantly, a cost-benefit analysis is required to use a method that is sustainable and reduces the cost of feed production, which is the reason for using insects as an alternative source of protein. Given that not everyone has access to ovens, let alone ovens, as well as the costs of using power.

2. A good AMR surveillance system can help by identifying the presence of AMR in the insects for use in animal feed in order to ensure the safety of the feed to be produced.

3. The presence of bacteria and MDR in both methods calls for more research in MDR in BSFL and its relationship to human bacterial strains.

4. There is need to determine whether the MDR could be due to where the BSFL was collected from as a result of multiple antibiotics use in poultry whose wastes the BSFL was collected from or the coexistence of multiple AMR genes on the same plasmid in the bacteria in the BSFL. Future studies need to analyze these strains further by whole-genome sequencing.

5. Probably quantitative determination of fecal contamination would be valuable, but it was out of scope of this master thesis

6.4. Limitations

Despite lack of comparative data from the unprocessed BSFL and the low sample size in this study, the objective of the study was achieved since the samples for both methods were the same and comparison between the two methods was possible.

REFERENCES

1. Abro, Z., Menale, K., Chrysantus, T., Dennis, B. & Gracious, D. (2020). Socio-economic and environmental implications of replacing conventional poultry feed with insect-based feed in Kenya. Journal of Cleaner Production, 265(April), p. 121871.
2. Acco, M., Ferreira, F., S., Henriques, J., A., P. & Tondo, E., C. (2003). Identification of multiple strains of *Staphylococcus aureus* colonizing nasal mucosa of food handlers. Food Microbiol; 20:489-493.
3. Arru, B., Furesi, R., Gasco, L., Madau, F.A. & Pulina, P. (2019). The introduction of insect meal into fish diet. Sustainability, 11(6), p.1697.
4. Raho, G.B. & Abouni, B. (2015). Escherichia coli and Staphylococcus aureus most common source of infection. In The Battle against Microbial Pathogens. Basic Science, Technological Advances and Educational Programs, Méndez-Vilas, A., Ed. Formatex Research Center: Badajoz, Spain.
5. Bogusz, R., Smetana, S., Wiktor, A., Parniakov, O., Pobiega, K. & Rybak, K. (2022). The selected quality aspects of infrared-dried black soldier fly (*Hermetia illucens*) and yellow mealworm (Tenebrio molitor) larvae pre-treated by pulsed electric field', Innovative Food Science and Emerging Technologies, 80(July).
6. Braide, W., Oranusi, S., Udegbunam, L., Oguoma, O., Akobondu, L. & Nwaoguikpe, K. (2011). Microbiological quality of an edible caterpillar of an emperor moth, Bunaea alcinoe. J. Ecol. Nat. Environ. 3:176–80.
7. Clinical and Laboratory Standards Institute/NCCLS. Performance standards for antimicrobial susceptibility testing. 30th informational supplement. Document M100-S30. Wayne, PA. CLSI. 2020
8. Deresinski, S. (2005). Methicillin-resistant *Staphylococcus aureus*: an evolutionary, epidemiologic and therapeutic odyssey. Clin Infect Dis. 2005; 40:562-573.
9. Durbin, R.J. & Senate, U.S. (2000). 'Report to the Honorable Richard J. Durbin, United States Senate', (September).
10. EFSA Journal. (2015). 13(10) Risk profile related to production and consumption of insects as food and feed.

11. Erickson, M.C., Islam, M., Sheppard, C., Liao, J. & Doyle, M.P. (2004). Reduction of *Escherichia coli* O157:H7 and Salmonella enterica serovar Enteritidis in chicken manure by larvae of the black soldier fly. Journal of Food Protection 67: 685-690.
12. Fegan, N. & Gobius, K. S. (2013). Pathogenic Escherichia coli and One Health implications. Current topics in microbiology and immunology, 366, 49–62.
13. Gabriela-Allegretti, Edson T., Veranica S., Paulo C., B. & Enrique O. (2018). Insect as feed: An emergy assessment of insect meal as a sustainable protein source for the Brazilian poultry industry, Journal of Cleaner Production, Volume 171, Pages 403-412.
14. Garofalo, C., Milanović, V., Cardinali, F., Aquilanti, L., Clementi, F. & Osimani, A. (2019). Current knowledge on the micro biota of edible insects intended for human consumption: A state-of-the-art review. Food Research International, 125, p.108527.
15. Grabowski, N.T., & Klein, G. (2017). Microbiology of processed edible insect products—Results of a preliminary survey. International Journal of Food Microbiology, 243: 103–107.
16. Graham, D.W., Begeron, G., Bourassa, M., W., Dickson, J., Howe, A., Kahn, l., h., Morley, P.S., Scoft, H.M., Simjee, S., Singer, D.S., Smith, T. C., Strokes, C., & Wittum, T.E. (2019). 'Complexities in understanding antimicrobial resistance across domesticated animal, human, and environmental systems', Annals of the New York Academy of Sciences, 1441(1), pp. 17–30.
17. Graveland, H.J.A., Wagenaar, K., & Bergs. (2011). Persistence of livestock associated MRSA CC398 in humans is dependent on intensity of animal contact. PLoS One 6: e16830.
18. Gutierrez, M., Somoskövi, A., Natarajan, K. & Bell, D. (2018). Need for better adherence to optimal incubation temperature for quality laboratory diagnostics and antibiotic resistance monitoring. African Journal of Laboratory Medicine. 7. 10.4102/ajlm.v7i2.789.
19. Gwenzi, W., Chaukura, N., Muisa-Zikali, N., Teta, C., Musvuugwa, T., Rzymski, P. & Abia, A.L.K. (2021) 'Insects, Rodents, and Pets as Reservoirs, Vectors, and Sentinels of Antimicrobial Resistance',*Antibiotics*, 10(1), 68.
20. Halloran, A., Hanboongsong, Y., Roos, N. & Bruum, S. (2017) Life cycle assessment of cricket farming in north-eastern Thailand, Journal of Cleaner Production, 156, pp. 83–94.
21. https://www.cdc.gov/laboratory/specimen-submission/pdf/Specimen-Packing-and-Shipping-Guidance-Infectious-Diseases-Laboratories.pdf

22. Hussein, M., Pillai V.V., Goddard, J.M., Park, H.G., Kothapalli, K.S. & Ross, D. (2017). Sustainable production of housefly (*Musca domestica*) larvae as a protein-rich feed ingredient by utilizing cattle manure. PLoS ONE 12(2): e0171708.
23. Jarlier, V., Nicolas, M., Fournier, G. & Philippon, A. (1988). Extended broad-spectrum βeta-lactamases conferring transferable resistance to newer βeta-lactam agents in Enterobacteriaceae: hospital prevalence and susceptibility patterns. Rev. Infect. Dis. 1302.
24. Kaper, J., B., Nataro, J., P. & Mobley, H., L. (2004). Pathogenic Escherichia coli. Nature Rev Microbiol.; 2:123-40.
25. KEBS, (2017). Dried Insect Products for Compounding Animal Feeds—Specification KS 2117: ICS 65.120; Kenya Bureau of Standards: Nairobi, Kenya, p. 9.
26. Kim, K.S. (2012). Current concepts on the pathogenesis of Escherichia coli meningitis: implications for therapy and prevention. Curr Op in Infect Dis. 2012; 25:273-8.
27. Kim, T. K., Yong, H. I., Kim, Y. B., Kim, H. W. & Choi, Y. S. (2019). Edible Insects as a Protein Source: A Review of Public Perception, Processing Technology, and Research Trends. Food science of animal resources, 39(4), 521–540.
28. Klunder, H.C., Wolkers-Rooijackers, J., Korpela, J.M. and Nout, M.J.R. (2012). Microbiological aspects of processing and storage of edible insects *Food Control*, 26: 628–6.
29. Knight-Jones, T., Hang'ombe, M., Songe, M., Sinkala, Y. and Grace, D. (2016). Microbial Contamination and Hygiene of Fresh Cow's Milk Produced by Smallholders in Western Zambia. International Journal of Environmental Research and Public Health, [online] 13(7), p.737.
30. Krejcie, R.V. & Morgan, D.W. (1970). Determining sample size for research activities. Educational and psychological measurement, 30(3), pp.607-610.
31. Lancellotti, M., de-Oliveira, M.P. & de-Avila, F.A. (2007) Research on Staphylococcus sp. in biofilm formation in water pipes and sensibility to antibiotics. Braz J Oral Sci. 6: 1283-1288.
32. Larouche & Jennifer (2017). Effects of Killing Methods on Lipid Oxidation, Colour and Microbial Load of Black Soldier Fly (*Hermetia illucens*) Larvae. Animals: an open access journal from MDPI vol. 9, 4 182. 21.
33. Larry M., & James, T.P. (2021). BAM Chapter 3: Aerobic Plate Count.
34. Lievens, S., Poma, G., Smet, J., Van-Campenhout, L., Covaci, A. & Van-Der B.M. (2021). Chemical Safety of Black Soldier Fly Larvae (*Hermetia Illucens*), Knowledge

Gaps and Recommendations for Future Research: a Critical Review. Journal of Insects as Food and Feed. 7. 1-14. 10.3920/JIFF2020.0081.

35. Makkar, H.P.,Tran, G., Heuze, V. & Ankers, P. (2014). State-of-the-art on use of insects as animal feed. Anim. Feed Sci. Technol. 197:1–33.
36. Mujuru, F.M., Kwiri, R., Nyambi, C., Winini, C. & Moyo.N.M. (2014). Microbiological quality of Gonimbrasia belina processed under different traditional practices in Gwanda, Zimbabwe. Int. Journal. Curr. Microbiol. App. Sci. 3:1085–94.
37. Mutungi, C., Irungu, F.G., Nduko, J., Mutua, F., Affognon, H., Nakimbugwe, D., Ekesi, S. & Fiaboe, K.K.M. (2017). Postharvest processes of edible insects in Africa: A review of processing methods, and the implications for nutrition, safety and new products development, Critical Reviews in Food Science and Nutrition.
38. Mukonka, (2020) Roadmap to phase out non-therapeutic antibiotic use and critically important antibiotics in food-animals in Zambia.
39. Nilsson, O. (2015). Hygiene quality and presence of ESBL-producing *Escherichia coli* in raw food diets for dogs. Infect. Ecol. Epidemiol. 5, 28758.
40. Nyangena, D., N., Mutungi, C., Imathiu, S., Kinyuru, J., Affognon, H., Ekesi, S., Nakimbugwe, D. & Fiaboe, K.K. (2020). Effects of traditional processing techniques on the nutritional and microbiological quality of four edible insect species used for food and feed in East Africa. Foods, 9: 574
41. Ortega-Paredes, D., de-Janon, S., Villavicencio, F., Ruales, K.J., De-La-Torre, K., Villacís, J.E., Wagenaar, J.A, Matheu, J., Bravo-Vallejo, C., Fernández-Moreira, E. & Vinueza-Burgos, C. (2020). Broiler Farms and Carcasses Are an Important Reservoir of Multi-Drug Resistant *Escherichia coli* in Ecuador. Front. Vet. Sci. 7:547843.
42. Osama E., Yuanpu Z., Xiaopeng X., Minmin Cai, Longyu Z., Heather R.J., Jeffery, K. Tomberlin, Feng H., Ziniu Y. & Jibin, Z. (2022) Inhibition of Zoonotic Pathogens Naturally Found in Pig Manure by Black Soldier Fly Larvae and Their Intestine Bacteria. Research, 4(4), 1297.
43. Pitout, J., D. (2012). Extraintestinal pathogenic *Escherichia coli*: an update on antimicrobial resistance, laboratory diagnosis and treatment. Expert Rev Anti Infect Ther. 10:1165-76.
44. Pomba, C., M. Rantala, C. & Greko. (2017). Public health risk of antimicrobial resistance transfer from companion animals. J. Antimicrob. Chemother. 72: 957–968.
45. Rumpold, B.A. & Schl€uter, O., K. (2013a). Nutritional composition and safety aspects of edible insects. Mol. Nutrition. Food Res. 57:802–23.

46. Rumpold, B. A. & Schl€uter, O.K. (2013b) Potential and challenges of insects as an innovative source for food and feed production. Innov. Food Sci. & Emerg. Technol. 17:1–11.
47. Sandec, (2019) Small-scale Drying Methods for Black Soldier Fly Larvae, Eawag Aquatic Research, (a), pp. 1–4.
48. Shumo, M., Khamis, F.M., Ombura, F.L., Tanga, C.M., Fiaboe, K.K.M., Subramanian, S., Ekesi, S., Schlüter, O.K., van-Huis, A. & Borgemeister, C., A. (2021). Molecular Survey of Bacterial Species in the Guts of Black Soldier Fly Larvae (*Hermetia illucens*) Reared on Two Urban Organic Waste Streams in Kenya. Front Microbiol. Sep 22; 12:687103.
49. Sogari, G., Amato, M., Biasato, I., Chiesa, S. & Gasco, L. (2019). The potential role of insects as feed: A multi-perspective review. Animals, 9(4), p.119.
50. Stefani, S. & Goglio, A. (2010) Methicillin-resistant *Staphylococcus aureus*: related infections and antibiotic resistant. Int. Journal. Infect. Dis. 14 (Suppl. 4): S19-S22.
51. Thanner, S., D. Drissner & Walsh, F. (2016). Antimicrobial resistance in agriculture. mBio 7: e02227. 12.
52. Marshall, B.M. & Levy, S.B. (2011). Food animals and antimicrobials: impacts on human health. Clin. Microbiol. Rev. 24: 718–733. 13.
53. Tschirner, M. & Simon, A. (2015). Influence of different growing substrates and processing on the nutrient composition of black soldier fly larvae destined for animal feed. Journal of Insects Food Feed, 1: 249–259.
54. Vandeweyer, D., De Smet, J., Van-Looveren, N. & Van-Campenhout, L. (2021). Biological contaminants in insects as food and feed. Journal of Insects as Food and Feed, 7(5), pp.807-822.
55. Van-Huis, A. (2003) Insects as Food in Sub-Saharian Africa. Insect Science and Application, 23, 163-185.
56. Van-Huis, A. (2013) Potential of Insects as Food and Feed in Assuring Food Security. Annual Review of Entomology, 58, 563-583.
57. Van-Huis, A., Van-Itterbeeck, J., Klunder, H., Mertens, E., Halloran, A.,Muir, G. & Vantomme, P. (2013). Edible insects: Future prospects for food and feed security (p. 201). Rome, Italy: Food and Agriculture Organization of the United Nations (FAO).
58. Verraes, C., Van-Boxstael, S., Van-Meervenne, E., Van-Coillie, E., Butaye, P., Catry, B., de Schaetzen, M.A., Van-Huffel, X., Imberechts, H., Dierick, K., Daube, G., Saegerman. C., De-Block J., Dewulf J. & Herman, L. (2013). Antimicrobial resistance

in the food chain: a review. Int Journal Environ Res Public Health. Jun 28; 10(7):2643-69.

59. Voss, A., Loeffen, F. & Bakker, J. (2005). Methicillin resistant *Staphylococcus* aureus in pig farming. Emerg. Infect. Dis. J. 11: 1965. 14**.**
60. Wang, Y.S., & Shelomi, M. (2017). Review of black soldier fly (*Hermetia illucens*) as animal feed and human food. Foods 6: 91.
61. Wulf, M. & Voss, A. (2008) MRSA in livestock animals: an epidemic waiting to happen? Clin. Microbiol. Infect. 14: 519-521.
62. Wynants, E., Crauwels, S., Lievens, B., Luca, S., Claes, J., Borremans, A., Bruyninckx, L. & Van-Campenhout, L. (2017). Effect of post-harvest starvation and rinsing on the microbial numbers and the bacterial community composition of mealworm larvae (Tenebrio molitor). Innovative food science & emerging technologies, 42, pp.8-15.
63. Wynants, E., Frooninckx, L., Crauwels, S., Verreth, C., De Smet, J., Sandrock, C., Wohlfahrt, J., Van Schelt, J., Depraetere, S., Lievens, B., Van Miert, S., Claes, J. & Van Campenhout, L. (2018). Assessing the microbiota of black Soldier fly larvae (*Hermetia illucens*) reared on organic waste streams on four different locations at laboratory and large scale. Microbial Ecology 77: 913-930.
64. Wynants, E., Frooninckx, L., Van-Miert, S., Geeraerd, A., Claes, J. & Van-Campenhout, L. (2019). Risks related to the presence of Salmonella sp. during rearing of mealworms (Tenebrio molitor) for food or feed: survival in the substrate and transmission to the larvae. Food Control 100: 227-234.