ASSESSING BACTERIOLOGICAL QUALITY OF FRESH BLACK SOLDIER FLY LARVAE

BY

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A dissertation submitted to the University of Zambia in partial fulfillment of the requirements for the award of the degree of Master of Science in Tropical Infectious Diseases and Zoonosis (TDZ).

The University of Zambia

School of Veterinary Medicine

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DECLARATION

I, **Ester Laiser**, do hereby declare that the contents of this dissertation being submitted herein are my original work and have not been previously submitted to any University for the award of a degree or any other qualification.

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

CERTIFICATE OF APPROVAL

The University of Zambia approves this dissertation submitted by Ester Laiser, as fulfilling the requirements for the award of the degree of Master of Science in Tropical Infectious Diseases and Zoonosis.

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(Board of Examiners)

DEDICATION

This work is dedicated to my family**, my daughters, Maureen and Nancy,** for their patience, encouragement, and emotional and spiritual support during my study. I also thank my mother Natang'amuaki Royan, my nephew Mr. Simel Mollel and his family for their support and encouragement during my studies. More importantly worth mentioning my late brother Mr. Solomon Wavi Laiser who showed me the way to go in life. Above all, I thank **God Almighty** for making all things possible.

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ABSTRACT

Black Soldier fly larvae (BSFL) is an insect larva that is used as a source of proteins for animals, including pigs, poultry, and fish. It is also used to decompose organic matter. This study aimed to assess the bacteriological quality of fresh BSFL and investigate the occurrence of antimicrobial resistance using *Escherichia coli* and *Staphylococcus* as potential model pathogens.

A total of 17 BSFL samples were collected from a dumpsite in May 2022. Then washed, disinfected with 70% ethanol before rinsed twice with distilled water, 1g weighed and homogenized then inoculated to MacConkey and Blood agar media and incubated at 37 °C for 24 hours. Bacteria isolated from BSFL were identified using colony morphology, Gram staining, Catalase test, Mannitol Salt Agar for *Staphylococcus* and Eosin Methylene Blue for *E. coli*, and the isolates obtained were selected based on their characteristics. *E. coli* and *Staphylococcus* were subjected to Antimicrobial Susceptibility Test (AST) using the Kirby-Bauer disk diffusion method. Antibiotics-resistant isolates were further assessed for extended-spectrum beta-lactamase-encoding genes using Polymerase Chain Reaction.

From 17 samples the process of culturing yielded 79 isolates based on colony morphology. Out of 79 isolates, only 20 (25%) were Gram-negative, while the remaining 59 (75%) were Gram-positive. Among the 20 Gram-negative strains, 95% (19/20) were *E. coli,* while Gram-positive isolates were dominated by *Staphylococcus* at 47.5% (28/59). All 19 *E. coli* isolates were resistant to three or more antibiotics classes (multidrug-resistant), exhibiting resistance to gentamicin, tetracycline, chloramphenicol, nalidixic acid, cefotaxime, cotrimoxazole, ciprofloxacin, streptomycin, and ampicillin. Furthermore, three out of 19 (3/19, 16%) *E. coli* isolates tested positive for bla_{TEM} and $bla_{\text{CTX-M}}$ drug resistance genes that encode class A extended spectrum β-lactamases (ESBLs) and can be responsible for therapeutic problems due to the resistant conferred. While all *Staphylococcus* isolates showed resistance to cloxacillin, none of them was positive for the *mecA* gene. The study revealed that fresh BSFL harbour bacteria of zoonotic significance.

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LIST OF ABBREVIATIONS

AMR	Antimicrobial Resistance
ARG	Antibiotic Resistant Gene
AST	Antimicrobial Susceptibility Test
AMP	Ampicillin
AMPs	Antimicrobial Peptides
BSFL	Black Soldier Fly Larvae
CBU	Copperbelt University
CFU	Colony Forming Unit
CHL	Chloramphenicol
CIP	Ciprofloxacin
CTX	Cefotaxime
DLP 2	Defensin-like peptide 2
DLP 4	Defensin-like peptide 4
DNA	Deoxyribonucleic Acid
ECOFF	Epidemiological cut-off values
EMB	Eosin Methylene Blue
ERES	Excellence Research Ethics and Sciences
ESBL	Extended Spectrum Beta-lactamase
EUCAST	European Committee on Antimicrobial Susceptibility Test
FEP	Cefepime
GEN	Gentamicin

LB	Luria Bertani
MSA	Mannitol Salt Agar
MIC	Minimum Inhibitory Concentration
MRSA	Methicillin Resistant *Staphylococcus aureus*
MDR	Multidrug Resistant
MHA	Muller Hinton Agar
NAL	Nalidixic Acid
PCR	Polymerase Chain Reaction
PBP	Penicillin binding Proteins
STR	Streptomycin
SXT	Cotrimoxazole
TET	Tetracycline
UNZA	University of Zambia

CHAPTER ONE

INTRODUCTION

1.1 Background

Black soldier fly (BSF) *Hermetia illucens* (Diptera: Stratiomyidae) is a tropical fly that inhabits tropical and warm-temperature regions worldwide. It is known to feed on a wide range of biological wastes at its larval stage (Nyakeri et al., 2019). BSF are ideal for industrial applications due to their rapid growth, and they are not disease vectors to humans (Klammsteiner et al., 2020). However, the growing insect industry and increasing use of insect products require a high understanding of the BSF's biology and its associated microbes in order to improve waste degradation and meet hygienic standards (De Smet et al., 2021; Klammsteiner et al., 2020).

Black soldier fly larvae (BSFL) can be reared in organic waste and can convert low-quality materials into valuable biomass (Bruno et al., 2019). Production of BSFL is simple, cost-effective, and environmentally sustainable (Müller et al., 2017; Wang and Shelomi, 2017). These larvae have many positive properties as they produce bioactive substances that could potentially be used for human and animal welfare. For example, BSFL proteins can potentially replace soy and fish meal commonly used for animal feed (Müller et al., 2017). They could thus contribute to reducing deforestation resulting from clearing land for soy production, also used in other components, such as lipids for biodiesel production and biochemicals for cosmetics (Müller et al., 2017; De Smet et al., 2021) . In addition, insect lipids may replace palm kernel oil and contribute to the conservation of tropical forests (Müller et al., 2017).

As the microbial safety of the larvae is of great importance if they are used as feed ingredients (Wang and Shelomi, 2017), the selection of the substrates on which BSFL are fed can be an important factor in assuring food/feed safety. Food pathogens that may be present in the substrate may be transferred to the larval intestinal tract and subsequently cause illness in the animals given a BSFL-based feed or in people consuming the animal products (Wynants et al., 2019). For example, *Salmonella* and *Escherichia coli* may be

found in BSFL-rearing substrates and can infect the animals that feed on BSFL, resulting in zoonotic diseases in a human consumer (Erickson et al., 2004). Therefore, BSFL has the potential to recycle the pathogens.

According to Nairuti et al. (2022), adult BSF do not feed; BSF only feed at the larvae stage and are used as animal feed at this stage. In the BSF lifecycle (figure 1.1), the development of the larval stage has six instars; the sixth instar (prepupal stage) is used as feed. The larval stage takes 13 to 18 days under optimal conditions, which can increase if the conditions are unfavorable (De Smet et al., 2018; Shumo et al., 2019). The optimum temperature required by BSFL ranges between 26ºC and 27ºC, and the moisture between 60% and 80%.

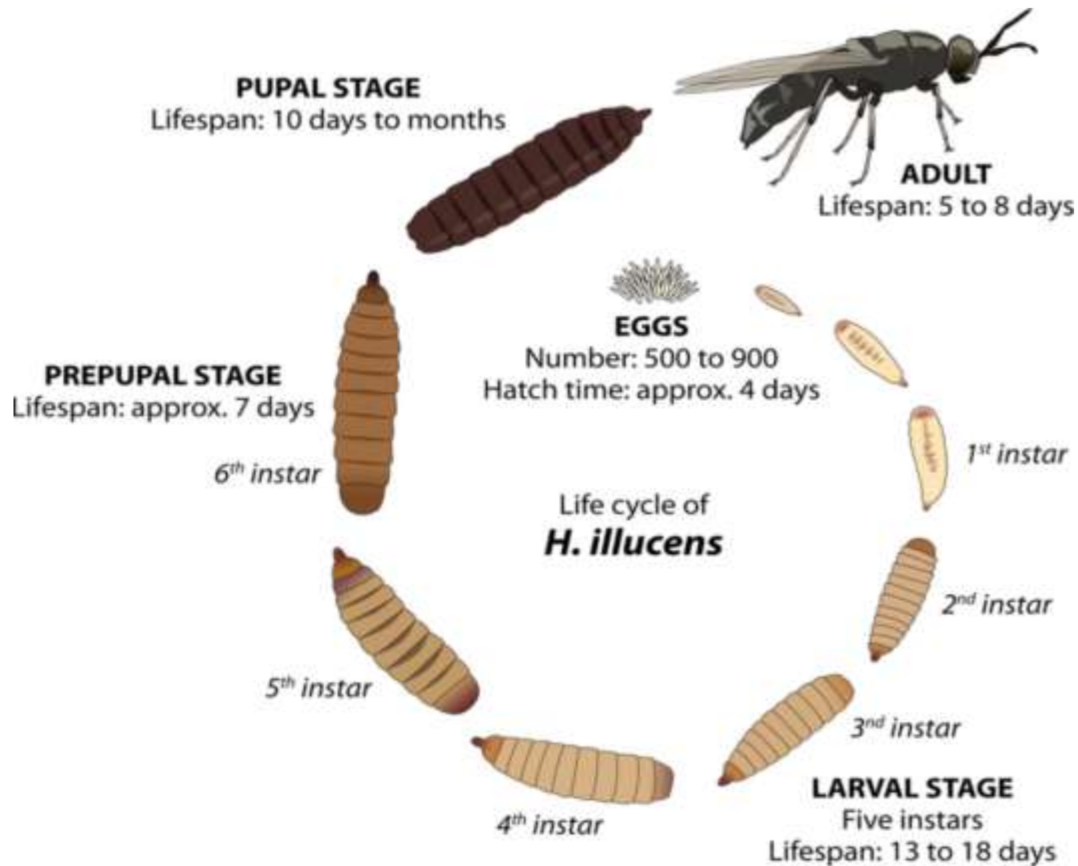

Figure 1.1 The lifecycle of BSF showing its stages of development and the average duration for each stage (De Smet et al., 2018)

BSFL are naturally found around the fecal waste of livestock, poultry, swine, and humans (Nairuti et al., 2022). A community of bacteria present in these wastes decomposes organic matter making nutrients more accessible to the larvae (Dortmans et al., 2017). BSFL contain microbes in the gut, for example, *Enterococcus, Providencia, Morganella,* and *Dysgonomonus* (Gorrens et al., 2021; Khamis et al., 2020; Klammsteiner et al., 2020). These microbes have many functions that are important to the growth and development of larvae, including the digestion of complex substrates (Gold et al., 2018; Cai et al., 2018). BSFL can convert a large amount of organic waste, such as food waste, poultry manure, straw, and sewage sludge, into biological fertilizers (Choi et al., 2009; Lee et al., 2014).

1.2 Statement of the problem

BSFL can be reared in waste organic matter and have been used as a source of proteins for animals, including pigs, fish, and poultry (Nyakeri et al., 2019). Therefore, there is a high risk of zoonotic diseases transmission through animal feed as the animal may feed on larvae reared on waste materials containing pathogenic bacteria. This means larvae may recirculate pathogens in the food cycle. Therefore, there is a need to understand the bacteriological quality of the larvae, especially in localities such as Zambia, where the idea of BSFL mass production is just starting.

Moreover, animal waste, including poultry manure is one of the substrates used in rearing BSFL. This manure may contain drug-resistant bacteria and antibiotic residues from those used as growth promoters and to treat animal disease. This may lead to antimicrobial resistance (AMR), potentially spreading among bacteria using mobile genetic elements like plasmids and transposons (Liu et al., 2020). This waste is regarded as a reservoir of many pathogens, including *Staphylococcus, E. coli, Listeria,* and *Salmonella,* that require further investigation on disease causation. Furthermore, the AMR characteristics of such pathogens on BSFL is unknown in Zambia.

1.3 Study Justification

Fresh BSFL are currently considered an alternative source of proteins for animals and the aquaculture industry while simultaneously being agents to decompose organic wastes (Kinasih et al., 2018). Therefore, it is crucial to determine the hygienic quality of fresh BSFL to minimize zoonotic pathogens that may spread through animal feed or human food especially in regions where the concept of using BSFL for feed is starting. Characterizing AMR patterns in pathogens such as *Escherichia coli* and *Staphylococcus* may guide risk assessment studies.

1.4 Study objectives

1.4.1 Main objective

To assess the **bacteriological quality of fresh BSFL, and investigate the presence of AMR in isolated *E. coli* and *Staphylococcus*** as potential pathogenic bacteria.

1.4.2 Specific Objectives

- To determine the bacterial load in fresh BSFL grown in untreated poultry wastes.
- To detect the phenotypic and genotypic resistance profiles of Gram-positive *Staphylococcus* and Gram-negative *E. coli* bacteria isolated on fresh BSFL.

CHAPTER TWO

LITERATURE REVIEW

2.1 BSFL

Black soldier fly is a valuable insect species which has various potentials at its larval stage. It is used for converting organic waste into compost, while the larval biomass generated could be harvested for its protein and fatty acid content (Banks et al., 2014). Unlike any other alternative protein source, BSFL produces valuable materials economically whilst requiring low water and land usage (Siddiqui et al., 2022). Large quantities of BSFL need to be produced to meet demands for its use as a source of protein for animal feed. In order to meet the increasing demands for the larvae, it is crucial to understand the pathogenic bacteria associated with BSFL. Although there has been research focusing on isolation and identification of bacteria on BSFL, there is no research in AMR of the bacteria associated with BSFL and their gene of resistance.

2.2 BSFL Production

BSFL can be reared in a controlled environment, where temperature and humidity are regulated to meet the larvae requirements (Gorrens et al., 2021). The larvae production is carried out in two steps; the first is in an insectarium consisting of adult and egg production. The second is done in a larvarium where the larvae can feed and decompose the organic waste matter (Gorrens et al., 2021).

Some microbes, like *Bacillus subtilis,* promote the growth and development of BSFL in poultry manure (Yu et al., 2011). Furthermore, using BSF as an alternative source of proteins at the larvae stage improves the sustainability of food or feed systems (Gougbedji et al., 2021). Therefore, to meet the global protein demand, there should be mass production of *H. illucens* larvae for animal feed. To this end, recent development shows that these insects are produced on food, animal, and brewery wastes (Yang and Tomberlin, 2020).

Insect farming, particularly BSF, needs access to water and feed (substrates) to supply energy and nutrients for growth and excrete intestinal content such as frass used as fertilizer (Nairuti et al., 2022). A review by Wynants et al. (2019) emphasized that special attention should be given to BSFL-rearing substrates since larvae may take pathogenic organisms from them, for instance, *Salmonella* species and *Bacillus cereus.* Therefore, these pathogens can be detected in larvae and residue samples and transmitted to animals through feed.

The European Food Safety Authority has restricted edible insect production including BSFL on animal manure, catering waste, or foodstuff containing meat and fish. This is to avoid zoonotic pathogens and ensure food security and safety according to the regulation EC No 1069/2009(EFSA Committee, 2015). Yet there are no specific regulations on the use of insects as food or feed in Zambia since this idea of BSFL mass production is new.

2.3 BSFL rearing substrates

BSFL can be reared in a wide range of substrates; regardless of the origin, any vegetable, fruit, honey, egg, and dairy products can be used to formulate a feed diet for BSFL (Ravi et al., 2020). In addition, food waste and agri-industry products such as breweries and the food processing industry can be used (Lalander et al., 2019; Nyakeri et al., 2017). A review conducted by De Smet et al. (2018) showed that BSFL growth is twice as rapid in hog manure compared to poultry manure due to differences in pH. Hog manure (6.0 to 6.2) decreased in pH over time, while the pH of poultry manure (7.4 to 8.2) increased.

2.4 Benefits of Black Soldier Fly larvae

BSF has never been known to transmit pathogens (Choi et al., 2009; Klammsteiner et al., 2020). Moreover, BSFL exhibit antibiotic properties by inhibiting the growth of some pathogenic bacteria (Awasthi et al., 2020). Therefore, BSFL can reduce zoonotic pathogens significantly in livestock manure (Elhag et al., 2022). A review by Erickson et al. (2004) reported the reduction of the pathogenic bacteria *E. coli* O157:H7 and *Salmonella* species in poultry manure by *H. illucens*. Also, Choi et al. (2012) noted that the larvae of *H. illucens* have antibacterial activity against Gram-negative bacteria such

as *Klebsiella pneumoniae, Shigella sonnei,* and *Neisseria gonorrhoeae*. Furthermore, BSFL has been used in Europe and America to treat skin damage such as burns and promote wound healing (Choi and Jiang, 2014).

BSFL play a significant role in the organic waste management of various biodegradable wastes (Lalander et al., 2019; Nyakeri et al., 2017; Shumo et al., 2019). BSFL were highlighted as potent recyclers of many types of waste, including biological waste from abattoirs, food, and human feces (Lalander et al., 2019; Tahamtani et al., 2021) . They reduce organic waste quickly, have a lower carbon footprint, and have a high conversion of feed to body mass (Siddiqui et al., 2022). They also, reduce odorous compounds from poultry, swine, and dairy manures by up to 87% (Beskin et al., 2018).

Using BSFL in animal feed reduces the demand for soybean production, reducing deforestation and loss of natural resources (Tahamtani et al., 2021). Insect-derived compost can be an alternative to chemical fertilizers (Choi et al., 2009). Furthermore, BSFL can be used in bioremediation, as stated by De Smet et al. (2018). For instance, BSFL can grow in substrates contaminated by pesticides (Xenobiotic), and the presence of BSFL in contaminated substrates results in a shorter half-life of pesticides. Finally, BSFL are used in forensic entomology to estimate human postmortem interval (Pujol-Luz et al., 2008).

2.5 BSF larvae contaminants and gut microbiota

BSFL can be contaminated by microorganisms found in substrates. Since BSFL mostly grow in organic waste like livestock wastes and kitchen remains, which are known to harbor many organisms, the larvae become contaminated with bacteria found in their rearing substrates (Erickson et al., 2004). Moreover, animal wastes used as substrates for BSFL rearing can be pathogens reservoirs, for example, *Staphylococcus*, *E. coli*, *Listeria, Clostridium*, *Salmonella*, *Campylobacter*, *Streptococcus*, *Corynebacterium*, and *Mycobacterium* which contaminate the larvae (Sheppard et al., 2002; Chung et al., 2021). According to Klammsteiner et al. (2020), BSFL contain microbes that exist naturally in their gut which help degrade substrates and protect the larvae against invasive pathogens.

The most abundant microorganisms in the BSFL gut are *Gammaproteobacteria (Enterobacteriaceae* and *Morganella)* species and *Bacilli (Enterococcus* species*, Lactococcus* species*, Lactobacillus* species*, Weissella* species); others like *Bacteroides* and *Actinobacteria* are less abundant (Klammsteiner et al., 2020). In addition, a study by Zhang et al. (2022) reported that some of these BSFL gut-associated microorganisms could inhibit the growth of some bacterial spp, including *S. aureus* and *Salmonella* species.

2.6 Antimicrobial peptides found in BSFL

BSFL produce antimicrobial peptides (AMPs) molecules that exist in nature and contain antibacterial effects on some bacteria, including Methicillin Resistant *Staphylococcus Aureus* (MRSA) (Park et al., 2014; Xia et al., 2021). A study conducted by Li et al. (2017) reported antibacterial compounds isolated and identified in *H. illucens* larvae, such as Defensin-like peptide 2 (DLP2) and Defensin-like peptide 4 (DLP4), which showed antimicrobial activities against bacteria, specifically MRSA. Antimicrobial Peptides in BSFL are the first line of defense against pathogen invasion (Moretta et al., 2020). These molecules can be used as alternatives to antibiotics to prevent and treat diseases in animals, and they have a low propensity for resistance (Xia et al., 2021).

Park and Yoe. (2017) studied the cecropin family of AMPs from *H. illucens* and demonstrated the inhibition of *E. coli* activity by cecropin-like peptide 1. Defensin-like proteins 1, 2, 3 and 4 showed antimicrobial activities against *S. aureus* (Park et al., 2015; Park and Yoe, 2017; Liu et al., 2017). Elhag et al. (2017) detected seven new gene fragments of three types of AMPs from *H. illucens*. Among these genes, StomoxynZHI was potentially active in controlling antibiotic-resistant pathogens. The detected Hexanedioic acid from BSFL extracts showed a selective inhibitory effect against the growth and proliferation of *S. aureus,* MRSA, *K. pneumoniae,* and *S. dysentriae* (Choi and Jiang, 2014).

Most AMPs can kill microbial pathogens directly, but others modify the host defense systems to prevent infection (Elhag et al., 2022).In addition, these AMPs may help to

block the horizontal transfer of resistance genes in the environment; hence they can be a source of antibiotics to control infections (Xia et al., 2021). Some substrates seem to support *H. illucens* larvae to reduce some pathogenic bacteria. In contrast, other substrates accelerate the growth of the same bacteria, as shown by Erickson et al. (2004), who reported that BSFL inactivated *E. coli* and *Salmonella* species in chicken manure, while at the same time, the larvae accelerated the growth of the same pathogenic bacteria in hog manure.

2.7 Antimicrobial-resistant bacteria

Antimicrobial resistance (AMR) is the condition in which microorganisms withstand a drug that would normally kill them or inhibit their growth. (Sah et al., 2021). AMR occurs when microorganisms evolve new survival mechanisms as they are exposed to antimicrobial drugs. As a result, drug-resistant pathogens spread and continue to threaten the ability of antibiotics to treat common bacterial infections. Not only the overuse of antibiotics but also the inappropriate use contributes to the release of large amounts of antibiotics into the environment, which select for antibiotic-resistant bacteria and antibiotic-resistance genes (ARGs). Consequently, multidrug-resistant bacteria have spread worldwide, causing untreatable infections (Yuan et al., 2020). Antibiotic resistance genes spread among bacteria by horizontal gene transfer via mobile genetic elements such as transposons, integrons, plasmids, and bacteriophages, resulting in the spreading of multidrug-resistant bacteria, which is a challenge for infectious disease control (Yuan et al., 2020).

The overuse and misuse of antibiotics contribute to the spread of AMR in the community (Fair and Tor, 2014). The most frequently used antibacterial agents are β-lactam antibiotics. However, some Gram-negative bacteria have developed resistance to β-lactams by producing enzymes that can hydrolyze bonds in the β-lactam ring. These enzymes are called β-lactamases because they can inactivate β-lactams (Sah et al., 2021). Other mechanisms of β-lactam resistance include an efflux pump, reduced permeability, and altered transpeptidases (Sawa et al., 2020). On the other hand, MRSA is an example

of Gram-positive bacteria that carries the *mec*A *g*ene, which causes resistance to methicillin and many other anti-Staphylococcal drugs, including cloxacillin.

2.7.1 Extended Spectrum β-lactamases

Extended-spectrum β-lactamases (ESBLs) are enzymes produced mainly by the *Enterobacteriaceae* family of Gram-negative bacteria, making bacterial infections challenging to treat with antibiotics (Yousefipour et al., 2019). *E. coli* is among the most common bacteria that produce ESBLs that can inactivate β-lactam antibiotics. Although its infections are usually treated with common antibiotics like penicillin and cephalosporins, when these bacteria produce ESBLs, they become resistant by breaking down and destroying the antibiotic target, making it useless against an infection. Also, ESBL-producing bacteria exhibit resistance to other antibiotic classes, such as aminoglycosides, quinolones, and sulfonamides (Pishtiwan and Khadija, 2019; Yousefipour et al., 2019).

ESBLs are often encoded on plasmids that carry genes for resistance to other antimicrobial agents like aminoglycosides, sulfonamides, tetracyclines, and chloramphenicol. The common ESBLs in *E. coli* strains include CTX-M, TEM, and SHV. Among these types of ESBLs, the CTX-M type is more common and considered a pandemic enzyme in the community (Akpaka and Legall, 2010; Literacka et al., 2009). Production of β-lactamases in Gram-negative bacteria is the common resistance mechanism to β-lactam antibiotics which catalyze the hydrolysis of the amide bond found in the β-lactam ring. β-lactamases are grouped into four classes based on amino acid sequence homology, i.e., class A, B, C, and D. Class A, C, and D are serine hydrolases. In contrast, class B ESBLs are metalloenzymes containing zinc ions (Zn^{2+}) at the enzyme's active center, which hydrolyze carbapenems (Palzkill, 2018). Some reviews reported the detection of CTX-M ESBLs in isolates obtained from human and livestock wastes (Birgy et al., 2013; Valverde et al., 2008). Therefore, it may be possible for ESBL-producing *E. coli* to disseminate among animals through BSFL feed and cause zoonotic diseases in humans if ESBL-producing *E. coli* contaminates rearing substrates.

2.7.2 Methicillin Resistant *Staphylococcus*.

Staphylococcus is a genus of Gram-positive bacteria that belongs to the family *Staphylococcaceae.* Most of them are commensals, but some can cause severe infection in animals and humans, for example, *S. aureus*. Most *S. aureus* can cause resistance to methicillin and other related antibiotics, leading to MRSA, which is usually pathogenic to human and animals. The *mecA* gene determines methicillin resistance, which is widely distributed in *Staphylococcus* and encodes a penicillin-binding protein (PBP2a). The resistance gene *mec*A can spread among *Staphylococcus* species through *Staphylococcal* cassette chromosomes *mec*, a mobile genetic element that spreads through horizontal gene transfer (Deurenberg and Stobberingh, 2008; Rocchetti et al., 2018).

Altogether, resistance to β-lactam antibiotics in *Staphylococcus* can be due to β-lactamase production or the production of a new PBP with low affinity to antibiotics (Rocchetti et al., 2018).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study area

This study was conducted in Zambia's Copperbelt Province in Kitwe District at a poultry waste dump site. The manure comes from a commercial layer poultry farm with a capacity of 100,000 birds that are kept in cages. The samples collected from one dumpsite with different heaps where the Copperbelt University (CBU) were collecting the larvae as a starter for large-scale production of BSFL for commercial purposes.

3.2 Study design

A cross-sectional study design was used in this study. Seventeen BSFL samples were collected simultaneously on different heaps depending on the availability of larvae. Each BSFL sample had an average weight of 30g.

3.3 Sampling technique

The sampling technique used in this study was purposive sampling. While there are several dump sites in Kitwe, samples were purposely collected from the sites where larvae had previously been collected for the introduction of the large-scale BSFL production plant at CBU. The larvae were collected using gloved hands, small clean buckets, and forceps. After collection, the samples were transferred to the laboratory at CBU and washed with running tap water using a sieve (1 mm mesh size) for 1 minute to remove residues from the surface of the larvae. Then the samples were stored in clean zip locks on icepacks then transported to UNZA, School of Veterinary Medicine, for further processing.

3.4 Bacterial isolation and Identification

The bacterial isolation and identification process is summarized in the Figure 3.1 below:

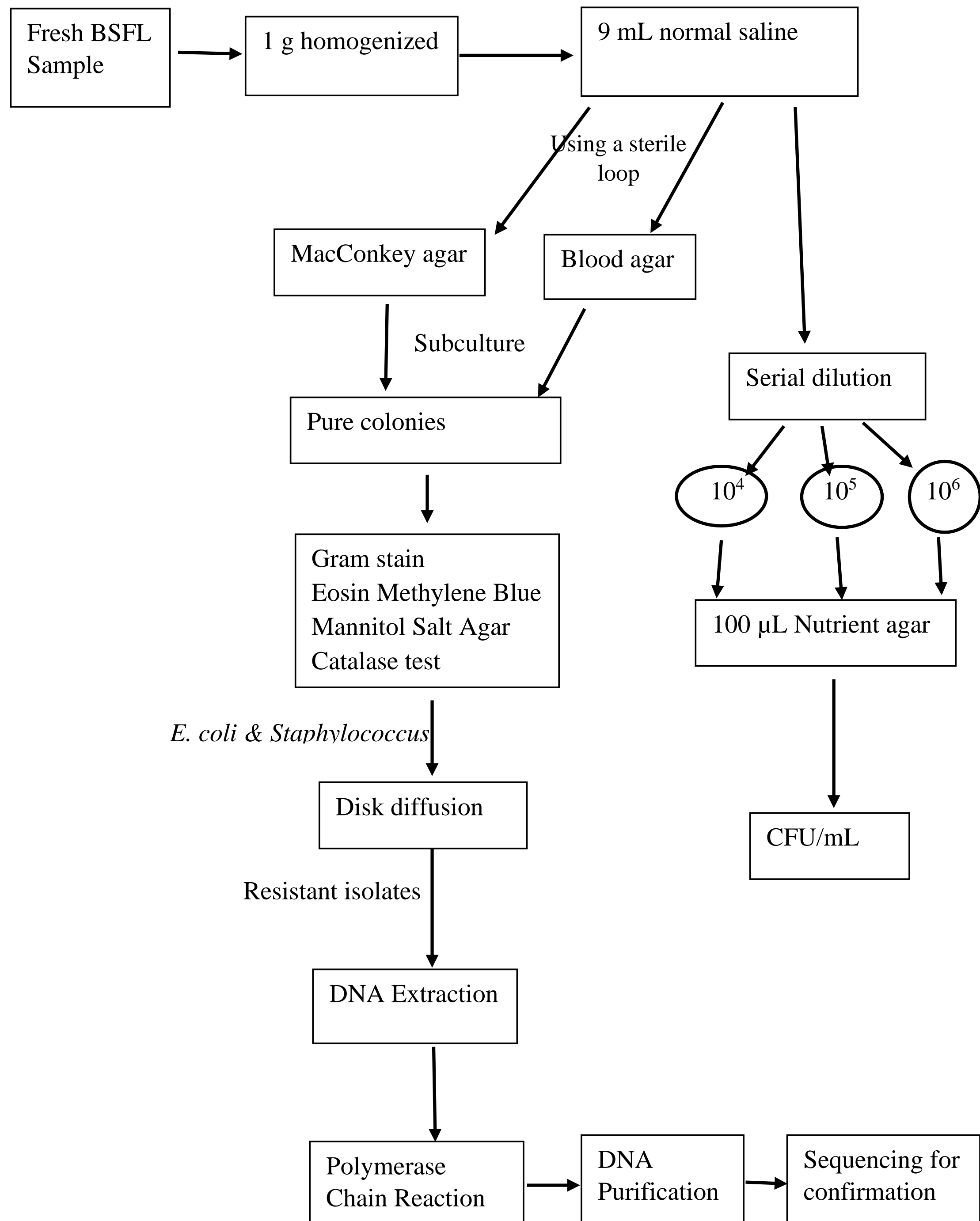

Figure 3.1 The schematic methodology used to identify some bacteria and their AMR genes

3.4.1 Bacterial culture

A total of 17 samples of BSFL collected from the field were disinfected with 70% ethanol to remove the surface microorganisms and then rinsed twice with distilled water. One gram of the larvae weighed and homogenized using a homogenizer to obtain the mixture, the resulting homogenate was transferred into 9 mL of normal saline. Then 10-fold serial dilutions were carried out, and 100 μL were aseptically surface-plated on Nutrient agar (HI Media M001), then spread using a spreader. Plates obtained from dilutions starting at 10^{-4} to 10^{-6} with countable microorganisms were considered for quantification. The bacterial colonies were counted then the number of colony-forming units (CFU/mL) was calculated. MacConkey agar was then used to isolate and differentiate non-fastidious gram-negative bacteria and blood agar for fastidious bacteria growth (particularly *Staphylococcus*). The media were plated by streaking directly followed by incubation at 37 °C for 24 hours. The colonies, which were morphologically different, were picked and streaked on a new culture media from MacConkey to a new MacConkey agar and blood agar to new blood agar, then incubated at 37 °C for 24 hours to obtain pure cultures as shown in figure 3.1.

The pure culture was picked from each plate, inoculated to Luria Bertani (LB) liquid broth media, and then incubated aerobically in a shaker at 37 °C for 24 hours and stored in glycerol at -80 °C. From the nutrient agar plates, the colonies were counted for the bacterial load. The gram staining technique, Catalase test, Mannitol Salt Agar (MSA), and Eosin Methylene Blue (EMB) further characterized the bacteria. MSA is selective and differential medium, the high concentration of salt selects the members of the genus *Staphylococcus.* The pure colonies were taken from blood agar and inoculated into MSA then incubated at 37 °C for 24 hours. EMB is a selective agar medium for isolation of Gram-negative bacteria, pure colonies were inoculated into EMB then incubated at 37 °C for 24 hours.

3.4.2 Gram staining

The Gram staining technique was done as described by Tripathi and Sapra, (2022). The pure colonies were then Gram-stained to determine the bacteria's gram-positive and gram-negative nature. Each pure colony was first emulsified in sterile normal saline to make a smear on a well-labeled, clean, dry glass slide. Then the slides were air-dried and fixed under a Bunsen burner. The slides were stained with Crystal Violet solution for 1 minute and then washed with running tap water. Next, the slides were flooded with Iodine solution to fix the microorganisms for one minute and then gently washed with running tap water. This was followed by acetone decolorizer and gentle washing with running tap water. Lastly, the slides were counter-stained with safranin solution for 1 minute and then washed gently with tap water and-air dried then viewed at X100 magnification under immersion oil.

3.4.3 Catalase test

The Catalase test was performed aseptically using a glass slide, dropper, and the disposable inoculating loop. Using a loop, a small amount of 24 hours colonies placed onto a glass slide, one drop of 3% hydrogen peroxide was added onto the organisms on the glass slide by using a dropper. The bubbles were observed for catalase positive bacteria as described by American society for microbiology (2016).

3.5 Antimicrobial susceptibility tests (AST)

The AMR profiles of *Staphylococcus* and *Escherichia coli* isolates observed were determined using the Kirby-Bauer method based on antibiotic disk diffusion on Mueller-Hinton Agar (MHA). First, the pure cultures were aseptically applied to the MHA plates using sterile cotton swabs. Then the antibiotic disks were added to the inoculated MHA plate gently using sterile forceps, and incubated at 37 °C for 24 hours. The selection of antibiotics was based on the common antibiotics used in hospitals and recommended by the World Health Organization (WHO). Antibiotics used were cefotaxime (CTX, 30 µg), cefepime (FEP, 30 µg), streptomycin (STR, 10 µg), tetracycline (TET, 30 µg), nalidixic acid (NAL, 30 µg), gentamicin (GEN, 10 µg), ciprofloxacin (CIP, 5 µg), ampicillin (AMP, 10 µg), co-trimoxazole (SXT, 25 µg) and chloramphenicol (CHL, 30 µg). The

zone of inhibition (diameter) of each antibiotic disk was measured in millimeters (mm) using a ruler, and the results were defined according to the Clinical Laboratory Standards Institute (CLSI) and epidemiological cut-off (ECOFF) under European Committee on Antimicrobial Susceptibility testing (EUCAST) guidelines**.** The bigger the zone of inhibition the higher the resistance based on the breakpoints of both EUCAST and CLSI guidelines

3.6 Multidrug-resistance (MDR) on *E. coli* and *Staphylococcus*

An organism is said to be MDR if it shows resistance to three or more antibiotics of different classes, as explained by Magiorakos et al., (2012). MDR was observed in both *E. coli* and *Staphylococcus* bacteria.

3.7 Genotypic determination of ESBL-production

Nineteen isolates selected based on their resistance to antibiotics especially cefotaxime, suspected ESBL-producing isolate*s* were subjected to DNA extraction using the QIAamp® DNA Mini Kit. Polymerase Chain Reaction (PCR) was carried out using TaKaRa Ex Premir™ DNA Polymerase and the primers shown in Table 3.1 under the conditions shown in Table 3.2 for the individual amplification and detection of AMR genes, namely $bla_{CTX\text{-}M}$, bla_{TEM}, bla_{SHV}, and bla_{OXA}. Then PCR products were purified using the Wizard® SV Gel and PCR Clean-Up System (Promega, USA) based on the instructions provided by the manufacturer. The purified products were subjected to Big Dye, 5X sequencing buffer, primers, and nuclease-free water; then, loading under the conditions outlined in Table 3.3. The PCR products were sequenced to determine the alleles of bla_{TEM} and $bla_{CTX\text{-}M}$ genes.

Electrophoresis was performed in agarose S gel stained with ethidium bromide to identify expected amplicons. The Gene Ladder Wide 1 was used as a standard molecular weight. Each reaction was separated by gel electrophoresis for 30 minutes in 1X TAE buffer. A UV transilluminator was used to visualize the amplified DNA bands with sizes 516 bp and 544 bp for bla_{TEM} and $bla_{CTX\text{-}M}$, respectively.

Table 1.1: Selected primers used in this study

Name	Sequence	Size (bp)	Target	Reference
CTX-MA1	*SCSATGTGCAG≠YACCAGTAA	544	*bla*$_{CTX-M}$	(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*$_{SHV}$	(Colom et al., 2003)
SHV-R2	ATTTGCTGATTTCGCTCG			
TEM-C	ATCAGCAATAAACCAGC	516	*bla*$_{TEM}$	(Mabilat & Courvalin, 1990)
TEM-H	CCCCGAAGAACGTTTTC			
OXA-F	ATATCTCTACTGTTGCATCTCC	619	*bla*$_{OXA}$	(Colom et al., 2003)
OXA-R	AAACCCTTCAAACCATCC			
MRSA$_1$	AAAATCGATGGTAAAGGTTGGC	533	*mec*A	(Rocchetti et al., 2018)
MRSA$_2$	GTTCTGCAGTACCGGATTTGC			

Table 3.2 PCR master mix for *bla*$_{CTX-M}$, *bla*$_{TEM}$, *bla*$_{SHV}$, and *bla*$_{OXA}$ genes detection

PCR Master mix	***Volume per sample (µL)***
Nuclease free water	9.5
Forward primer	0.75
Reverse primer	0.75
DNA template	1.5
Takara enzyme	12.5
Total volume	25.0

The PCR conditions

The initial denaturation temperature 94°C for one minute, denaturation temperature of 94°C for 10 seconds and the annealing temperature of 59.5°C for 15 seconds and the tension temperature 68°C for 30 seconds under 35 cycles then temperature hold at 4°C to infinite.

Table 3.3 Big Dye method for sequencing reaction

Reagent	***Volume per sample (µL)***
Big dye	1
Forward primer	0.32
Reverse primer	0.32
DNA template	5
5x sequencing buffer	3.8
Nuclease free water	9.88
Total volume	20.0

Conditions for Big Dye kit

The initial denaturation temperature was 96°C for one minute, denaturation temperature was 96°C for ten seconds, the annealing temperature of 50°C for five seconds and the extension temperature 60°C for four minutes under 35 cycles. Then the temperature kept on hold at 4°C to infinite.

3.8 Determination of *mec*A gene

Eleven isolates catalase-positive *Staphylococcus* were inoculated on LB agar supplemented with 64 µg/mL of cloxacillin, then incubated at 37°C for 24 hours. Next, a single colony was transferred to LB broth supplemented with 64 µg/mL of cloxacillin and incubated overnight at 37 ℃. The overnight cultures were then subjected to DNA extraction using the Quick-DNA^{TM} Miniprep Plus Kit according to the manufacturer's

instructions. DNA concentration was measured using Nanodrop 1000, and PCR was carried out using KOD ONE enzyme under the conditions shown in table 3.3.

Table 3.4 PCR master mix for *mec*A genes detection

Reagent	*Volume per sample (µL)*
Nuclease free water	10
Forward primer	0.75
Reverse primer	0.75
DNA template	1.0
KOD ONE	12.5
Total volume	25.0

PCR conditions

The initial denaturation temperature was 98° C for 2 minutes, denaturation temperature was 98° C for 10 seconds, annealing temperature of 59.5° C for 5 seconds and the extension temperature 72° C for 3 seconds under 35cycle. Then, the temperature kept on hold at 4°C to infinite

3.9 Data Processing and Analysis

Data on AST were recorded in Excel 2007 and imported into R for manipulation, analysis, and visualization using packages such as dplyr and ggplot2.

3.10 Data interpretation

AST results were interpreted based on both Epidemiological cut off (ECOFF) under European committee on antimicrobial susceptibility test (EUCAST) and Clinical laboratory standard institute (CLSI) guidelines. EUCAST developed a disk diffusion test with zone diameter breakpoints correlating with the EUCAST chemical MIC breakpoints. EUCAST introduced the epidemiological cut-off value (ECOFF) concept to describe the MIC above which bacterial isolates have phenotypically detectable acquired resistance

mechanisms (Turnidge et al., 2006). Similarly, CLSI helps laboratories to deliver standardized high-quality test results. In the present study EUCAST-ECOFF standards were used since samples were collected from the wild environment in comparison to CLSI guidelines that improves the quality of medical laboratory testing.

3.11 Ethical consideration

Ethical approval has been sought from the Excellence in Research Ethics and Science (ERES) with Reference number 2022-march-021

CHAPTER FOUR

RESULTS

4.1 Isolation and identification of bacteria

Six bacterial genera with 79 isolates were isolated from 17 samples based on the colony morphology, more than one isolate has been isolated from one sample. The majority (59/79, 75%) of the identified bacteria were gram-positive, including *Staphylococcus, Bacillus, Streptococcus,* and *Clostridium*. Gram-negative bacteria (20/79, 25%) included *E. coli* and *Proteus*, shown in Table 4.1. *Staphylococcus* (11) and *Escherichia coli* (19) isolates showed resistance and 3 *E. coli* isolates confirmed carrying the genes of resistance.

The number of bacterial cells found in fresh BSFL was estimated based on the colony-forming units per mL (CFU/mL). The estimated mean, median and standard deviation for the bacterial CFU/mL were 2.9×10^7, 2.2×10^7 and 1.96×10^7 respectively.

Table 4.1 Examples of bacteria genera isolated from fresh BSFL

Identified bacterial genera	***Number of isolates n=79***
Bacillus	20
Clostridium	1
Escherichia	19
Proteus	1
Staphylococcus	28
Streptococcus	8
Sporulated Bacillus	2

4.2 Antimicrobial Susceptibility Test (AST) results for *E. coli*

The 19 isolates of *E. coli* isolated from BSFL were then subjected to disk diffusion tests for resistance against antibiotics commonly used for treatment in humans. These antibiotics belonged to seven classes, namely cephalosporins (CTX), quinolones (CIP), aminoglycosides (GEN), penicillin (AMP), sulfonamides (SXT), and phenols (CHL). The AST breakpoints used were both EUCAST-ECOFF and CLSI guidelines. Generally, there was not much difference in the two standards. The percentage resistance on SXT and CIP, were the same at 74% and 63% respectively in both standards breakpoints, as shown in Figure 4.2. There was a difference in resistance percentage for CTX, GEN, and CHL observed using the two standards. Specifically, CLSI resulted in a higher resistance rate for CTX, while EUCAST-ECOFF gave a higher rate for GEN and CHL.

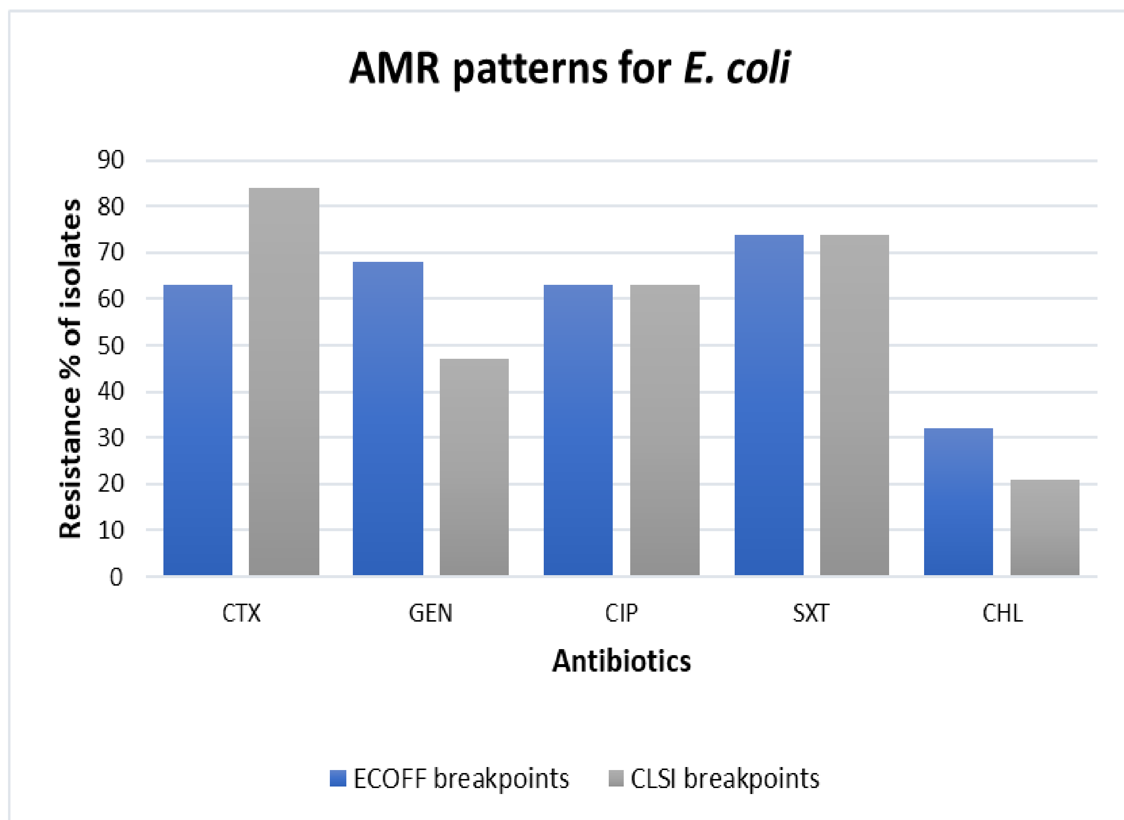

Figure 4.1: Showing the percentage resistance of *E. coli* against antibiotics commonly used.

4.3 Multidrug-resistance (MDR) on *E. coli* isolates

All 19 *E. coli* isolates were 100% multidrug resistant to antibiotics commonly used such as cefotaxime, tetracycline, gentamycin, nalidixic acid, cefepime, chloramphenicol, Streptomycin, ciprofloxacin, ampicillin and cotrimoxazole as shown on figure 4.3.

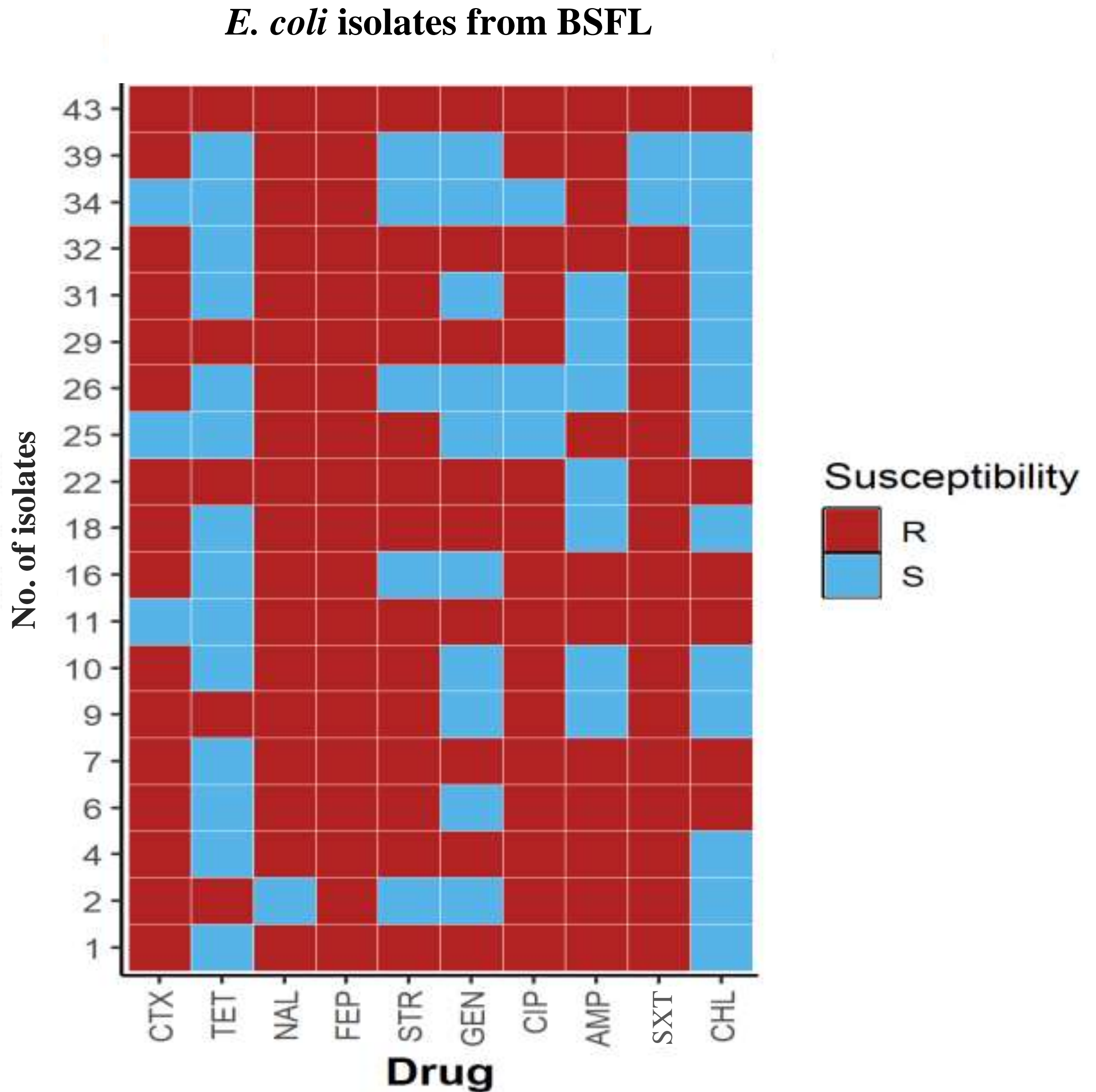

Figure 4.2 Showing *E. coli* isolates as MDR to the antibiotics tested based on CLSI guidelines

4.5 AMR patterns in *Staphylococcus*

Most *Staphylococcus* isolated from BSFL were susceptible to the common antibiotics. Specifically, the isolates were susceptible to CHL (88%), GEN (88%), and CIP (72%), followed by SXT (64%) and TET (52%) (Figure 4.4). AMP showed high resistance to *Staphylococcus* species.

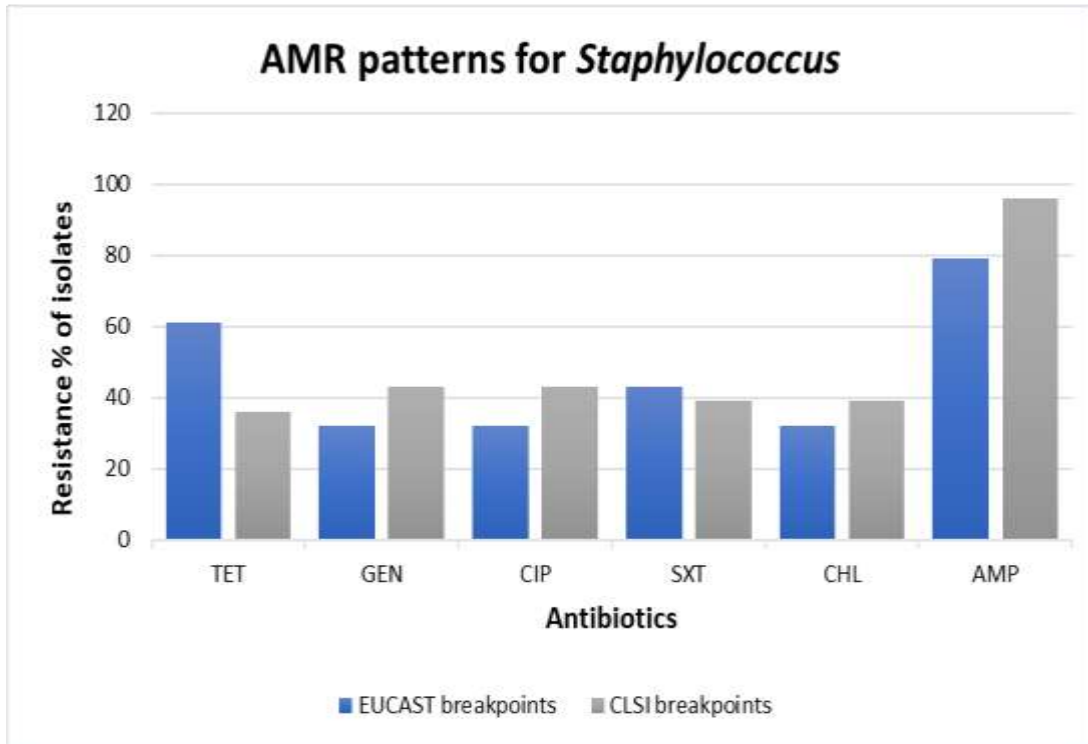

Figure 4.3 Showing the proportion of AMR profile for *Staphylococcus*.

4.6 Multidrug-resistance in *Staphylococcus* isolates

Some of the *Staphylococcus* isolated from fresh BSFL showed MDR. For example, in Figure 4.5 below, six isolates showed MDR to most of the antibiotics tested; three of them (number 27, 44, and 47) showed resistance to four antibiotics from different classes, and the other three (number 17, 28, and 46) displayed resistance to three antibiotic classes. Most *Staphylococcus* isolates showed resistance against one or two antibiotics. Some isolates were susceptible to all antibiotics tested.

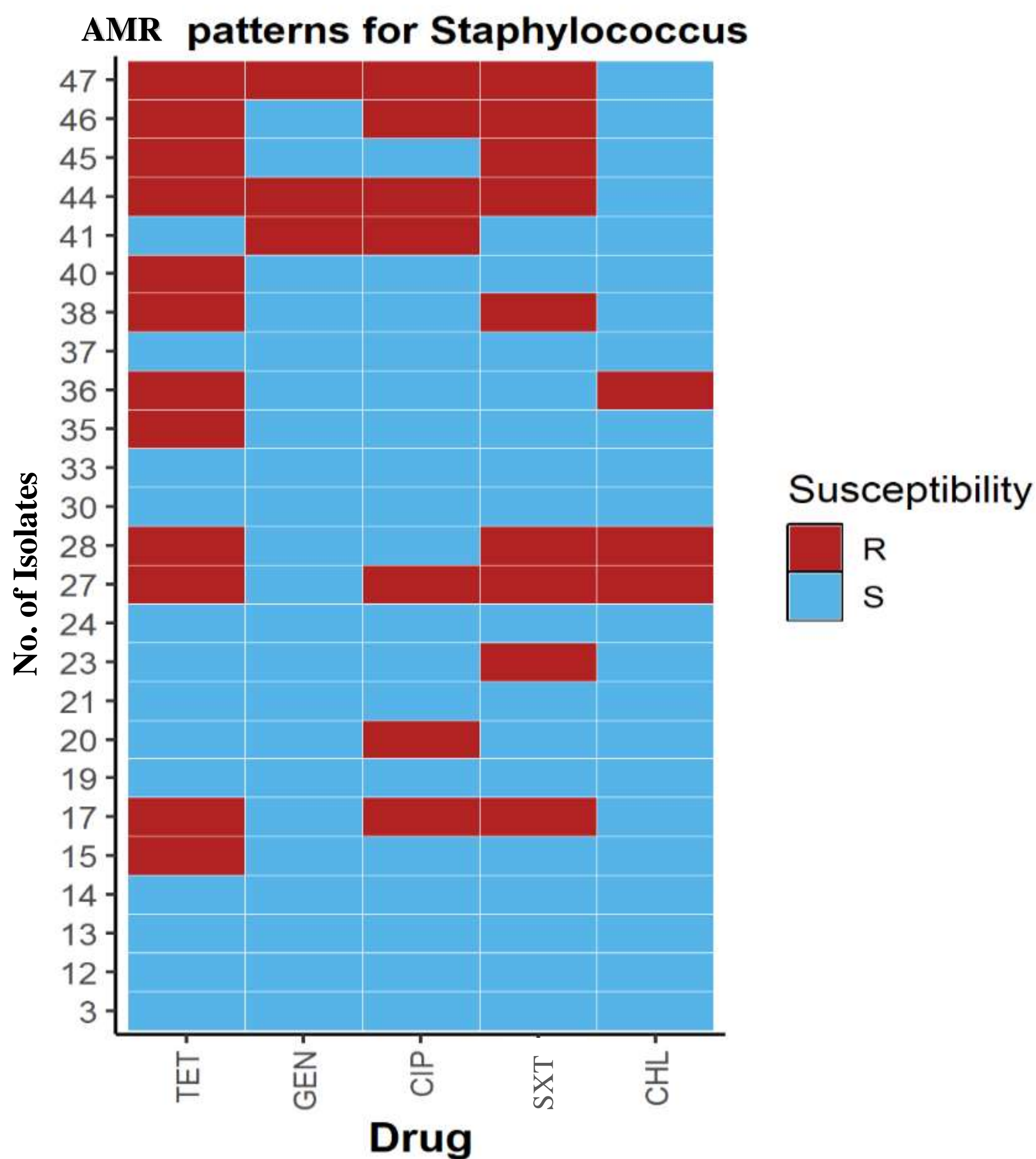

Figure 4.4 MDR results for *Staphylococcus* based on CLSI guidelines

4.7 Molecular detection of AMR genes

PCR was performed to detect genes associated with ESBL among *E. coli* isolates, and bands of the expected size were regarded as positive results. Despite the high MDR rates, only three out of 19 (3/19, 16%) *E. coli* isolates were positive for the tested AMR genes, two of which tested *bla*$_{CTX\text{-}M}$ only and one tested both *bla*$_{CTX\text{-}M}$ and *bla*$_{TEM}$ genes. The *bla*$_{CTX\text{-}M}$ and *bla*$_{TEM}$ genes on agarose gel under UV light were detected with band sizes of 544 bp and 516 bp, respectively, as shown in Figure 4.6. In addition, confirmation was achieved by Sanger sequencing of the amplicons.

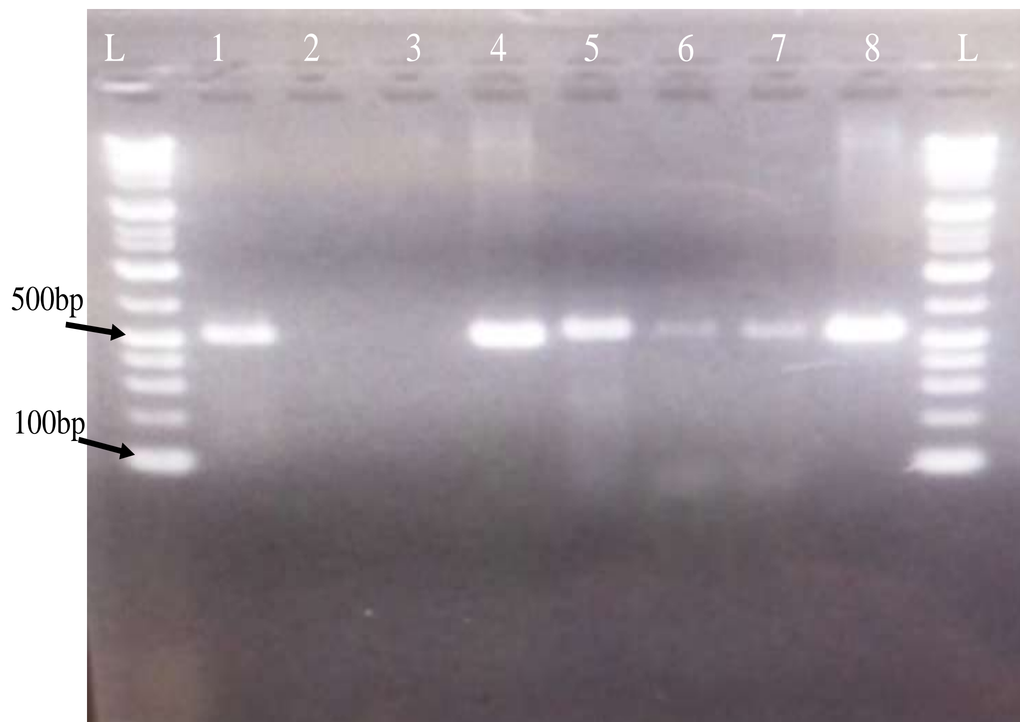

Figure 4.5 DNA maker (L), 1, is positive bla$_{TEM,}$ (516 bp) gene, 2 & 3 are negative, 4 & 8 are positive controls; 5, 6, and 7 are bla$_{CTX\text{-}M}$ (544bp) genes.

Table 4.2 Molecular Detection of ESBL Producer *E. coli* gene

Sample number	Organism name	E Value	Percentage identification	Accession number
E12	*E. coli*(*bla*$_{CTX-M}$) gene	0.0	99.13	KF448073.1
E12	*E. coli*(*bla*$_{TEM}$) gene	0.0	97.45	OL629041.1
E14	*E. coli* (*bla*$_{CTX-M}$) gene	2e-140	85.07	NG081695.1

The results obtained from sequencing were compared in the GenBank by BLAST, and the observed genes are shown in table 4.2 above.

CHAPTER FIVE

DISCUSSION

Among all bacteria isolated on BSFL, *E. coli* and *Staphylococcus* were selected as Gram-negative and Gram-positive model for AST and detection of their AMR genes. This is because some *E. coli* are ESBL producers that break down the β-lactam ring of cephalosporins and penicillin, leading to resistance (Yousefipour et al., 2019). In addition, some *E. coli* are commensals, but others are potentially pathogenic that may cause serious infections including Urinary tract infection, enteritis in human and also associated with diarrhea in pets and farm animals as described by (Allocati et al., 2013). Also, the AMR genes from *E. coli* can be transferred from commensal bacteria to pathogenic bacteria by horizontal gene transfer through plasmids for example bla_{CTX-M}, bla_{TEM} as described by (Rozwandowicz et al., 2018), transposons, and insertion sequences, limiting treatment options.

Staphylococcus was selected since some strains are potentially pathogenic and contain the *mec*A gene that encodes resistance to β-lactam antibiotics as shown by Rocchetti et al., (2018). A typical example is MRSA, a zoonotic pathogen that causes severe infections in humans for example pneumonia, endocarditis, skin and soft tissue infections as described by Li et al. (2018). and also, it causes mastitis, dermatitis in farm animals that can be difficult to treat as described by Saeed et al. (2022).

The average bacterial load was 2.9×10^7 CFU/mL showing that BSFL carry different types of bacteria depending on the type of rearing substrates since BSFL can pick bacteria from their rearing substrates as reported by Erickson et al, (2004). Gram-positive bacteria were more diverse (59/79, 75%) than Gram-negative isolates (20/79, 25%) in fresh BSFL samples. This may be because BSFL contain AMPs that affect the growth and proliferation of Gram-negative bacteria as described by (Choi et al., 2012). However, to a lesser extent, even the growth of Gram-positive species can be inhibited by BSFL AMPs, especially *S. aureus* (including MRSA) (Choi & Jiang, 2014; Kinney et al., 2022).

E. coli is among the most common pathogens in hospital and community bacterial infections (Toval et al., 2014). Therefore, AMR in *E. coli* threatens public health as it leads to adverse health outcomes among patients. In this study, some of the *E. coli* isolated from *H. illucens* larvae were resistant to the most used antibiotics such as CTX, NAL, STR, CIP, AMP, and SXT and possessed ESBL genes. Since the BSFL in this study were collected from a dumpsite including poultry wastes, the ESBL-producers could have come from poultry. In Zambia, ESBL-producing *E. coli* has been reported in cloacal swabs obtained from chickens (Munang'andu et al., 2012), probably because antibiotics are frequently used for growth promotion in the poultry industry (Agyare et al., 2019). Furthermore, the clonal transmission of ESBL producers between poultry and humans was recently reported in Zambia (Shawa et al., 2022). However, the strains presented in this study will need further characterization to determine their relationship with poultry and human strains previously reported.

The isolates 1, 4, and 32 of *E. coli* in Figure 4.3 showed identical resistance patterns and were all susceptible to TET and CHL; these isolates probably came from the same origin. In this study, all CTX-resistant *E. coli* isolates displayed MDR as they showed resistance to three or more groups of antibiotics tested. For instance, isolate 43, as shown in Figure 4.3, showed resistance against all seven antibiotic classes tested. This could imply that poultry waste (BSFL rearing substrate) contains multiple antibiotic residues that select for MDR among *Enterobacteriaceae*. However, MDR can also be maintained by a single drug since most AMR genes coexist on plasmids, which require only a single antibiotic for selection. Therefore, further studies are needed to determine the presence of antibiotic residues in BSFL rearing substrates.

Out of 19 *E. coli* isolates analyzed, three (3/19, 16%) were positive for AMR genes; among the three positive isolates, two tested positive for $bla_{\text{CTX-M}}$ only, and one had both $bla_{\text{CTX-M}}$ and bla_{TEM}. This result highlights the limitation of our genotypic characterization approach. While $bla_{\text{CTX-M}}$ and bla_{TEM} are the commonest β-lactamase genes in Zambian clinical and poultry strains (Shawa et al., 2021), the current results could suggest a

different picture in BSFL. The discrepancy between phenotypic and genotypic AMR observed here could be explained by point mutations, such as those modifying PBPs. Furthermore, the diversity of ESBLs involving about ten classes could suggest the presence of other types in these strains. Also, the observed difference could be fostered by the inhibitory effects of AMPs produced by BSFL, which block the horizontal gene transfer among bacteria in the environment, as described by (Xia et al., 2021). Therefore, there is a need to characterize the current *E. coli* strains using robust methods like whole-genome sequencing.

Meanwhile, *Staphylococcus* species isolated from *H. illucens* larvae showed high susceptibility to GEN (22/25, 88%), CHL (22/25, 88%), CIP (18/25, 72%), SXT (16/25, 64%), and TET (13/25, 52%). Gram-positive bacteria isolated from BSFL in this study were more diverse than gram-negative bacteria isolated from the same sample. This observation could be explained by the inhibitory effect of AMPs, which is more pronounced in gram-negative species. Despite the predominance of *Staphylococcus* in this study (Table 4.1), none of the isolates tested positive for the *mec*A gene even after selection on cloxacillin. While the *Staphylococcus* isolates in this study were catalase-positive, it is possible that species other than *S. aureus* were involved, which could highlight the inhibitory effects of BSFL AMPs against the growth of the pathogenic *S. aureus* and MRSA, as described by (Choi & Jiang, 2014; Kinney et al., 2022). Therefore, further work on the current strains will involve sequencing of the 16S rRNA gene to allow classification to the species level.

CHAPTER SIX

CONCLUSION AND RECOMMENDATION

6.1 Conclusion

Gram-positive bacteria were more diverse than Gram-negative bacteria, since there were four genera of Gram-positive bacteria isolated from BSFL that include *Bacillus, Staphylococcus, Streptococcus* and *Clostridium* but were only two Gram-negative bacteria genera isolated such are *E. coli* and *Proteus*. Despite the low diversity of gram-negative bacteria, MDR *E. coli* were detected, including ESBL-producing bacteria positive for $bla_{CTX\text{-}M}$ and bla_{TEM} genes. This study revealed that BSFL harbor bacteria of Zoonotic significance.

6.2 Recommendation

Processing methods need to be developed if BSFL should be used as feed for animals in order to minimize the spread of zoonotic pathogens, AMR surveillance in dumpsites serving as BSFL sources for large-scale production is crucial, Further studies is required on characterization of potentially pathogenic bacterial species from BSFL in Zambia. Finally, Zambia require specific regulation on edible insects' production and processing including BSFL to minimize zoonotic pathogens and ensure feed safety and security.

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