CHARACTERIZATION AND ANTIMICROBIAL ACTIVITY OF *Serratia marcescens* ENDOSYMBIONTS OF RHABDITIS NEMATODES AGAINST SELECTED BACTERIAL AND FUNGAL PATHOGENS

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DECLARATION

I ALEYO ROSELYNE, hereby declare that this is my original work and has never been presented for examination purposes at a degree program in this or any other institution to the best of my knowledge. Information from other sources has been acknowledged.

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ABBREVIATIONS AND ACRONYMS

ABR: Antibiotic-resistant

AMR: Antimicrobial resistance

BCA(s): Biological Control Agent (s)

BLAST: Basic Local Alignment Search Tool

CDC: Centre for Disease Control

CFU: Colony forming units

CO_2: Carbon dioxide

DJs: Dauer juveniles

E.A: East Africa

EPN(s): Entomopathogenic nematode (s)

ESKAPE: *Escherichia coli*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumanii*, *Pseudomonas aeruginosa*, and *Enterococcus* bacteria

IAA: Indole-3-acetic acid

IJ(s): Infective juveniles

IPM: Integrated pest management

KALRO: Kenya Agricultural and Livestock Research Organization

LB: Luria Bertani

MAC: MacConkey

MDR: Multi-drug resistant

MHA: Mueller Hinton Agar

MOH: Ministry of Health

NBTA: Nutrient bromothymol blue 2, 3, 5-triphenyltetrazolium chloride agar

NCBI: National Centre for Biotechnology Information

NRF: National Research Fund

OTC: Over-the-counter

PCR: Polymerase chain reaction

RPM: Revolutions per minute

SM: Secondary metabolites

TTC: Triphenyltetrazolium chloride

UNITID: University of Nairobi Institute of Tropical and Infectious Disease

WHO: World Health Organization

ABSTRACT

Serratia is a genus of gram-negative non-sporulating gamma proteobacteria of the family Enterobacteriaceae. They are the newest addition to the well-known nematode-bacterium association with the two most common endosymbionts being *Xenorhabdus* and *Photorhabdus* species. *Serratia marcescens* is associated with the entomopathogenic nematodes of the genus *Oscheius*. They produce many secondary metabolites including antibiotics, enzymes, prodigiosin, serrawettins, siderophores, bacteriocins, anticancer agents, acids and toxins which have shown cytotoxicity against various microbes. This study aimed to characterize the nematode-associated bacteria and determine their efficacy against selected antibiotic-resistant bacteria as well as phytopathogenic fungal species. To obtain the bacteria, five late instars of *Galleria mellonella* larvae were placed on the surface of a Whatman filter paper lined in a Petri dish followed by the addition of 5 drops of nematode suspension per Petri dish, sealed with parafilm and incubated in the dark at room temperature for 4 days. The hemolymph from dead larvae was streaked on Nutrient bromothymol blue 2, 3, 5-triphenyltetrazolium chloride agar (NBTA) and MacConkey agar, making five replicates and incubated at 28°C for 48hrs. Pure colonies obtained were used in the subsequent bioassay involving antimicrobial susceptibility tests against selected pathogens. All the bacterial isolates were gram-negative with green circular, smooth, colonies on NBTA media. They were all positive for motility test on NBTA and MAC media pathogenicity test, catalase test, Simmons citrate agar (SCA) test, dye adsorption (on both NBTA and MAC) as well as pigment production test. Other biochemical tests including oxidase test, urease test and bioluminescence (visualization in the dark) gave negative results in all four bacterial isolates. The Cetyl Trimethyl Ammonium Bromide (CTAB) method was employed in the isolation of the total genomic DNA followed by amplification of the 16S rRNA gene using universal primers, B27F and 1492R as the forward and reverse primers, respectively. The PCR products were sent for sequencing at the University of Nairobi's Institute of Tropical and Infectious Disease (UNITID) laboratories. Molecular analysis results established that the four sampled enteric bacteria were as follows; isolate 93A had a 99.26% identity to *Klebsiella oxytoca* strain 127, while isolates 93B and 93C scored 99.26% and 98.22% identity to *Serratia marcescens* strain B195 and *Serratia marcescens* strain RS, respectively and finally isolate 93D had a 99.80% similarity to *Citrobacter freundii* strain UIS1115. The two species of *Serratia* in the current study were thus named *Serratia marcescens* strain 93B and *S. marcescens* strain 93C. All the bacterial isolates did not significantly differ with respect to

antimicrobial efficacy towards the tested bacterial and fungal pathogens based on the size of the inhibition zones. Isolate 93A showed the lowest inhibition on *P. aeruginosa* (15.33 mm), *S. aureus* (18.00 mm), MRSA (21.00 mm), *E. coli* (23.33 mm), *C. albicans* (27.00 mm) and highest activity on *B. cereus* (34.00 mm). The cell-free suspension (100% concentration) of isolate 93B resulted in inhibition zones with the following diameters: MRSA (16.00mm), *P. aeruginosa* (16.33 mm), *S. aureus* (18.33 mm), *E. coli* (26.67 mm), *C. albicans* (27.67 mm) and *B. cereus* (33.67 mm). Additionally, the diameters of the zones of inhibition obtained from the effect of the 100% concentration of isolate 93C were, *P. aeruginosa* (17.33 mm), MRSA (17.67 mm), *S. aureus* (20.67 mm), *E. coli* (25.67 mm), *B. cereus* (31.33 mm) and *C. albicans* (36.33 mm). Isolate 93D inhibited the growth of bacterial test pathogens as follows: *P. aeruginosa* (16.00 mm), MRSA (19.67 mm), *S. aureus* (19.67 mm), *E. coli* (24.00 mm), *B. cereus* (33.33 mm) and *C. albicans* (35.33 mm). The susceptibility of phytopathogenic fungi to the 100% concentration of isolate 93A were as follows: *F. verticillioides* (0.00 mm), *F. oxysporum* (13.33 mm), *F. solani* (14.33 mm), *P. chrysogenum* (17.00 mm), *Penicillium* spp. (19.00 mm) and *A. flavus* (21.33 mm). Isolate 93B inhibited fungal growth with diameters as follows: *F. verticillioides* (0.00 mm), *F. solani* (15.00 mm), *A. flavus* (15.33 mm), *P. chrysogenum* (17.00 mm), *Penicillium* spp. (18.00 mm) and *F. oxysporum* (18.33 mm). The inhibitory effects of the full-strength concentration of isolate 93C against the six test fungi were: *F. verticillioides* (4.67 mm), *F. solani* (12.67 mm), *A. flavus* (13.33 mm), *Penicillium* spp. (16.67 mm), *P. chrysogenum* (18.33 mm) and *F. oxysporum* (19.67 mm). The 100% cell-free suspension of isolate 93D showed the following inhibitory diameters against the six pathogenic fungal species: *F. verticillioides* (0.67 mm), *A. flavus* (15.33 mm), *F. solani* (16.00 mm), *F. oxysporum* (17.67 mm), *P. chrysogenum* (18.33 mm) and *Penicillium* spp. (18.67 mm). The current study concluded that the nematode-associated enterobacteria have potential to control both bacterial human pathogens as well as the plant pathogenic fungal species and should therefore be exploited in integrated pest management of plant pathogens and control of antibiotic resistant human pathogens.

CHAPTER ONE: INTRODUCTION

1.1 Background to the study

Nematodes are pseudocoelomate, invertebrate worms that have neither segments nor jointed appendages (Poinar, 2006). These nematodes are usually parasitic to plants, insects, animals and other invertebrates. They comprise 12 clades inhabiting nearly all environmental niches from marine to below and above the land (De Ley, 2006; Holterman *et al*., 2006; Borgonie *et al*., 2011). The phylum Nematoda consists of entomopathogenic nematodes (EPNs) which are minute soil inhabitants that grow parasitically in insects, using them as hosts, where they feed and reproduce ultimately killing them then emerging in high numbers (Asif e*t al*., 2013). The two major groups of EPNs are *Heterorhabditis* and *Steinernema* formed through parallel evolution (Ferreira and Malan, 2014). These two genera exist in association with endosymbiotic bacteria of the genus *Photorhabdus* and *Xenorhabdus*, respectively. The bacteria are mutually exclusive and as such only one genus can be found within the nematode (Ferreira and Malan, 2014). Entomopathogenesis has over the years been considered to have evolved twice at the minimum in these nematodes (Blaxter *et al*., 1998): once in the *Heterorhabditis* - *Photorhabdus* duo and once in the *Steinernema* - *Xenorhabdus* lineage.

However, direct isolation methods and *Galleria* trap experiments have revealed new nematodes besides *Steinernema* and *Heterorhabditis* which have similar attributes (Young-Keun *et al*., 2007; Zhang *et al*., 2008). For instance, Zhang *et al*., 2009 in their study reported a new species of *Serratia nematodiphila*, a symbiont of the newly discovered entomopathogenic nematode, *Heterorhabditidoides chongmingensis* (Zhang *et al*., 2008; Abebe *et al*., 2010). Hence, the genus *Oscheius*, previously called *Heterorhabditidoides*, has now been added as a third EPN group (Al-Zaidawi *et al*., 2019). *Oscheius* nematodes are mutually associated with *Serratia* species of bacteria (Al-Zaidawi *et al*., 2019). For instance, *Oscheius carolinensis* was found to be in a symbiotic relationship with *Serratia marcescens* and just like the other EPNs, was found to be a lethal parasite of insect pests (Torrini *et al*., 2015). The nematode was also reported to support a dual lifestyle of the bacterium (*Serratia* spp.) within it (Torres-Barragan *et al*., 2011; Dillman *et al*., 2012; Al-Zaidawi *et al*., 2019).

Increased research on entomopathogenic nematodes is being done as the years go by, from 13 species of *Steinernema* (10) and *Heterorhabditis* (3) in the 1980s (Kaya and Gaugler, 1993) to 66 spp. of *Steinernema* (55) and *Heterorhabditis* (11) in 2007 (Nguyen and Hunt,

2007; Nguyen *et al*., 2007). The number of described EPNs stands at 116 comprising 100 species of *Steinernema* and 16 species of *Heterorhabditis* (Hunt and Subbotin, 2016). The genus *Serratia* has 18 spp. that have been isolated from different surroundings such as contaminated soils, water, marine environments, plants, animals as well as hospitalized patients (Grimont and Grimont, 2006; Su *et al*., 2016). A large number of EPNs has been employed in the biological control of crop pests with great success (Hominick, 2002) due to their prolonged persistence in the fields as opposed to synthetic pesticides (Grewal *et al*., 2005; Lang *et al*., 2011; Del Valle *et al*., 2013). Successful utilization of the *Rhabditis* (*Oscheius*)-*Serratia* relationship has been demonstrated in controlling rice yellow stem borer, *Scirpophaga incertulas* (Padmakumari *et al*., 2007) and control of areca nut spindle bug in the field (Mohandas *et al*., 2004) without any detrimental effects. They were found to kill several important insect pests within 24 - 72 hours in the laboratory (Sangeetha *et al*., 2016). Native EPN species have also been successfully used in Brazil to control the fall armyworm (*Spodoptera frugiperda*) thus avoiding over-reliance on chemical pesticides (Salvadori *et al*., 2012). *Serratia* sp. strain TEL isolated from the gut of infective juveniles of the EPN *Oscheius* sp. TEL-2014 exhibited 100% mortality of *G. mellonella* in 72 hours (Lephoto *et al*., 2015).

Before the introduction of antibiotic therapy, human health was under threat from many different pathogens. Alexander Fleming, in 1928, paved the way for the subsequent development of antibiotics following the isolation of Penicillin from *Penicillium chrysogenum*. Antimicrobial resistance (AMR) has over the years been on the rise in various parts of the world particularly in Africa, Kenya included (Kimang'a, 2012). This is attributed to abuse and misuse of antibiotics through high prescription in hospitals and over-the-counter (OTC) medication (26.4%). This misuse of drugs increases antibiotic resistance leading to high mortality, morbidity and economic constraints (Kimang'a, 2012). *Escherichia coli*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumanii*, *Pseudomonas aeruginosa* and *Enterococcus* species (ESKAPE bugs) pose the greatest threat in the health sector (Rice, 2008). For this reason, many studies on EPNs and their enteric bacteria are underway for their possible application in the medical field; for instance, autoimmune disease treatment (Kijk and Van Die, 2010; Correale and Farez, 2011).

In addition, studies are being carried out on the potential application of these symbiotic bacteria and their secondary metabolites in combating economically important plant pathogens. Many plants ranging from trees, herbs as well as crops suffer from pathogenic

microbes. There are more than 50,000 different plant pathogens including nematodes, bacteria, viruses and fungi (Stock *et al*., 2017). This is a problem that affects many crops in the tropics and temperate regions where these pathogenic microorganisms thrive because the climatic conditions are suitable for their growth and survival. Over the years, many different pathogenic microbes have spread from their native habitats to other new environments. These new environments do not have natural antagonists to fight the new pathogens thus making it very difficult to curb these pathogens. These microbial pathogens result in low yields in agricultural crops, stunted growth, death of plants and seedlings. Of great significance are the fungal pathogens since they account for the highest losses in agriculture.

1.2 Problem statement

Scientific studies on EPNs are steadily increasing over the years. To date, atleast 100 valid *Steinernema* species and 21 *Heterorhabditis* species have been reported from different countries of the world (Bhat *et al*., 2020). As a result, this remains a vast field to be explored particularly the newly added genus *Oscheius* (*Heterorhabditidoides*) which offers greater potential in both human health and agricultural arenas (Torres-Barragan *et al*., 2011; Bao *et al*., 2017). This creates a need for more research into the associated bacterial endosymbionts and the antimicrobial agents they secrete. A relatively large number of reports, many of them in recent years, clearly demonstrate a growing interest in native EPNs in Africa (Abate *et al*., 2018). The discovery of new native EPN species, their endosymbiotic bacteria and most importantly their secreted bioactive compounds are a promising source for the development and use of these secondary metabolites (SM) as biocontrol agents in the medical and agricultural industries.

Most developing countries lack policies that can effectively limit antimicrobial resistance (AMR) both in the health and the agricultural sectors. This leaves the people exposed to poor health, mortality and poverty. Some previously effective and affordable antibiotics like cotrimoxazole have become less effective (Ampaire *et al*., 2016). East Africa carries the burden of life-threatening antimicrobial resistance because of its limited testing capacity (Ampaire *et al*., 2016). About 54% resistance to third-generation carbapenems and cephalosporins has been reported (WHO, 2014). There are very few reports documenting AMR in East Africa and this hinders proper studies on these pathogens (Ampaire *et al*., 2016).

Additionally, many agricultural crops suffer from the great burden of fungal pathogens. Fungi are a great menace to plants because they attack all parts of crops and at any stage of

development ranging from seedling, flowering, maturity stages and further persist during storage. Many fungal pathogens persist in the fields and therefore damage the produce from one growing season to another. This eventually leads to poor growth of crops, death of young crops and low agricultural yields leading to a shortage of food. Most losses in maize yields are accounted for by pathogenic microbes and past research indicates that over sixty different types of diseases are caused by various pathogens affecting the maize crop (Anon, 2007) with the highest prevalence rates being from fungi of various genera such as *Aspergillus*, *Penicillium, Fusarium* and *Helminthosporium* which occur right from planting, ripening, through harvest and storage (Tsedaley and Adugna, 2016). Maize, being a staple food in Kenya has been faced with high levels of mycotoxins. Three million bags of maize grain from coastal and eastern Kenya were destroyed in 2010 as a result of fungal contamination. Such actions curtail the government's efforts in achieving food security and vision 2030. This leads to reduced crop yields leading to food shortages hence imparting constraints on the developing nations and especially in Kenya where there are fewer food alternatives (Almeida *et al*., 2000). For instance, recurrent outbreaks of acute aflatoxicosis have been periodically reported in Kenya, majorly in Makueni, Kitui and Machakos districts in 1982, 2004 and 2005 (Afla Control, 2010). There is a need for the isolation of effective antimicrobial compounds to combat these fungal phytopathogens and save lives.

The United Nations report projects an increase in human population to 9.7 billion people by 2050, with sub-Saharan Africa accounting for more than half of the total population (UN, 2019). This, coupled with the Covid-19 virus and the locust invasion in the East African region, will be catastrophic to humans resulting in hunger and increased mortality. In a bid to increase agricultural production to sustain this growing population, chemical pesticides and fungicides have been continually used over the years albeit being environmentally hazardous. They result in underground water pollution, damage to both target and non-target organisms, are very expensive and result in resistance. They remain protractedly in the food chain (Bussaman *et al.,* 2012) and once consumed by both humans and other animals cause diseases such as food poisoning, allergies and cancer (Nyatuame and Ampiaw, 2015).

1.3 Justification

Many scientific researchers focus on the isolation of EPNs and not their symbiotic bacteria. As a result, isolation, identification and characterization of these enteric bacteria is needed in order to evaluate their activity against bacterial, cancerous, fungal, insecticidal and

nematicidal agents (Webster *et al*., 2002). This calls for discovery of endemic EPN species with their associated bacteria (Serepa *et al*., 2015) in the East African region. This could solve the problem of pathogen resistance in health and agricultural pests (Rice, 2010) including fungal phytopathogens such as *Aspergillus flavus*, *Fusarium oxysporum*, *Fusarium solani*, *Fusarium verticillioides* and *Penicillium chrysogenum.*

Current chemical formulations including antibiotics and antifungal agents are losing their efficacy due to the high emerging resistance among microbial populations (Hillocks, 2012). The recent overuse and misuse of chemical pesticides in agriculture coupled with chemical poisoning warrant the need for new effective environmentally friendly applications to improve present integrated pest management (IPM) management systems (Chitwood, 2003). A significant number of reports show that resistance to common bacteria has reached alarming levels in many parts of the world indicating that many of the available treatment options for common infections in some settings are ineffective (WHO, 2014). This offers an incentive for the development of indigenous antibiotics and antimicrobials against these pathogens. Secondary metabolites, biomolecules and virulence factor protein-coding genes from *Serratia* species are yet to be fully described.

The most optimistic approach to developing more efficient treatment agents is by discovering and studying new and native microorganisms including their secondary metabolites followed by evaluating their antimicrobial activity against various pathogens in order to ensure that they can tolerate local climatic conditions (Pervez *et al*., 2013). Various bioactive metabolites obtained from different beneficial microbes have shown invaluable results for the discovery of therapeutic drugs and agricultural agents (Webster *et al*., 2002). For instance, some secondary metabolites from *Pseudomonas* spp. are known to confer plants with protection against various soil-borne fungal phytopathogens (Haas *et al*., 1992). The use of biological control agents against pathogens is a better alternative to chemical control since it is environmentally friendly, sustainable and cheap because the application is not seasonal and has no detrimental effects on the non-target organisms (Fukruksa *et al*., 2017). The current study focuses on the characterization and antimicrobial efficacy of *S. marcescens* against selected ABR bacterial pathogens (*Staphylococcus aureus,* Methicillin-resistant *S. aureus* (MRSA), *Escherichia coli*, *Bacillus cereus, Pseudomonas aeruginosa*) including the yeast *Candida albicans* and phytopathogenic fungi (*Aspergillus flavus, Fusarium oxysporum*, *Fusarium verticillioides*, *Fusarium solani* and *Penicillium* species).

1.4 Objectives

1.4.1 Broad objective

The broad objective of this study was to characterize and determine the antimicrobial activity of symbiotic *Serratia* bacterial species isolated from *Oscheius* (*Heterorhabditidoides*) nematodes against selected economically important bacterial and fungal pathogens.

1.4.2 Specific objectives

The specific objectives of this study were:

i. To determine the phenotypic and biochemical characteristics of *Serratia* and other bacteria isolated from *Heterorhabditidoides* (*Oscheius*) nematodes.
ii. To determine the phylogeny of the isolated bacteria (the genus *Serratia*) using the 16S rRNA gene.
iii. To evaluate the antimicrobial activity of the isolated (*Serratia*) bacteria against selected bacterial and fungal pathogens.

1.5 Hypotheses

The following were the hypotheses for this study:

i. The isolated bacterial species have phenotypic attributes that are characteristic of the genus *Serratia.*
ii. Phylogenetic reconstruction of the genus *Serratia* will identify novel Kenyan *Serratia* isolates.
iii. The isolated *Serratia* species have effective antimicrobial activity against selected economically important bacterial and fungal pathogens.

CHAPTER TWO: LITERATURE REVIEW

2.1 Distribution and occurrence of entomopathogenic nematodes

The phylum Nematoda has various functional groups comprising some nematodes which parasitize insects and are called entomopathogenic nematodes (EPNs). Reports indicate that nematodes have a worldwide distribution (Nyasani *et al*., 2008). Entomopathogenic nematodes have been reported to occur in almost all continents except Antarctica (Muangpat *et al*., 2017). They are frequent soil inhabitants including the marine sediments where they occur as aggregates rather than as randomly distributed isolates within the soil (Nyasani *et al*., 2008). Entomopathogenic nematodes have a vast distribution including forests, agricultural/crop fields, uncultivated soils, river banks and national parks (Mwaniki *et al*., 2008; Muangpat *et al*., 2017). The intensity and prevalence of EPNs in soil differ with time, type of soil and the crops grown (Hummel *et al*., 2002; Nyasani *et al*., 2008). The survival and motility of dauer juveniles are influenced by soil type (clay, loam, and sandy soils), texture, moisture, pH, temperature and biotic factors in the soil (Glazer, 2002).

Nyasani *et al*. (2008) reported the abundance of EPNs in less disturbed soils. Their findings additionally showed the dominance of *Heterorhabditis* species over *Steinernema* species in cultivated soils. Likewise, the few reported cases of *Oscheius* have been isolated in undisturbed soils (Torres-Barragan *et al*., 2011; Lephoto *et al*., 2015). Mwaniki *et al*. (2008) reported that in Kenya, *Steinernema* and *Heterorhabditis* species isolation was in the ratio of 9:1, respectively. *Oscheius* being a newly accepted EPN has limited literature and therefore warrants more research into the genus and the associated entomopathogenic bacterium *Serratia* (Serepa *et al*., 2015).

The three EPNs (*Steinernema*, *Heterorhabditis* and *Heterorhabditidoides* (*Oscheius*)) are similar in structure (Figure 1) and have developed mutualistic associations with *Xenorhabdus*, *Photorhabdus* and *Serratia*, respectively. These bacteria are Gram-negative, flagellated, rod-shaped bacilli of the family Enterobacteriaceae. The associations of these entomopathogenic nematodes with their respective bacteria are fatal to many insects. Once inside the IJs, *Xenorhabdus* bacteria are located in the intestinal vesicles (Bird and Akhurst, 1983). *Photorhabdus* species on the other hand occupy the entire intestines of Heterorhabditids (Endo and Nickle, 1991) whereas *Serratia* spp. inhabit the intestines and cuticle of adult females as well as areas near the pharyngeal bulb (Kaya and Gaugler, 1993; Zhang *et al*., 2009; 2012; Torres-Barragan *et al*., 2011; Bao *et al*., 2017). Some species

of *Oscheius* nematodes are associated with only one bacterial endosymbiont while other members associate with multiple bacterial species (Fu and Liu, 2019). Approximately 19 different genera of bacteria associate with Rhabditis (*Oscheius*) nematodes (Sangeetha *et al*., 2016). Some of these bacteria are *Acinetobacter*, *Comamonas*, *Klebsiella*, *Brucellaceae*, *Achromobacter* (Deepa *et al*., 2011); *Bacillus* (Mohandas *et al*., 2007); *Alcaligenes*, *Providencia*, *Flavobacterium* (Park *et al*., 2011); *Serratia*, *Microbacterium*, *Staphylococcus*, *Rheinheimera* (Tambong, 2013); *Proteus*, *Pseudomonas*, *Stenotrophomonas*, *Enterobacter*, *Lysinibacillus* and *Enterococcus* (Padmakumari *et al*., 2007; Sangeetha *et al*., 2016).

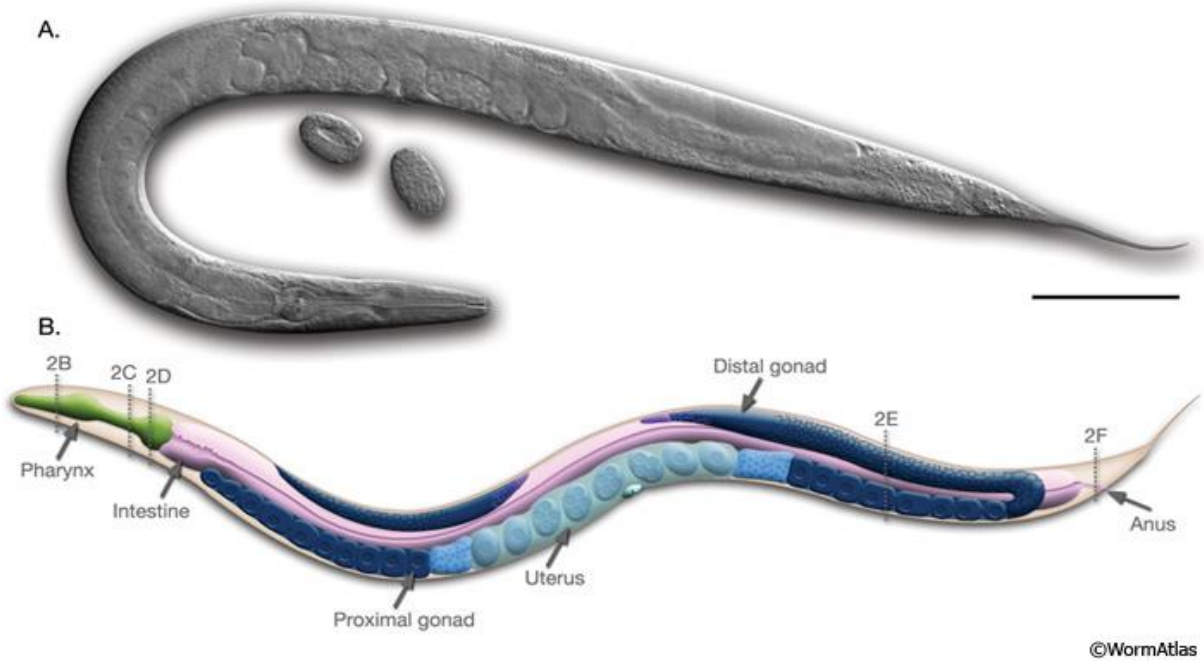

Figure 1: The anatomy of a hermaphrodite entomopathogenic nematode

Source: Dillman, 2013

This nematode-bacterium complex is preferred because of its ease of culturing, high virulence potential and high reproductive efficiency which has led to the nematodes' successful integration into pest management programs (IPM) to control soil pests and diseases (Chowdhury *et al*., 2015). The importance of using EPNs in biological control has shown promise and encouraged further surveys of EPNs on a global basis. The results of the surveys indicate that EPNs are widely distributed all over the world (Hominick, 2002; Adams *et al*., 2006). *Oscheius* nematodes, the newest addition to the EPN group, share many attributes with

Steinernema and *Heterorhabditis* spp. (Ye *et al*., 2010; Liu *et al*., 2012 and Torres-Barragan *et al*., 2011; Zhang *et al*., 2008, 2012; Dillman *et al*., 2012; Lephoto *et al*., 2015). Some of the species in the genus are *O. chongmingensis* (Liu *et al*., 2012), *O. carolinensis* (Torres-Barragan *et al*., 2011), *O. gingeri* (Pervez *et al*., 2014) and *O. rugaoensis*. Application of entomopathogenic nematodes in biological control has been effective in the European countries for over half a century with tremendous efficacy against plant-boring insects/pests, soil-dwelling insects, among others (Bao *et al*., 2017).

2.1.1 Foraging behavior of entomopathogenic nematodes

Entomopathogenic nematodes exhibit different strategies for searching and entering insect host bodies. These EPN species only infect their hosts as infective juveniles (IJs). Only these IJs are capable of living in the soil freely, actively seeking out their target insect hosts and infecting them. The other developmental stages exist exclusively inside the insect hosts but the dauer juveniles (DJs) are capable of free-living in the soil since they are a non-feeding, developmentally-arrested third larval stage (Hallem *et al*., 2011). Heterorhabditids are cruisers, that is, they actively traverse the soil scavenging their hosts in an upward-downward and horizontal movement while the Steinernematids are ambushers which stand on their tails in a stationary manner called nictation and can attach to hosts as they pass (Labaude and Griffin, 2018). This continuous movement exhibited by *Heterorhabditis* spp. poses a threat to cruisers since they are eaten by other organisms in soil (Lortkipanidze *et al*., 2016). While researching the method used by the three EPN genera in host-seeking, Dillman *et al*. (2012) reported that the mechanism used by *O. carolinensis* in host-seeking behavior has not yet been characterized (Dillman *et al*., 2012).

Despite the differences in host-seeking methods of these nematodes, the consensus is that they all employ olfactory cues to locate their hosts like carbon dioxide (CO_2) (Hallem *et al*., 2011; Lortkipanidze *et al*., 2016). Carbon (iv) oxide is excreted as a by-product of respiration by all animals. Chemotaxis to CO_2 may enable migration of dauers towards invertebrate hosts, thereby enabling dispersal to new niches. The BAG sensory neurons are used in mediating attraction to carbon (iv) oxide (Hallem *et al*., 2011; Dillman *et al*., 2012). These neurons are sensitive to low levels of oxygen (Liu and Cai, 2013). Insect hosts release various compounds which facilitate host-seeking behaviour. Some of these substances include acetic acid, dimethylsulfone, 2-butanone, trimethylamine, α-pinene, propanol, propionic acid, 3-hydroxy-2-butanone and some sex pheromones (Hallem *et al*., 2011; Labaude and Griffin, 2018). *In*

vitro experiments, therefore, employ this mechanism by using insects to multiply and maintain the viability of entomopathogenic nematodes. The most widely used insect host is the *Galleria mellonella* larvae. This insect species represents a generally accepted insect host for studies involving host-pathogen interactions, mechanisms of virulence, as well as antimicrobial efficacy bioassays (Jorjão *et al*., 2018). *Galleria mellonella* larvae confer a selective advantage in terms of wide availability, fast life cycle, high susceptibility to many nematode species, high yields, low maintenance cost and ease of rearing (Shapiro-Ilan and Gaugler, 2002; Jorjão *et al*., 2018).

2.2 Bacteria associated with Rhabditid nematodes

2.2.1 *Klebsiella* species

Bacteria of the genus *Klebsiella* are rod-shaped, Gram-negative, non-motile bacteria of the Enterobacteriaceae family. The *Klebsiella* genus contains bacterial species that are common in nature and often implicated in hospital and community-acquired infections (Trivedi *et al*., 2015). In human beings, these bacteria inhabit the digestive tract where they do not generally cause problems. However, they are amongst the leading causative agents of hospital-acquired infections particularly *Klebsiella pneumoniae* and *Klebsiella oxytoca*. The most commonly Rhabditis-associated *Klebsiella* species is *K. oxytoca* which has been obtained from Tumian strains of *O. chongmingensis* (Liu *et al*., 2012).

2.2.2 *Citrobacter* species

The most widely isolated Rhabditid-*Citrobacter* association occurs between *Oscheius* nematodes and *C. freundii*. The *Citrobacter* species belong to Enterobacteriaceae and are characterized by Gram-negative, rods of approximately 1.0 × 2.0 - 6.0 μm in size. They lack a capsule, occur singly or in pairs and are flagellated. *Citrobacter freundii* species form small, circular, convex, dark pink colonies on MacConkey agar. Approximately 11 species are found in this genus: *C. freundii, C. farmer, C. braakii, C. sedlakii, C. amalonaticus, C. rodentium, C. kosei, C. werkmanii, C. youngae, C. gillenii* and *C. braakii* (Petrella *et al*., 2001).

2.2.3 *Enterococcus* species

Enterococci bacteria are hardy, Gram-positive, facultatively anaerobic bacteria usually occurring in short and medium chains. The genus comprises commensals which are members of the gut microbiota inhabiting nearly all living organisms ranging from invertebrates (insects, nematodes) and land animals including human beings (Van-Tyne *et al*., 2013; Selleck *et al*.,

2019). *Enterococcus mundtii* has been isolated from the *O. chongmingensis* nematodes (Torres-Barragan *et al*., 2011).

2.2.4 *Achromobacter* species

Achromobacter species are non-motile, rod-shaped, non-pigmented, non-fermenting aerobes that are implicated in meat spoilage. They are positive for catalase and oxidase tests (Dijksterhuis and Samson, 2006). These bacteria have been associated with several opportunistic human infections (Barrado *et al*., 2013). The bacterium *Achromobacter xylosoxidans* has been reported to be associated with *O. chongmingensis* nematodes (Torres-Barragan *et al*., 2011).

2.2.5 *Providencia* species

The genus *Providencia* belongs to the gamma-proteobacteria (Sangeetha *et al*., 2016). The genus *Providencia* is made up of Gram-negative straight rods and has at least six known species, among them, *P. rustigianii*, *P. alcalifaciens*, *P. rettgeri*, *P. heimbachae* and *P. stuartii*. The bacterium *P. rettgeri* is associated with *O. chongmingensis* nematodes (Torres-Barragan *et al*., 2011). Their colonies are relatively large (0.6-0.8µm by 1.5-2.5µm), usually non-swarming and appear colourless on most growth media.

2.2.6 *Bacillus* species

Bacillus bacteria are Gram-positive, endospore-forming, rod-shaped, aerobic or facultatively anaerobic microorganisms. Some cultures of *Bacillus* spp. turn Gram-negative with age. Most species within the genus show a wide range of physiological attributes that enable them to live and thrive in all natural environments including extreme conditions (Sangeetha *et al*., 2016).

2.2.7 The genus *Serratia*

Serratia bacteria belong to the Enterobacteriaceae family. *Serratia marcescens* is the type species (Grimont and Grimont, 2006). The *Serratia* genus comprises Gram-negative, short (0.8 - 1.3 x 60.6 - 0.7 mm) non-sporulating, psychrophilic, facultatively anaerobic bacilli usually motile with a single flagellum (Boemare and Akhurst, 2006; Zhang *et al*., 2009; Serepa and Gray, 2014; Payelleville *et al*., 2019). *Serratia* colonies that grow on nutrient agar appear red, circular with entire smooth margins. Their temperature growth ranges from 4 - 42℃ (optimum 30 - 37 ℃) (Zhang *et al*., 2009). They mutually associate with *Heterorhabditidoides* nematodes (Bao *et al*., 2017). The genus name is coined after Serafino Serrati, who was an Italian physicist (Mai, 2018).

Table 1: Some described entomopathogenic nematode-bacteria associations

Nematode host	Symbiotic bacteria	Country of isolation
Oscheius carolinensis	*S. marcescens*	USA
O. chongmingensis	*S. marcescens, S. nematodiphila*	China
Oscheius spp.	*S. marcescens* MCB	South Africa
Oscheius spp.	*Serratia* spp.	Hawaii
Heterorhabditis bacteriophora	*P. luminescens*	Trinidad; Kenya
H. noenieputensis	*P. luminescens* subsp. *noenieputensis*	South Africa
H. indica	*Photorhabdus* spp.	Kenya
H. safricana	*P. luminescens* subsp. *laumondii*	South Africa
H. downesi	*P. temperata* subsp. *temperata, P. temperata* subsp. *cinerea*	Ireland, Hungary
H. megidis	*P. temperata* subsp. *cinerea*, *P. temperata* subsp. *temperata, P. temperata* subsp. *khanii*	Hungary, UK, Netherlands, Russia, USA, Hungary
H. zealandica	*P. temperata* subsp. *tasmaniensis*	New Zealand, Australia
Steinernema carpocarpsae	*X. nematophila*	France, Poland, USA
S. karii	*Xenorhabdus* spp.	Kenya
S. yirgalemense,	*Xenorhabdus* spp.	Kenya
S. weiserii	*Xenorhabdus* spp.	Kenya
S. poinari	*X. bovienii*	Poland
S. feltiae	*X. bovienii*	France
S. glaseri	*X. poinarii*	Kenya; USA

Source: Bhat *et al*., 2020

Over 70 species of this genus are associated with insects (Serepa and Gray, 2014). They have been implicated in various human infections as well as food spoilage (Mai, 2018). This nematode-bacterium relationship has many similarities to those exhibited by *Steinernema* - *Xenorhabdus* and *Heterorhabditis* - *Photorhabdus* duo. Various species of EPNs, all symbiotic with strains of *Serratia,* have been isolated from the *Heterorhabditidoides* group. These include

O. chongmingensis, *O. rugaoensis* and *O. carolinensis* (Ye *et al*., 2010; Liu *et al*., 2012; Zhang *et al*., 2012; Bao *et al*., 2017) (Table 1). Some species of the nematode *Oscheius* that are associated with *Serratia* have shown parasitism to insects (Lephoto *et al*., 2015; Torrini *et al*., 2015). Some species of *Serratia* include, *S. fonticola*, *S. nematodiphila*, *S. plymuthica*, *S. marcescens*, *S. rubidaea*, *S. grimesii*, *S. liquefaciens*, *S. proteamaculans*, *S. marnorubra* and *Serratia odorifera* (Bao *et al*., 2017; Mai, 2018).

2.2.7.1 *Serratia marcescens*

Serratia marcescens is a Gram-negative, motile, rod-shaped, facultatively anaerobic bacterium in the gamma-proteobacteria and family *Enterobacteriaceae.* It has been known to cause nosocomial infections and other opportunistic diseases in humans. *Serratia marcescens* is the most significant pathogenic species of the *Serratia* genus (Abdullah *et al*., 2017). This species has several strains such as *Serratia marcescens* subsp. *sakuensis, Serratia marcescens* MCB to mention but a few (Karthick *et al*., 2015). Over the past few years, reports documenting the mutualistic association of *Serratia* strains with EPNs including *Oscheius* and *Caenorhabditis* have been published (Zhang *et al*., 2009; Abebe *et al*., 2010; Torres-Barragan *et al*., 2011; Tambong, 2013). Many of the *Serratia* species associated with *Caenorhabditis* share close genetic history with *S. marcescens*, whilst those that associate with *O. carolinensis* represent *S. marcescens* strains that have lost their pathogenicity towards nematodes (Petersen and Tisa, 2013).

2.2.7.2 *Serratia plymuthica*

Like all *Serratia* species, *S. plymuthica* is a Gram-negative, red-pigmented, facultatively anaerobic bacilli often isolated from plants, animals, soil and water. *Serratia plymuthica* strains are important agents in biological control due to their extracellular enzymes and metabolites which effectively hinder the growth of various plant pathogens (de Vleesschauwer and Höfte, 2007; Weise *et al*., 2014).

2.2.7.3 *Serratia nematodiphila*

Serratia nematodiphila is a red-pigmented, motile, Gram-negative, non-sporulating, fluorescent, rod-shaped species with a single flagellum. These symbiotic enteric bacteria have been isolated from the intestines of the nematode *Oscheius chongmingensis* formally, *Heterorhabditidoides chongmingensis* (Zhang *et al*., 2009; Petersen and Tisa, 2013).

2.4 Pathogenicity and life cycle of the nematode-bacterium association

This is an example of a cyclic life cycle (Figure 1) starting and ending with infective juveniles (Hurst *et al*., 2015). Species within the genus *Oscheius*, like those of *Heterorhabditis*, can have more than one associated bacteria unlike the *Steinernema* genus whose nematode-bacterium relationship is specific (Table 1) and only relates with a single bacterium at any given time (Hurst *et al*., 2015). Infective juveniles protect endosymbionts against harsh soil environments and other competing organisms in the ground. The free-living 3rd stage IJs (Stock *et al*., 2017) can survive outside their hosts for days or weeks. This is also the only stage that is capable of causing infection, hence the name infective juvenile (Dillman, 2013). These third larval stages of the EPNs are stress-tolerant, bacteria vectoring, soil-inhabiting invertebrates that actively search, infect and kill insect hosts (Adams and Nguyen, 2002; Hinchliffe *et al*., 2010; Dillman *et al*., 2012; Dillman, 2013). Once a host is located, the nematodes penetrate through natural openings like the mouth, spiracles, cuticle or anus (Goloboff *et al*., 2008) into the hemolymph, releasing up to 200 bacteria within five hours (Ciche and Ensign, 2003; Waterfield *et al*., 2009). These enteric bacteria are met with the fast-acting innate immune system of the insect host such as humoral defenses, phagocytic destruction by killer proteases and free radicals, lysozymes and other insect antimicrobial compounds (Waterfield *et al*., 2009).

The symbiotic bacteria rapidly grow and multiply within 48 hours (Watson *et al*., 2005), reaching the stationary phase and secreting toxins, secondary metabolites and enzymes. This exponential growth is attributed to these secondary bioactive molecules and toxins which suppress the insects' immune defenses (Kenney *et al*., 2019) and also cause injury to the host tissues (Goodrich-Blair and Clarke, 2007). Death of the insects ultimately occurs within 24 - 48 hours via toxaemia and septicaemia. The symbiotic relationship occurs whereby nematodes are supplied nutrients by the endosymbionts while providing them with a habitat to grow and develop (Hurst *et al*., 2015). Additionally, the bacteria secrete several antimicrobial compounds including antibiotics which inhibit putrefaction of the insect cadaver, outcompete other microflora and prevent colonization of the insect cadaver by other secondary invading pathogens (Hurst *et al*., 2015; Mai, 2018).

Availability of food and nutrients encourages larval growth from the 3rd stage dauer juveniles (DJs) to 4th stage juveniles (J4) then pupa which then lay eggs which eventually hatch in 8 – 10 days, forming the first generation (J1) of female hermaphrodite adult infective juveniles

through the process of self-fertilization (*endotokia matricida*) (Ponnusamy and Belur, 2015). Self-fertilized females form amphiteric male and female IJs as well as self-fertile hermaphrodite females (Ponnusamy and Belur, 2015). Nematode multiplication continues forming three generations of IJs. Their increased numbers deplete nutrients in the insect carcass. This stage usually signifies the end of the pathogenic phase and commencement of the symbiotic state (Ffrench-Constant *et al*., 2003; Chitra *et al*., 2017). The IJs reassociate with the endosymbiotic bacteria differentiating into free-living, IJs which emerge from the dead insect (Figure 2) after 7 - 10 days in search of new hosts (Ciche and Ensign, 2003; Koppenhöfer, 2007; Ciche *et al*., 2008). Due to their insecticidal properties, this nematode-bacterium complex has now been successfully used in integrated pest management programs and biological control programs worldwide (Stock *et al*., 2017). Different metabolites have been reported from *Serratia* species, including but not limited to serrawettins, prodigiosin, lipases, hemolysins, chitinases, serralysin, toxins, nucleases, indole acetic acids, protease and amylases (Grimont *et al.,* 1979; Williamso *et al*., 2005; Dhar *et al*. (2018); Mai, 2018).

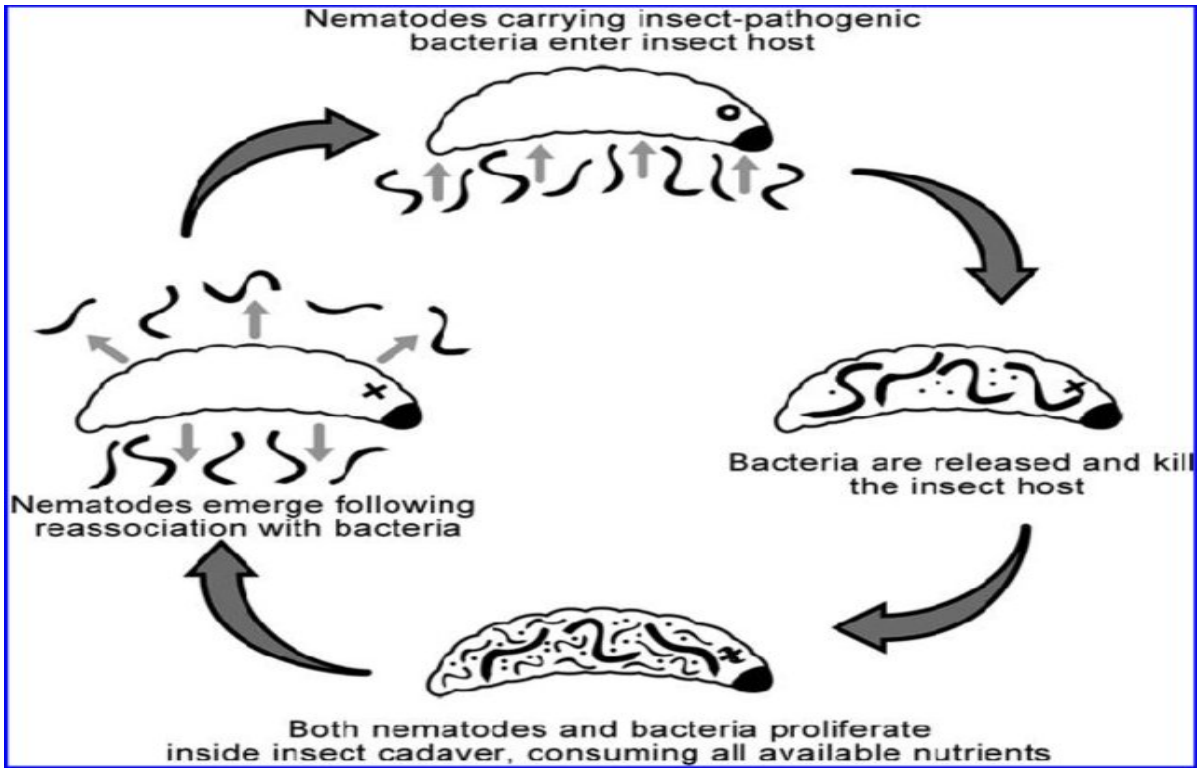

Figure 2: Generalized life cycle of nematode-bacterium association signifying the sequence of events involved.

Source: Petersen and Tisa, 2013.

2.5 Phase variation within the genus *Serratia*

Phase variation is a mechanism of bacterial adaptation used by some species of bacteria to adapt to environmental conditions. The bacteria undergo frequent reversible phenotypic phases as a result of DNA sequence plasticity, which generates a reversible switch between 'on' and 'off' phases to express one or more protein-encoding genes (Gaudriault *et al*., 2008). Nematode-associated endosymbiotic bacteria occur in two forms i.e. primary (Phase I) and secondary form (Phase II) (Derzelle *et al*., 2004). Infective juveniles carry the 1° form but the 2° phase usually develops from the 1° state *in vitro* (Adams *et al*., 2006). Phase II variants are therefore incapable of supporting nematode growth and development. These two phases have distinct colony morphologies that can be visualized. Phase I variants absorb dyes, for instance, NTBA cultures give a red core overlaid by a dark blue area usually encompassed by a clear zone signifying adsorption of bromothymol blue and tetrazolium chloride (TTC). The same happens to MacConkey agar where they adsorb neutral red. The 1° form produces more secondary compounds (Derzelle *et al*., 2004; Gaudriault *et al*., 2008).

This change from one state to another occurs from the primary to the secondary state (Derzelle *et al*., 2004). The two phases have also shown differences in pathogenicity capacity with the primary form being more virulent than the secondary form. Reversion of the phases is unidirectional from the primary to the secondary state in *Photorhabdus* spp. However, *Xenorhabdus* spp. can reverse from the secondary to the primary form (Adams *et al*., 2006). More research needs to be conducted on *Serratia* species to ascertain whether the phases are reversible or unidirectional. Joyce and Clarke (2003) used these two phenotypic variants to explain the molecular basis behind this bacterium switch between the symbiotic and pathogenic phases. They reported the activity of a gene homologous to hexA gene from *Erwinia* spp. that has repressive activity towards symbiosis in Phase II cells. This, therefore, suggests the role played by this gene in controlling and regulating pathogenicity and mutualism. Additionally, the HexA gene is involved in the temporal separation of pathogenicity and mutualism (Joyce and Clarke, 2003).

2.6 Antimicrobial compounds and toxins produced by *Serratia* species

Bacteria of the genus *Serratia* are inhabitants of diverse environmental niches with many of the strains regarded as reservoirs of unique antimicrobial metabolites with various activities. The production of most of these biomolecules is due to the presence of polyketide synthase (PKS) genes (Mai, 2018). Over the years, pigments from microbes have been researched for

their many functions. *Serratia* species can secrete bioactive secondary metabolites mediated by quorum sensing (Su *et al*., 2016).

The most studied antimicrobial agent from the *Serratia* genus is prodigiosin. It is a red, water-insoluble, non-diffusible substance on the cell envelope of some strains of *Serratia* such as *S. marcescens, S. plymuthica* and *S. rubideae* (Mai, 2018) (Table 2). Albeit, prodigiosin is soluble in organic solvents like methanol (Jafarzade *et al*., 2013; Darshan and Manonmani, 2015; Elkenawy *et al*., 2017). It belongs to the prodiginines family and its structure comprises three pyrrole rings that form a pyrrolo-pyrrole-ethene linkage (Moons *et al*., 2006; Venil and Lakshmanaperumalsamy, 2009; Ibrahim *et al*., 2014). Many of the reported strains of *S. marcescens* are of clinical origin, mostly non-pigmented whereas many environmental strains are pigmented. Production of prodigiosin is optimal at 28℃ - 30℃ (Tanaka *et al*., 2004; Weise *et al*., 2014; Su *et al*., 2016; Faraag *et al*., 2017) and is controlled by a cluster of operon genes called pigA-O. Prodigiosin has been suggested to aid in the overflow of metabolic waste products from bacteria, assist in surface adherence and increasing the dispersal mechanism of bacteria and act as storage for excess proline (Harris *et al*., 2004; Fineran *et al*., 2005). The pigment exhibits immunosuppressive, plasmid curing, antimalarial, antibacterial, antiprotozoal, antioxidant, antifungal, anticancer and anti-tumor activities (Table 2) (Slater *et al*., 2003; Williamson et al. 2005; Giri *et al*., 2004; Wang *et al*., 2013; Mathlom *et al*., 2018). Prodigiosin extracts purified from *S. marcescens* IBRL and *S. marcescens* B10 VKM USM 84, *Serratia marcescens B2* are active against *S. aureus*, *P. aeruginosa*, *B. subtilis*, *B. cereus*, *Salmonella* spp., *Shigella* spp., *C. albicans*, *C. utilis*, *Cryptococcus* as well as algal blooms (Thomson *et al*., 2000; Pore *et al*., 2016).

Table 2: Presently identified secondary metabolites produced by *Serratia* species and their antimicrobial activity.

Secondary metabolites	Derivatives	Activity
Prodigiosin	Prodiginines	Immunosuppressive, plasmid curing, antimalarial, antibacterial, antiprotozoal, antioxidant, antifungal, cytotoxic
Biosurfactants		Antifungal, antiviral, antibacterial and anti-algal
Serrawettins	Sw1, Sw2	Antibacterial, anticancer
Antibiotics	Althiomycin, andrimid, zeamine	Antibacterial, bacteriostatic
Antimycotic	Oocydin A, haterumalides	Antifungal, antimycotic
Indole	Indole acetic acid (IAA)	Nematicidal, plant growth promoting
Carbapenems	1-carbapen-2-em-3-carboxylic acid	Antibacterial
Siderophore		Antibacterial, plant growth promoting
Pyrrolnitrin		Antifungal
Chlorpyrifos		Insecticidal
Acids	Heptadecanoic acid, nonanoic acid, octadecanoic acid	Antioxidant, antifungal, antibacterial
Enzymes	Proteases, chitinases, lipases, serralysins, nucleases, hemolysins, amylases	Hydrolysis, biodegradation
Toxins	Ssp1 and Ssp2 toxins	Antibacterial

Source: Zhang *et al*., 2014; Su *et al*., 2016; Schmidt *et al*., 2017; Mai, 2018.

Biosurfactants are antimicrobial compounds secreted by bacterial cells and have hydrophobic and hydrophilic moieties conferring the ability to reduce surface and interfacial tension

(Mathlom *et al*., 2018). The chemical composition of a biosurfactant comprises polysaccharide-protein complexes, protein-like substances, glycolipids, lipopolysaccharides, lipopeptides, phospholipids, neutral lipids and fatty acids (Figure 3). Bacterial biosurfactants are the best alternatives to synthesized medicines and may be used as safe and effective therapeutic agents (Singh and Cameotra, 2004). The efficacy of biosurfactants against fungi, viruses, bacteria and algae has been reported by Cameotra and Makkar (2004); Mathlom *et al*., 2018 among others (Table 2).

Some strains of *S. marcescens* such as *S. marcescens* IBBPo15 and *S. marcescens* strain NSK-1 secrete serrawettins. These are lipopeptide compounds synthesized by polyketide synthases and are thus biosurfactants with potent anticancer agents against T-cell leukemia and Burkitt's lymphoma (Su *et al*., 2016) as well as broad-spectrum antibacterial compounds (Clements *et al*., 2019). The opportunistic pathogen *Serratia marcescens* additionally secretes serratamolide, a small cyclic amino-lipid, involved in hemolysis and swarming motility (Shanks *et al*., 2013). Other antibacterial compounds secreted by *Serratia* include the broad spectrum β-lactam antibiotic carbapenem, bacteriocins, the antibiotic andrimid, zeamine antibiotics as well as the antibacterial toxins Ssp1 and Ssp2. The antibiotic zeamine has broad-spectrum bactericidal efficacy against yeast and multidrug-resistant bacteria (Slater *et al*., 2003; Abebe-Akele *et al*., 2015; Mai, 2018). Althiomycin, a white crystalline antibiotic compound secreted by *S. marcescens* has inhibitory effects on the growth of both Gram-positive and Gram-negative bacteria (Gerc *et al*., 2012).

Oocidin A, a chlorinated macrocyclic lactone has also been isolated from some strains of *Serratia marcescens*. This lactone contains one atom of chlorine, a carboxyl group and a tetrahydrofuran ring that is internal to a larger macrocyclic ring (Figure 3). Antifungal activity of oocydin A has been reported against various phytopathogenic oomycetes including *Phytophthora parasitica*, *Pythium ultimum, Phytophthora citrophora* and *Phytophthora cinnamomi* (Strobel *et al*., 1999). Oocydin A may therefore have potential as an antimycotic agent in agricultural applications, especially for crop protection. Other effective antifungal agents like haterumalides have also been isolated from *Serratia proteamaculans* and *Serratia* sp. ANU101. Haterumalides were the very first polyketides to be discovered in the genus *Serratia* (Stock *et al*., 2003; Lim *et al*., 2015; Adam *et al*., 2016). Dhar *et al*. (2018) also reported the secretion of siderophores and plant growth-promoting compounds like indole-acetic acids from *S. marcescens*.

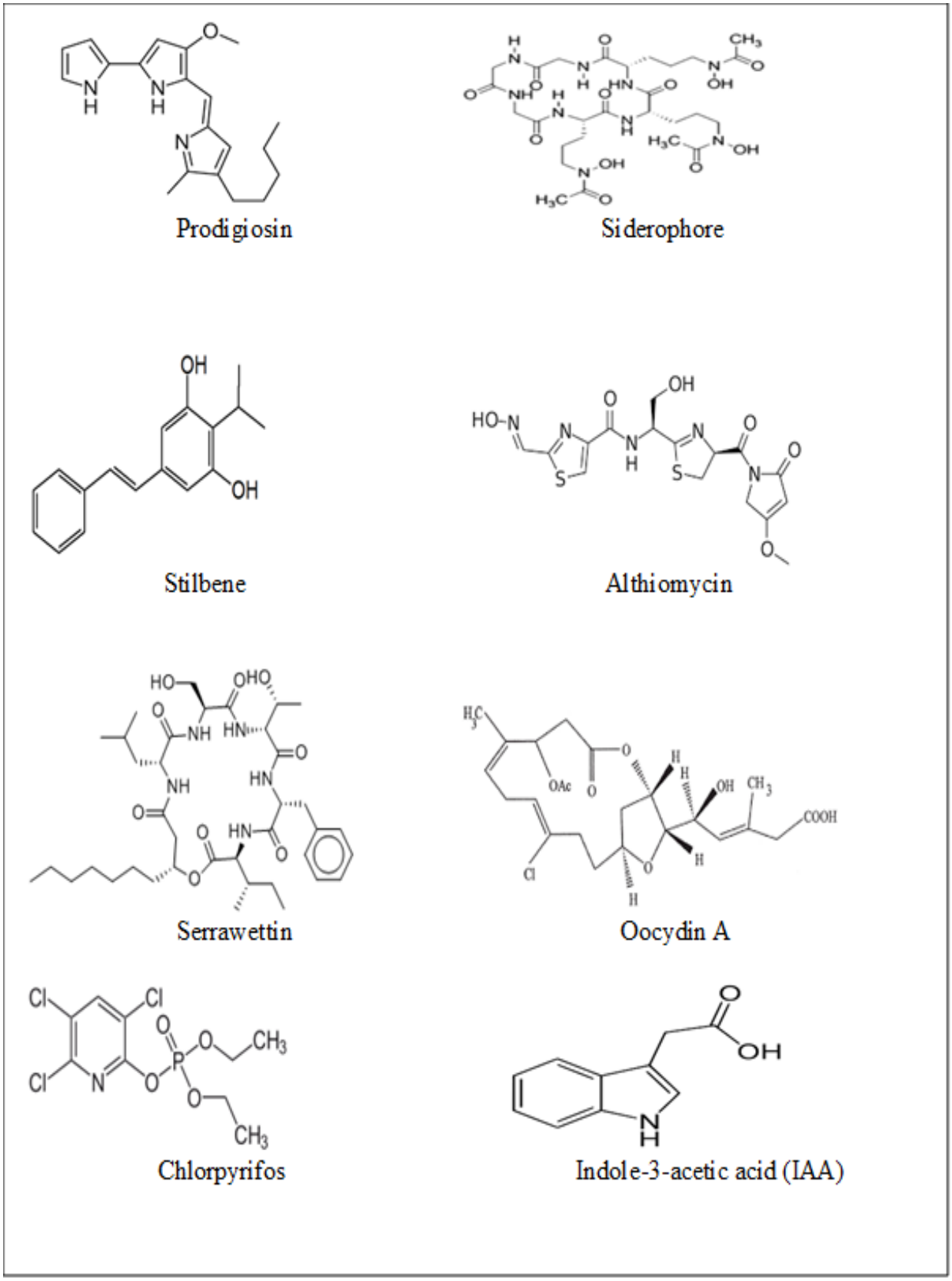

Figure 3: Secondary antimicrobial compounds produced by *Serratia* species

Source: Karthick *et al*., 2015

Furthermore, amylases, lipases, serralysins, nucleases, hemolysins, protease and chitinases are among other commercially significant biomolecules produced by *Serratia* spp. (Grimont *et al.*, 1979; Williamson *et al*., 2005; Dhar *et al*., 2018; Mai, 2018). *Serratia marcescens* has proved to be one of the most efficient bacteria for the degradation of chitin in the environment (Sezen *et al*., 2001). Two major chitinases are secreted by *S. marcescens*, ChiA and ChiB working synergistically with a chitin-binding protein CBP21 and chitobiase to completely degrade chitin (Petersen and Tisa, 2013). Chitinase B, is an enzyme characterized by very high thermal stability. It is believed that the production of enzymes in this genus is highly favored by their ability to colonize various environmental niches (Abdou, 2003; Williamson *et al*., 2005). The bacterium *S. marcescens* B742 produces protease and chitosanase enzymes and further hydrolyses shrimp shells into water-soluble protein hydrolysates (Zhang *et al*., 2014). *Serratia* spp. ISTVKR1 has been reported to have a high biodegradable ability of various chemical contaminants among them, methyl parathion, organophosphorus pesticides and p-nitrophenol (Gupta and Thakur, 2015). Many strains of *Serratia marcescens* isolated from petroleum-contaminated sites have shown potential in the bioremediation of petroleum spills (Mai, 2018). Serepa *et al*. (2015) have also documented the presence of indole-3-acetic acid and tryptophan from *S. marcescens* MCB. They also concluded that this strain shares many similar characteristics with *Photorhabdus* and *Xenorhabdus* species, hence suggested that its host nematode ought to be accepted as an entomopathogenic nematode. Strains of *S. odorifera, S. marcescens* and *S. plymuthica* produce sodorifen, dimethyl trisulfide and methanethiol and terpenoids which are volatile organic compounds. They have exhibited cytotoxicity and broad bacteriostatic inhibitory effects against nematodes, fruit flies, bacterial and fungal pathogens (Kwak *et al*., 2015; Schmidt *et al*., 2017).

Karthick *et al*. (2015) also extracted various biomolecules from *S. marcescens* which showed efficacy against both fungal and bacterial pathogens. Analysis of the fractioned ethyl acetate extract showed the presence of phenol, 2, 4-bis (1, 1- dimethyl ethyl), an antibacterial compound as well as 9-oxo methyl ester, nonanoic acid and octadecanoic acid which showed both antibacterial and antifungal agents (Table 2). Insecticidal biomolecules chlorpyrifos and heptadecanoic acid which are antioxidants were also extracted. Besides these, other compounds like beta 1, 5 dibenzoylribofuranose, dimethyl silanediol, tetradecanol, 4-dimethyl-amino-benzoic acid methyl ester, decanoic acid, benzene acetic acid, docosanolide and 1-ethoxy, 2, 2, 2-bicyclo octane were also found to be present (Karthick *et al*., 2015).

2.7 Phylogenetic and taxonomic reconstruction

Phylogeny refers to the study of the evolutionary relationships in species of living organisms. The purpose of constructing a phylogenetic tree is to describe organisms based on relatedness to common ancestry. (Harrison and Langdale, 2006). Recent classification of bacteria uses 16S rRNA gene in addition to phenotypic tests (Stock and Goodrich-Blair, 2008). The 16S rRNA gene has better resolution than 16S rDNA making it preferred (Stackebrandt *et al*., 2002; Stock and Goodrich-Blair, 2008). Most available bacterial rRNA sequences are founded on analysis and similarity of gene sequences of *Escherichia coli* rRNA as a standard (Evguenieva-Hackenberg, 2005). The first *Photorhabdus* genome to be sequenced was that of *P. luminescens* subspecies *laumondii* strain TT01 in the year 2003. The TT01 strain has a genome sequence of 5,688,987 base pairs (bp) with 4,839 protein-coding genes (Duchaud *et al.*, 2003). It is an enterobacterium associated with the vector *Heterorhabditis bacteriophora*. Presently there are about 33 genome sequences in the NCBI GenBank that are associated with the various *Photorhabdus* spp. (Somvanshi *et al*., 2019).

2.7.1 16S rRNA gene structure

The ribosomal RNA (rRNA) is the most conserved gene in cells of living organisms. Bacterial rRNA sequences gained more value in the last two decades following their application in taxonomic determination studies as well as for direct identification of particular bacteria in medical and environmental samples (Evguenieva-Hackenberg, 2005). Bacterial rRNA consists of two parts, the small and large subunits comprising 16S rRNA and (23S and 5S rRNAs), respectively (Figure 4). The 16S rRNA gene is approximately 1.5kb in size, single-stranded and can fold into a secondary structure made of unbound and bound regions of nucleic acids (Mathews *et al.,* 2000). The unbound, also known as hypervariable regions bend into loop-like sections interrupted by double-stranded stems representing the conserved sequences (Klug *et al*., 2009). Conserved areas have very minimal differences among closely related species (Mathews *et al.,* 2000) serving as catalysts in ribosomes for the synthesis of peptides hence maintain the 2° structure of rRNA. These sequences in addition facilitate amplification of 16S rRNA genes thus allowing comparisons between and among species (Cox and Nelson, 2008). Hypervariable regions are named from the high level of variability occurring across and within different species. These regions enable differences between species to be determined because of the existing mismatches (Wiley *et al.,* 2008).

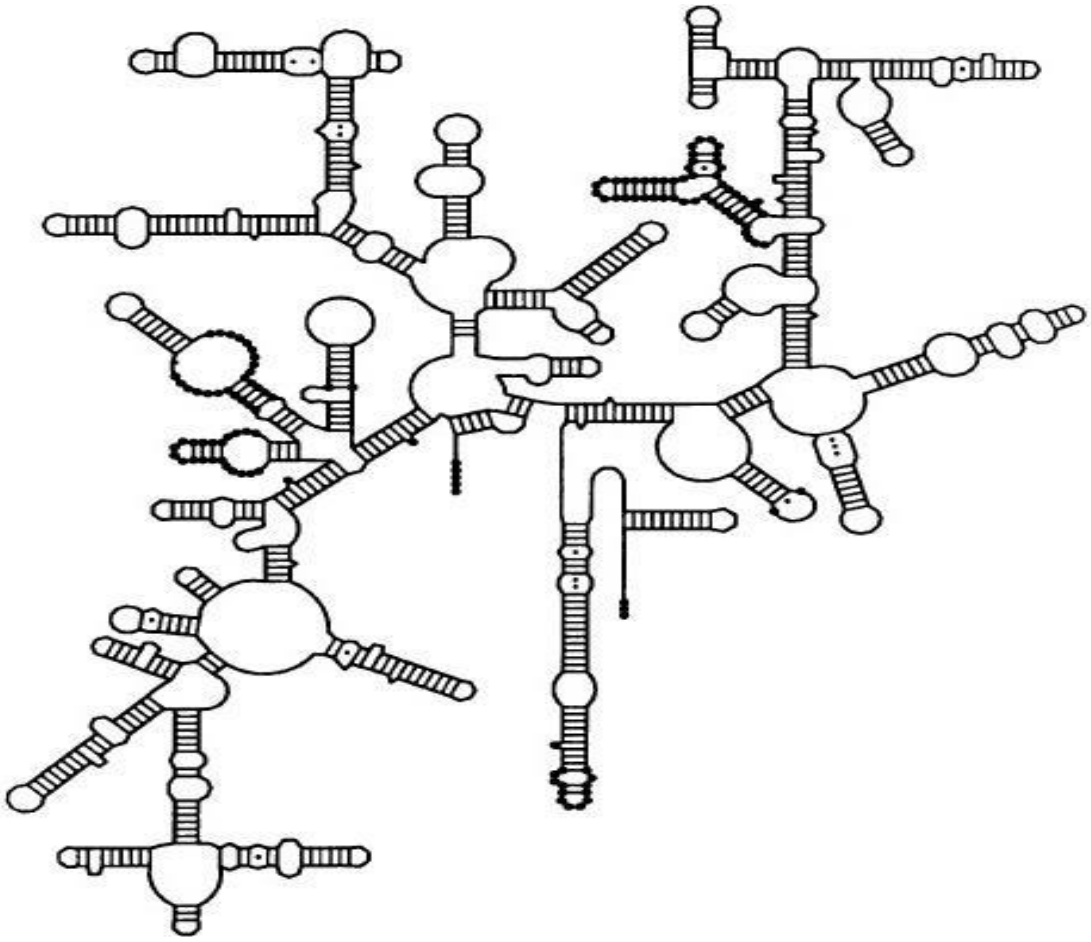

Figure 4: The secondary structure of bacterial 16S rRNA gene

Source: Woese, 1987.

2.8 Antimicrobial resistance

Antimicrobial resistance (AMR) simply refers to a reduction in susceptibility of pathogens towards agents that previously showed efficacy, such as, antibiotics, antivirals, antiparasitic and antifungal treatments (Madigan *et al*., 2009). As a result, standard treatments become ineffective, infections persist and may spread to others. When such resistance to previously treatable infections occurs, these pathogens are known as "superbugs". Alexander Fleming discovered penicillin, the first-ever antibiotic in 1928. Following the discovery, several other antibiotics were developed. This was very important particularly in Africa where these infections were taking a toll on peoples' lives. However, the continued use of antibiotics has resulted in the development of resistance by some bacterial species. Resistance to antimicrobial agents occurs naturally in pathogenic microorganisms as a result of genetic variations within their genomes. However, abuse, misuse and overuse of these agents have also resulted in higher levels of resistance (WHO, 2016).

Following antibiotic discovery, increased synthesis of counterfeit drugs has been observed in African countries (Slavcovici *et al*., 2015). This results in flooding the market with these substandard antibiotics which are sold at cheaper prices, easily accessible over the counter and have high consumption rates. Inaccessibility to quality primary care also plays a major role in exacerbating this menace in low and middle-income countries (WHO, 2018). The most misused antimicrobial agents are antibiotic drugs since they are the first prescribed medications even in viral infections like the flu and colds in humans, as growth promoters in plants, animals, poultry and fish. These can still be passed down to humans through the consumption of the products as food. These pathogens can also be spread through travel, trade and migration of people and animals. As resistance accumulates, the once efficient treatments have become less and less effective rendering them useless resulting in pandemic diseases with high mortality rates in the once easily curable infections (WHO, 2014; WHO, 2016). Between the years 2020 - 2015, antibiotic use increased by 65% worldwide and doubled in low and middle-income countries. This is affecting many sectors including the suffering of newborns from sepsis and new hospital infections to those who have undergone surgery, cancer treatments or transplant care (WHO, 2018).

Enterococcus spp., *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterobacter* spp. pose the greatest threat in the fight against AMR (Rice, 2008). They are the leading causative agents of nosocomial infections and easily evade the biocidal activity of antibiotics. Once these bugs are successfully controlled, the knowledge can be applied for in controlling other pathogens with similar resistance (Rice, 2008). Increased levels of transmitted anti-HIV drug resistance have been detected among patients starting antiretroviral therapy, thereby making efforts to combat the disease even harder (WHO, 2016). In addition to the mentioned ESKAPE bugs, other pathogenic bacteria showing resistance include *Streptococcus pneumonia*, nontyphoidal *Salmonella*, *Shigella* species and *Neisseria gonorrhea*. The reports on the high occurrence of resistance to third-generation cephalosporins in *E. coli* and *K. pneumoniae* imply that treatment of severe infections caused by these bacteria in many countries now has to rely on carbapenems, which are the last resort for treatment of severe community and hospital-acquired infections. These antibiotics are more expensive, limited in resource-constrained settings and may further accelerate the development of resistance within microorganisms. Of great worry is the reported resistance of *K. pneumoniae* to carbapenems which may render the pathogen uncontrolled in the near future (WHO, 2016; 2018).

2.9 Phytopathogenic fungi

Plant pathogens include bacteria, fungi, viruses, nematodes and other parasitic plants. However, about 85% of plant diseases are caused by pathogenic fungi and the remaining portion by the other pathogenic microbes (Kumar *et al*., 2016). Fungi are non-chlorophyte, sporulating, eukaryotic (cells having true nuclei) organisms usually filamentous and branched in most of the species. Fungi account for the greatest destruction of agricultural plants than any other group of phytopathogens. Out of about 100,000 species of the fungal species known, more than 10,000 species are capable of causing disease in plants while only about 50 species cause diseases in animals and humans (Kumar *et al*., 2016). Fungal pathogens indiscriminately affect any part of the plants ranging from the fruits, leaves, stems and the roots in seedlings, mature plants as well as stored foods (Fletcher *et al*., 2010). Some of the most devastating fungal pathogens are: *Aspergillus flavus*, *Fusarium oxysporum*, *Fusarium solani*, *Penicillium* spp. and *Botrytis* spp. (Almeida *et al*., 2000). Within a single plant pathogenic fungal species, several other varieties exist. For instance, in the case of *Fusarium oxysporum*, nearly 120 different varieties are recorded (Kumar *et al*., 2016).

These phytopathogens cause tremendous losses in crops as well as producing toxic secondary metabolites such as mycotoxins which are extremely harmful to humans once contaminated food is ingested. Mycotoxins produced by fungal pathogens result in infections referred to as mycotoxicoses in humans and animals. The most important mycotoxins are aflatoxins, fumonisins, zearalenone, deoxynivalenol, patulin, ochratoxin A, T-2 toxin, penicillic acid and trichothecenes (Erkekoğlu *et al*., 2008; Ismaiel and Papenbrock, 2015; Perczak *et al*., 2018). Aflatoxins are potent carcinogens and result in high mortality especially in association with hepatitis B virus (Pitt, 2000). They result in acute liver damage, liver cirrhosis, attack on the central nervous system, skin disorders, hormonal effects, mutations and induction of cancerous tumors (Erkekoğlu *et al*., 2008; Perczak *et al*., 2018). *Serratia* bacteria have shown antifungal activity against other pathogens.

CHAPTER THREE: MATERIALS AND METHODS

3.1 Retrieval and maintenance of *Oscheius* nematodes

Entomopathogenic nematodes (ENPs) of *Oscheius* species were obtained from stock cultures maintained at the Horticultural Research Institute (HRI) at the Kenya Agricultural and Livestock Research Organization (KALRO), Thika, Kenya. These nematodes were stock cultures that were baited from the soil in Kenya. A total of 10 nematode samples already baited from the soil were collected from the Nematode Collection Centre using sterile universal bottles after confirmation of their viability under the light microscope by observing their movement. The EPNs were maintained and cultured following the *in vivo* procedure described by Kaya and Stock (1997) where last instars of *Galleria mellonella* larvae were inoculated with 5 drops of the nematode suspension on Petri dishes each lined with a white absorbent cotton cloth and incubated for 5 days at room temperature (23 ± 2 ºC) in the dark. Once dead, only the odorless cadavers (indicating infection with EPNs) were selected and transferred to a larger harvest dish known as a White trap. The trapping technique was set up in such a way that the dead cadavers were placed on the surface of a moist absorbent cloth in an inverted position and placed inside the larger harvest dish containing 30 ml sterile distilled water (Shapiro-Ilan *et al*., 2002). When the infection cycle was complete the infective juveniles emerged from the larvae into the water and were harvested by decanting. The nematodes were stored at room temperature in sterile distilled water in wide troughs and re-infection was done after every 5 months to maintain viability.

The greater wax moth (*Galleria mellonella*) larvae were used for the initial infection with the entomopathogenic nematodes. The *G. mellonella* larvae were also obtained from the same Institute from cultures maintained on an artificial diet (in g/Kg: 464.0 g wheat bran, 69.0 g dried yeast, 67.0 g beeswax, 207.0 g glycerine and 193.0 g honey beeswax) as described by Realpe *et al.* (2007). The components were weighed, thoroughly mixed using a sterile spatula and heated on moderate heat while gently stirring until the final product formed a fine strong diet which was fed to the larvae and changed after every 14 days when the nutrients had already been depleted (Jorjão *et al*., 2018). For the experiment, only late instars of *G. mellonella* were selected and used for the study. The nematodes were multiplied and maintained in the greater wax moth, *Galleria mellonella*. Five *G. mellonella* larvae were placed in each of the three Petri dishes lined with a clean sterile moist absorbent white cotton cloth followed by the addition of 5 drops of nematode suspension per plate and incubated at

room temperature for 5 days in the dark. Red pigmented dead *G. mellonella* larvae were then transferred to white traps for the emergence of the infective juveniles subsequently used for re-infection and isolation of symbiotic bacteria (Aiswarya *et al*., 2017).

3.2 Isolation of *Serratia* spp. and other nematode-associated endosymbiotic bacteria from infected *Galleria mellonella* larvae

In a laminar airflow cabinet, five late instars of *G. mellonella* larvae were placed on the surface of a clean sterile absorbent cotton cloth in a Petri dish followed by the addition of 5 drops of nematode suspension per Petri dish and made in triplicate. All the Petri dishes were sealed with parafilm and incubated in a dark cupboard at room temperature for 4 days. The red-pigmented dead insect larvae were collected and surface sterilized by submerging in 70% ethanol for 1 minute. They were rinsed thrice in sterile distilled water and dried on blotter paper in a laminar airflow cabinet (Sangeetha *et al*., 2016). The oozing hemolymph was collected using a sterile loop after dissecting the cadavers with a sterile scalpel and streaked on a differential medium, Nutrient bromothymol blue 2,3,5- triphenyltetrazolium chloride (NBTA) containing (25.0 mg bromothymol blue, 40.0 mg 2,3,5-triphenyltetrazolium chloride and 8.0 g nutrient agar in one litre sterile distilled water) and MacConkey agar (MAC) containing (1.5 g pancreatic digest of casein, 1.5 g peptic digest of animal tissue, 17.0 g pancreatic digest of gelatin, 10.0 g lactose, 5.0 g sodium chloride, 0.03 g crystal red, 0.001 g crystal violet, 1.5 g bile salts and 13.5 g agar per liter sterile distilled water), (Aatif *et al*., 2012). This procedure was repeated to make 3 replicates and the inoculated plates were incubated at 28 °C for 48 hours. Only reddish colonies on MAC and NBTA (representing Phase 1 culture cells of *Serratia* bacteria) were sub-cultured onto fresh media (NBTA and MAC) to obtain pure colonies of bacteria used in the subsequent steps (Wang *et al*., 2008; Sigh *et al*., 2012; Muangpat *et al*., 2017). For long-term preservation of all the isolates, 5 ml volume of each bacterial isolate grown overnight in Luria-Bertani (LB) broth (10.0 g tryptone, 10.0 g sodium chloride, 5.0 g yeast extract in 1 litre sterile distilled water) was mixed with an equal volume of sterile double-distilled glycerol and stored at -20 °C for future use (Babic *et al*., 2000). The LB media was used because it encourages faster growth of bacteria.

3.3 Phenotypic and biochemical characterization of endosymbiotic bacterial isolates

3.3.1 Cultural and morphological characterization

Cultural and morphological traits of the bacterial isolates were observed after 48 hours of incubation at 28 °C on MAC and NBTA media for confirmation of phase variation in the

bacterial endosymbionts (Sigh *et al*., 2012). Phenotypic properties of each colony were studied and recorded based on several features including colour, size, shape, form, elevation and margin of the colonies observed under X 40 magnification (Sangeetha *et al*., 2016). Gram staining was also performed using 24-hour bacterial culture grown on nutrient broth. First, a loopful of an overnight bacterial suspension was added onto a drop of sterile distilled water on a sterile glass slide and gently spread using the loop to obtain a very thin film which was then heat-fixed by gently passing it over a flame 3 times. The slide was flooded with crystal violet and left to stand for 1min before rinsing with sterile distilled water. A drop of Gram's iodine solution was added and left to stand for 1 min, rinsed with sterile distilled water and decolorized by the addition of 95% ethyl alcohol drop by drop for 10 seconds and immediately rinsed with water. The smear was then flooded with safranin as a counter-stain and left to stand for 1 minute, rinsed with a gentle stream of sterile distilled water and blot-dried with bibulous paper (air-dried) then observed under a light microscope under oil immersion after covering the slide with a coverslip (Kammar *et al*., 2016).

3.3.2 Biochemical tests

The isolated bacterial species were tested for their positive or negative response to the following biochemical tests and compared to different representative genera in Bergey's Manual of Determinative Bacteriology (Cowan, 1948). Each test was conducted in triplicate.

3.3.2.1 Catalase test

Petri dishes containing NBTA agar were inoculated with the bacterial isolates by streaking, sealed with parafilm and incubated at 28 °C for 24 hours followed by the addition of a drop of 3% hydrogen peroxide (H_2O_2). Production of bubbles (positive) was indicative of the presence of catalase enzyme which broke down H_2O_2 releasing water and oxygen which is seen by bubbles and vice versa (Kammar *et al*., 2016).

3.3.2.2 Oxidase test

Agar plates with NBTA were inoculated with overnight cultures of the bacterial endosymbionts by streaking and incubated at 28 °C for 24 hours after sealing with parafilm. Three drops of tetramethyl-phenylenediamine dihydrochloride (dye/reagent) were added to the culture growth surface. The presence of a deep purple colour indicated the presence of cytochrome C oxidase enzyme (oxidase positive), which oxidized the dye to a dark purple

colour. However, oxidase-negative organisms had no effect on the reagent, which remained colourless (Kammar *et al*., 2016).

3.3.2.3 Starch hydrolysis test

Starch agar (23.0 g nutrient agar, 10.0 g potato starch in 1litre sterile distilled water) plates were prepared and streaked with bacterial cultures then incubated at 28 °C for 48 hours. The plates were flooded with Gram's iodine solution and allowed to stand for 10 minutes. A zone of clearing around the growth colonies indicated the presence of the enzyme amylase (positive results) which was able to break down starch while a dark blue color indicated an absence of amylase enzyme (negative results) (Sigmon, 2008).

3.3.2.4 Urease test

Sterile universal bottles containing Stuart's urease broth (Himedia) (0.10 g yeast extract, 9.10 g potassium dihydrogen phosphate, 9.50 g dipotassium hydrogen phosphate, 0.01 g phenol red in 1 litre sterile distilled water) were inoculated with an overnight culture of each bacterial isolate and incubated for 48 hours at 28 °C. Usually, the urea broth contains urea and the indicator phenol red. When the inoculated test organism produces the enzyme urease, it breaks down urea in the presence of water releasing carbon dioxide and ammonia which combine to form ammonium carbonate. The ammonium carbonate raises the pH of the broth resulting in a colour change of the phenol red indicator from orange-yellow to bright pink (positive results). Failure of an organism to produce the enzyme urease does not affect the area and the colour remains orange-yellow (negative results).

3.3.2.5 Triple Sugar Iron (TSI) Agar test

Slant cultures were prepared using Triple Sugar Iron agar (10.0 g peptone, 10.0 g tryptone, 3.0 g yeast extract, 3.0 g HM peptone B, 10.0 g lactose, 10.0 g sucrose, 1.0 g dextrose, 5.0 g sodium chloride, 0.20 g ferrous sulphate, 0.30 g sodium thiosulphate, 0.024 g phenol red, 12.0 g agar in 1 litre sterile distilled water at a final pH of 7.4). Each isolate was inoculated into the TSI slants in triplicate and incubated at 28 °C for 48 hours. The expected results obtained from the test are outlined:

1. If lactose or sucrose is fermented, a large amount of acid is produced, which turns the phenol red indicator yellow both in the butt and in the slant.

2. If glucose but not lactose is fermented, the oxygen-deficient butt will be yellow (butt has comparatively more glucose than slant i.e. more media more glucose), while the slant will be red since the acid produced (less acid produced in slant as media is less) will be oxidized to carbon dioxide and water.

3. If neither lactose, sucrose nor glucose is fermented, both the butt and the slant will be red. The slant can become a deeper red-purple (more alkaline) as a result of the production of ammonia from the oxidative deamination of amino acids (since peptone is a major component of TSI agar).

4. If hydrogen sulphide (H_2S) is produced, the black colour of ferrous sulphide is observed in the slant or butt.

3.3.2.6 Simmons Citrate Agar (SCA) test

Petri dishes containing Simmons Citrate Agar (0.20 g magnesium sulphate, 1.00 g ammonium dihydrogen phosphate, 1.00 g dipotassium hydrogen phosphate, 2.00 g sodium citrate, 5.00 g sodium chloride, 0.08 g bromothymol blue and 15.0 g agar in 1 litre sterile distilled water at pH 6.8) were streaked with each endosymbiont isolate in triplicate and incubated at 28 °C for 48 hours. Citrate utilization results in alkaline pH (above 7.6) due to the production of carbonates and bicarbonate bi-products confirmed by the change in color of the bromothymol blue from green to blue, indicative of positive results and vice versa.

3.3.2.7 Motility and pigment production tests

For the motility test, a single colony of each isolate was inoculated at the center of NBTA plate and incubated for 48 hours at 28 °C. Motility was determined by visualization of the growth pattern exhibited by the bacterial isolates. A single colony from each isolate was inoculated in 20 ml nutrient broth (NB) in sterile universal bottles in order to determine pigment production by the various isolates. The cultures were incubated for 72 hours at 28 °C in a shaking incubator at 180 rpm.

3.4 Molecular characterization of *Serratia* spp. and other nematode-associated bacterial endosymbionts

3.4.1 Extraction of bacterial genomic DNA

Extraction of genomic DNA from the bacterial isolates was accomplished by the Cetyl Trimethyl Ammonium Bromide (CTAB) method of bacterial isolation, following the

procedure outlined by Minas *et al*. (2011). A single colony of each isolate grown on NBTA was transferred from the plates into 50 ml sterile Falcon tubes containing 5 ml Luria Bertani broth, Himedia (10.0 g Casein enzymic hydrolysate, 5.0 g Yeast extract, 10.0 g sodium chloride in 1 litre sterile distilled water at a final pH of 7.5) and incubated overnight in a dark shaking incubator at 180 rotations per minute (rpm). The bacterial cells were harvested by centrifugation at 14,000 rpm for 15 minutes to pellet the cells and the supernatant was discarded. The cell pellet was resuspended in 540 µl of DNA extraction/lysis buffer (5 ml 1M Tris.Cl pH 8.0, 10 ml 1M EDTA, 2.5 ml 5M NaCl and 250 µl proteinase K (20 mg/ml) in 50 ml sterile distilled water), thoroughly mixed by vortexing and the whole solution transferred to 1.5 ml Eppendorf tubes followed by addition of 60 µl 10% SDS (sodium dodecyl sulphate) and incubated at 65 °C for 30 minutes to achieve maximum lysis of the bacterial cells. This was followed by the addition of 100 µl of 5M NaCl solution and 80 µl of CTAB (2% CTAB + 0.7 M NaCl) to get rid of the cell debris and proteins. The components were thoroughly mixed by gentle inversions to avoid shearing of the DNA then the mixture was incubated at 65 °C for 10 minutes and centrifuged at 12,000 rpm at 4 °C for 10 minutes. The supernatant was transferred into a new vial. The DNA of interest was extracted twice by addition of an equal volume (780 µl) of chloroform: isoamyl alcohol (24:1; Sigma - Aldrich) and mixed thoroughly by gentle inversions (to avoid shearing of the DNA) of the tube forming a milky solution. The cellular fractions were separated by centrifugation at 12,000 rpm for 15 minutes forming another supernatant (containing the DNA of interest) separated by a white interphase, usually the protein layer. The upper aqueous phase containing the DNA of interest was carefully transferred into another vial using a sterile pipette, taking care not to interfere with the protein interphase (this step was done twice). To precipitate the DNA of interest, a three-fold volume of ice-cold isopropanol (pre-stored at -20 ℃) was added to each Eppendorf tube and incubated overnight at -20 ℃ after gentle mixing. The resulting mixture was centrifuged for 15 minutes at 12,000 rpm at 4 °C. The supernatant was discarded and the pellet was rinsed with 1 ml 70% ethanol (stored at room temperature) twice followed by a spin at maximum speed for 2 minutes. The supernatant was discarded and the pellet air-dried for 2 hours before resuspending the gDNA in 50 µl of DNase-free sterile distilled water followed by the addition of 60 µl RNase A (10mg/ml) then incubated for 1 hour at 37 ℃ and stored at -20 ℃ for future use (Minas *et al*., 2011; William *et al*., 2012).

The presence of isolated bacterial genomic DNA was confirmed by running gel electrophoresis using a 5 µl aliquot of the DNA sample on 0.8% agarose gel in 1X TAE buffer

(0.04 M Tris Acetate, 0.01 M EDTA, pH 8) supplemented with 1.5 µl 10 mg/ml ethidium bromide (EtBr). The electrophoresis was carried out for 60 minutes at 100V and the gel was visualized under UV radiation. The gel images were taken using a Samsung Galaxy A70 phone.

3.4.2 PCR amplification of 16S rRNA gene

Amplification of the 16S ribosomal RNA (rRNA) gene of the nematode associated bacterial species was done using a set of forward and reverse universal primers, B27F (5' - AGA GTT TGA TCM TGG CTC AG - 3') and 1492R (5' - GGT TAC CTT GTT ACG ACT T - 3') respectively in a Programmable Thermal Controller (PTC-100^{TM}) following the procedure outlined by New England BioLabs. The amplification was performed in two different reactions in final volumes of 50 µl. The first reaction was done using a ready-made Taq 2X Master mix (AMPLIQON) supplemented with $MgCl_2$. A final volume of 50 µl composed of 25 µl of the master mix, 2 µl of each 10 mM forward and reverse Primers, 2 µl of template DNA and 19 µl nuclease-free water. The PCR cycling conditions were as follows: 1 cycle at 95 ℃ for 5 minutes for the initial denaturation followed by 35 cycles of subsequent denaturation at 95 ℃ for 30 seconds, annealing at 5 2℃ for 45 seconds, initial extension at 72 ℃ for 30 seconds and a final extension at 72 ℃ for 5 minutes following the manufactures instructions. This was termed reaction 1. The second reaction was performed in a final volume of 50 µl containing 10 µl of 10X Taq reaction buffer, 4 µl of 25 mM $MgCl_2$, 2 µl of 10 mM dNTP mix, 2 µl of each 10 mM F and R Primers, 1 µl of high fidelity Taq DNA polymerase (Platinum), 4 µl of template DNA and 30 µl nuclease-free H_2O (Chowdhury *et al*., 2015). The PCR cycling conditions were as follows: 1 cycle at 95 ℃ for 5 minutes followed by 35 cycles of subsequent denaturation at 95 ℃ for 1 minute, annealing at 48 ℃ for 1 minute, initial extension at 72 ℃ for 2 minutes and a final extension at 72 ℃ for 7 minutes (Muangpat *et al*., 2017). This was termed reaction 2.

Separation of the amplified PCR products was then carried out by agarose gel electrophoresis using 1% (w/v) agarose dissolved in TAE buffer stained with ethidium bromide at a final concentration of 0.5 ug/ml. Finally, 2.5 µl of 1kb plus DNA ladder and 5 µl of the PCR products were loaded into each well of the gel. Running of the gel was carried out at 100V for 60 minutes in 1X TAE buffer (0.04 M Tris Acetate, 0.01 M EDTA, pH 8). Visualization of the bands was done under ultraviolet radiation (UV) and their sizes were identified by comparing to 1kb plus molecular DNA ladder (ThermoFisher Scientific) map (Al-Zaidawi

et al., 2019). The amplified samples were then sent for sequencing at the University of Nairobi Institute of Tropical and Infectious Disease (UNITID) laboratories.

3.4.3 Phylogenetic analysis of *Serratia* spp. and other associated enteric bacteria

The obtained sequences of the 16S rRNA gene of isolated bacterial endosymbionts were compared to other closely related members from sequences in the database. Following the maximum likelihood (ML) method, a phylogenetic tree was constructed (Tamura *et al*., 2013) using Geneious tree builder with specified out-groups in the Geneious Prime software (Version 2020.2.4). For maximum likelihood inferences, any incomplete or missing data, as well as any gaps, were deleted then the "nearest-neighbour-interchange" (NNI) method was preferentially selected so that the initial tree will be automatically created. BLAST analysis was performed using 100 Bootstrap replicates. The resulting tree provided useful information regarding the clustering of sequences which in turn increased the obtained information from the BLAST search.

3.5 Preparation of bacterial cell-free suspension

Each of the four bacterial isolates was prepared for screening of its activity against selected pathogenic microbes. A single colony of each isolate was picked using a sterile loop from NBTA culture and transferred into 25 ml LB in a flask and incubated at 28 °C in a shaking incubator at 180 rpm for 24 hours. The LB media was chosen because it encourages faster growth and higher volumes of bacteria. The whole-cell suspension was adjusted to the 0.5 McFarland standard concentration according to the optical density at a wavelength of 600 nm, equivalent to 1 x 10^8 Colony Forming Units (CFUs)/ml (Muangpat *et al*., 2017). For the preparation of the cell-free suspension, 1 ml of each whole-cell suspension was centrifuged (to pellet debris so as not to clog the filter) at 12,000 g for 5 minutes then filtered through a 0.22 µm filter in the laminar flow and the filtrate stored at -20 °C in sterile universal bottles further use (Han and Ehlers, 2001; Bussaman *et al*., 2012; Muangpat *et al*., 2017).

3.6 Effect of bacterial isolates on selected bacterial and fungal test pathogens

A total of six human bacterial pathogens were used in the study namely; *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, methicillin-resistant *Staphylococcus aureus*, *Bacillus cereus* and the yeast *Candida albicans*. The following six plant pathogenic fungal species were also used as test organisms; *Aspergillus flavus*, *Fusarium solani*, *Fusarium verticillioides*, *Fusarium oxysporum* and *Penicillium chrysogenum* and *Penicillium*

spp. The test pathogens were obtained from the Microbiology Culture Collection Center at the Department of Biology, University of Nairobi. Confirmation of the identity of fungal pathogens was based on their cultural and morphological characteristics (Nelson *et al*., 1994; Klich, 2002; Samson *et al*., 2014).

3.6.1 Antibacterial activity against selected antibiotic-resistant bacteria

Six species of drug-resistant bacteria were tested; *Escherichia coli*, *Staphylococcus aureus*, Methicillin-resistant *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Bacillus cereus* and the pathogenic yeast *Candida albicans*. The test bacteria were prepared by streaking each strain on Mueller Hinton agar (MHA) (2.0 g beef extract, 17.5 g casein hydrolysate, 1.5 g starch and 17.0 g agar dissolved in 1 litre of distilled water) and incubated at 37 °C for 24 hours. A colony of each isolate was dissolved in phosphate-buffered saline solution (0.9 % NaCl) and adjusted to the 0.5 McFarland standard concentration of 0.08 - 0.13 according to the optical density at a wavelength of 600 nm (Muangpat *et al*., 2017). Using a sterile pipette, approximately 20 µl of each test bacterium was dispensed into a Petri dish followed by the addition of MHA at 45 °C, thoroughly mixed using circular motions and allowed to dry. The experiment was done in triplicate. Using a cork borer in a laminar flow cabinet, four wells each measuring 6 mm in diameter were made in each agar plate.

To screen the entomopathogenic bacterial isolates against the selected bacterial test pathogens, 20 µl of each of the two concentrations (50 % and 100 %) of an overnight culture of each cell-free suspension were transferred using a sterile pipette into the wells on MHA plates that had been plated with ABR test bacteria (Balouiri *et al*., 2016). The broad-spectrum antibiotic ampicillin (100 mg/ml) was used as the positive control while sterile distilled water was used as the negative control. The MHA plates were incubated at 28 °C for 48 hours. The diameter of the zone of inhibition was measured and recorded in millimeters (Aiswarya *et al*., 2017: Muangpat *et al*., 2017).

3.6.2 Antifungal activity against selected pathogenic fungi

Pure cultures of the plant pathogenic fungi *Fusarium oxysporum*, *Fusarium verticillioides*, *Fusarium solani*, *Aspergillus flavus*, *Penicillium chrysogenum* and *Penicillium* spp. were obtained from the Microbiology Culture Collection Center in the Department of Biology, University of Nairobi. They were cultured on Potato dextrose agar (PDA) agar (20.0 g dextrose, 15.0 g agar and 4.0 g potato starch in 1litre distilled water) and incubated for 7 days at 25 °C before being used for the antimicrobial bioassay.

The fungal suspension was prepared in the laminar flow cabinet by cutting a piece of each fungus grown on PDA using a sterile scalpel and growing them in 20 ml potato dextrose broth, (PDB) (20.0 g dextrose and 200.0 g potato infusion in 1litre distilled water) for 4 days at room temperature (23 ± 2 ºC). At the end of the incubation period, each fungus was thoroughly mixed in the broth and filtered through a sterile double-layer cheesecloth in the laminar flow. Twenty microliters of the fungal suspension were spread on Petri dishes with PDA agar making three replications of each test pathogen. After drying the plates for twenty minutes in a laminar airflow cabinet, four equidistant 6mm diameter wells were made in each Petri dish using a cork borer. Exactly 20 µl of the cell-free suspension of each nematode-associated bacterial isolate (isolates 93A, 93B, 93C and 93D) was added in two concentrations (50 %, 100 %) and made in triplicate (Balouiri *et al*., 2016). Apron Star® 42 WS (mefenoxam 200 g/kg, thiamethoxam 200 g/kg and difenoconazole 20g/kg), a broad-spectrum plant pathogenic antifungal agent was prepared in the laminar flow cabinet following the manufacturer's instructions by dissolving 1g of the fungicide in 1L of sterile distilled water and thoroughly mixing it before use. Apron Star® 42 WS was used as the positive control while sterile distilled water was used as the negative control. The Petri dishes were incubated in the dark at 25 °C for 5 days. Suppressive activity of the isolates was determined by measuring the zone of inhibition of the fungi and recorded in millimetres.

3.7 Data analysis

3.7.1 Antimicrobial bioassay data analysis

Data analysis was performed in the R software version 3.5.3. Data were first checked for normality using the Shapiro test and found to be 3.416×10^{-9} indicating they were not normally distributed and therefore were log-transformed Log_{10} (mu + 1). Two-way analysis of variance (ANOVA) was performed on the transformed data and significant differences were detected among the test pathogens (bacteria and fungi) and concentration. In this regard, data sub-setting was done. Separation of means (posthoc) was done using the lsmeans package (Lenth, 2016) and adjusted using Tukey multcompview package (Graves *et al*., 2015) at $\alpha = 0.05$. The 95 % confidence level used was adjusted using the sidak method.

3.7.2 Molecular sequence analysis

The sequences were opened in the Geneious software version 11.1.5 (Kearse *et al.*, 2012) where they were assessed for complementarity using the local pairwise alignment for each sequence set (forward and reverse). The algorithm scored all the alignments and necessary

cleaning and editing were done in the same Geneious software involving trimming of the ends and filling of the gaps using the MUSCLE algorithm with default parameters. Consensus sequences were then generated where applicable (samples 93A, 93B and 93C) except for sample D_1492 (R) which was not clean enough and therefore only the reverse sequence was used. Consequently, each of the curated sequences was pasted into the NCBI GenBank sequence similarity search database using the Basic Local Alignment Search Tool (BLAST: https://blast.ncbi.nlm.nih.gov/Blast.cgi) where searches were optimized for highly similar sequences (Megablast). The resultant hits were checked for high similarity by judging the query cover and the percentage (%) identity score. Query sequences that had the highest query cover and likewise highest identity score were favored.

A phylogenetic tree for each sequence was then constructed from this alignment by using the MEGA X (Kumar *et al*., 2018) with specified out-groups. The evolutionary history of the nematode-associated bacterial isolates was inferred by using the Maximum Likelihood (ML) method and Tamura-Nei model (Tamura and Nei, 1993). Initial tree (s) for the heuristic search were obtained automatically by applying the Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach and then selecting the topology with a superior log-likelihood value. The Tamura-Nei model was implemented for the determination of genetic distances between groups after which trees were plotted using the Neighbor-Joining method. Resampling was optimized with the default parameters; bootstrap over 100 replicates after which the consensus tree (the average and most suitable) was computed by the majority greedy clustering method and plotted. The trees were drawn to scale, with branch lengths measured in the number of substitutions per site. This analysis involved 12 to 16 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. The final datasets had a total of 1739, 1653, 1710 and 1705 positions arranged in an ascending order for each of the samples from sample 93A to sample 93D.

CHAPTER FOUR: RESULTS

4.1 Viability and multiplication of entomopathogenic nematodes

All the ten nematode samples collected (representing 100 %) in solution form showed active motility by rapid wiggling movements (Figure 5) when viewed under X40 magnification using the light microscope. This positive response confirmed their viability and warranted further multiplication, maintenance and use. The nematodes were moderately long and slender with very rapid movements (Figure 5). The emergence time of the nematodes from the dead *Galleria mellonella* larvae cadavers into the sterile distilled water in the trough varied greatly during multiplication using the White trap method. Nine of the samples representing 90 % of the total had all their nematodes emerge by the 5^{th} day of incubation while the remaining 10 % had emerged between the 8^{th} and 10^{th} day post-infection. This 10% of the samples represented *Oscheius* nematodes (Figure 6). This was confirmed by morphological identification under the microscope and by observation of the color of the dead larval cadavers after 5 days of incubation following reinfection. The brick-red to maroon pigmented cadavers were recorded as *Oscheius* species after visualization of the *G. mellonella* cadavers in a dark room to rule out the *Heterorhabditis* nematodes. This darkening of the dead cadavers is due to the characteristic red pigment known as prodigiosin secreted by their associated endosymbiotic *Serratia* species. The remaining 90 % (cream to whitish) of the nematodes were discarded since they were representatives of the *Steinernema* group of EPNs.

The larvae though dead remained moist with no foul smell at all and were not infested by any other opportunistic pathogens. However, a majority (90 %) of the cadavers retained a whitish color without any pigmentation and these were characterized *Steinernema* species of nematodes (Figure 6). All *G. mellonella* larvae died by the 10^{th} day post-infection, indicating the pathogenic and high virulence nature of the nematodes-bacteria association against the *Galleria mellonella* larvae.

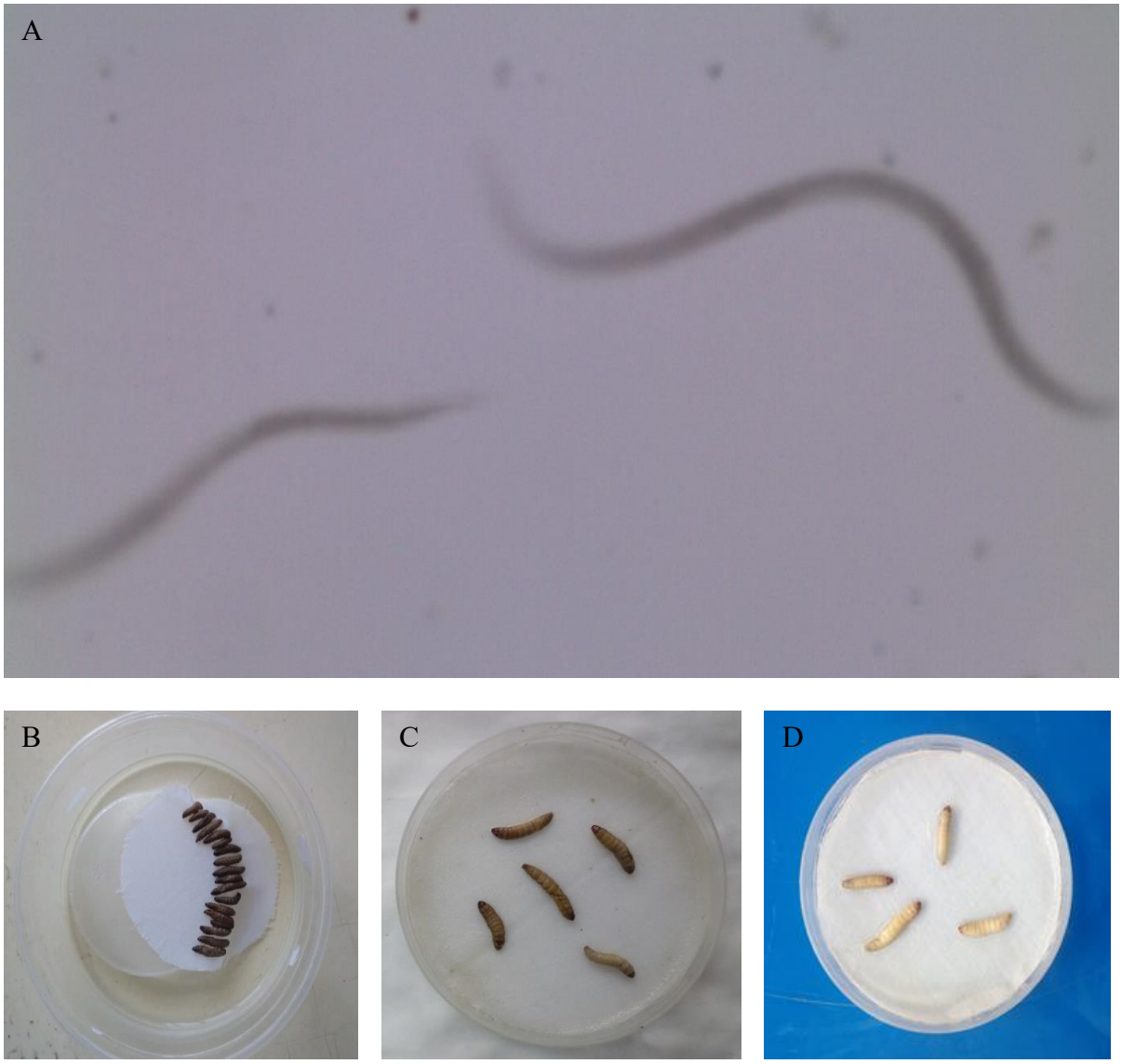

Figure 5: Entomopathogenic nematode multiplication and infection of the *Galleria mellonella* larvae. A – Wiggling motion of entomopathogenic nematodes in sterile distilled water under X40 magnification. B – White trap multiplication method of EPNs. C – *Galleria mellonella* larvae infected with *Oscheius* nematodes (brick-red). D – *Galleria mellonella* larvae infected with *Steinernema* nematodes (cream-whitish).

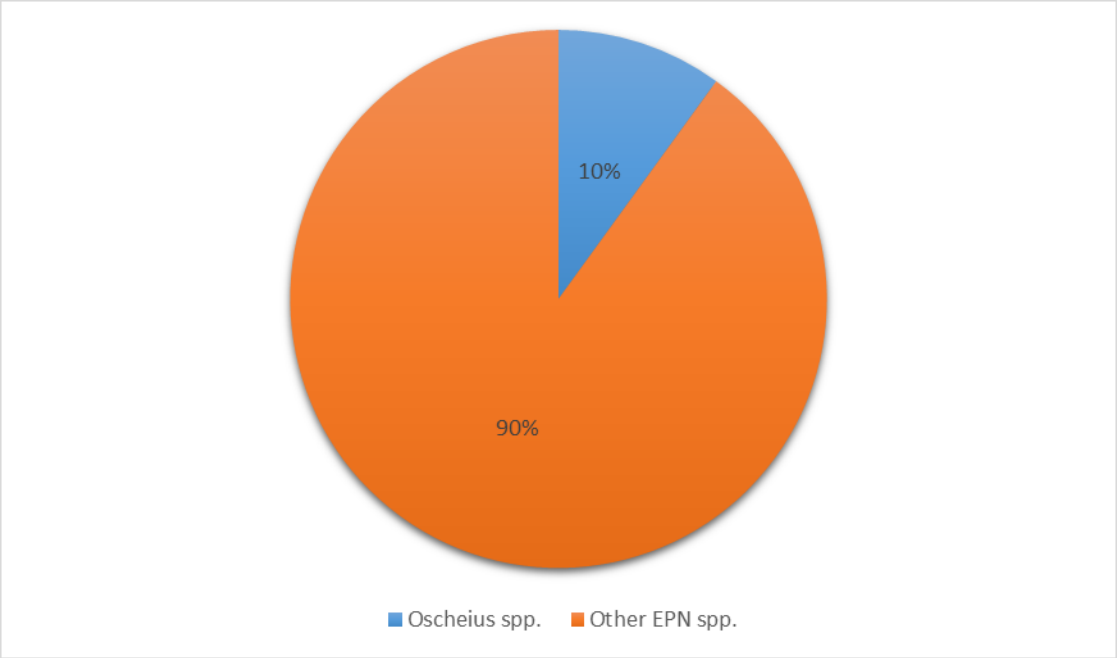

Figure 6: Percentage yield of entomopathogenic nematodes from *Galleria mellonella* larvae following multiplication using the White trap method.

4.2 Isolation and morphological characteristics of *Serratia* and other nematode associated bacteria

4.2.1 Cultural and morphological characteristics of nematode enteric bacterial species

The four enteric bacterial isolates were obtained from Rhabditis nematodes. Preliminary identification of the endosymbiotic bacterial species at the genus level was based on colony morphology on differential NBTA media, to distinguish the *Serratia* spp. (which are known endosymbionts) and the other Rhabditis-associated bacterial species (with green colonies) from *Xenorhabdus* species (with blue colonies). All the enterobacterial isolates had colonies that were green in color representing the primary form of the bacteria when cultured on NBTA media. However, the intensity of the green color varied greatly from deep green to a light green shade. Isolate 93A had deep green, medium-sized, dome-shaped, smooth, glistening colonies with a somewhat sticky appearance (Table 3). Isolates 93B and 93C had light green, smooth, shiny, circular, opaque, raised (convex), umbonated colonies with regular, entire margins which were surrounded by a clear margin but completely turned off-white after prolonged incubation (7 days). The colonies belonging to these two bacterial isolates were neither shiny nor sticky. Isolate 93D had circular, green, translucent, medium-sized colonies with a glossy surface that was not surrounded by any margin. All the colonies except for isolate 93D turned

to a dark red (maroon) color surrounded by white clear margins after 4 days of incubation at 28 ℃. All the colonies were generally medium-sized with an average diameter of approximately 2mm except for isolate 93A which had comparatively larger colonies of between 2.5 to 4 mm in diameter when not crowded.

Table 3: Cultural and morphological characteristics of isolated bacterial endosymbionts

Isolate	Colour		Colony shape	Colony margin	Colony Elevation	Motility	Gram stain
	Phase I	Phase II					
93A	DG	Br	Rod	Smooth	Raised	-	-
93B	LG	Br	Rod	Smooth	Raised	+	-
93C	GW	Br	Rod	Smooth	Raised	+	-
93D	G	W	Rod	Smooth	Raised	+	-

Key: DG = deep green, LG = light green, GW = greenish-white, G = green, W = White, Br = brick-red, + = positive and - = negative

All the four isolates were gram-negative rod-shaped (Figure 7) bacteria that appeared in pairs. The isolates also showed positive utilization of substrates within the media (NBTA, NA and MAC) by clearing the color of the media around the growth areas compared to the areas without the colonies. All the isolates appeared red/pink on MAC and cream on NA (Figure 8). However, phase II cells showed increased yellow intensity on NA and reduced intensity in the pink colour on MAC. The secondary form turned from green to maroon on NBTA media (Figure 8). There was also visible pigment production and dye absorption within the solid media on NBTA, MacConkey agar and nutrient agar (Figure 8). When the isolates were cultured on half-strength NBTA media, swarming motility was visibly observed whereby the bacteria were able to move with clearly distinct serrated margins in all the bacterial isolates except for isolate 93A.

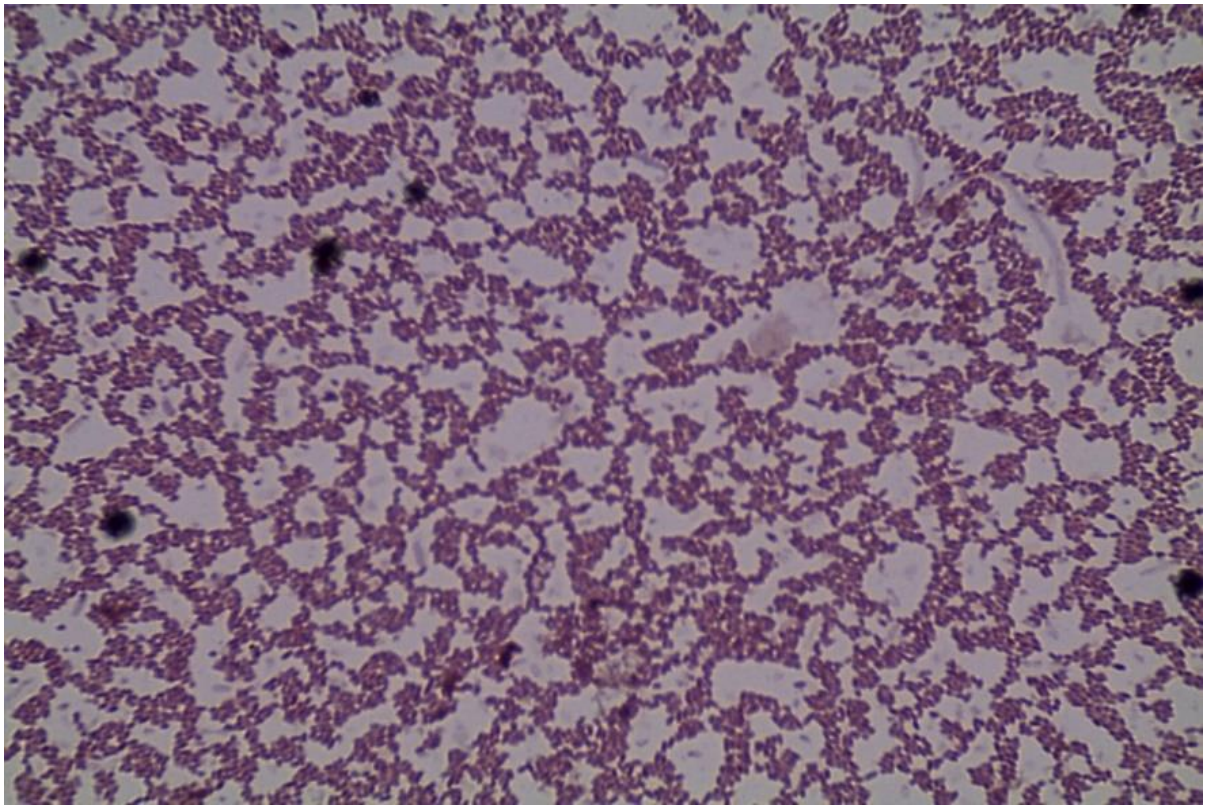

Figure 7: Gram stain reaction of a 24-hour culture of *Serratia marcescens* isolate 93B in LB broth viewed under oil immersion X100 magnification.

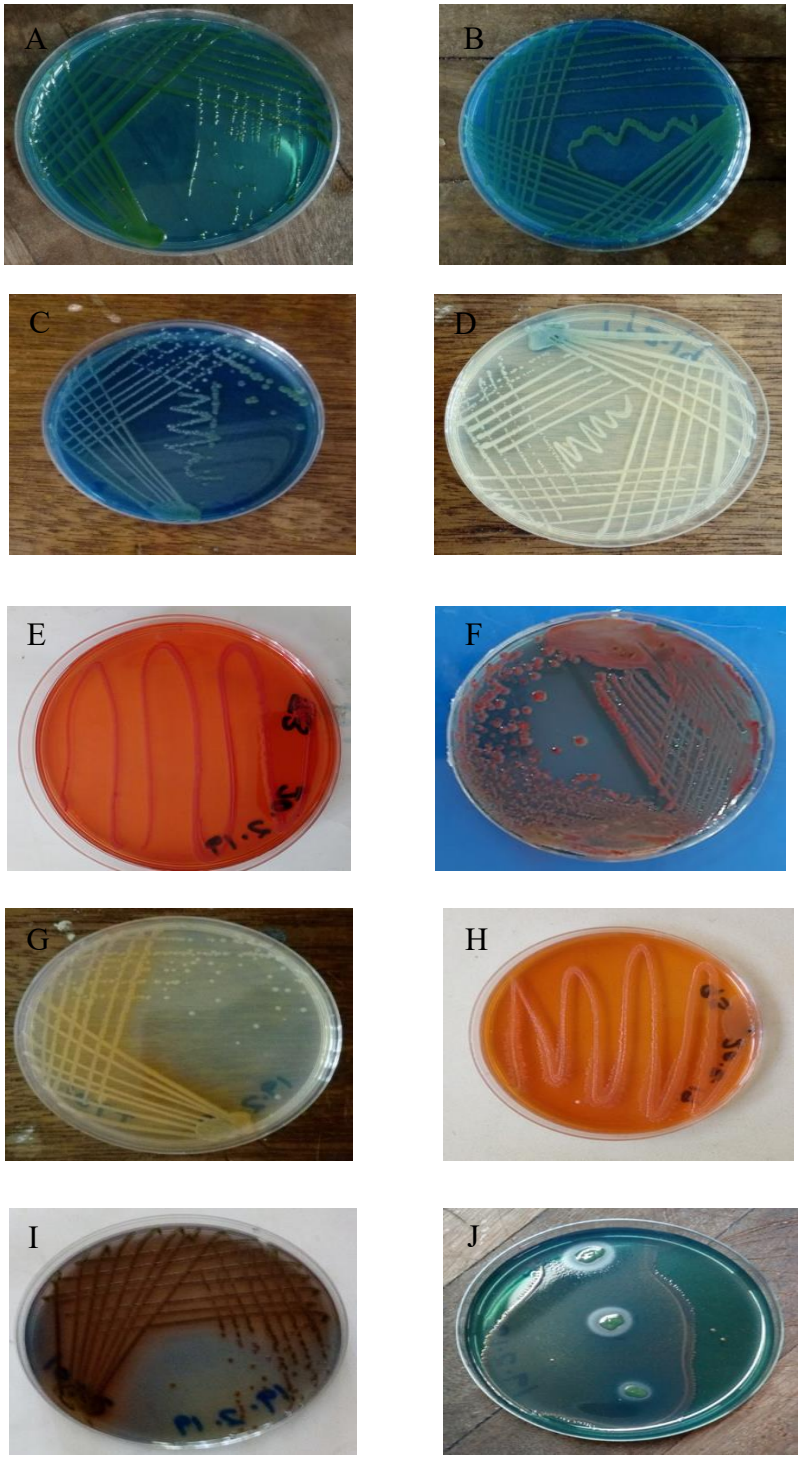

Figure 8: Colony characteristics of *Serratia marcescens* 93B isolate on different culture media. A, B and C – Phase I cells on NBTA media after 48 hrs. D and E – Phase I cells on NA and MAC media after 48 hrs, respectively. F, G and H – Phase II cells on NBTA, NA and MAC after 4 days of incubation, respectively. I – NBTA plate with isolate showing dye absorption and pigment production. J – Swarming motility of bacterial isolates on half-strength NBTA media. In all cases, incubation was done at 28℃.

4.2.2 Biochemical characteristics of nematode enteric bacterial species

Several biochemical tests were performed on all four endosymbiotic bacterial isolates. All isolates were positive for the following tests; motility test on NBTA and MAC media, pathogenicity test, catalase test, Simmons citrate agar (SCA) test, dye adsorption (on both NBTA and MAC) as well as pigment production test (Table 4; Figure 9). The pathogenicity of the isolates was demonstrated upon infection of the *G. mellonella* larvae in which case *Serratia* endosymbionts proved to be more pathogenic than the other bacterial endosymbionts by killing their hosts within 3 days of infection as opposed to 5 days. The Triple Sugar Iron (TSI) Agar test was also positive in all the samples tested. Two isolates (93A and 93C) had a red slant (alkaline) and a yellow butt (acid) with no gas production. Isolates 93B and 93D had a yellow slant and butt with the production of gas. However, after 48 hours, this color started blackening in the tube with isolate 93B. Other biochemical tests including oxidase test, urease test and bioluminescence (visualization in the dark) gave negative results in all four bacterial isolates (Table 4).

Table 4: Summary of the biochemical tests on the four endosymbiotic bacterial isolates

Biochemical test	Bacterial isolate			
	Isolate 93A	Isolate 93B	Isolate 93C	Isolate 93D
Catalase	+	+	+	+
Oxidase	-	-	-	-
Urease test	-	-	-	-
Simmons citrate test	+	+	+	+
TSI test (slant/butt)	R/Y	Y/Y	R/Y	Y/Y
Pathogenicity	+	+	+	+
Motility	-	+	+	-
Bioluminescence	-	-	-	-
Dye adsorption on NBTA	+	+	+	+
MacConkey agar	R	R	R	R
Pigment production test	O	Y	Y	Y
Lactose fermentation	+	+	+	+

All tests were carried out at 28°C.

Key: R = Red, O = Orange, Y = yellow, y = light yellow, + = positive and - = negative.

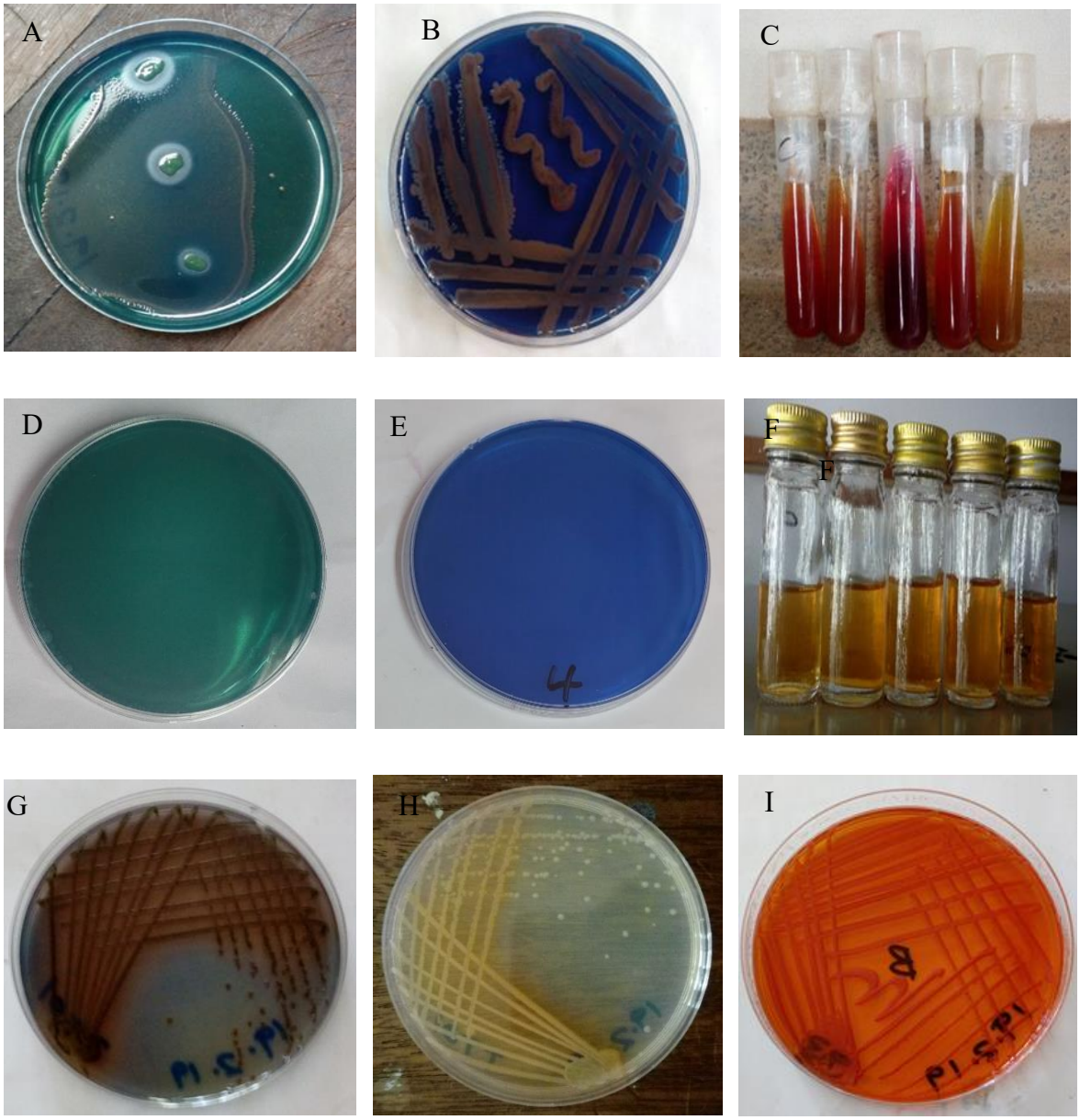

Figure 9: Biochemical tests on entomopathogenic-associated bacterial isolates (*S. marcescens* 93C). A - Swarming motility test of primary cells on NBTA media. B - Swarming motility of secondary cells on NBTA media. C – Triple Sugar Iron test – (Change in colour of slant/butt from maroon to either yellow or red, or black - positive result). D - SCA test (negative control, the color remains green). E – Simmons Citrate Agar test (blue - positive result). F - Urease test (orange-yellow - negative result). G, H and I - Dye adsorption and pigment production on NBTA, NA and MAC respectively (clearing of the color of the media at the growth areas - positive results for dye adsorption; release of colored pigments into the growth media - positive result for pigment production).

4.3 Molecular characteristics of *Serratia* spp. and other associated enterobacterial isolates

4.3.1 Genomic DNA and PCR amplification of partial 16S rRNA gene

The CTAB method of extraction of genomic DNA was very effective in obtaining the DNA of interest from the isolated bacteria as seen by the high concentration and bright bands on the DNA micrograph (Figure 10). The partial 16S rRNA genes were successfully amplified by PCR from all the bacterial isolates (Figure 11). Partial 16S rRNA gene fragments of an interpolated fragment length of approximately 1500 base pairs were isolated. This was corroborated with the two primers used, B27 forward primer (5' - AGA GTT TGA TCM TGG CTC AG - 3') and 1492 reverse primer (5' - GGT TAC CTT GTT ACG ACT T - 3') as their target sequence of amplification was 1500 base pairs (Lane, 1991). All fragments were approximately aligned at one level denoting the uniformity in length.

The fragment length was extrapolated by comparing the position of DNA bright bands to the ThermoFisher Scientific 1kb plus gene ruler DNA molecular ladder

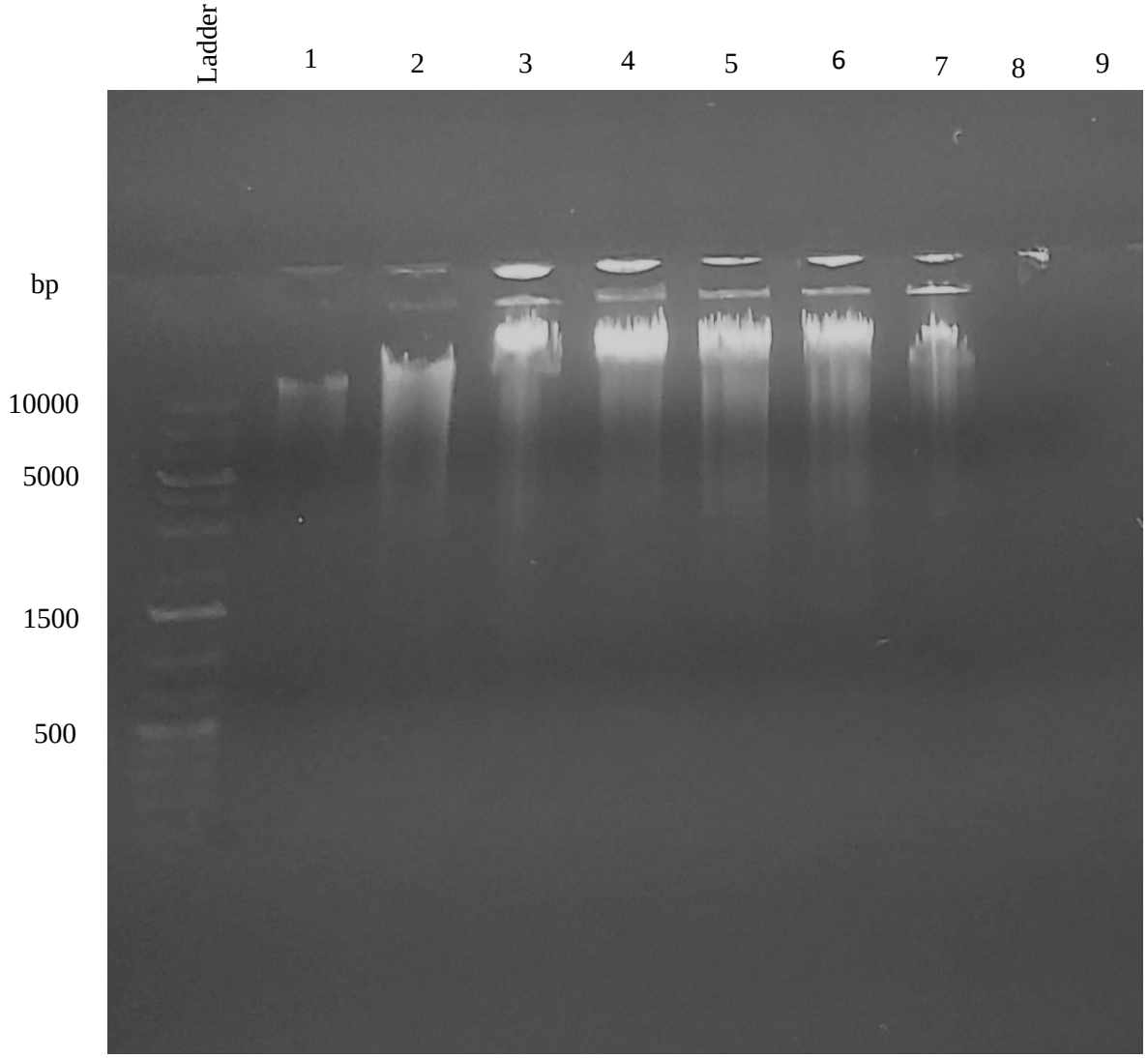

Figure 10: Autoradiograph of agarose gel electrophoresis with genomic DNA products of each sample done in duplicate. Lane M was with the 1kb plus molecular marker: lane 1 and 2: isolate 93A. Lane 3 and 4: isolate 93B. Lane 5 and 6: isolate 93C. Lane 7 and 8: isolate 93D. Lane 9: negative control – without template DNA.

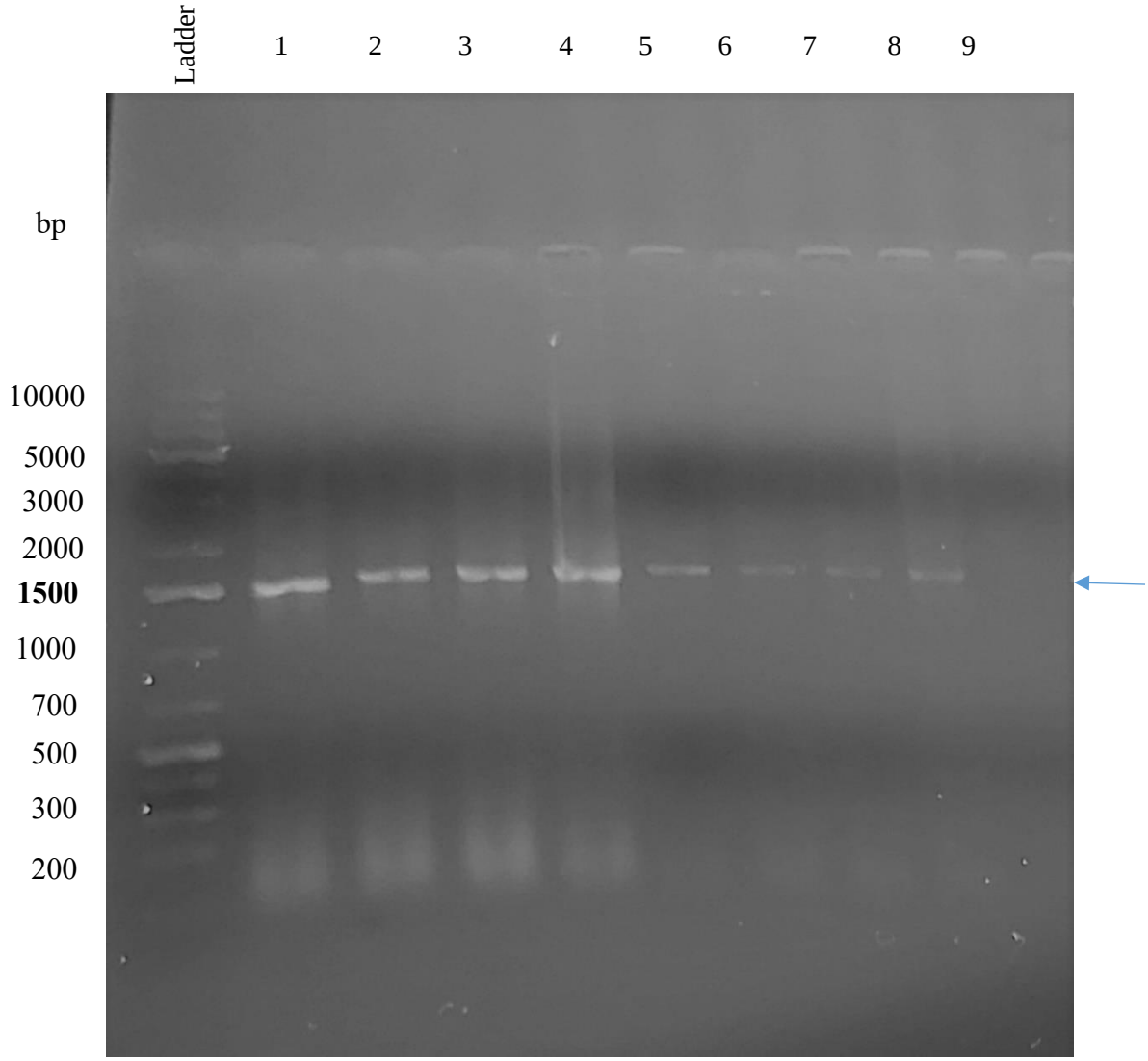

Figure 11: Autoradiograph of agarose gel electrophoresis with PCR products of the samples. Lane M was with the 1kb plus molecular marker: Lane 1 to 4: isolates 93A, 93B, 93C and 93D of reaction 1 respectively. Lane 5 to 8: isolates 93A, 93B, 93C and 93D of reaction 2 respectively. Lane 9: negative control - without template DNA.

.

4.3.2 Characterization of 16S rRNA gene

After checking the quality of the sequences, they were cleaned by trimming the edges, filling in the gaps and edited using the Geneious software version 11.1.5 (Kearse *et al.*, 2012). Consensus sequences for isolates (93A, 93B and 93C) were then generated except for isolate 93D where only the reverse sequence was used. The sequences once cleaned had a length of 1220 base pairs, 1213bp, 1172bp and 498bp for the samples 93A, 93B, 93C and 93D, respectively (Figures 12 - 15).

>TATTCACCGTGGCATTCTGATCCACGATTACTAGCGATTCCGACTTCATGGAG
TCGAGTTGCAGACTCCAATCCGGACTACGACATACTTTATGAGGTCCGCTTGC
TCTCGCGAGGTCGCTTCTCTTTGTATATGCCATTGTAGCACGTGTGTAGCCCTA
CTCGTAAGGGCCATGATGACTTGACGTCATCCCCACCTTCCTCCAGTTTATCAC
TGGCAGTCTCCTTTGAGTTCCCGACCGAATCGCTGGCAACAAAGGATAAGGGT
TGCGCTCGTTGCGGGACTTAACCCAACATTTCACAACACGAGCTGACGACAGC
CATGCAGCACCTGTCTCAGAGTTCCCGAAGGCACCAAAGCATCTCTGCTAAGT
TCTCTGGATGTCAAGAGTAGGTAAGGTTCTTCGCGTTGCATCGAATTAACCAC
ATGCTCCACCGCTTGTGCGGGCCCCCGTCAATTCATTTGAGTTTTAACCTTGCG
GCCGTACTCCCCAGGCGGTCGACTTAACGCGTTAGCTCCGGAAGCCACTCCTC
AAGGGAACAACCTCCAAAGTCGACATCGTTTACAGCGGTGGACTACCAGGGT
ATCTAATCCTGTTTGCTCCCCACGCTTTCGCACCTGAGCGTCAGTCTTTGGTCC
AGGGGGCCGCCTTCGCCCAACCGGTATTCCTCCAGAATCTCTACGCATTTCAC
CGCTACACCTGGAATTCTACCCCCCCTCTACAAGACTCCAGCCTGCCAGTTTCG
AATGCAGTTCCCAGGTTGAGCCCGGGGATTTCACATCCGACTTGACAGACCGC
CTGCGTGCGCTTTACGCCCAGTAATTCCGATTAACGCTTGCACCCTCCGTATTA
CCGCGGCTGCTGGCACGGAGTTAGCCGGTGCTTCTTCTGCGGGTAACGTCAAT
GAATAAGGTTATTAACCTCACTCCCTTCCTCCCCGCTGAAAGTACTTTACAACC
CGAAGGCCTTCTTCATACACGCGGCATGGCTGCATCAGGCTTGCGCCCATTGT
GCAATATTCCCCACTGCTGCCTCCCGTAGGAGTCTGGACCGTGTCTCAGTTCCA
GTGTGGCTGGTCATCCTCTCAGACCAGCTAGGGATCGTCGCCTAGGTGAGCCG
TTACCTCACCTACAAGCTAATCCCATCTGGGCACATCCGATGGCAAGAGGCCC
GAAGGTCCCCCTCTTTGGTCTGCGACGTTATGCGGTATTAGCTACCG
>

Figure 12: Sequence listing of partial 16S ribosomal RNA gene of *Klebsiella oxytoca* strain 127 with 1220 base pairs

>

ACCGTAGCATTCTGATCTACGATTACTAGCGATTCCGACTTCATGGAGTCGA
GTTGCAGACTCCAATCCGGACTACGACATACTTTATGAGGTCCGCTTGCTCTC
GCGAGGTCGCTTCTCTTTGTATATGCCATTGTAGCACGTGTGTAGCCCTACTC
GTAAGGGCCATGATGACTTGACGTCATCCCCACCTTCCTCCAGTTTATCACTG
GCAGTCTCCTTTGAGTTCCCGGCCGAACCGCTGGCAACAAAGGATAAGGGTT
GCGCTCGTTGCGGGACTTAACCCAACATTTCACAACACGAGCTGACGACAGC
CATGCAGCACCTGTCTCAGAGTTCCCGAAGGCACCAAAGCATCTCTGCTAAG
TTCTCTGGATGTCAAGAGTAGGTAAGGTTCTTCGCGTTGCATCGAATTAAAC
CACATGCTCCACCGCTTGTGCGGGCCCCCGTCAATTCATTTGAGTTTTAACCT
TGCGGCCGTACTCCCCAGGCGGTCGATTTAACGCGTTAGCTCCGGAAGCCAC
GCCTCAAGGGGCACAACCTCCAAATCGACATCGTTTACAGCGTGAACTACCA
GGGTATCTAATCCCTGTTTGCTCCCCACGCTTTTCGCACCTGAGCGTCAGTCT
TCGTCCAGGGGGGGCGCCTTCGCCACCGGTATTCCTCCAGATCTCTAACCGC
ATTTCACCGGCTACACCTGGAATTCTACCCCCCTCTACGAGACTCTAGCTTGC
CAGTTTCAAATGCAGTTCCCAGGTTGAGCCCGGGGATTTCACATCTGACTTA
ACAAACCGCCTGCGTGCGCTTTACGCCCAGTAATTCCGATTAACGCTTGCAC
CCTCCGTATTACCGCGGCTGCTGGCACGGAGTTAGCCGGTGCTTCTTCTGCG
AGTAACGTCAATTGATGAGCGTATTAAGCTCACCACCTTCCTCCTCGCTGAA
AGTGCTTTACAACCCGAAGGCCTTCTTCACACACGCGGCATGGCTGCATCAG
GCTTGCGCCCATTGTGCAATATTCCCCACTGCTGCCTCCCGTAGGAGTCTGGA
CCGTGTCTCAGTTCCAGTGTGGCTGGTCATCCTCTCAGACCAGCTAGGGATC
GTCGCCTAGGTGAGCCATTACCCCACCTACTAGCTAATCCCATCTGGGCACA
TCTGATGGCAAGAGGCCCGAAGGTCCCCCTCTTTGGTCTTGCGACGTTATGC
GGTATTAGCT

Figure 13: Sequence listing of partial 16S ribosomal RNA gene of *Serratia marcescens* strain B195 with a sequence length of 1213bp

>

ATTCACCGTAGCATTCTGATCTACGATTACTAGCGATTCCTACTTCATGGAGTC
GAGTTGCAGACTCCAATCCGGACTACGACATACTTTATGAGGTCCGCTTGCTC
TCGCGAGGTCGCTTCTCTTTGTATGTGCCATTGTAGCACGTGTGTAGCCCTACT
CGTAAGGGCCATGATGACTTGACGTCATCCCCACCTTCCTCCAGTTTATCACT
GGCAGTCTCCTTTGAGTTCCCGGCCGAACCGCTGGCAACAAAGGATAAGGGT
TGCGCTCGTTGCGGGACTTAACCCAACATTTCACAACACGAGCTGACGACGCC
ATGCAGCACCTGTCTCAGAGTTCCCGAAGGCACCAAAGCATCTCTGGTAAGTT
CTCTGGATGTCAAGAGTAGGTAAGGTTCTTCGCGTTGCATCGAATTAAACCAC
ATGCTCCACCGCTTGTGCGGGCCCCCGTCAATTCATTTGAGTTTTAACCTTGCG
GCCGTACTCCCCAGCGGTCGATTTAACGCGTTAGCTCCAGAAGCCACGCCTCA
GGGCACAACCTCCAAATTCGACATCGTTTACTAGCGTGGACTACCAGGGTATC
TAATCCTGTTTGCTCCCACGCTTTCGCACCTGAGCGTCAGTCTTTCGTCCAGGG
GCCGGCCTTTCGCCACCGGTATTCCTCCAGATCTCTACGCATTTCACGCTACAC
CTGGGAATTCTACCCCCCCTCTACGAGACTTTAGCTTGCCAGTTTCAATGCAG
TTCCCAGGTTGAGCCCGGGGATTTCACATCTGACTTACAACCGCCTGCGTGCG
CTTTACGCCCAGTAATTCCGATTAACGCTTGCACCCTCCGTATTACCGCGGCT
GCTGGCACGGAGTTAGCCGGTGCTTCTTCTGCGAGTAACGTCAATTGATGAGC
GTATTAAGCTCACCACCTTCCTCCTCGCTGAAAGTGCTTTACAACCCGAAGGC
CTTCTTCACACACGCGGCATGGCTGCATCAGGCTTGCGCCCATTGTGCAATAT
TCCCCACTGCTGCCTCCCGTAGGAGTCTGGACCGTGTCTCAGTTCCAGTGTGG
CTGGTCATCCTCTCAGACCAGCTAGGGATCGTCGCCTAGGTGAGCCATTACCC
CACCTACTAGCTAATCCCATCTGGGCACATCTGATGGCAAGAGGCCCGAAGG
TCC

Figure 14: Sequence listing of partial 16S ribosomal RNA gene of *Serratia marcescens* strain RS with a sequence length of 1172bp

>
TATTCACCGTGGCATTCTGATCCACGATTACTAGCGATTCCGACTTCATGGAG
TCGAGTTGCAGACTCCAATCCGGACTACGACATACTTTATGAGGTCCGCTTGC
TCTCGCGAGGTCGCTTCTCTTTGTATATGCCATTGTAGCACGTGTGTAGCCCTA
CTCGTAAGGGCCATGATGACTTGACGTCATCCCCACCTTCCTCCAGTTTATCA
CTGGCAGTCTCCTTTGAGTTCCCGGCCGAACCGCTGGCAACAAAGGATAAGG
GTTGCGCTCGTTGCGGGACTTAACCCAACATTTCACAACACGAGCTGACGAC
AGCCATGCAGCACCTGTCTCAGAGTTCCCGAAGGCACCAAGGCATCTCTGCT
AAGTTCTCTGGATGTCAAGAGTAGGTAAGGTTCTTCGCGTTGCATCGAATTAA
ACCACATGCTCCACCGCTTGTGCGGGCCCCCGTCAATTCATTTGAGTTTTAAC
CTTGCGGCCGTACTCCCCAGGCG

Figure 15: Sequence listing of partial 16S ribosomal RNA gene of *Citrobacter freundii* strain UIS1115 with a sequence length of 498bp

The sequences once cleaned were then queried against the National Centre for Biotechnology Information (NCBI) GenBank database using the Basic Local Alignment Search Tool (BLAST) parameters (NCBI; https://blast.ncbi.nlm.nih.gov/Blast.cgi). The resultant blast identified the four sampled enteric bacteria as follows: Sample 93A had a 99.26% identity to *Klebsiella oxytoca* strain 127, while samples 93B and 93C scored 99.26% and 98.22% identity to *Serratia marcescens* strain B195 and *Serratia marcescens* strain RS, respectively and finally sample 93D had a 99.80% similarity to *Citrobacter freundii* strain UIS1115 (Table 5). All the 16S rRNA sequences had a query cover of 100% to the subject sequences which further proved the identities of these enterobacteria. The evolutionary history of the nematode-associated bacterial isolates was inferred by using the Maximum Likelihood (ML) method and Tamura-Nei model (Tamura and Nei, 1993). The tree (s) with the highest log-likelihood for each bacterium is shown in section 4.3.3 (Phylogenetic reconstruction).

Table 5: Identity and percentage scores of the sequences of the isolated nematode-associated enterobacterial species.

Query sequence	Subject		Score		
	AC	16S rRNA gene	Query cover (%)	E value	Identity (%)
A (B27_1492R)	KF254665.1	*K. oxytoca* strain 127	100.00	0.00	99.26
B (B27_1492R)	MN532689.1	*S. marcescens* strain B195	100.00	0.00	99.26
C (B27_1492R)	MN396719.1	*S. marcescens* strain RS	100.00	0.00	98.22
D (1492R_H08)	MT081644.1	*C. freundii* strain UIS1115	100.00	0.00	99.80

Key: AC - Accession number, % - Percentage

4.3.3 Phylogenetic reconstruction

In terms of identifying the clade to which the Kenyan isolates belonged, three clades were proposed. The first was named the *Klebsiella oxytoca* clade represented by isolate 93A having a 99.26% identity to *Klebsiella oxytoca* strain 127. This Kenyan bacterial isolate had a sequence length of 1220bp and was named *K. oxytoca* strain 93A (Figure 16). Two isolates (93A and 93B) representing 50% of the total sample and the isolates of interest, showed a 99.26% and 98.22% identity to *Serratia marcescens* strain B195 and *Serratia marcescens* strain RS respectively. The two *Serratia* species in the current study were thus named *Serratia marcescens* strain 93B and *S. marcescens* strain 93C and their nucleotide sequences were 1213base pairs and 1172 base pairs respectively. These were thus placed in the clade *Serratia marcescens* (Figure 17 and 18, respectively). Isolate 93D exhibited 99.80 percentage identity to *Citrobacter freundii* strain UIS1115 and had a sequence length of 498 base pairs since only the reverse sequence was used in the analysis as opposed to a consensus sequence being

generated. This isolate was referred to as *Citrobacter freundii* strain 93D and formed the *Citrobacter* clade (Figure 19).

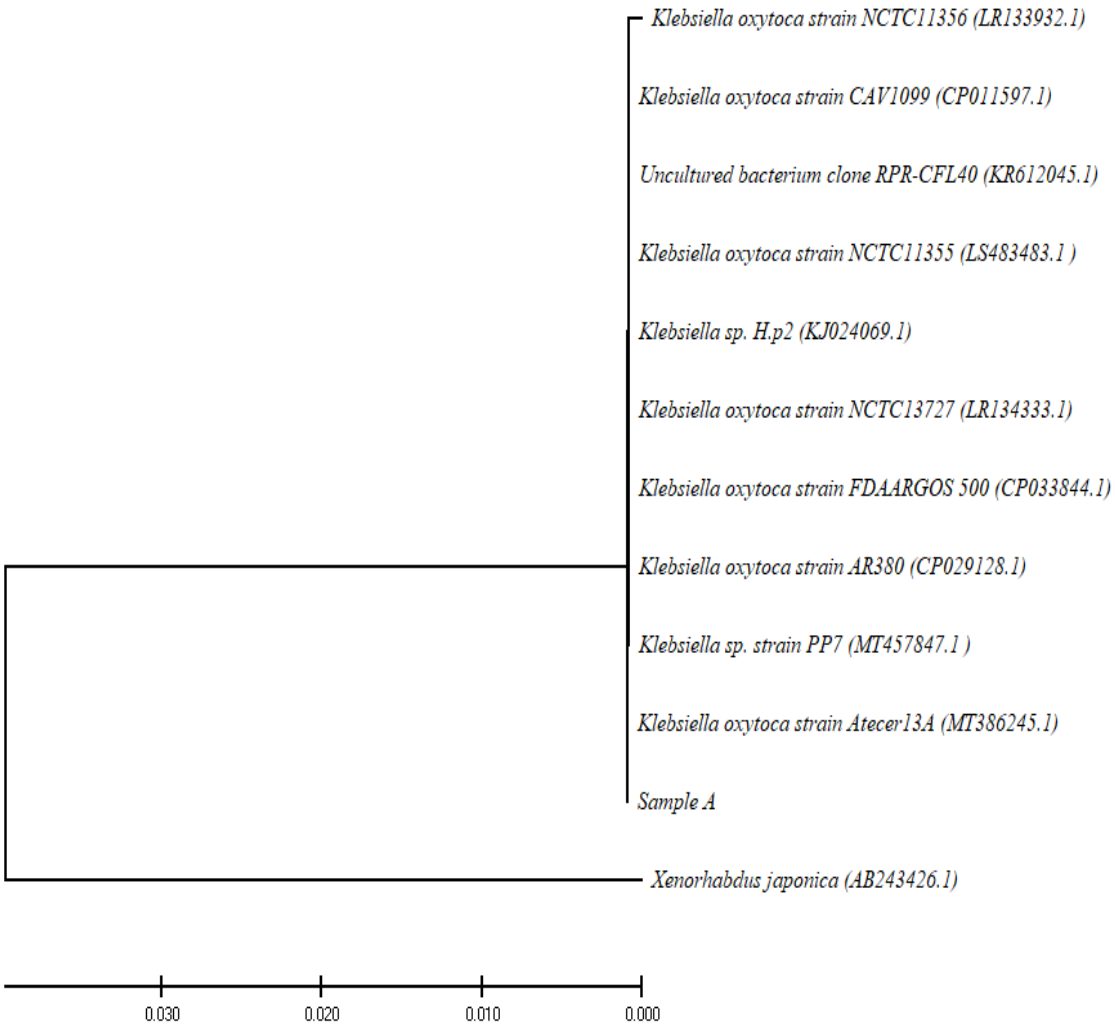

Figure 16: *Klebsiella oxytoca* 93A clade

This new Kenyan isolate (Sample A) – *Klebsiella oxytoca* strain 93A, with a 99.26% identity to *Klebsiella oxytoca* strain 127. Evolutionary analyses were conducted in MEGA X (Kumar *et al*., 2018).

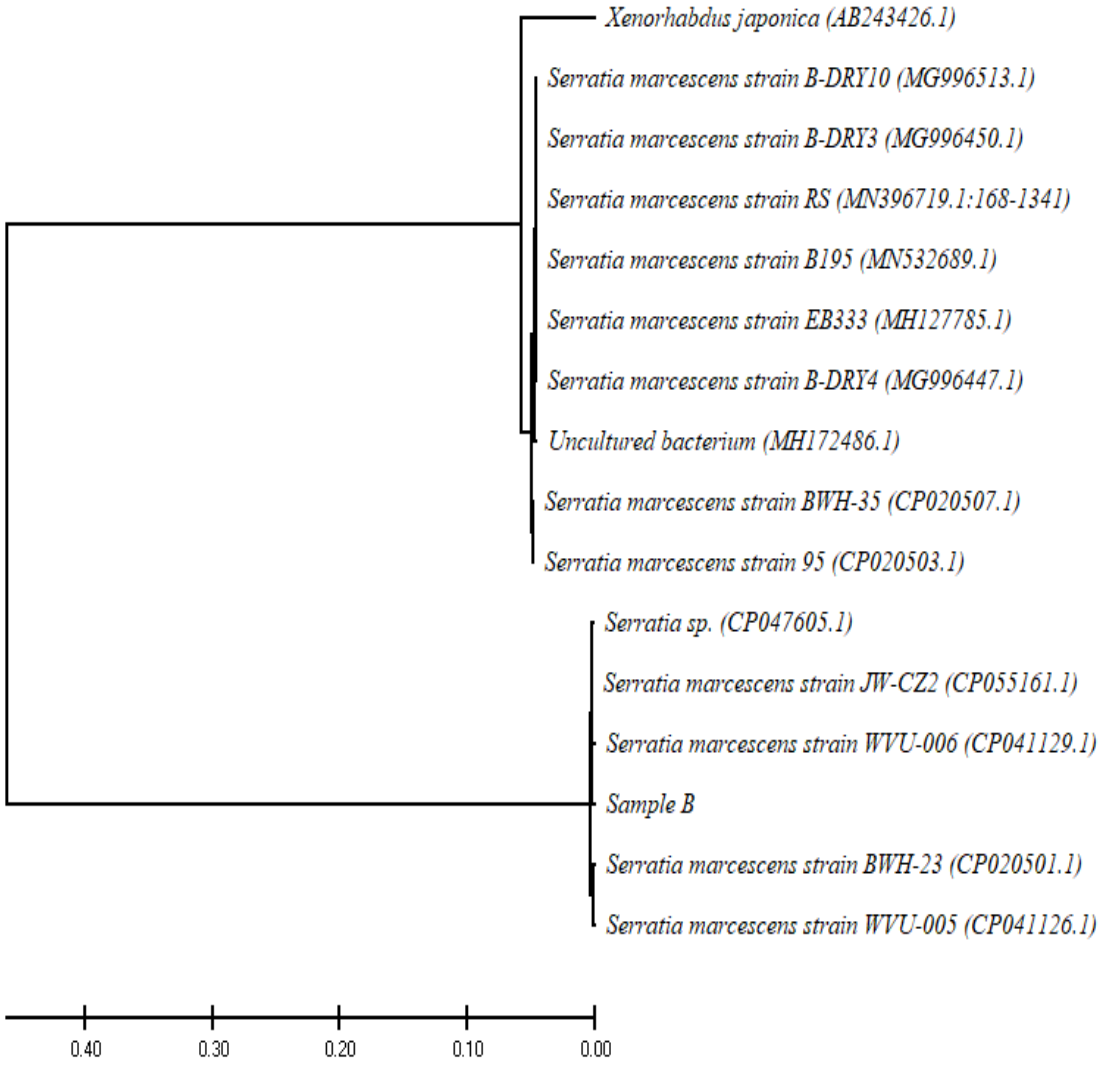

Figure 17: *Serratia marcescens* 93B clade

This new Kenyan isolate (Sample B) – *Serratia marcescens* strain 93B, had a 99.26 percentage identity to *Serratia marcescens* strain B195. Evolutionary analyses were conducted in MEGA X (Kumar *et al*., 2018).

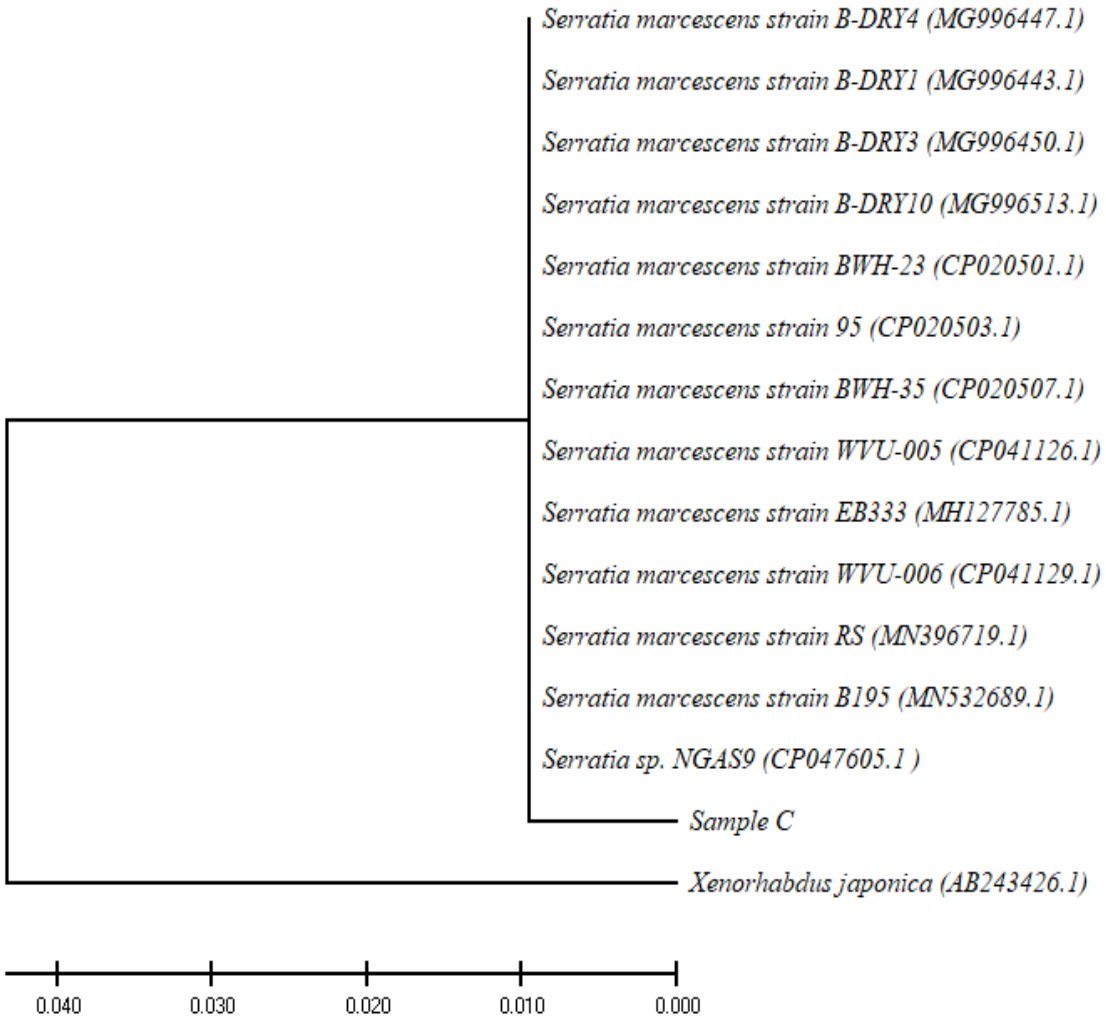

Figure 18: *Serratia marcescens* 93C clade

This new Kenyan isolate (sample C) – *Serratia marcescens* strain 93C, had a 98.26 % identity to *Serratia marcescens* strain RS. Evolutionary analyses were conducted in MEGA X (Kumar *et al*., 2018).

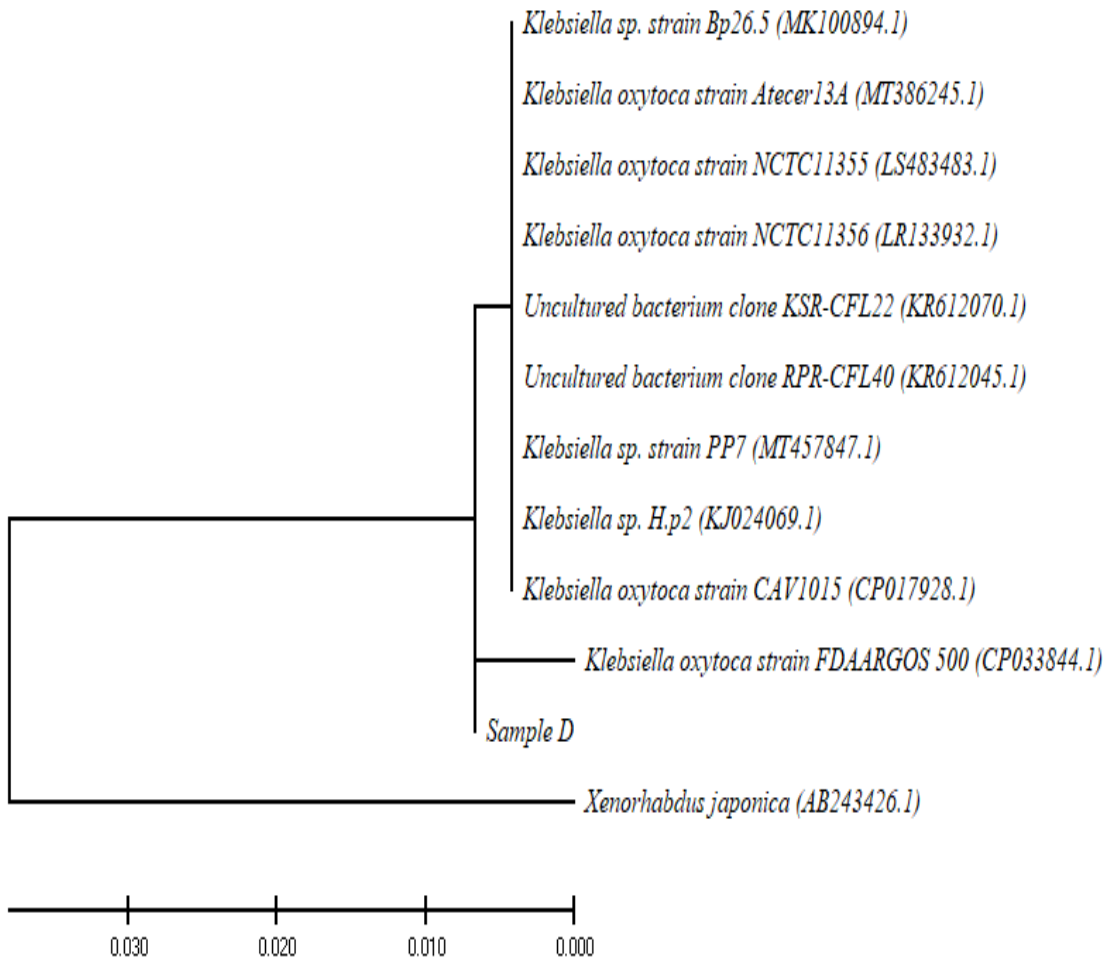

Figure 19: *Citrobacter freundii* 93D clade

This new Kenyan isolate (sample D) – *Citrobacter freundii* strain 93D, had a 99.80 % identity to *Citrobacter freundii* strain UIS1115. Evolutionary analyses were conducted in MEGA X (Kumar *et al*., 2018).

4.4 Antimicrobial activity of *Serratia* spp. and other endosymbiotic bacteria against selected pathogens

4.4.1 Antimicrobial activity against selected bacterial pathogens

Cell-free suspensions of all the four isolates showed activity against all the six human test bacterial pathogens (Figure 19). This activity was clearly shown by the significant reduction in the growth of the bacterial pathogens at $p<0.005$ as opposed to the rapid growth of the same bacterial pathogens on the negative control. However, after 3 days of incubation, the diameter of the inhibition zones reduced.

The susceptibility of the test bacterial pathogens in ascending order to the cell-free suspension of the bacterial isolate 93A was as follows: *P. aeruginosa* (14.00 mm; 15.33 mm), *S. aureus* (14.33 mm; 18.00 mm), MRSA (18.67 mm; 21.00 mm), *E. coli* (21.33 mm; 23.33 mm), *C. albicans* (21.33 mm; 27.00 mm) and *B. cereus* (30.67 mm; 34.00 mm) for the 50% and 100% concentrations, respectively. The cell-free suspension obtained from isolate 93B resulted in inhibition zones with diameters as follows: MRSA (12.67 mm; 16.00 mm), *P. aeruginosa* (12.67 mm; 16.33 mm), *S. aureus* (13.67 mm; 18.33 mm), *E. coli* (21.33 mm; 26.67 mm), *C. albicans* (21.33 mm; 27.67 mm) and *B. cereus* (30.67 mm; 33.67 mm) for the 50% and 100% concentrations, respectively. The diameters of the inhibition zones measured against the test bacteria with respect to the cell-free suspension of bacterial isolate 93C were *P. aeruginosa* (13.33 mm; 17.33 mm), MRSA (14.33 mm; 17.67 mm), *S. aureus* (18.00 mm; 20.67 mm), *E. coli* (21.67 mm; 25.67 mm), *B. cereus* (29.00 mm; 31.33 mm) and *C. albicans* (21.67 mm; 36.33 mm) for the 50% and 100% concentrations respectively. The cell-free suspensions (50% and 100% concentrations) of bacterial isolate 93D showed efficacy results with the following diameters against the six test bacterial pathogens respectively: *P. aeruginosa* (13.33 mm; 16.00 mm), MRSA (16.33 mm; 19.67 mm), *S. aureus* (17.00 mm; 19.67 mm), *E. coli* (20.67 mm; 24.00 mm), *B. cereus* (30.67 mm; 33.33 mm) and *C. albicans* (20.67 mm; 35.33 mm) (Table 6; Figure 20).

In general, the least susceptible pathogen was *P. aeruginosa* with a mean diameter of 13.00 mm followed by MRSA (14.83 mm), *S. aureus* (15.92 mm), *E. coli* (21.09 mm), *B. cereus* (26.92 mm) and finally the yeast *C. albicans* with a mean diameter of 30.34 mm. Both concentrations of the cell-free suspension maintained the same efficacy trend against the test bacterial pathogens with the only difference being a reduction in the diameter of the zones with the half-strength concentration. *Pseudomonas aeruginosa* exhibited the least zones of

inhibition across all the bacterial suspensions (except isolate 93B which had the least inhibition with MRSA than *P. aeruginosa*) hence susceptibility. Three species, *C. albicans* (30.34 mm), *B. cereus* (26.92 mm) and *E. coli* (21.09 mm) showed the highest zones of inhibition and were classified as extremely sensitive (mean diameters of inhibition zones larger than 20.00 mm) while *S. aureus* (15.92 mm) was ranked as being very sensitive (mean diameters of inhibition zones between 15.00 and 19.00 mm) while MRSA (14.83 mm) and *P. aeruginosa* (13.00 mm) were the least susceptible species and ranked as sensitive (mean diameters of inhibition zones between 13.00 and 15.00 mm) after 48 hours of incubation. The cell-free bacterial suspensions competed effectively when compared to the positive control which showed the highest inhibition in controlling the growth of *B. cereus* (32.00 mm) followed by *C. albicans* (29.82 mm), *E.coli* (23.09 mm), *S. aureus* (17.45 mm), *P. aeruginosa* (14.09 mm) and finally methicillin-resistant *S. aureus* (13.64 mm) (Table 6).

There were no significant differences among the treatments (cell-free bacterial suspensions) ($F_{4, 265} = 1.55$, $p=0.19$). However, higher significant differences were detected among the test bacterial species ($F_{5, 265} =219.10$, $p<0.0001$) compared to the differences shown between the two concentrations ($F_{1, 265} =34.55$, $p<0.0001$). Interactions between treatment and bacteria showed high significance differences ($F_{20, 216} =4.06$, $p<0.0001$) and so did interactions between treatment and concentration ($F_{4, 216} =11.74$, $p<0.0001$) when compared to interactions between bacteria and concentration ($F_{5, 216} =1.99$, $p=0.09$). There were no significant differences detected in interactions among treatments, concentrations and bacteria ($F_{20, 216} =0.92$, $p=0.56$). The two concentrations used also showed significant differences with the 100% concentration resulting in larger inhibition zones across all the test bacteria than the half-strength concentration. All the bacteria showed significant differences with respect to concentration as follows (arranged from most susceptible to least susceptible): *C. albicans* ($F_{1, 44} =10.49$, $p=0.002$), *E.coli* ($F_{1, 44} =8.53$, $p=0.005$), *P. aeruginosa* ($F_{1, 44} =7.11$, $p=0.011$), *B. cereus* ($F_{1, 44} =5.72$, $p=0.021$), *S. aureus* ($F_{1, 44} =5.38$, $p=0.025$) and finally MRSA ($F_{1, 44} =3.02$, $p=0.089$) which showed no significant difference with respect to the concentration levels used (Table 6).

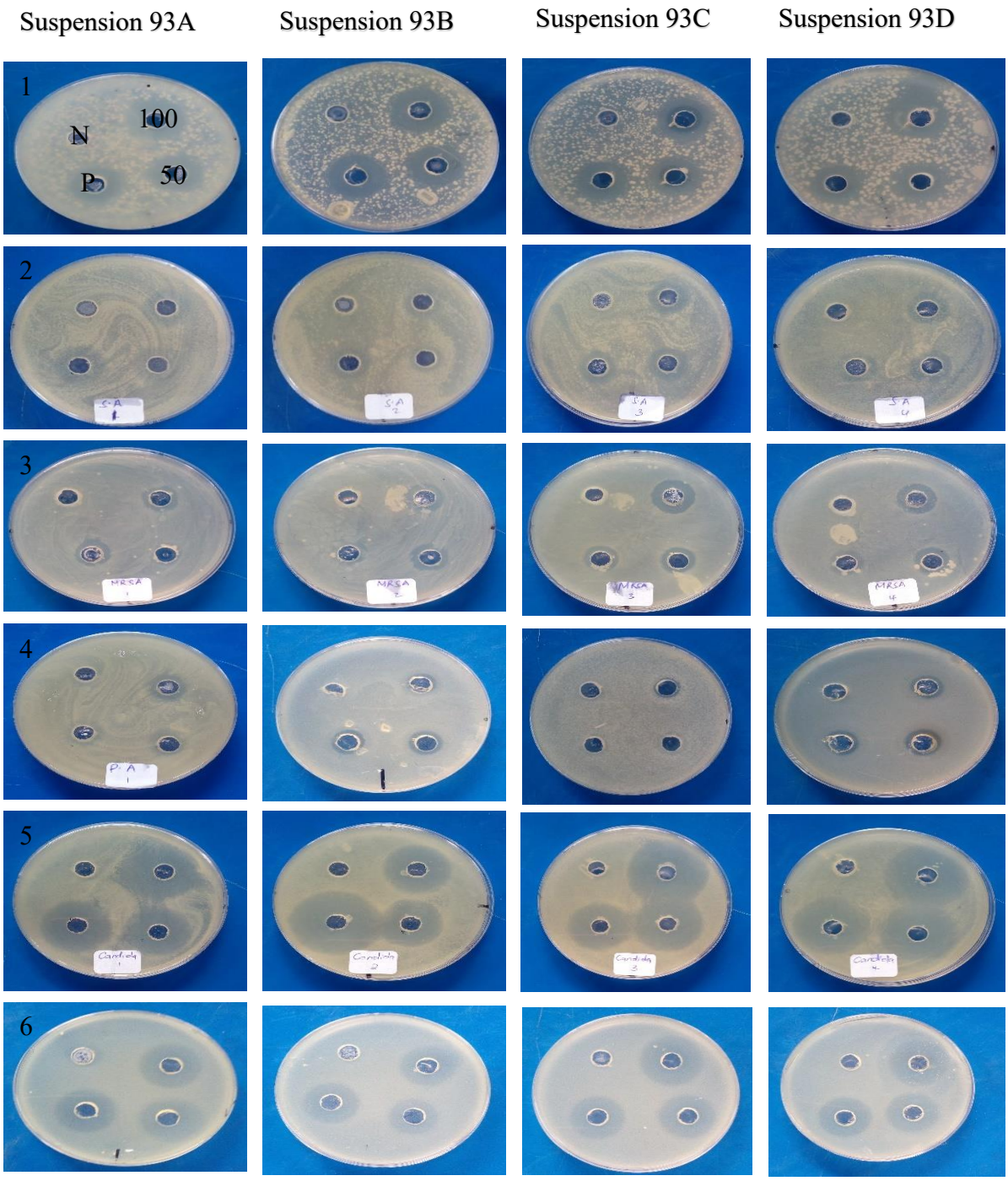

Figure 20: Inhibition zones of four bacterial cell-free suspensions (93A, 93B, 93C and 93D) on six bacterial test pathogens. Row 1: *E.coli*, Row 2: *S. aureus*, Row 3: MRSA, Row 4: *P. aeruginosa*, Row 5: *C. albicans* and Row 6: *B. cereus* after 48 hours of incubation at 28°C on MHA. (N - Negative control; P - Positive control; 100 - 100% concentration; 50 - 50% concentration and represents the same arrangement for all the plates).

Table 6: Diameter of inhibition zones (mm) of four bacterial cell-free suspensions on six bacterial pathogens after 48 hrs of incubation at 28°C on MHA.

Treatment	%Conc.	Diameter of inhibition zone (mm) of test bacteria					
		E.coli	*S. aureus*	MRSA	*P. aeruginosa*	*B. cereus*	*C. albicans*
Suspension 93A	50	21.33±0.7b	14.33±0.8a	18.67±0.7ab	14.00±0.6a	30.67±0.3c	21.33±1.4b
	100	23.33±0.3bc	18.00±0.7ab	21.00±0.9abc	15.33±0.6a	34.00±0.4c	27.67±1.1cd
Suspension 93B	50	21.33±0.3b	13.67±0.9a	12.67±0.9a	12.67±0.6a	30.67±0.3c	21.33±1.2b
	100	26.67±0.3bc	18.33±0.7a	16.00±0.9a	16.33±0.5a	33.33±0.4c	27.67±0.9b
Suspension 93C	50	21.67±1.2cd	18.00±0.5bc	14.33±0.6ab	13.33±0.5a	29.00±0.3d	21.67±1.2cd
	100	25.67±0.3bc	20.67±0.6ab	17.67±0.7a	17.33±0.4a	31.33±0.4cd	36.33±1.1
Suspension 93D	50	20.67±0.7bA	17.00±0.6ab	16.33±0.6ab	13.33±0.4a	30.67±0.3c	20.67±0.5b
	100	24.00±0.4b	19.67±0.5ab	19.67±0.5ab	16.00±0.4a	33.33±0.4c	35.33±1.1c
+ve Control		23.09±0.4c	17.45±0.8b	13.64±0.5a	14.09±0.5a	32.00±0.4d	29.82±1.0d
Mean (±1.0)		21.09	15.92	14.83	13.00	26.92	30.34

Values represent the mean ± standard error of mean for bioassay conducted in triplicate. Means followed by the same alphabetical letter(s) within and across rows are not significantly different.

4.4.2 Antimicrobial activity against selected phytopathogenic fungal species

The cell-free suspensions of all the enterobacterial isolates showed activity against at least five of the six plant pathogenic fungal species except for *Fusarium verticillioides* (Figure 21) which exhibited very minimal to no inhibition when treated with the isolates. This activity was clearly shown by the difference in diameter of the zones of inhibition against each phytopathogenic fungi after 5 days of incubation at room temperature. In addition, the isolates also significantly decreased the growth rate of the fungal pathogens. However, after 10 days of incubation, the diameter of the inhibition zones reduced greatly.

The susceptibility of the test fungal plant pathogens in ascending order concerning the cell-free suspension of the bacterial isolate 93A were as follows: *F. verticillioides* (0.00 mm; 0.00 mm), *F. oxysporum* (10.67 mm; 13.33 mm), *F. solani* (12.33 mm; 14.33 mm), *P. chrysogenum* (15.33 mm; 17.00 mm), *Penicillium* spp. (14.00 mm; 19.00 mm) and *A. flavus* (15.33 mm; 21.33 mm) for the 50% and 100% concentrations, respectively. The cell-free suspension obtained from isolate 93B resulted in inhibition zones with diameters as follows: *F. verticillioides* (0.00 mm; 0.00 mm), *F. solani* (13.33 mm; 15.00 mm), *A. flavus* (11.67 mm; 15.33 mm), *P. chrysogenum* (13.00 mm; 17.00 mm), *Penicillium* spp. (12.67 mm; 18.00 mm) and *F. oxysporum* (13.00 mm; 18.33 mm) for the 50% and 100% concentrations respectively. The diameters of the inhibition zones measured against the test fungi for the cell-free suspension of bacterial isolate 93C were *F. verticillioides* (4.00 mm; 4.67 mm), *F. solani* (11.00 mm; 12.67 mm), *A. flavus* (11.33 mm; 13.33 mm), *Penicillium* spp. (13.67 mm; 16.67 mm), *P. chrysogenum* (14.67 mm; 18.33 mm) and *F. oxysporum* (17.33mm; 19.67mm) for the 50% and 100% concentrations respectively. The cell-free suspensions (50% and 100% concentrations) of bacterial isolate 93D showed efficacy results with the following diameters against the six test fungal pathogens respectively: *F. verticillioides* (0.00 mm; 0.67 mm), *A. flavus* (12.67 mm; 15.33 mm), *F. solani* (12.33 mm; 16.00 mm), *F. oxysporum* (14.67 mm; 17.67 mm), *P. chrysogenum* (15.00 mm; 18.33 mm) and *Penicillium* spp. (16.33 mm; 18.67 mm) (Table 7; Figure 21).

Generally, the most susceptible pathogens to the cell-free extracts in order of decreasing susceptibility were *F. oxysporum* (19.74 mm), *P. chrysogenum* (16.55 mm), *Penicillium* spp. (15.93 mm), *A. flavus* (14.81 mm), *F. solani* (13.81 mm) and finally *F. verticillioides* (0.96 mm) which had the least zone of inhibition hence susceptibility (Figure 21; Table 7). The most resistant pathogen was *F. verticillioides* with a mean diameter of 0.96 mm. The only bacterial suspension which showed some mild efficacy against this phytopathogen was that obtained from isolate 93C (*Serratia marcescens* strain 93C) with an inhibition zone of 4.00mm and 4.67

mm with 50% and 100% concentrations respectively (Table 7). These test fungi were thus classified into three categories as extremely sensitive (mean diameter of inhibition zones larger than 15.00 mm), moderately sensitive (mean diameter of inhibition zones between 10.00 and 15.00 mm), or resistant (mean diameter of inhibition zones below 1.00mm). Three plant pathogenic species, *F. oxysporum* (19.74 mm), *P. chrysogenum* (16.55 mm) and *Penicillium* spp. (15.93 mm) showed the highest zones of inhibition and were classified as extremely sensitive whilst two fungal species, *A. flavus* (14.81 mm) and *F. solani* (13.81 mm) were ranked as being moderately sensitive and finally *F. verticillioides* (0.96 mm) which was classified as resistant after 5 days of incubation. The cell-free bacterial suspensions competed effectively when compared to the positive control which showed the highest inhibition in both *F. oxysporum* and *P. chrysogenum* (20.33 mm) followed by *Penicillium* species (18.67 mm), *F. solani* (17.33 mm), *A. flavus* (17.00 mm) and finally *F. verticillioides* (0.00 mm).

High significant differences were detected among the fungal test phytopathogens ($F_{5, 169} = 364.78$, $p<0.0001$). Similarly, higher significant differences were detected between the concentrations ($F_{1, 169} = 10.50$, $p = 0.0014$) unlike the treatments which showed the least significant differences ($F_{4, 169} = 2.81$, $p = 0.027$). Only the interaction between treatment and fungal species showed significant differences ($F_{20, 120} = 2.81$, $p=0.0003$) but the interactions between fungi and concentration ($F_{5, 120} = 0.43$, $p = 0.83$) and those between treatment and concentration ($F_{4, 120} = 0.26$, $p=0.90$) were not significantly different. Likewise, the interactions among treatments, fungi and concentration were not significantly different ($F_{20, 120} = 0.11$, $p=1.00$). The two concentrations used also showed significant differences with the 100% concentration resulting in larger inhibition zones across all the test bacteria than the half-strength concentration. All the fungi showed significant differences with respect to concentration as follows (arranged from most susceptible to least susceptible): *Penicillium* spp. ($F_{1, 28} = 15.33$, $p=0.0005$), *F. oxysporum* ($F_{1, 28} = 9.90$, $p=0.0039$), *P. chrysogenum* ($F_{1, 28} = 8.27$, $p=0.0076$), *A. flavus* ($F_{1, 28} = 7.30$, $p=0.0116$), *F. solani* ($F_{1, 28} = 5.42$, $p=0.0273$) and finally *F. verticillioides* ($F_{1, 28} = 0.002$, $p=0.970$) which showed no significant difference with respect to the concentration levels used (Table 7).

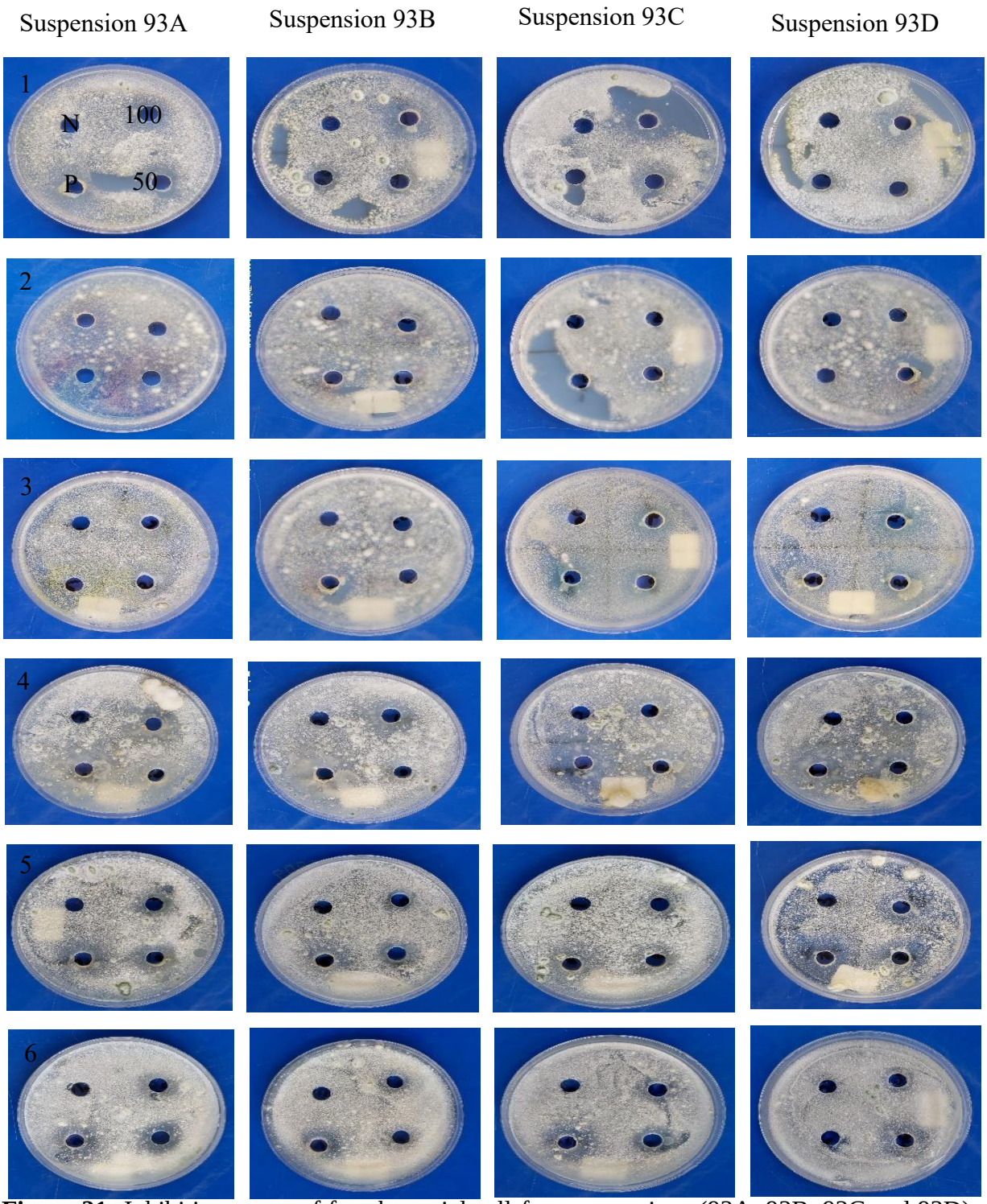

Figure 21: Inhibition zones of four bacterial cell-free suspensions (93A, 93B, 93C and 93D) against six test fungal species grown on potato dextrose agar: Row 1: *A. flavus*, Row 2: *F. solani*, Row 3: *F. verticillioides*, Row 4: *F. oxysporum*, Row 5: *P. chrysogenum* and Row 6: *Penicillium* spp. after 5 days of incubation at room temperature. (N - Negative control; P - Positive control; 100 - 100% concentration; 50 - 50% concentration and represents the same arrangement for all the plates).

Table 7: Diameter of inhibition zones (mm) of four bacterial cell-free suspensions on six phytopathogenic fungi after 5 days of incubation at 24°C on PDA.

Treatment	%Conc.	Diameter of inhibition zone (mm) of test fungi					
		A. flavus	*F. solani*	*F. oxysporum*	*F. verticillioides*	*P. chrysogenum*	*Penicillium spp.*
Suspension 93A	50	15.33±0.5c	12.33±0.3bc	10.67±0.4b	0.00±0.0a	15.33±0.4c	14.00±0.3c
	100	21.33±0.8c	14.33±0.6bc	13.33±0.7b	0.00±0.0a	17.00±0.4bc	19.00±0.6bc
Suspension 93B	50	11.67±0.2b	13.33±0.9b	13.00±0.3b	0.00±0.0a	13.00±0.5b	12.67±0.2b
	100	15.33±0.1b	15.00±0.4b	18.33±0.4b	0.00±0.0a	17.00±0.5b	18.00±0.2b
Suspension 93C	50	11.33±0.4ab	11.00±0.3ab	17.33±0.3b	4.00±0.0a	14.67±0.3b	13.67±0.4b
	100	13.33±0.4ab	12.67±0.5ab	19.67±0.5b	4.67±0.0a	18.33±0.3b	16.67±0.4b
Suspension 93D	50	12.67±0.3ab	12.33±0.3ab	14.67±0.3b	0.00±0.0a	15.00±0.5b	14.33±0.4b
	100	15.33±1.2b	16.00±0.5b	17.67±0.3b	0.00±0.0a	18.33±0.5b	16.33±0.5b
+ve Control		17.00±0.46b	17.33±1.0b	20.33±0.7a	0.00±0.0b	20.33±0.5b	18.67±0.7b
Mean (±1.0)		14.81	13.81	19.74	0.96	16.55	15.93

Values represent the mean ± standard error of mean for bioassay conducted in triplicate. Means followed by the same alphabetical letter(s) within and across rows are not significantly different.

CHAPTER FIVE: DISCUSSION

The primary objective of this study was to characterize the entomopathogenic symbiotic bacterium *Serratia* spp. and other associated bacteria isolated from Rhabditid nematodes. Additionally, the specific objectives were to isolate and determine the morphological characteristics of symbiotic *Serratia* spp. and other associated bacterial species as well as determining their phylogeny using 16S rRNA gene and finally, evaluation of antimicrobial efficacy of the bacterial isolates against selected human bacterial pathogens and fungal phytopathogens. This section of the discussion will therefore be founded on these factors.

5.1 Multiplication of entomopathogenic nematodes

All the ten nematode samples collected were viable as confirmed by their rapid wiggling motion. The White trap method of nematode multiplication was employed. The emergence time of the nematodes from the dead larval cadavers into the sterile distilled water in the trough varied greatly during multiplication, such that in nine out of the ten samples, the nematodes emerged by the 5^{th} day of incubation with only one sample having the nematodes emerge between the 8^{th} - 10^{th} day after incubation in the dark. Only 10% of the samples represented *Oscheius* nematodes while the rest (90%) were *Steinernema* nematodes. This was confirmed by morphological identification under the microscope and by observation of the color of the dead larval cadavers after 5 days of incubation following reinfection. The *Oscheius* nematodes were long and slender with very rapid movements. Only this one sample had brick-red to darkly pigmented cadavers as opposed to the whitish/cream cadavers representing the Steinernematids. The *Heterorhabditis* nematodes associated with *Photorhabdus* species have also been reported to have brick-red to darker *G. mellonella* cadavers due to the presence of anthraquinones (Pankewitz and Hilker, 2008; Stock *et al*., 2017). The *Oscheius* genus has been reported to be more closely related to the Heterorhabditid nematodes than to the Steinernematids (Zhang *et al*., 2008). When visualized in the dark, no bioluminescence was observed and this further confirmed that the nematode was *Oscheius* species because this is a characteristic that has only been observed within the *Heterorhabditis* genus (Gerrard *et al*., 2003). This darkening of the *Oscheius*-victimized cadavers is due to the characteristic red pigment known as prodigiosin secreted by their characteristic endosymbiotic *Serratia marcescens* B195 bacteria. These results were similar to those reported by Myers *et al*. (2015) where they documented that *Steinernema*-killed larvae were cream to greyish-yellow in colour while *Oscheius*-victimized cadavers showed a brick-red to dark brown coloration and retained their original flaccid shape. Similar results were also

reported by Zhang *et al*. (2008) during their study on *Heterorhabditidoides chongmingensis* (Hang and Uža 2012; Alhussaini, 2018).

5.2 Isolation, morphological and biochemical characterization of symbiotic *Serratia* spp. and other associated bacterial species

Species of *Serratia* are known endosymbionts of the *Oscheius* nematodes (Lephoto *et al*., 2015). As a result, the nematodes which had already been selected and multiplied by the White trap technique were used for the isolation of these bacteria. In this study, the *Oscheius* (*Heterorhabditidoides*) nematodes were found pathogenic to the *Galleria mellonella* larvae causing 90% death within 24 hours and 100% mortality within 48 hours post-infection. This rapid death can be attributed to the many antimicrobial agents secreted by the nematode-associated bacterial species. These antimicrobial agents include prodigiosin, biosurfactants, ethyl acetate, phenol and nonanoic acid which are known to inhibit invasion of the dead larvae by opportunistic microbiota (Serepa and Gray, 2014; Karthick *et al*., 2015; Mathlom *et al*., 2018) as well as the insecticidal compound chlorpyrifos (Karthick *et al*., 2015). During larval infection, usually, the entomopathogenic bacterium, such as *Serratia marcescens* is released from the nematode into the insect haemocoel where the bacterium causes rapid host death exhibited by loss of movement, feeding incapacitation and death of the insect (Han and Ehlers, 2001), usually within 48 hours following infection.

The first and foremost phenotypic evidence in the identification of this bacterium was the discoloration of the *G. mellonella* larval cadavers, which showed a dark brown colour as compared to the creamish colour of the uninfected larvae. Similar results were reported by Myers *et al*. (2015) and Hover *et al*. (2016). They reported that larvae killed by Steinernematids display a cream to greyish-yellow colour while *Oscheius*-victimized cadavers exhibited brick-red to dark brown or maroon coloration while remaining moist with no odour and retaining their original shape (Myers *et al*., 2015). The darkening of colour observed in *Oscheius*-victimized cadavers can be attributed to the pigment production and secretion capacity of the nematode-associated *Serratia* bacteria particularly the well-known prodigiosin which is a red coloured pigment (Karthick *et al*., 2015; Myers *et al*., 2015; Hover *et al*., 2016). This phenomenon is similar to characteristics induced by other EPN mutualistic bacteria (Myers *et al*., 2015; Bisel, 2016). This change in colour of *Galleria mellonella* larvae from cream to dark brown supports the idea that the genus *Serratia* is a source of diverse and

efficient hydrolytic metabolites and enzymes among pathogenic microorganisms (Karthick *et al*., 2015; Myers *et al*., 2015). In all cases, the cadavers exhibited a gummy consistency.

In addition, the *G. mellonella* larvae exhibited a flaccid body with no foul smell and were uncontaminated from other competing microbes as is the case with *Xenorhabdus* and *Photorhabdus* species symbiotically associated with *Steinernema* and *Heterorhabditis* nematodes respectively. These results were in synch with those reported by Myers *et al*. (2015) and Bisel (2016). The results in the current study were also similar to those reported by Hang and Uža (2012) when studying *Oscheius chongmingensis* and its entomopathogenic activity. The dead larvae were completely devoid of any foul smell and no growth of other opportunistic pathogens was observed, proving that indeed death was caused by the nematode endosymbiotic enterobacteria *Serratia marcescens* strain 93B and *Serratia marcescens* strain 93C and the other nematode-associated bacteria (*Klebsiella oxytoca* strain 93A and *Citrobacter freundii* strain 93D). The observations were in agreement with those reported by Hang and Uža (2012) and Bisel *et al*. (2012). This, therefore, creates the need to further exploit this bacterial endosymbiont as a source of potential antimicrobial agents and antibiotics against diseases as well as biological control agents especially because the *Oscheius-Serratia* association has only been recently included as one of the entomopathogenic nematodes group (Liu *et al*., 2012; Torres- Barragan *et al*., 2011; Ye *et al*., 2010). There are thus vast areas of research to be conducted on this nematode-bacterium duo hence the need for this study.

MacConkey and NBTA agar were used to differentiate and select only for gram-negative bacterial species (Jung and Hoilat, 2020). All nematode-associated bacterial colonies had varied shades of green color (phase I) on NBTA which later turned to maroon in phase II. This phase variation is typical of many bacteria, whereby the secondary form (Phase II) exhibits a color change due to the production of various secondary metabolites during the stationary phase of bacterial growth. Primary variant cells absorb bromothymol blue on NBTA plates, resulting in green colonies, while the secondary variant colonies appear red or maroon because of the triphenyltetrazolium chloride (TTC) (Akhurst, 1980). They also adsorb neutral red from MacConkey agar. Phase II variants reduce TTC but not bromothymol blue hence resulting in the formation of red colonies without a clear zone. The primary form produces more bioluminescence, proteases, antibiotics, pigmentations, lipases and phospholipases (Gaudriault *et al*., 2008). The difference in the intensity or shade of the green color could be attributed to differences in the production of metabolites by the various

bacterial species (Derzelle *et al*., 2004; Gaudriault *et al*., 2008). The isolated bacterial colonies were also Gram-negative, shiny, circular, opaque, raised (convex), or umbonated with regular, smooth entire margins and were in agreement with the results reported by Thanwisai *et al*. (2012); Myers *et al*. (2015). Similar results were documented by Mathlom *et al*. (2018) where they reported the studied *Serratia marcescens* G10 as a red-pigmented, gram-negative rod-shaped bacterium. The colonies had an average diameter of approximately 2mm. Swarming motility was also observed when the isolates were cultured on half-strength NBTA media, whereby the bacteria were able to move with clearly distinct serrated margins except for *Klebsiella oxytoca* strain 93A. This is because isolates *Serratia marcescens* strain 93B, *Serratia marcescens* strain 93C and *Citrobacter freundii* strain 93D possess flagella which aid in movement unlike *Klebsiella oxytoca* strain 93A which is unflagellated, hence immotile. Pradel *et al*. (2007) reported similar results on the ability of *Serratia* species to move and attributed this to their capacity to secrete the biosurfactant serrawettin W2. This biosurfactant has shown its ability to disperse bacteria such as *Caenorhabditis elegans* (Pradel *et al*., 2007). All the endosymbiotic bacteria were gram-negative, positive for pathogenicity test, catalase test, starch hydrolysis test, Simmons citrate agar (SCA) test and negative for urease and oxidase just like the results reported by Karthick *et al*. (2015) and Abdullah *et al*. (2017) during their studies on *Serratia* species.

There was positive utilization of substrates within the growth media (NBTA, NA and MAC) indicated by visible fading in the colour of the media (dye absorption) around the growth areas compared to the areas without the colonies. All the isolates appeared red/pink on MAC agar (proving that the bacteria are lactose fermenters) and cream on NA. On MacConkey plates, the primary variant cells bind to neutral red, generating red colonies (Jung and Hoilat, 2020). However, phase II cells showed decreased intensity in the green, pink and yellow colors on NBTA, MAC and NA plates. Similar results were reported by Abdullah *et al*. (2017) when studying the susceptibility of *Serratia marcescens* to various antibiotics. Pigment production within the solid media on both NBTA and NA as well as the broth was confirmed by changes in color to orange or yellow. This further confirmed the presence of secondary metabolites. These results agreed with those reported by Karthick *et al*. (2015) while investigating the activity of *Serratia* species strain AAI which was a close relative of *S. marcescens* subsp. *sakuensis* and *S. nematodiphila*. This could be attributed to the increase in the concentration of secreted compounds in the secondary phase. This observation was similar to that given by Nambiar *et al*. (1974) who reported visible growth of *Serratia* spp. in the nutrient broth after

18 hours with no visible production of pigments even after 24 hours. These findings were similar to those reported by Williams (1973) who confirmed that prodigiosin, being a secondary metabolite, is only produced in the log phase of growth. However, this property to produce pigments was lost with subsequent culturing which only produced non-pigmented strains with completely similar properties except for pigmentation (Nambiar *et al*., 1974).

Since the viability of the nematodes was maintained by reinfection of *G. mellonella* larvae every 3 - 4 months, it demonstrated that *Serratia marcescens* 93B and *S. marcescens* 93C are capable of completing the cycle of parasitism by penetrating, reproducing and killing the new insect host. These observations were in agreement with reports in well-studied nematode-bacteria associations (Torres-Barragan *et al*., 2011). This demonstrates that this relationship between Rhabditid nematodes and *Serratia marcescens* strains 93B and 93C is potentially entomopathogenic and that both the nematode (for penetration) and the bacteria (for insect pathogenesis) appear to be necessary and sufficient to effect insect death. A similar approach was used by Abebe *et al*. (2010) to confirm that *C. briggsae* KT0001 was an entomopathogenic nematode associated with the symbiotic bacterium *Serratia* sp. SCBI. In their study, Torres-Barragan *et al*. (2011) also isolated four species of bacteria associated with the nematode *O. carolinensis*, namely; *Enterococcus mundtii*, *Achromobacter xylosoxidans*, *Serratia marcescens* and *Providencia rettgeri*. They reported that *Serratia marcescens*, is carried on the cuticle of the nematodes and through its association, is capable of providing the *Oscheius* nematodes the entomopathogenic potential. *Serratia marcescens* also killed fourth-instar larvae of *Helicoverpa zea* within 24 hours at concentrations as low as 330µg/ml. *Enterococcus mundtii*, only inflicted mortality of 33.3% after mechanical wounding of the larvae while *Achromobacter xylosoxidans*, resulted in no mortality even at the highest concentration of 500mg/ml with or without wounding. *Providencia rettgeri*, the fourth species isolated, resulted in low mortality of about 25%. They concluded that *P. rettgeri* in combination with *S. marcescens* are responsible for the facultative insect colonization ability of *O. carolinensis*. They also suggested that the association of *O. carolinensis* with its bacterial endosymbiont, *S. marcescens*, enables the nematode a dual lifestyle as an entomopathogen (Torres-Barragan *et al*., 2011). This further confirms the results in the current study that *S. marcescens* 93B and *S. marcescens* 93C are indeed nematode-associated symbiotic bacteria.

5.3 Phylogenetic analysis of *Serratia* species and other associated enteric bacterial spp.

In terms of identifying the clade to which the Kenyan isolates belonged, three clades were proposed. The first clade was named the *Klebsiella oxytoca* clade containing the *K. oxytoca* strain 93A from this study and had a 99.26% identity to *Klebsiella oxytoca* strain 127. This Kenyan bacterial isolate had a sequence length of 1220bp. Two isolates (93A and 93B) representing 50% of the total sample and the isolates of interest, showed a 99.26% and 98.22% identity to *Serratia marcescens* strain B195 and *Serratia marcescens* strain RS respectively. This *S. marcescens* B195 strain was isolated in Turkey from *Rhizopus oryzae* (Abaci *et al*., 2019) while the *S. marcescens* RS strain was reported in China when it was isolated from the heads of termites (*Reticulitermes speratus*) by Xiao-Tong *et al*. (2019). The two *Serratia* species in the current study were thus named *Serratia marcescens* strain 93B and *S. marcescens* strain 93C and their nucleotide sequences were 1213 base pairs and 1172 base pairs respectively. These were thus placed in the second clade, *Serratia marcescens* clade. The final isolate (93D) with 99.80% identity to *Citrobacter freundii* strain UIS1115 was placed in the *Citrobacter* clade. This Kenyan isolate was called *Citrobacter freundii* strain 93D. Abebe-Akele *et al*. (2015) reported similar results by isolating and sequencing a South African entomopathogenic bacterium *Serratia marcescens* strain SCBI from *Caenorhabditis briggsae*. Similar results were documented by Torres-Barragan *et al*. (2011) when they isolated four bacterial species from *Oscheius carolinensis*. Their reported bacterial symbionts were *Serratia marcescens, Enterococcus mundtii, Achromobacter xylosoxidans* and *Providencia rettgeri* and concluded that only *S. marcescens* had a symbiotic relationship with the nematode species (Torres-Barragan *et al*., 2011).

All the nematode-associated enterobacteria that were isolated in this study i.e. *Klebsiella oxytoca* 93A, *S. marcescens* 93B, *S. marcescens* 93C and *C. freundii* 93D are gammaproteobacteria belonging to the family Enterobacteriaceae (Hoenigl *et al*., 2012; Abebe-Akele et al., 2015). However, only the Serratia genus has been shown to have a symbiotic relationship with its nematode host and thus accepted into the EPN group (Torres-Barragan *et al*., 2011), hence the focus on *Serratia marcescens* endosymbionts in the current study. Abebe *et al*. (2010) reported the isolation of *Serratia* species from *Caenorhabditis elegans* and confirmed that this bacterium was capable of penetrating, killing and reproducing in an insect host and that the bacterial associate could induce this insect pathogenic life cycle in other species of *Caenorhabditis*. Our research has shown that different bacterial genera

associate with Rhabditid nematodes which is in agreement with results reported by Sangeetha *et al*. (2016).

5.4 Antimicrobial activity of *Serratia* spp. and other Rhabditid-associated bacteria against selected pathogens

5.4.1 Antimicrobial activity against selected bacterial pathogens

All the isolates, namely; *Serratia marcescens* strain 93B, *S. marcescens* 93C and the other two associated endosymbiotic bacteria (*Klebsiella oxytoca* strain 93A and *Citrobacter freundii* strain 93D) were found to have potent antibacterial activity against all the human test bacterial pathogens, namely; *Bacillus cereus, Escherichia coli*, *Pseudomonas aeruginosa, Staphylococcus aureus*, methicillin-resistant *Staphylococcus aureus* (MRSA) and the pathogenic yeast *Candida albicans*. The antimicrobial activities of the cell-free suspensions of the isolated bacterial endosymbionts varied greatly from one test bacterium to another. Three species, *C. albicans* (30.34 mm), *B. cereus* (26.92 mm) and *E.coli* (21.09 mm) were extremely sensitive to the cell-free suspension and therefore showed the highest diameters of inhibition. *Staphylococcus aureus* (15.92 mm) was ranked very sensitive while MRSA (14.83 mm) and *P. aeruginosa* (13.00 mm) showed the least growth inhibitions hence more resistance. The cell-free bacterial suspensions used in the current study competed effectively when compared to the positive control. Comparable findings were reported by Mathlom *et al*. (2018) when studying the effect of prodigiosin and biosurfactants isolated from *S. marcescens* G10 on *S. aureus* and *E. coli*. The inhibition zones against *S. aureus* and *E. coli* were (9.80 to 19.00 mm) and (11.00 to 18.00 mm) respectively with prodigiosin and increased to (10.60 to 25.00 mm) and (11.20 to 24.00 mm) respectively after the addition of biosurfactant (Mathlom *et al*., 2018). Previous studies by Irfan *et al*. (2015), have also reported effective antimicrobial activity of *Serratia marcescens* compounds against *S. aureus*. Lependa *et al*. (2015) also noticed greater sensitivity of *S. aureus* to prodigiosin in comparison to the standard antibiotics (chloramphenicol and gentamicin). The results in this study are further confirmed by several other reports which document the bacteriostatic effects of prodigiosin against several pathogenic bacteria strains such *Acinetobacter* species, *E. faecalis*, *E. coli* and *S. pyogenes* (Lapenda *et al*., 2015; Wang *et al*., 2016; Mai, 2018). Purified prodigiosin extracts obtained from *Serratia marcescens* B2, *S. marcescens* IBRL USM 84 and *S. marcescens* B10 VKM have shown activity against algal blooms as well as *B. cereus*, *B. subtilis*, *C. utilis*, *C. albicans*, *S. aureus*, *P. aeruginosa*, *Cryptococcus* spp., *Shigella* and *Salmonella* species (Thomson *et al*., 2000; Pore *et al*., 2016).

The results of the present study demonstrate the inherent ability of bacterial species to secrete various antimicrobial compounds. Many studies have documented the capacity of endosymbiotic bacteria to produce various secondary metabolites that are both bacteriostatic and bactericidal towards pathogenic bacteria (Mathlom *et al*., 2018). The results obtained in the current study agreed with the reported promising antibacterial activity of nematode-associated enterobacteria of *Serratia*, *Klebsiella* and *Citrobacter* against a wide range of human pathogenic bacteria of economic importance (Su *et al*., 2016). They reported that the inhibition zones of prodigiosin at concentrations of (50 to 100) µg/ml were measured from (11.00 to 18.00 mm) and (9.80 to 19.00 mm) with *E.coli* and *S. aureus*, respectively. Their results similarly reported inhibitory activity of serrawettin W2 against the growth of various tested bacterial pathogens including 21 strains of *S. aureus*, *B. subtilis* A47, *M. luteus* CGMCC 1.2299, *P. aeruginosa* A62, *R. rhodochrous* CGMCC 4.1815 and *S. dysenteriae* CGMCC 1.1869 (Su *et al*., 2016). Clements *et al*. (2019a) also reported broad-spectrum antimicrobial activity of secondary metabolites produced by *Serratia marcescens* strains P1 and NP1 crude extracts against food, environmental and clinical bacterial pathogens, such as multidrug-resistant *Pseudomonas aeruginosa*, *Cryptococcus neoformans* and methicillin-resistant *Staphylococcus aureus*.

Cyclic lipopeptides from *Serratia* spp. with antibacterial activities, such as syringomycin, massetolide, viscosin, orfamide and arthrofactin have been reported as possible candidates for the development of new antibiotics by Bionda and Cudic (2011) and Bionda et *al*. (2013). Serrawettin W2 is a broad spectrum lipopeptide with suppressive abilities to both Gram-positive and Gram-negative bacteria (Su *et al*., 2016). In their report, Su *et al*. (2016) documented the cytotoxic activity of Serrawettin W2 on cancer cells and its selectivity for different cancer cell lines. The first isolation of Serrawettin W2 from *S. marcescens* was in 1986 (Matsuyama *et al*., 1986; Matsuyama *et al*., 2011) and displayed antimicrobial activity against *Staphylococcus aureus* (Gerc *et al*., 2014). Kadouri and Shanks (2013) investigated the antimicrobial activity of a serrawettin W1 produced by *Serratia marcescens* and reported that this compound exhibited efficacy mainly against Gram-positive bacteria, such as methicillin-resistant *Staphylococcus aureus* (MRSA). Likewise, serrawettin W2 has been documented to show activity against Gram-positive bacteria, like *Micrococcus* spp. and *S. aureus* whilst also exhibiting antibacterial activity against a few Gram-negative bacteria such as *Shigella* spp. and *Pseudomonas* species (Su *et al*., 2016). The genus *Serratia* has also been reported to secrete carbapenems, long known antibiotic compounds (Wilf and Salmond, 2012). *Serratia*

marcescens Db10 has been shown to use a Type 6 secretion system to deliver multiple antibacterial effectors, conferring it with potent activity against both closely and distantly related competitors (Murdoch *et al*., 2011). The sulfur-containing antibiotic althiomycin, has shown inhibition against both Gram-negative and positive bacteria. Some of the bacterial pathogens, for instance, *Escherichia coli*, *Bacillus anthracis*, *S. typhosa* and *S. paratyphi* were very sensitive while *P. aeruginosa* was less sensitive. This antibiotic althiomycin did not show inhibition against any fungi tested including *A. niger* and *S. cerevisiae* (Yamaguchi *et al*., 1957). The antibacterial activity was not weakened by the addition of the horse serum to the medium. The current experiment employed the use of cell-free suspensions, suggesting that some if not all of the secondary metabolites were responsible for the efficacy. Therefore, serrawettins, prodigiosin, althiomycin among others could be isolated, purified and used as valuable antibiotics in the future.

All four bacterial isolates were equally effective and showed no significant differences in their efficacy against the six test bacterial pathogens. This can be attributed to the fact that all four bacterial isolates are capable of secreting antimicrobial compounds with activity against pathogenic microbes. *Klebsiella oxytoca* and *Citrobacter freundii* have been known to produce these compounds as well (Shanks *et al*., 2012). Differences in concentration of the cell-free suspensions resulted in larger inhibition zones with the full-strength (100%) concentration compared to the half-strength (50%) concentration, because the latter was diluted hence subsequence reduction in the amount/concentration of the secondary antimicrobials. Generally, all the treatments (bacterial endosymbionts) were as effective as the positive control (broad-spectrum antibiotic ampicillin). This creates the need to further extract each of these antibiotic compounds and purify them for use in the control of human bacterial pathogens.

5.4.2 Antimicrobial activity against selected phytopathogenic fungal species

Some living organisms and microorganisms possess the ability to limit the adverse effects of many pathogenic microbes. This deterrence of pathogenesis is known as biological control (biocontrol) which is the best known alternative approach in the control of phytopathogens within sustainable agricultural systems (Johansson *et al*., 2004; Bale *et al*., 2008). Interactions involving prokaryotes and eukaryotes are ubiquitous. *Serratia marcescens* is a very unique microorganism among the enteric group of bacteria in various aspects. This bacterium secretes extracellular deoxyribonuclease (DNase), lipase, gelatinase, proteases, hydrolytic enzymes, plant growth-promoting metabolites like indole-3-acetic acid (IAA) and siderophores,

cellulase, 3 chitinases and a chitin-binding protein (Vaaje-Kolstad *et al*., 2013; Hover *et al*., 2016).

In the bacterial-fungal confrontation assay, all tested phytopathogenic fungi (namely; *A. flavus*, *F. oxysporum*, *F. solani*, *F. verticillioides*, *P. chrysogenum* and *Penicillium* spp.) were inhibited by at least one of the bacterial extracts. Three plant pathogenic species, *F. oxysporum*, *P. chrysogenum* and *Penicillium* spp. had the least growth and were classified as extremely sensitive whilst *A. flavus* and *F. solani* were moderately sensitive. The phytopathogen *F. verticillioides* was classified as resistant since it had the highest growth after 5 days of incubation. This resistant pathogen recorded no inhibition from three out of the four isolates used. The only bacterial suspension which showed some mild efficacy against this fungus was 93C (*Serratia marcescens* strain 93C) with inhibition zones of 4.00 mm and 4.67 mm with 50% and 100% concentrations, respectively. The low activity of bacterial cell-free suspensions on *F. verticillioides* can be attributed to the dual lifestyle of *F. verticillioides* which makes it difficult to effectively control (Bacon *et al*., 2008). The cell-free bacterial suspensions competed effectively when compared to the positive control which showed the highest inhibition in both *F. oxysporum* and *P. chrysogenum* followed by *Penicillium* species, *F. solani*, *A. flavus* and finally *F. verticillioides* whose growth was not inhibited at all. Similar results showing the interaction of *S. marcescens* with different fungal species were reported by Hazarika *et al*. (2020). In their study, *S. marcescens* was able to spread highly and effectively in the cultures of *Mucor irregularis* SS7 and *Fusarium oxysporum* SC7.1. However, the bacterium spread was very low in *Fusarium solani* hyphae but no significant spreading of the bacterium was detected inside the fungal hyphae of the rest of the strains; *Aspergillus flavus*, *Aspergillus nomius*, *Penicillium citrinum*, *Coprinellus sp*., *Pleurotus ostreatus*, *Pycnoporus coccineus*, *Chlorophyllum molybdites*, *Sarcodon sp*., and *Leucocoprinus sp*. (Hazarika *et al*., 2020). This high spreading capability of *S. marcescens* in *F. oxysporum* might have been the reason why this fungus was the most inhibited during the current bioassay while *F. solani* and *A. flavus* recorded lower inhibitions.

Additionally, the isolates also significantly decreased the rate of growth of the fungal pathogens when compared with the negative control. However, after 10 days of incubation, the diameter of the inhibition zones reduced greatly. This can be attributed to weakening of the antimicrobial agents after the stationary phase of growth due to accumulation of toxins and depletion of nutrients. Results of the present study were in agreement with those reported by Someya *et al*. (2000), whereby *S. marcescens* strain B2 suppressed two fungal diseases of cyclamen plants

mainly the Fusarium wilt caused by *Fusarium oxysporum* f. sp. *cyclaminis* and the damping-off disease caused by *Rhizoctonia solani* under greenhouse conditions. This highly chitinolytic bacterium has also been reported to effectively control *Botrytis fabae* the causative agent of broad bean chocolate spot (Iyozumi *et al*., 1993; Okamoto *et al*., 1998), *Botrytis* spp. (Akutsu *et al*., 1993) and *B. cinerea* which causes cyclamen grey mold (Iyozumi *et al*., 1996). Similar results reported by Hazarika *et al*. (2020) showed that *S. marcescens* D1 could invade and spread over the culture of *F. oxysporum* and resulted in mycelial death. This mechanism was facilitated by the red-pigmented prodigiosin which increases the permeability of fungal cell membranes (Hazarika *et al*., 2020). Similar results proving the antifungal activity of *Serratia marcescens* were reported by Strobel *et al*. (1999) when they extracted oocydin A from this bacterium which later showed efficacy against various phytopathogenic fungal species including as *Phytophthora parasitica, Phytophthora cinnamomi, Phytophthora citrophora* and *Pythium ultimum*. They further concluded that Oocydin A could be used in crop protection as an antifungal and antimycotic agent in agriculture (Strobel *et al*., 1999).

The 100% suspension of test bacteria (*Klebsiella oxytoca* strain 93A, *Serratia marcescens* strain 93B, *Serratia marcescens* strain 93C and *Citrobacter freundii* strain 93D) showed larger inhibition zones across all the test fungal pathogens compared to the half-strength (50%) suspension. This is because the concentration of antimicrobial compounds in the 50% concentration was lower (due to dilution with water) than the 100% concentration which was undiluted. Conversely, the cell-free bacterial suspensions showed no significant differences in their activity against the fungal pathogens indicating that all the bacterial suspensions did not vary. Only the interaction between treatments (bacterial cell-free suspensions) and fungal species showed significant differences but the interactions between fungi and concentration and those between treatment and concentration were not significantly different. Likewise, the interactions among treatments, fungi and concentration were not significant indicating that all the bacterial isolates resulted in similar antifungal activity. Generally, all the treatments (cell-free bacterial suspensions) were as effective as the positive control (Apron Star®) which is a broad-spectrum antifungal agent. This supports the findings that *Serratia* species secrete a wealth of volatile organic compounds which influence the growth of phytopathogenic fungi and bacteria (Kai *et al*., 2009; 2010). *Serratia marcescens* is known to be one of the most efficient chitin-degrading bacteria in the environment (Vaaje-Kolstad *et al*., 2013). This study concluded that *Serratia marcescens* strains 93B and 93C and the other nematode-associated bacteria (*K. oxytoca* strain 93A and *C. freundii* strain 93D) were capable of inhibiting the

growth of the tested fungal plant pathogens. This creates the need to further extract and purify each of these antifungal or antimycotic biomolecules for possible application as biological control agents in agriculture. The current study sets a base for the formulation of endosymbiotic bacteria such as *Serratia marcescens* for greenhouse/field testing to demonstrate their potential use as biological control agents.

This is the first study reported in Kenya documenting the isolation and antimicrobial activity of entomopathogenic *S. marcescens* strain 93B, *S. marcescens* strain 93C, *K. oxytoca* strain 93A and *C. freundii* strain 93D on fungal phytopathogens and human bacterial pathogens. The study, therefore, lays down the significant groundwork for a more comprehensive study on the potential applications of new EPN associated bacteria such as *S. marcescens* in the development of novel, eco-friendly and affordable biopesticides for the management of economically important pathogens.

6.0. CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

1. The current study isolated four nematode enteric bacteria that were morphologically identified based on 16S r RNA gene sequences, namely; *Klebsiella oxytoca* strain 93A, *Serratia marcescens* strain 93B, *Serratia marcescens* strain 93C and *Citrobacter freundii* strain 93D.
2. The findings obtained from this study showed that the isolated entomopathogenic bacteria (*Serratia marcescens* strain 93B, *Serratia marcescens* strain 93C, *Citrobacter freundii* strain 93D and *Klebsiella oxytoca* strain 93A) were effective in controlling both the test bacterial human pathogens as well as the plant pathogenic fungal species. All the nematode-associated bacterial species showed high efficacy against all the tested human test pathogens and the fungal species except for *Fusarium verticillioides* which was resistant and only showed mild inhibition when treated with *Serratia marcescens* strain 93C.
3. In general, this study exhibited promising antimicrobial activities of *Serratia marcescens* strains as well as the other associated endosymbiotic bacteria (*Klebsiella oxytoca* strain 93A and *Citrobacter freundii* strain 93D) against the test phytopathogens and bacteria. All the bacterial test pathogens were sensitive towards the isolated bacterial species.

6.2 Recommendations

Based on the findings of this study, the following recommendations are made:

i. *Serratia marcescens* antimicrobials should be formulated and used to control antibiotic resistant human bacterial pathogens as well as plant pathogenic fungal pathogens.
ii. The antibiotic and antifungal efficacy of *S. marcescens* strains were observed *in vitro*. Similar experiments should be conducted under greenhouse and field conditions.
iii. There is need for more research on the mechanism of action and secretion of antimicrobials by symbiotic *S. marcescens* to fully understand this new genus.
iv. Research on the enterobacteria *Serratia marcescens* 93B and *Serratia marcescens* strain 93C by use of other molecular markers such as Restriction fragment length polymorphism (RFLP) is required to understand and describe their phylogeny.
v. There is need for further investigation and bioassay-guided isolation and characterization of pure active compounds from *S. marcescens* strain 93B and *Serratia marcescens* strain 93C antimicrobial compounds that confer the observed antimicrobial efficacy.

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