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Antibacterial activity and susceptibility testing of bacterial isolates from nematodes (*Cruznema* spp.)

By

Maletjema Magdeline Mothapo

(859667)

Dissertation

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Supervisor: Dr. Tiisetso E. Lephoto

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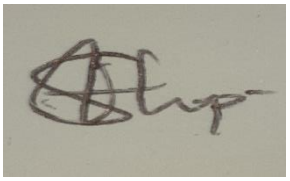
Abstract

Nematodes are unsegmented worms found in different niches associated with a diverse range of bacteria. Various types of nematodes exist including those that are parasitic to insects, known as entomopathogenic nematodes (EPNs). EPNS of genera *Steinernema, Heterorhabditis* and *Oscheuis* are symbiotically associated with *Xenorhabdus*, *Photorhabdus* and *Serratia,* respectively. The symbiotic bacteria of EPNs have been reported to produce a broad spectrum of antimicrobial compounds active against human pathogens. The aim of this study was to isolate and identify nematodes and their associated bacteria from soil samples collected from a vegetative farm in Lesotho and study their antimicrobial activity against four species of pathogenic bacteria (*E. coli, S. aureus, E. faecalis and P. aeruginosa*). An uncharacterized species of *Cruznema* was isolated and named *Cruznema* NTM-2021 (GenBank 18S rDNA accession number: OQ408141). Based on the BLASTN search incorporating the phylogenetic analysis of the 16S rDNA region, three genera of bacteria were identified as *Alcaligenes* sp.*, Enterobacter* sp. and *Elizabethkingia* sp. The study revealed that all three bacterial isolates were pathogenic to *Tenebrio molitor*. Symbiosis tests, using lipid agar method demonstrated the ability of the host nematodes to develop and reproduce in the presence of their associated bacteria. Bacterial supernatants of *Alcaligenes sp.* and *Enterobacter sp.* showed some inhibitory activity against *Escherichia coli* and *Enterococcus faecalis*, by disk diffusion method. *Staphylococcus aureus* and *Pseudomonas aeruginosa* were the most resistant bacteria to supernatants of the three isolates. This study also showed that the *Alcaligenes, Enterobacter*, and *Elizabethkingia s*pecies isolated from *Cruznema* NTM-2021 were resistant to ampicillin, amoxicillin, cefuroxime/sodium, vancomycin and cephalothin but susceptible to gentamicin.

Declaration

I, Maletjema Magdeline Mothapo (859667), declare that this dissertation is my own, unaided work. It is submitted in fulfilment of the Master of Science degree in Microbiology and Biotechnology at the University of the Witwatersrand, Johannesburg. The dissertation has not been submitted for any other degree at the university.

Maletjema Magdeline Mothapo

Signature: 

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Above all, I give thanks to God.

Dedication

I dedicate this dissertation to my beloved daughter, Zenande Mothapo. I express my deepest love and profound gratitude to her, as she has been a constant source of joy and inspiration in my life. Furthermore, I extend my heartfelt appreciation to my esteemed mother, Betty Mothapo, whose unwavering belief in me, unwavering support, and boundless love have propelled me forward.

I would also like to express my sincerest gratitude to my partner, Thabiso Mbali, whose presence and support have been instrumental in my journey. Their support and guidance have given me the strength to overcome challenges and pursue my aspirations.

To my cherished siblings, Jacky, Joyce, Lina, and Matome Mothapo, I offer my deepest thanks for their support throughout this endeavor. Their encouragement and presence have been invaluable to me, and I am grateful for their belief in my abilities.

In conclusion, I am immensely grateful to all the individuals who have played a significant role in my life and supported me throughout the process of completing this dissertation. Their love, support, and encouragement have been essential to my success, and I extend my heartfelt appreciation to each and every one of them.

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Conferences

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- **Poster Presentation title**: Investigating the antimicrobial activity of insect pathogenic bacteria associated with entomopathogenic nematodes.

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Chapter 1: Literature Review

Introduction

Nematodes are microscopic, non-segmented roundworms that belong to the phylum Nematoda (Hodda, 2022). They are abundant, diverse and found in habitats such as the soil, freshwater, and marine habitats (Koppenhofer, 2000; Vashisth et al., 2013a).They are found in various geographical zones and are usually affected by change in climatic conditions (Bautista-Garfias et al., 2022). Nematodes are either free living, predaceous, or parasitic (Hodda, 2022). Free- living nematodes are considered as either bacterivores, fungivores or omnivores depending on what they feed on (Hodda 2022; Taylor 2019). This group of nematodes usually live in the soil but are dependent on their host for development and reproduction (Neher & Powers, 2004). *Caenorhabditis elegans* is an example of a free-living nematode that feeds on bacteria (Rae et al., 2010). Another example of free-living parasitic nematodes that feed on bacteria are *Cruznema* nematodes and are also known to affect cricket and mollusk/snail (d'Ovidio et al., 2019; Du et al., 2022). Parasitic nematodes infect several types of organisms including plants, insects, animals, and humans (Caron et al., 2020; Coyne et al., 2018; Jasmer et al., 2003; Taylor et al., 2010).

Nematodes play a crucial role in nutrient recycling, decomposition of organisms, and as bio-indicators of soil health and regulators of plants productivity (Gebremikael et al., 2016; Neher & Powers, 2004; Wasmuth et al., 2008). Furthermore, other beneficial species of nematodes linked with insects or arthropods are used as biological control agents of pest insects (Grewal, 1990). Previous studies have reported the use of nematodes or nematode secretions as therapy for autoimmune diseases in humans (Harnett et al., 2010; Karabowicz et al., 2022; Smallwood et al., 2017). Additionally, (Rae et al., 2008) reported the use of *Phasmarhabditis hermaphrodita* nematodes as potential biological control agent of mollusc pests. The association between nematodes and insects or arthropods ranges from phoresy, necromeny to parasitism (Dillman et al., 2012). Necromeny is an association where nematodes use dead insects as a source of food but do not take part in the insect death (Sudhuas, 2008). Phoresy is a relationship where nematodes use insects to move to new habitat or locate their hosts (Kiontke & Sudhaus, 2006). Parasitism is

a relationship where nematodes are modified to use living insects as food source, while harming the insect host (Dillman et al., 2012).

It was previously suggested that *Cruznema* nematodes are necrominic and feed solely on bacteria or bacteria found in dead insects, however, recent studies have shown that these nematodes can infect live insects (Doucet, 1994; Tahseen et al., 2012).The Mitogenome of *Cruznema tripartitum* has revealed that the nematode shares the same gene order with *Ocheius chongmingensis, Caenorhabditis* spp. and others (Du et al., 2022). *O. chongmingensis* has been acknowledged as a potential biological control agent (Fu & Liu, 2019). Given that *Cruznema* nematodes infect and kill insects, the one question that needs to be asked, however, is whether *Cruznema* nematodes can be classified as entomopathogenic nematodes. Data from several studies has identified entomopathogenic nematodes as a biological control agent against numerous pests (Aalaoui et al., 2022; Hazir et al., 2004; Loulou et al., 2022; Lulamba, Tshikala et al., 2019; Mahmoud, 2017; Vinay et al., 2014). However, little has been done on the use of *Cruznema spp*. as biological control agents. Figure 1 shows a microscopic image of one of the most commonly isolated species of *Cruznema.*

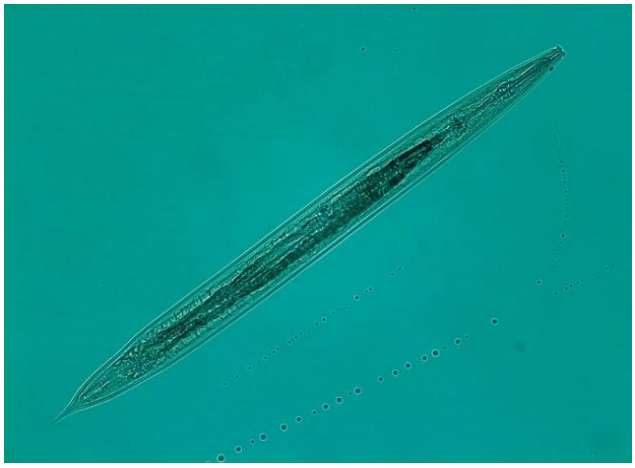

Figure 1 A representation of *Cruznema tripartium* found in soil samples: [adapted from https://www.wur.nl/en/Research-Results/Chair-groups/Plant-Sciences/Laboratory-of-Nematology/Nematode-in-the-picture/Nematode-Pictures/Cruznema-tripartitum.htm]

Entomopathogenic nematodes (EPNs)

Entomopathogenic nematodes (EPNs) are free-living obligate parasites of insects found in different types of habitats and abundant mostly in moist and clumped places (Acharya et al., 2020; Belien, 2018; Shapiro-ilan et al., 2018). EPNs have an infective juvenile stage that is free-living, non-feeding, and non-developing and is responsible for parasitizing insects (Koppenhöfer et al., 2020b; Platt et al., 2020). Due to infective juveniles' (IJs) ability to survive without a host, they have been mass cultivated, formulated, and used as biological control agents against insect pests (Devi, 2018; Manochaya et al., 2022; Platt et al., 2020). Furthermore, they have been considered an alternative to chemical pesticides due to their ability to cause infection in various economically important pests, their safety towards non-target organisms, and the environment (Koppenhöfer et al., 2020a; Kumar, Jaiswal et al., 2022; Nxitywa, 2021). EPNs are mutually associated with bacteria that aid in their pathogenicity to insects (Vashisth et al., 2013b). *Steinernema, Heterorhabditis, and Oscheius* are mutually associated with bacteria of the genus *Xenorhabdus, Photorhabdus,* and *Serratia* respectively (Kagimu et al., 2017; Vashisth et al., 2013a; Zhou et al., 2017). Not all species of *Oscheius* are mutually associated with *Serratia*, some are associated with *Bacillus* and/or *Alcaligenes faecalis* (Shan et al., 2019; Ye et al., 2010). The symbiotic bacteria are transmitted by the IJs into the hemocoel of the insects, where they replicate, produce secondary metabolites, and cause septicaemia, which causes the death of the insect (Ciche et al., 2006; Yooyangket et al., 2018). The symbiotic bacteria produce antibiotics and other toxins to assist the nematode in overpowering the defence mechanism of the insect hosts (Brivio & Mastore, 2018). *Xenorhabdus* is found in the vesicles of its host nematode (Fig. 2), while *Photorhabdus* lives in the gut of its nematode (Stock & Blair, 2008). *Oscheius* sp. are reported to live in association with several bacteria species (Park et al., 2011; Shan et al., 2019). The bacteria provide the nematodes with nutrition and also produce antibiotics (Cardoso et al., 2015; Ngugi et al., 2021). The bacteria produce antibiotics and other toxins to assist the nematode in overcoming the humoral and cellular defenses of the insect hosts (Ciche et al., 2006; Stock & Blair, 2008). Some of the toxins produced by *Photorhabdus luminiscens* are toxin complexes, the *Photorhabdus* insect related (Pir) proteins, the "makes caterpillars floppy" (Mcf) toxins and the *Photorhabdus* virulence cassettes (PVC) (Rodou et al., 2010). *Xenorhabdus* produces toxins such as Txp40 toxin, which plays a role in causing damage to insect hosts (Brown et al., 2006).

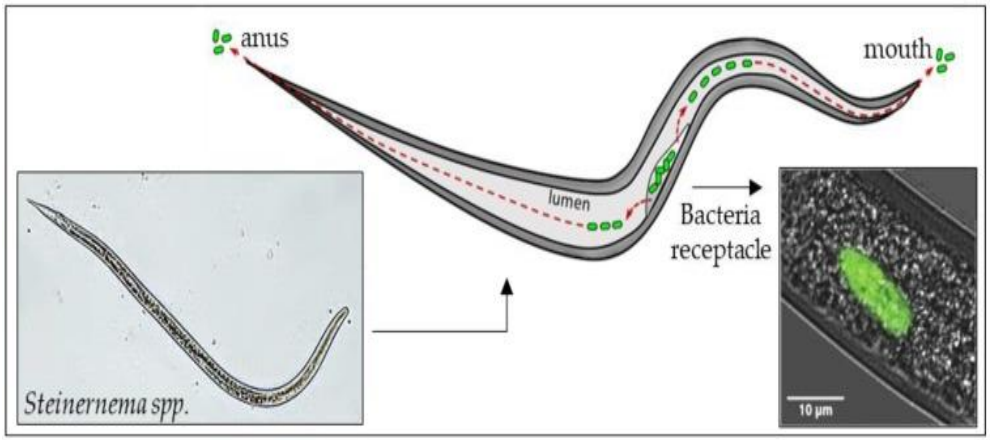

Figure 2: An image of *S. Carpocapsae* and its mutualistic bacteria *Xenorhabdus* sp. (camouflaged in green) (Brivio & Mastore, 2018).

Mechanism of Infection

Upon entering the host insect, the IJs secrete compounds and release their symbiotic bacteria in the hemolyph of the insect, thus activating the innate immunity of the insect, which induces antibacterial activity (Brivio & Mastore, 2018). To counter the action of the host immunity, the symbiotic bacteria prevent the upregulation of immune genes, thereby causing immune suppression (Eleftherianos, Shokal, & Yadav, 2016). Additionally, the symbiotic bacteria cause immune suppression through the inhibition of Phenoloxidase (PO), an important part of the immune system of insects found in the hemolymph plasma. This system is activated by a protease cascade following an invasion of foreign microbes that leads to the production of melanoic nodules surrounding the microbes (González-Santoyo & Córdoba-Aguilar, 2012). A study carried out by Eleftherianos et al., (2007), has shown that *Photorhabdus* sp. synthesizes an antibiotic molecule (E)-1,3-dihydroxy-2-(isopropyl)-5-(2-phenylethenyl) benzene (ST) that also inhibits phenoloxidase (PO) in the insect host. Furthermore, (Eliáš et al., 2020) discovered that *Heterorhabditis bacteriophora* produces bioactive compounds that inhibit phenoloxidase. Figure 3 shows how the toxins produced by both *Photorbadus* and *Xenorhabdus* inhibit phenoloxidase upon invasion of the insect host.

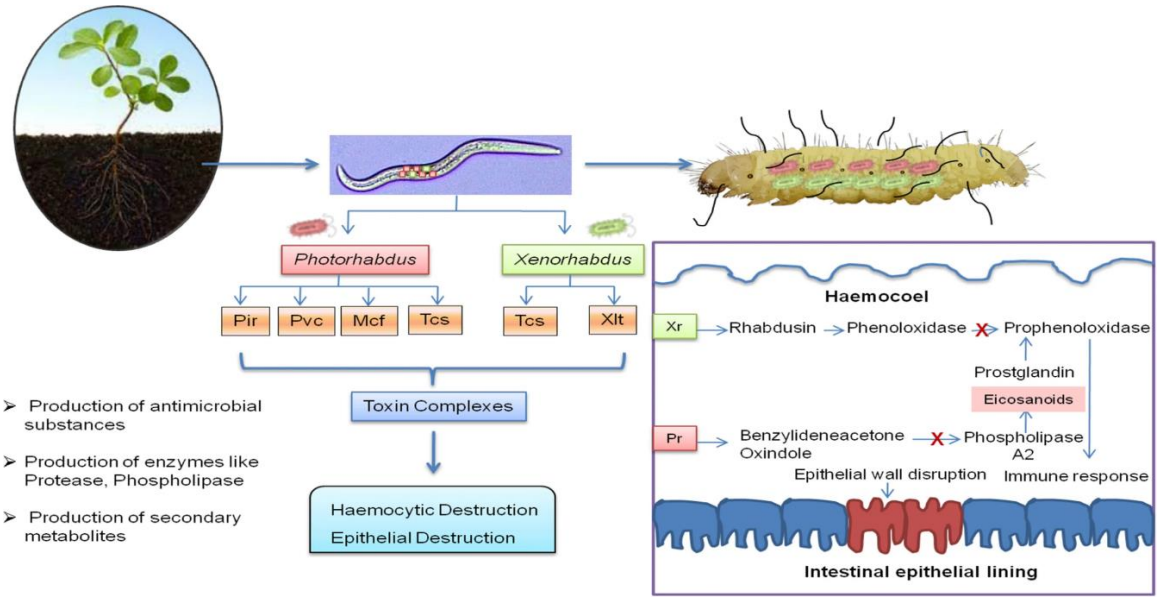

Figure 3 An image showing toxins of symbiotic bacteria of EPNs inhibiting the phenoloxidase activity (Tomar et al., 2022).

Signaling pathways such as Toll, Janus kinase (JAK)/signal transducers and activators of transcription (STAT) - JAK-STAT, Immune deficiency (IMD) and small RNA interference (RNAi) also play a significant role in the defense of insects against pathogenic microorganisms (Gregorio et al., 2002; He et al., 2021; Zeng et al., 2022). The JAK/STAT is activated by the unpaired (Upd) ligand, the receptor Domeless (Dome), the JAK tyrosine kinase Hopscotch (Hop), and the STAT (Stat92E) (Bang, 2019; Myllymäki et al., 2014). This pathway contributes to insects' gut immunity by expressing antimicrobial peptides following cell damage induced by pathogens (Buchon et al., 2009). The Imd signaling pathway is activated by the invasion of gram-negative bacteria, where antibacterial peptides such as diptericin are expressed (Agaisse & Perrimon, 2004). Several techniques, such as membrane rupture, disruption of bacterial metabolism, and targeting of cytoplasmic components, are used by antibacterial peptides to kill bacteria (Wu et al., 2018). During *Xenorabdus nematophila* infection of *Drosophila melanogaster* flies, the Imd pathway is activated, allowing the fly to survive (Yadav et al., 2018).

Biological control agents

Biological control is the reduction of a population of pathogens through the use of living organisms (Köhl et al., 2019). Biological control agents are used as an alternative to chemical pesticides, which pose a threat to the environment and other non-target organisms such as animals and humans

(Lushchak et al., 2018; Pirttilä et al., 2021)**.** Biological control agents are eco-friendly and safe for non-target organisms (Gozel & Cigdem, 2016).

Microbial biocontrol agents

Microorganisms used as biopesticides include bacteria, algae fungi, viruses, nematodes and protozoa (Dar et al., 2021). Microbial biocontrol agents work either by interacting with pathogens directly or indirectly, where they synthesize and release antimicrobial compounds, and interfere with the quorum sensing (QS) system of pathogens (Bonaterra et al., 2022). An example of a microbial biopesticide studied extensively is *Bacillus thurigiensis* (Bt), which is a gram-positive bacterium that produces *Cry* and *Cyt* proteins effective in killing insect pests (Bravo et al., 2011; Nair et al., 2018). Furthermore, it has been suggested that *Pseudomonas spp*. produce bioactive metabolites, such as antibiotics, cyclic peptides, and enzymes, which play an important role in biocontrol (Chin-A-Woeng et al., 2003). *Wolbachia*, an endosymbiont of onchocercid/filarial nematodes, can be utilized as a biocontrol agent to successfully control vector-borne infections including the malaria parasite *Plasmodium gallinaceum* as well as viral pathogens like the dengue, yellow fever, and chikungunya viruses (Manoj et al., 2021). Other bacteria used as microbial biopesticide include *Bacillus sp., Burkholderia sp., and Trichoderma sp.* (Ahmad et al., 2008; Lahlali et al., 2022). These species are used to treat plant pathogen infections caused by fungi (Palmieri et al., 2022). In addition to protecting crops against pathogenic diseases, some of these microorganisms promote plant growth (Sehrawat & Sindhu, 2019). *Beauveria bassiana*, is an example of fungi used as biocontrol agent against various crops (Mantzoukas & Eliopoulos, 2020). Viruses from the family *Baculoviridae* are commonly used as viral biopesticides (Haase et al., 2015). In addition, *Photorhabdus luminescens subsp. akhurstii* associated *Heterorhabditis sp*. has a potential to be used as a biological control agent of mosquitoes (Yooyangket et al., 2018).

Entomopathogenic nematodes as biological control agent

Entomopathogenic nematodes have successfully been used to control various economically important insects such as codling moth, black vine weevil, cutworms, leaf miners, (Lacey & Georgis, 2012; Platt et al., 2019; Steyn et al., 2020). There are approximately 17 species of *Hererhabditis* and 100 species of *Steinenermatiditis* that are reported to be effective in controlling insect pests (Tomar et al., 2022)**.** EPNs are used as biological control agent as they have benefits such as the ability to move, locate and kill their target host, easy mass culturing, high reproductive

potential, safety to non-target organisms and wide host range (Abd-Elgawad, 2019). **T**hey are reported to work effectively when integrated with other control methods (Hussaini, 2014**).** EPNs such as *S. carpocapsae, S. feltiae, S. glaseri, S. riobrave*, H. *bacteriophora* and *H. megidis* are successfully used to control some pests (Abate et al., 2017). Locally produced EPNs were able to control the Cape grapevine leafminer, *H. capensis*, in South Africa (Steyn et al., 2019). Additionally, species of *Steinenerma*, *S. carpocapsae* and *S. feltiae*, have been used as biological control agents against codling moth in orchards and for treatment of fruit bins (Malan et al., 2006). EPNs in the genus *Oscheui*s have also shown the potential to be used as biological control agents against several pests (Ghavamabad et al., 2021; Loulou et al., 2022; Torrini et al., 2015). A study by Lephoto & Gray (2019) revealed a new species named *Oscheius basothovii n. sp*., that was able to infect both *Galleria mellonela* and *Tenebrio Molitor* within 48h. Symbiotic bacteria of these EPNs secrete several metabolites, including lipase(s), phospholipase(s), protease(s), and several different broad-spectrum antibiotics as they enter stationary phase of their growth cycle (Forst & Nealson, 1996). Metabolites derived from symbiotic bacteria of nematodes (EPNs) have nematicidal activity, antibacterial activity, insecticidal and antimycotic effects (Glare et al., 2012; Stock et al., 2017).

Secondary metabolites produced by symbiotic bacteria of nematodes.

Secondary metabolites are small molecules produced by microorganisms that are not involved in the primary metabolic processes of the microorganisms (O'Brien & Wright, 2011; Singh et al., 2019). Secondary metabolites include antiparasitic agents, antibiotics, pigments, growth hormones, antitumor agents, enzyme inhibitors, and others, and these are synthesized during the stationary phase growth of microorganisms (Singh et al., 2017). They are produced by a wide range of organisms, including bacteria, fungi, and plants (Craney et al., 2013; Guerriero et al., 2018; Nawrot-chorabik & Sułkowska, 2022). Symbiotic bacteria of nematodes are known to produce a wide spectrum of secondary metabolites (Anju et al., 2015; Murfin et al., 2013; Stock et al., 2017). Some species of *Photorhabdus* and *Xenorhabdu*s were known to produce one type of antibiotic or metabolite. However, Li et al. (1995) described a strain of *Xenorhabdus sp,* Q1, that produced two types of metabolites, namely, xenorhabdins and xenocoumacins. Similarly, a

study by Dreyer et al., (2018) demonstrated that strains from the same species may produce active compounds that differ and inhibit dissimilar insects or bacteria. In addition, *Serratia* species produce a wide spectrum of bioactive compounds and have the potential to be used as a biological control agent (Soenens et al. 2019).

Secondary metabolites as biological control agent

Secondary metabolites derived from symbiotic bacteria or nematodes have been shown in numerous studies to have the potential to be used as biological control agents of plant pathogens and/or insects (Kumar et al., 2014; Stock et al., 2017). Kgosiemang (2022) extracted and tested secondary metabolites from *S. beitlechemi* SGI 197 for antifungal activity against *Fusarium graminearum*, an agent that causes Fusarium head blight in small grains, and discovered that the extracts were able to inhibit 92% of the fungi. Moreover, nematophin, with structure 3-indoleethyl (3'-methyl-2'-0xo) pentanamide isolated from strain BC1 of *X.nematophilus* exhibited bioactivity against a series of fungal and bacterial species (Abate et al., 2018; Ji et al., 2004). Furthermore, *X.nemathophilus* was able to kill *Manduca sexta* with only five bacterial cells injected into the hemocoel of the larvae (Forst & Nealson, 1996). *Wolbachia* produces antimicrobial peptides that have the potential to inhibit plasmodium development (Iturbe-ormaetxe et al., 2011). According to Vicente-Díez et al. (2021), *Steinernematid* species and bioactive compounds produced by their symbiotic bacteria could be used as a new agro-technological approach in controlling *Lobesia botrana* in vineyards. Moreover, secondary metabolites from *P. luminscences* have been reported to kill *Aedes aegypti* larvae (Da Silva et al., 2020; Ofori et al., 2020).

(Yip et al., 2021) proposed that *S. marcescens* produces prodigiosin, a heterocyclic bacterial secondary metabolite that belongs to the class of tripyrrole compounds produced by various types of bacteria in order to outcompete other bacteria and survive. Moreover, prodiogosin has the ability to degrade nucleic acid in biofilm-forming species such as *P. aeruginosa* (Kimyon et al., 2016). Additionally, a study conducted by (Heu et al., 2021) demonstrated that *S. marcescen*s Vectopole Amazonien inhibits the growth of several mosquito microbiota isolates using prodigiosin and other secondary metabolites. Other studies have also demonstrated that prodigiosin has shown efficacy against various pathogenic bacteria (Gohil et al., 2020; Soenens Amalia and Imperial Juan, 2019; Yip et al., 2021). Secondary metabolites derived from symbiotic bacteria of EPNs are not only active against insect or plant pathogens, but they are also active against several human pathogens, including drug - resistant bacteria such as *Methicillin-resistant Staphylococcus aureus*, *Bacillus*

subtilis, *E. coli* and others (Anju et al., 2015; Awori et al., 2017; Donmez Ozkan et al., 2019; Muangpat et al., 2020).

Antibacterial activity of secondary metabolites derived from symbiotic bacteria of nematodes

Numerous studies have demonstrated that nematode symbiotic bacteria are a novel source of antibiotics active against pathogens, including multidrug-resistant pathogens (De Mandal et al., 2021; Dreyer et al., 2018; Fodor et al., 2022). Considering the declining effectiveness of antibiotics against drug-resistant bacteria across the globe, novel sources of antibiotics need to be tapped into, and secondary metabolites from symbiotic bacteria of nematodes may be a promising alternative approach. Secondary metabolites produced by nematode endosymbionts can be used to inhibit the causative agents of a variety of diseases (Muangpat et al., 2020; Yimthin et al., 2021). According to (Clements et al., 2019), *S.marcensens* strains produce serrawettin, prodigiosin, and glucosamine derivatives that display broad-spectrum antimicrobial activity against pathogenic bacteria such as multidrug-resistant *Pseudomonas aeruginosa, methicillin-resistant Staphylococcus aureus,* and *Cryptococcus neoformans*.

X. nematophila, a gram negative symbiotic bacteria of *Steinernema,* has been reported to produce several compounds with antimicrobial activity, including indole derivatives, nematophin, benzylideneacetone, xenocoumacins and the major class of non-ribosomally produced secondary metabolites (Xenematides depsipeptides, lysine-rich cyclo PAX-peptides) (Gualtieri et al., 2009; Guo et al., 2017). PAX 3, a lysine-rich cyclolipopetide compound fermented by *Xenorhabdus nematophila* F1 strain is active against plants and human fungal pathogens, bacteria and yeast (Gualtieri et al., 2009). Additionally, *X.nematophilus* symbiotically associated with *S.carpocapsea* exhibited antibacterial effects on *E. coli, Ralstonia solanacearum* and *Pseudomonas aeruginosa,* however, it was unable to inhibit Pseudomonas putida and Pseudomonas fluorescens (Park et al., 1999).

Xenocoumacins 1 and 2 were first isolated and identified by McInerney et al. (1991) from *Xenorhabdus* species. These two metabolites are effective in inhibiting bacteria, fungi, and ulcers. Their chemical structure is shown in figure 4. Xenamoucacin and xenorhabdin belong to the same class of compounds with their structure and pharmacological activity like that of AI-

77/amicoumacin compounds (McInerney et al., 1991). They have a similar spectrum of antibacterial activity, and they may have the same inhibitory effects as amicoumacin, which is known to be a promising antibiotic (Maksimova et al., 2021; McInerney et al., 1991). Thaler et al. (1995) also carried out a study on *Xenorhabdus spp.* and isolated Xenorhabdicin from *X.nematophila*. The compounds demonstrated inhibitory effect against *Xenorhabdus spp*., *P.luminescens*, and *Proteus sp*. (Aiswarya et al., 2017) also suggested that Xenorhandicin is active against other *Xenorhabdus* species.

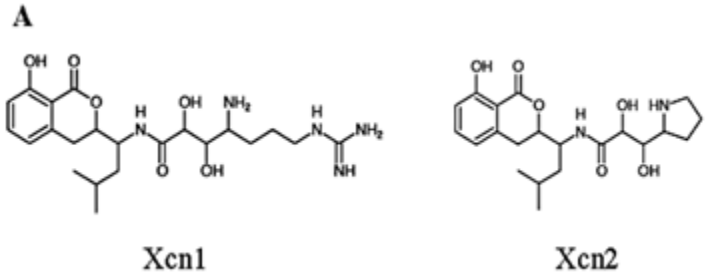

Figure 4: Chemical structure of xenocoumacin 1 and 2 extracted from a symbiotic bacteria associated entomopathogenic nematodes. (adapted from (McInerney et al., 1991).

(Paul et al., 1981) isolated and identified hydroxyl- and acetoxyl-bearing indole derivatives and two antibiotics, 4-ethyl- and 4-isopropyl-3, 5-dihydroxy-trans-stilbenes from two different strains of *Xenorhabdus spp*. *X.nematophilus* contained defective phages that may be responsible for lysis of *Bacillus cereus*. Additionally, a study conducted by (Lang et al., 2008) identified three new peptides, xenortide A, xenortide B, and xenematide, as well as the known compounds xenocoumacin II and nematophin. *Xenorhabdus* spp. isolated in Kenya demonstrated efficacy against Methicillin-resistant *staphylococcus aureus* – MRSA (Awori et al., 2016). These findings support the notion that *Xenorhabdus spp*. produce a broad-spectrum of compounds that inhibit a variety of pathogens including antibiotic resistant bacteria such as MRSA.

The most studied species of *Photorhabdus, P. luminescens,* can inhibit *Bacillus cereus* and *Bacillus subtilis* (Stock et al., 2017). In addition, some studies have also shown that *P.luminescens* strains inhibit plant pathogens such as *Erwinia carotovora* (Derzelle et al., 2002; Stock & Blair, 2008). Furthermore, *P. luminescens* has been reported to produce isopropylstilbene. This compound has shown activity against *E. coli, B. subtilis, S.pyogenes,* and *S.aureus* (Yimthin et al., 2021). Moreover, previous studies demonstrated that crude extracts from *P. temperata* have

antimicrobial activity against drug-resistant bacteria (Kang et al., 2004; Muangpat et al., 2017). However, *P. asymbiotica,* has emerged as a human pathogen (Gerrard et al., 2006). As much as some nematodes, such as *Steinenermatids* are known to interact with symbiotic bacteria deemed safe for non-target organisms, they are also known to carry bacteria that are potential pathogens for humans and animals.

Nematodes as vectors of human pathogens

Although symbiotic bacteria of nematodes have been reported to be harmless to other living organisms, several studies have revealed that some nematodes are a potential source of foodborne and waterborne diseases as most of them harbour bacteria that cause diseases in humans and animals (Anderson et al., 2003; Caldwell et al., 2003; Kenney et al., 2005). The pathogens are acquired from the environment via the infective juvenile of the nematodes which further transmit the pathogens to susceptible host (Lacharme-Lora et al., 2009).

It has been reported that *Heterorhabditis sp*. are associated *Photorhabdus asymbiotica*, which is the only species of the genus *Photorhabdus* that has emerged as a human pathogen (Costa et al., 2009; Mulley et al., 2015). Furthermore, a considerable literature has been published on *Heterorhabitis spp*. being associated with several other pathogens of humans and animals such as *Ochrobactrum spp., Pseudomonas aeruginosa, Alcaligenes faecalis* and *Stenotrophomonas maltophilia* (Ashwini et al., 2022; Razia et al., 2011; Shan et al., 2019). Moreover, another species of nematodes known to be a carrier of human pathogens such as *Salmonella*, *pseudomonas* and *E. coli* is *C. elegans* (Bichai et al., 2008; Caldwell et al., 2003).

According to studies, some of the bacteria isolated from nematodes are resistant to widely recommended antibiotics, while others are resistant to drugs from different classes (Horcajada et al., 2019; Huang, 2020; Nair et al., 2018). Antibiotic-resistant bacteria have become a major issue in public health, and this is as a result of bacteria becoming less susceptible to existing antibiotics (Friedman et al., 2016; Khan et al., 2019; Viens & Littmann, 2015). Bacteria develops resistance to antibiotics through natural recombination and integration into the genome of the bacteria or through horizontal gene transfer (Sultan et al., 2018). Only a few effective antibiotics have been developed against common antibiotic resistant bacteria (Cui et al., 2022; Terreni et al., 2021). This is the rationale behind the research for novel antibacterial compounds or sources with new mode of action.

Some of the bacteria that have developed resistance to multiple classes of drugs include nosocomial pathogens such as *Staphylococcus aureus*, *Actinobacter*, *Enterococcus spp. and Mycobacterium tuberculosis* (Dreyer et al., 2018; Pidot et al., 2014; Muangpant et al., 2020). The World Health Organization (WHO) forecasts that antibiotic resistance threatens the accomplishments made by modern drugs and this will lead to common infections and injuries causing death (World Health Organization, 2022). Additionally, the (World Health Organization, 2022) also noted a surge in drug resistance in developing and middle-income nations, which raises serious concerns about the development of resistance to common antibiotics. Resistance in bacteria is either as a result of overuse of antibiotics or lack of novel antibiotics (Ventola, 2015).

Mechanisms Of Action Of Antimicrobial Agents

Understanding how antimicrobial agents function is crucial for understanding the mechanisms of resistance. Antimicrobial drugs have limited or no influence on host functions while targeting key microbial processes with specificity. Different antimicrobial agents function in various ways. Understanding these pathways and the chemical makeup of the antimicrobial drugs is essential for comprehending how resistance to them arises. As shown in figure 5, antimicrobial agents target multiple sites of pathogen: 1. DNA replication, 2. protein synthesis, 3. metabolic pathway. 4. synthesis of the cell-wall/cell membrane (Hooper, 2001; Khameneh et al., 2019).

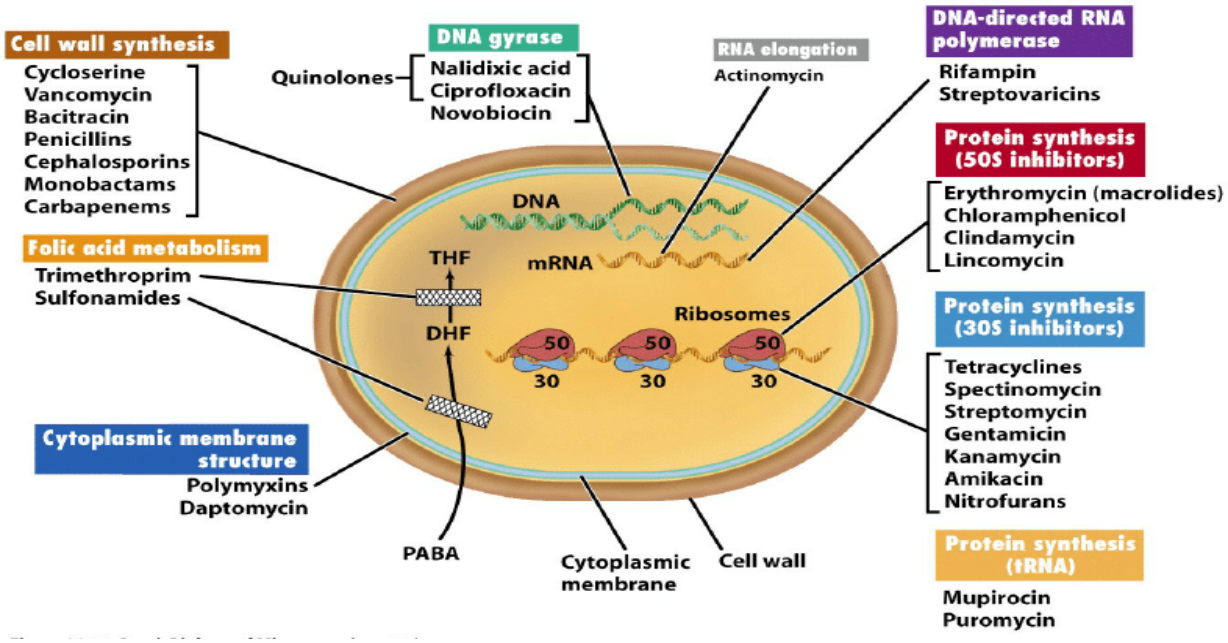

Figure 5 highlights different classes of antimicrobial agents and their modes of action on bacterial cell (Adapted from Bbosa et al., (2014)).

Cell wall synthesis

Bacterial cell wall synthesis has been perhaps the target area most extensively exploited for antimicrobial development. The components of the cell wall are appealing antimicrobial targets because of the absence of counterparts in eukaryotic cell (Mcdermott et al., 2003). The sequential nature of the steps involved in cell wall synthesis of bacteria, such as *Escherichia coli*, make it possible to determine the site in the pathway at which an antimicrobial function (Hooper, 2001). The most common cell wall synthesis inhibitors are β-lactams, which work by binding to penicillin-binding proteins (PBPs), enzymes involved in the final steps of peptidoglycan cross-linking in both Gram-negative and Gram-positive bacteria (Sarkar et al., 2017). β -lactams include narrow and broad-spectrum penicillin, cephalosporins, monobactams, and carbapenems (Bush & Bradford, 2016). It has been demonstrated that some classes of antibiotics such as β-lactams are used synergistically with β-lactamase to counteract resistance in bacteria that produce β-lactamase inhibitors (Van Hoek et al., 2011). Furthermore, Trojanowski et al., (2019) reported that β-lactams are combined with griselimycin or novobiocin to inhibit cell synthesis.

Protein synthesis

Protein synthesis inhibitors operate as transcription or translation inhibitors to stop the production of proteins. Numerous forms of antibiotics kill or hinder bacterial growth by stopping them from producing proteins by disrupting one or both processes (Uddin et al., 2021). Aminoglycosides inhibit synthesis of protein by interacting with the A-site on the 30S ribosome's 16S ribosomal RNA to prevent the production of new proteins (Krause et al., 2016). Additionally, resistance of aminoglycosides antibiotics also occurs through alteration of enzymes (Van Hoek et al., 2011). Examples of aminoglycosides include streptomycin, gentamicin, kanamycin, amikacin, neomycin, and paromomycin (Duijkeren et al., 2018).

DNA and RNA synthesis

Antibiotics target chromosome replication because the proteins that control bacterial DNA replication are different from those that control eukaryotic DNA replication. fluoroquinolones disrupt DNA synthesis and cause deadly double-strand DNA breaks during DNA replication (Trojanowski 2019). Antibiotics such quinolone and rifampin are reported to interfere with the synthesis of DNA and RNA (Kohanski et al., 2010).

Metabolic pathway

Antibiotics change the metabolic state of bacteria, which results in death (Stokes et al., 2019). The metabolic state of bacteria influences their susceptibility to antibiotics such as sulfonamides and trimethoprim (TMP) (Lobritz et al., 2015; Stokes et al., 2019). These antibiotics are known to impede the pathway for folic acid production, which limits synthesis of DNA (Feng et al., 2022). Additionally, sulfonamide and TMP are known to alter a microbial cell's energy metabolism (Lee et al., 2018; Mubeen et al., 2021; Van Hoek et al., 2011).

Mechanisms of resistance to antimicrobial agents

Several mechanisms have been highlighted to elucidate how antimicrobial resistance occurs (Arzanlou et al., 2017). Antimicrobial resistance can either be intrinsic, adaptive, or acquired. Intrinsic resistance refers to the ability of the bacteria to counteract antibiotics due to the bacteria's structure, such as efflux pumps (Langendonk et al., 2021). Although antibiotic resistant genes are naturally present in the bacteria, they are only expressed at levels that confer resistance after being exposed to an antibiotic (Reygaert, 2018). Other intrinsic mechanisms include alteration of the binding site, destruction of antibiotics, and synthesis of an alternate target protein to resist the effect of antibiotics (Jubair et al., 2021; Khameneh et al., 2019). As illustrated in Figure 6, it is commonly observed that intrinsic resistance, particularly in gram-negative bacteria, involves the utilization of outer cell membrane impermeability and active efflux pumps as mechanisms to resist antibiotics. These bacterial defense mechanisms contribute to the reduced effectiveness of antibiotics and present challenges in treating infections caused by such bacteria. (Cox & Wright, 2013). *P. aeruginosa*, which has low outer membrane permeability, efflux pumps, and resistant genes, is an example of a gram-negative bacteria that uses intrinsic mechanisms to resist antibiotics such as β-lactmas (Glen & Lamont, 2021). Furthermore, it is suggested that *Enterobacter sp*. uses the same mechanism to resist to β -lactams and carbapenems (Davin-Regli & Pagès, 2015). Adaptive resistance refers to the bacteria's ability to adapt and survive harsh conditions by constantly changing their transcriptome in response to environmental signals (Han et al., 2022). Acquired resistance refers to the ability of the bacteria to gain novel resistant genes through horizontal gene transfer (HGT) or mutations of specific chromosomal genes (Munita et al., 2016; Sandner-Miranda et al., 2018).

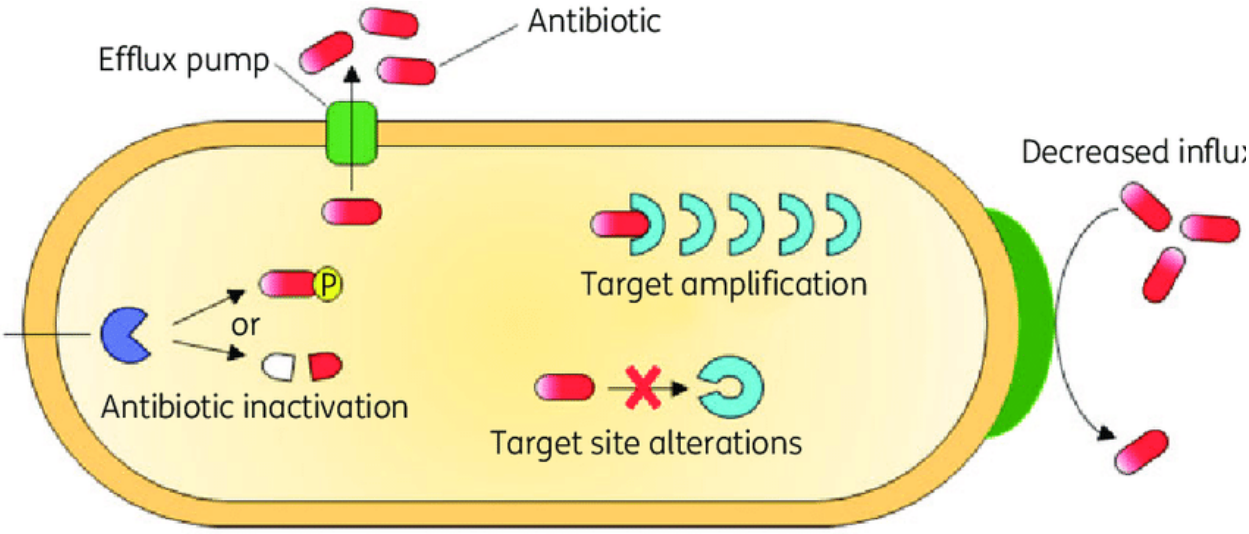

Figure 6 shows an image of a mechanism employed by bacteria to resist antimicrobial agents (Adapted from (Alav et al., 2018)).

Extensive literature has been published on EPNs and their symbiotic bacteria as potential biological control agents against economically important crops. However, not much has been published on other free-living nematodes such as *Cruznema* as potential biological control agents. Furthermore, little is known about their associated bacteria and their role in the survival, reproduction, pathogenesis, and development of *Cruznema* nematodes. Secondary metabolites produced from symbiotic bacteria of EPNs have shown antagonistic effects against pathogenic bacteria, including antibiotic-resistant bacteria, and they have been reported as a promising source of new antibiotics that may combat the issue of antibiotic resistance that poses a threat to public health. There is no literature on the secondary metabolites derived from bacteria associated with *Cruznema* and their ability to inhibit pathogenic bacteria. As a result, the goal of this study is to investigate the antimicrobial activity of bacteria associated with *Cruznema* NTM-2021 against *Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus,* and *Entercoccus faecalis*.

Summary

Nematodes were isolated from soil samples collected from a vegetative farm in Lesotho. Bacteria associated with the nematodes were isolated and identified using molecular techniques. The nematodes and their associated bacteria were pathogenic to *Tenebrio molitor larvae*. The disk diffusion method was used to test the potential use of the isolates' bacterial supernatants to inhibit human pathogens. The supernatants of the isolates showed a weak but promising potential

to inhibit two of the test pathogens, *Escherichia coli* and *Enterococcus faecalis*. The bacterial isolates were tested for their susceptibility to common drugs, and it was revealed that they were resistant to most antibiotics.

Research motivation

Antibiotic resistance is a persistent challenge in public health across the globe. Pathogenic bacteria are constantly evolving to resist commonly used antibiotics. The resistance of bacteria to antibiotics has led to researchers seeking different sources of antibiotics with novel modes of action. Bacteria associated with nematodes are a potential source of antibiotics that has not been explored enough. Bacteria associated with free-living parasitic nematodes, such as entomopathogenic nematodes, produce a broad spectrum of toxins and antimicrobial compounds. These antimicrobial compounds can inhibit some antibiotic-resistant bacteria that cause diseases in plants, animals, and humans. Antimicrobial compounds derived from bacteria linked to EPNs have been studied for their ability to inhibit antibiotic-resistant bacteria and other pathogens. However, inadequate literature exists on the potential use of bacteria associated with other free-living nematodes, such as *Cruznema* nematodes. As a result, the goal of this research was to determine the antimicrobial activity of bacteria associated with *Cruznema* nematodes against pathogenic bacteria.

Objectives

The objectives of this study were to:

1. Isolate nematodes from soil samples collected from a vegetative farm in the Leribe region of Lesotho.
2. Identify the nematodes based on molecular characterization of the 18S rDNA region by PCR amplification using universal primers and DNA sequencing.
3. Perform phylogenetic analysis.
4. Isolate the bacteria associated with the nematodes.
5. Phylogenetically analyse the bacterial isolates based on 16S rDNA sequences.
6. Test the pathogenicity of the bacterial isolates against *Tenebrio Molitor* larvae

7. Determine the antimicrobial activity of the bacterial isolates of *Cruznema* NTM-2021 against pathogenic bacteria using the Kirby-Bauer disk diffusion method.
8. Evaluate the susceptibility of the bacterial isolates to common antibiotics using the Kirby-Bauer disk diffusion method.

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Chapter 2: Isolation and identification of nematodes from a vegetable farm in Lesotho

Introduction

Nematodes are microscopic, non-segmented roundworms that belong to the phylum Nematoda (HODDA, 2022). They are abundant, diverse, and found in habitats such as the soil, freshwater, and marine habitats (Koppenhofer, 2000; Vashisth et al., 2013). They feed on bacteria, fungi, algae, yeasts, and other nematodes (Ingham, 1994). Nematodes play a crucial role in nutrient recycling, and they are also important indicators of soil health and quality (Wasmuth et al., 2008; Zhao & Neher, 2013). Additionally, some members of the *Mononchida* order are essential in the bioregulation of litter and soil ecosystems (Dipchikova et al., 2019).

Nematodes are either free-living, parasitic, or predatory (Kang et al., 2019). A few parasitic nematodes are known to cause substantial diseases in animals, plants, and humans (Bernard et al., 2017; Caron et al., 2020; Shah & Shahidullah, 2018). (Coyne et al., 2018) highlighted the impact of parasitic nematodes on crops, with emphasis on root knot nematodes (*Meloidogyne spp*.) being the major nematode pest across Africa. Additionally, (Pulavarty et al., 2021) elaborated on the effects plant parasitic nematodes have on economically important pests across the globe and their distribution.

Whereas parasitic nematodes have detrimental effects on plants and humans and animals, free-living parasitic nematodes have been commended for their helpful contribution to the rhizosphere and significance for agricultural productivity (Atandi et al., 2022; Divya & Sankar, 2009; Rae et al., 2008; Wilson, 2013). In the agricultural field, free-living parasitic nematodes such as entomopathogenic nematodes are used as biological control agents to combat or reduce the effects pests have on economically important crops (Belien, 2018; de Waal et al., 2018; James et al., 2018; le Vieux & Malan, 2013). Entomopathogenic nematodes are considered an alternative to chemical pesticides (Othman et al., 2015; Tomar et al., 2022). The most beneficial and well-studied entomopathogenic nematodes are from the families *Steinernematidae* and *Heterorhabditidae* (Dolinski et al., 2012; Lacey et al., 2015; Nthenga et al., 2014; Platt et al., 2020). *Oscheius* species have also been reported as entomopathogenic nematodes capable of infecting and killing various

pests (Foye & Steffan, 2020; Lephoto & Gray, 2019b; Loulou et al., 2022; Ye et al., 2010). *Steinernema* and *Heterorhabditis* have formed mutualistic relationship with bacteria *Xenorhabdus* and *Photorhabdus* respectively. *Oscheius* nematodes are symbiotically associated with *Serratia* (Serepa-Dlamini & Gray, 2018). The symbiotic bacteria are carried in the intestines of the infective juveniles (IJs). The infective juvenile of the EPNs finds a suitable host insect and enters by natural openings like the mouth, anus, or spiracles (Le Vieux & Malan, 2013). Previous studies have shown that symbiotic bacteria of EPNs, *Xenorhabdus*, *Photorhabdus* and *Serratia*, play a crucial role in the protection, development, pathogenesis of their respective nematode host (Abd-Elgawad, 2022; Sajnaga & Kazimierczak, 2020; Serepa-Dlamini & Gray, 2018).

Life cycle of entomopathogenic nematodes

In the first stage of the life cycle of the EPNs (Figure 7), the free-living infective juveniles (IJs) locate and infect insect host as depicted in figure 1. The IJs use chemical cues and signals to find their host (Zhang et al., 2021). During this stage, the IJs are resistant to extreme environments, and do not feed, develop nor reproduce (Shapiro-ilan et al., 2018). Upon penetrating a suitable insect host through its open circulatory system, the IJs release the bacteria by regurgitation into the insect's hemocoel (Mcmullen et al., 2017). Once inside the host, the bacteria multiply and creates a deadly septicaemia in the host by producing diverse toxins that effectively kill the insect within 24-48h (Abbas, 2020; Lu et al., 2017). In addition, the symbiotic bacteria produce compounds that result in cytotoxicity (Elbrense et al., 2021). The IJs feed, reproduce, develop, and complete their life cycle within the cadaver. Upon depletion of resources, a new generation of infective juveniles is produced, and these emerge from the cadaver in search of a susceptible host (Cardoso et al., 2015; Gulcu et al., 2017). *Steinenermatidae* and *Heterorhabditiae* have similar life cycle, nonetheless their bacterial symbionts are pathogenic to diverse types of insects (Chaston et al., 2011).

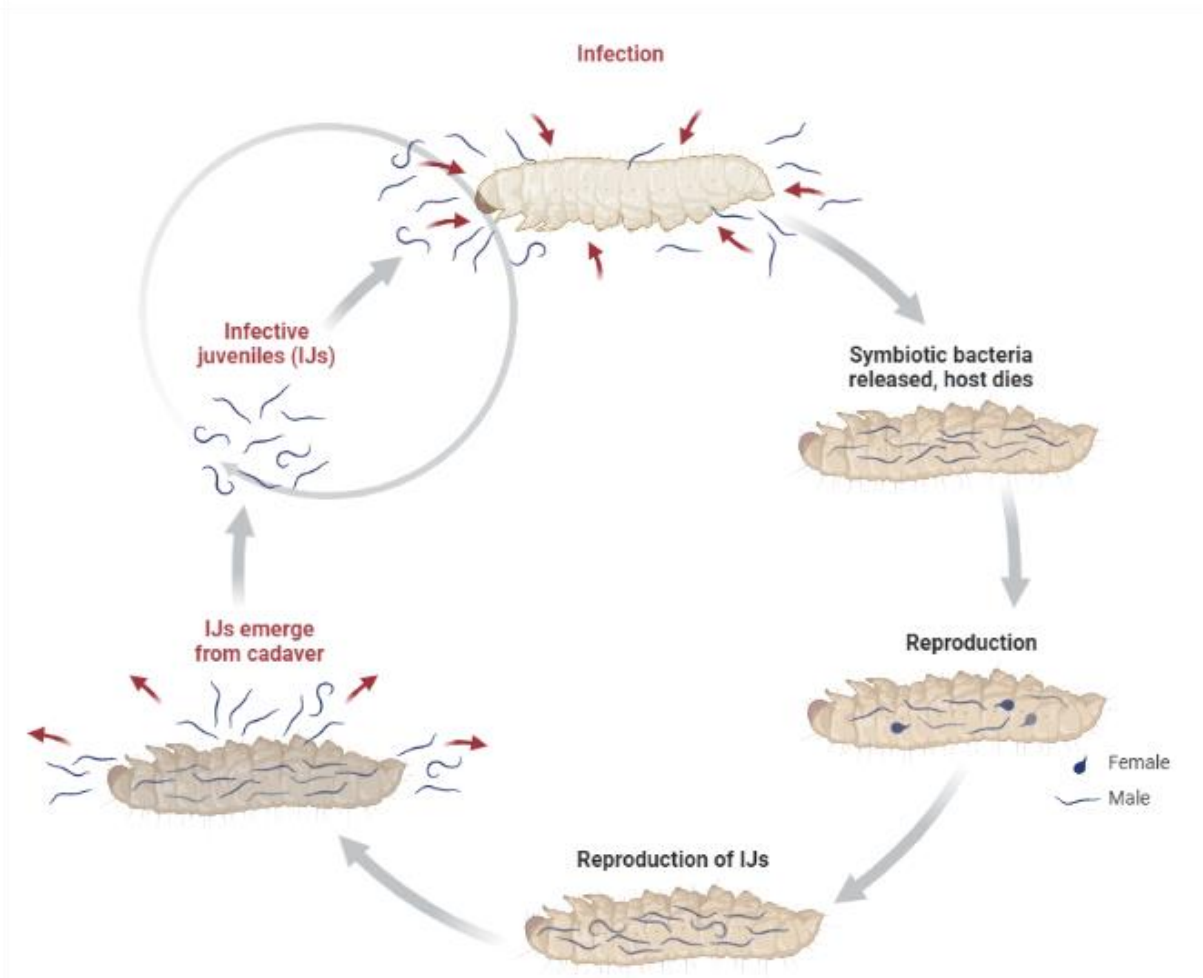

Figure 7 Life Cycle of entomopathogenic nematodes (adapted from Biorender.com)

Numerous studies have focused on the use of EPNs as biological control agents (Manochaya et al., 2022; Nxitywa, 2021). However, there are limited studies that have focused on the use of other nematodes as potential effective biological control agents (Askary 2009; Grewal, Elhers, & Shapiro-Ilan 2005). A noteworthy example of the possible use of the less well-studied nematode group in pest management programs is the facultative parasite of slugs, *Phasmarhabditis hermaphrodita*, whose dauer larva also serves as the infective stage in the parasitic life cycle (Rae et al., 2008).

In this study, we report a new *Cruznema* species, *Cruznema* sp. NTM-2021, which was isolated from a vegetable farm in the Leribe region, Lesotho. Molecular techniques such as PCR amplification and DNA sequencing of the 18S rDNA gene were used to characterize the species.

The 18S rDNA sequence of the *Cruznema* species was deposited in Genbank at the NCBI and given the accession number OQ408141.

Aim

The aim of the study was to isolate and identify nematodes from soil samples collected from a vegetative farm in Lesotho.

Objectives

The objectives of this study were to: 1) Isolate nematodes from soil samples collected from a vegetative farm in the Leribe region of Lesotho using soil baiting and white trapping techniques. 2) Confirm the cause of infection by completing Koch's postulate. 3) Extract genomic DNA from the isolated nematodes. 4) Identify the nematodes based on molecular characterization of the 18S rDNA region by PCR amplification using universal primers and DNA sequencing. 5) Perform phylogenetic analysis using the MEGA11 software application.

Methods and Materials

Rearing of *Tenebrio Molitor* larvae

Tenebrio molitor, mealworm, was used as a study model. It was maintained to produce larvae for isolation and culturing of nematodes from the collected soil samples. The larvae (both male and female) were placed in plastic tubs closed with lids that had holes to allow efficient aeration and prevent the larvae or beetles from escaping. The *T. molitor* larvae were constantly fed with a medium that consisted of wheat bran and apples. Tiny pieces of wood were added to the medium to allow the larvae to move, mate, and lay eggs. The apples were removed every two days. The tubs were stored in the dark at 25 °C, which is the optimum temperature for nematodes. Figure 8

(A and B) shows the way *T. molitor* larvae and beetles were reared. Fresh apples were added to provide the larvae and beetles with sugar and keep them hydrated.

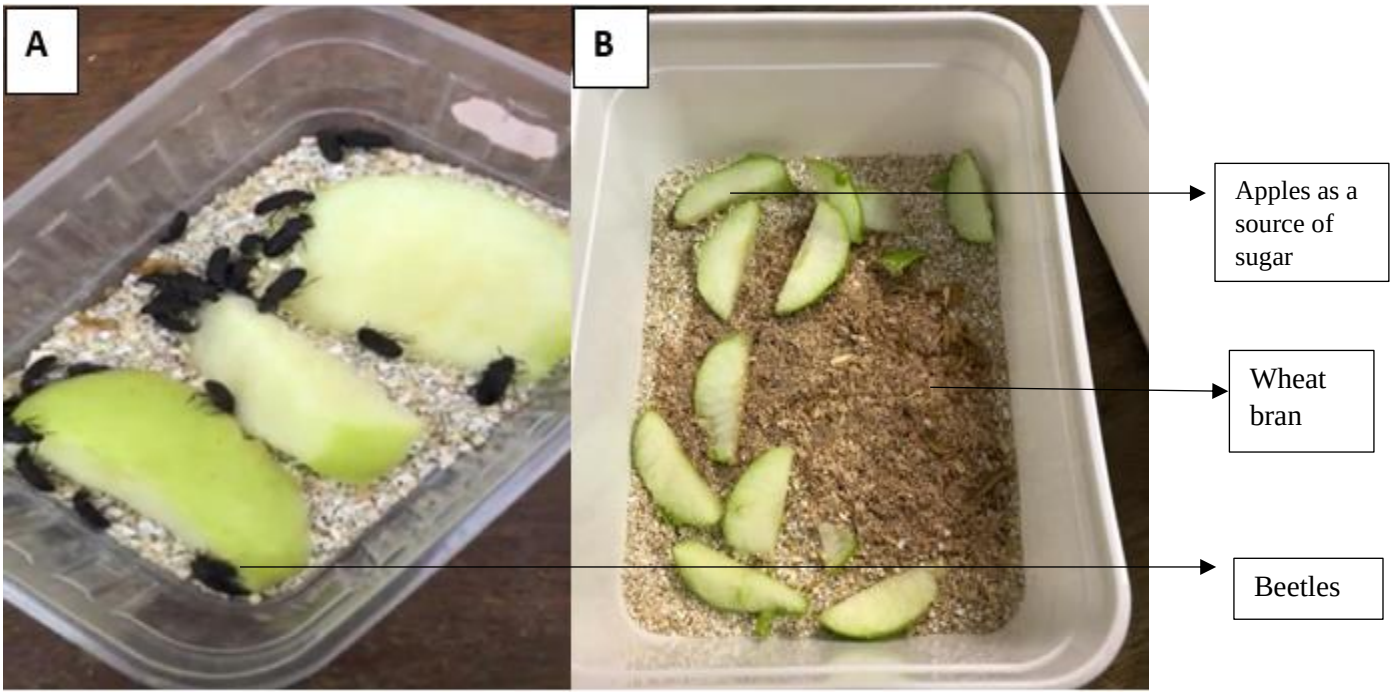

Figure 8 (A) shows in vivo rearing of beetles. (B) shows in vivo rearing of larvae of *Tenebrio molitor*

Soil Collection

Soil samples were collected from a vegetable farm in the Leribe region, in Lesotho: GSP coordinates 28˚59'17.411'S,28˚8'25.027" E. The soil was sandy loamy. A shovel was used to collect the soil to a depth of 15cm (Lephoto & Gray, 2019a). The soil samples were stored in separate plastic tubs and stored at 22-25°C during transportation. It is worth noting that the plastic tubs used were gouged with scissors to open holes that allowed aeration. Stones and other unwanted debris were removed by sieving the soil samples into clean tubs. Sterilized water was added to the soil samples before baiting to keep the soil hydrated. The samples were hydrated (moisture level of 8%) to enable the movement of the nematodes.

Isolation of nematodes

Soil baiting

The soil baiting technique was performed according to the method described by (Orozco et al., 2014). Sterile water was sprayed to hydrate the soil and facilitate the movement of nematodes. About 200 g of the hydrated soil was transferred to clean plastic tubs with lids. As seen in figure 9, five insect baits, *Tenebrio molitor* (Coleoptera: Tenebrionidae) the yellow meal worm, were added to each soil sample. The tubs were then covered with a lid and inverted. The tubs were stored at room temperature in the dark to allow the infective juveniles to infect the meal worm larvae. The samples were checked every 2-3 days, dead insects were removed, and five healthy insects were added for further baiting. The cadavers were rinsed with sterile water, patted with a paper towel sprayed with 70% (v/v) ethanol, and then placed on a white trap to allow the emergence of nematodes at room temperature. The soil was kept at 8% (v/v) moisture level to allow for the movement of the nematodes.

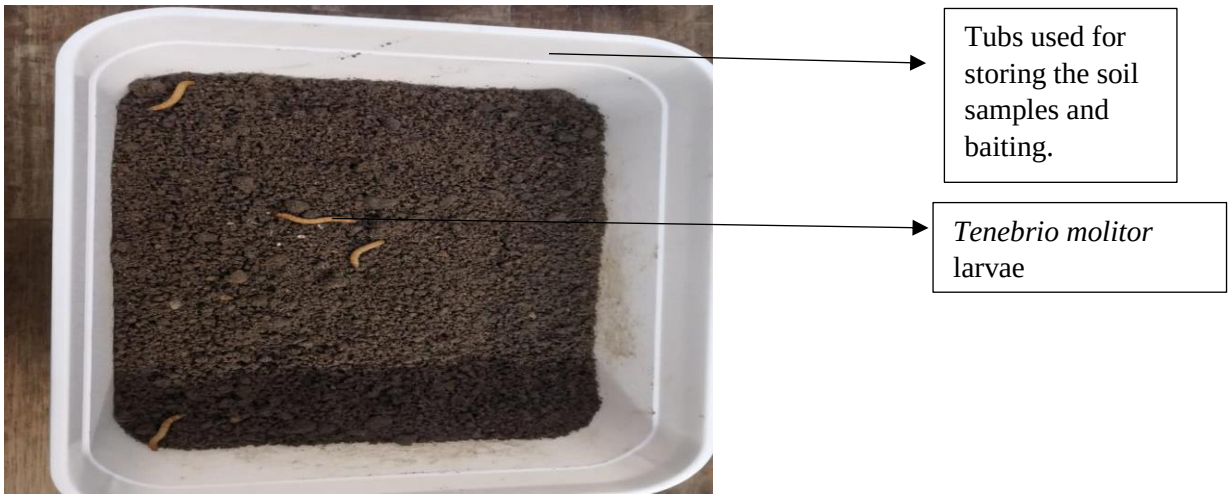

Figure 9 Soil sample from Lesotho baited with *T. molitor* larvae in a tub.

White trapping

The infective juveniles were harvested from the infected larvae through the White trapping method described by (White, 1927). The set up for the White trap method is shown in figure 9. A petri dish of 50 mm in diameter was placed inside a larger dish of 90 mm, and a Whatman No. 1 filter paper was placed inside the smaller dish. The cadavers were placed on the filter paper that was on top of the smaller dish, and the larger petri dish was filled with about 20 mL of sterile water. The petri

dish was then covered with a lid. The petri dish was kept at room temperature until the infective juveniles emerged, which occurred within 7–9 days. When the nematodes had emerged and moved through the filter paper into the water, they were then transferred into a sterile 50ml falcon tube. The nematodes were soaked in 0.1% sodium hypochlorite (NaOCl) for an hour and then rinsed three times with sterile water.

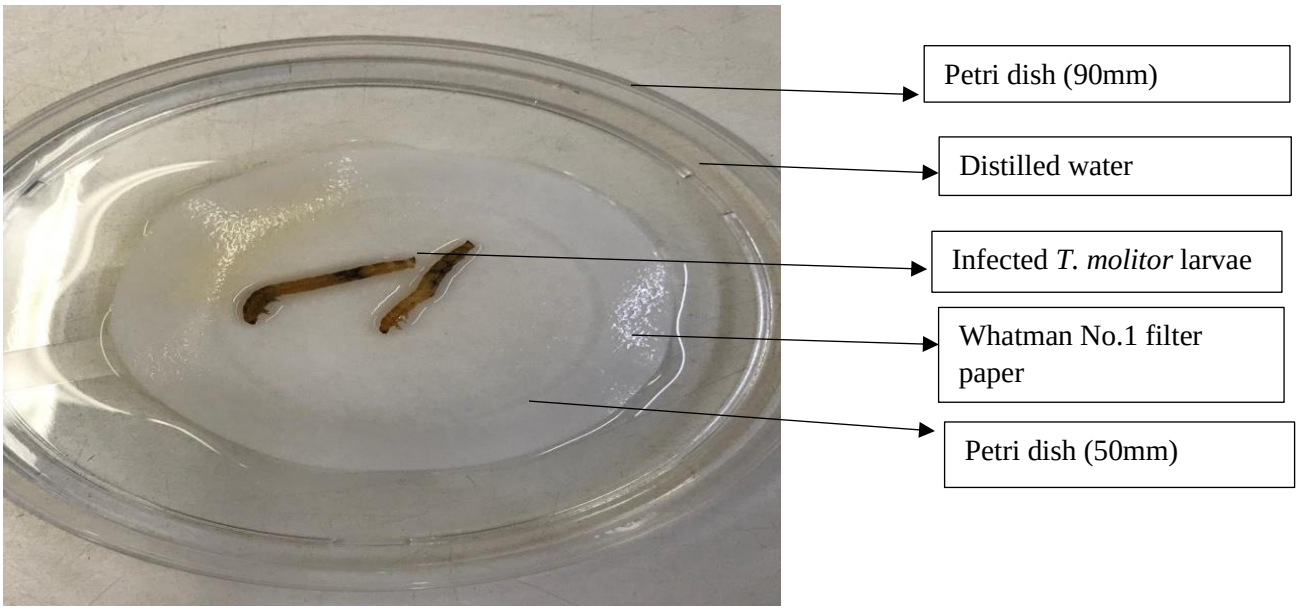

Figure 10 *T. molitor* cadaver infected by nematodes on a White trap.

Confirmation of Koch' s postulate

To confirm the infectivity and virulence of the IJs, Koch's postulates were completed as follows: The IJs collected from white traps were transferred to a clean falcon tube and rinsed with 0.1% v/v of sodium hypochlorite three times. This was done to ensure that the nematodes were free of contaminants. Approximately 45 g of autoclaved sand was weighed, transferred into a 90mm sterile petri dish, and inoculated with the sterilized IJs. Five fresh larvae were placed on top of the sand. The sand was regularly sprayed with sterile water. The sand was kept at an 8% moisture (w/v) level to avoid drying and to also enable the movement of the IJs. A negative and positive control were also performed, where the negative control included fresh larvae and the positive control included infected larvae. Observations were made to monitor colour changes and the mortality of the larvae (Schurkman & Dillman, 2021; Stock, 1995). To confirm that the infection

was caused by the same IJs, the same procedure was repeated several times. This procedure was also performed to maintain the nematodes.

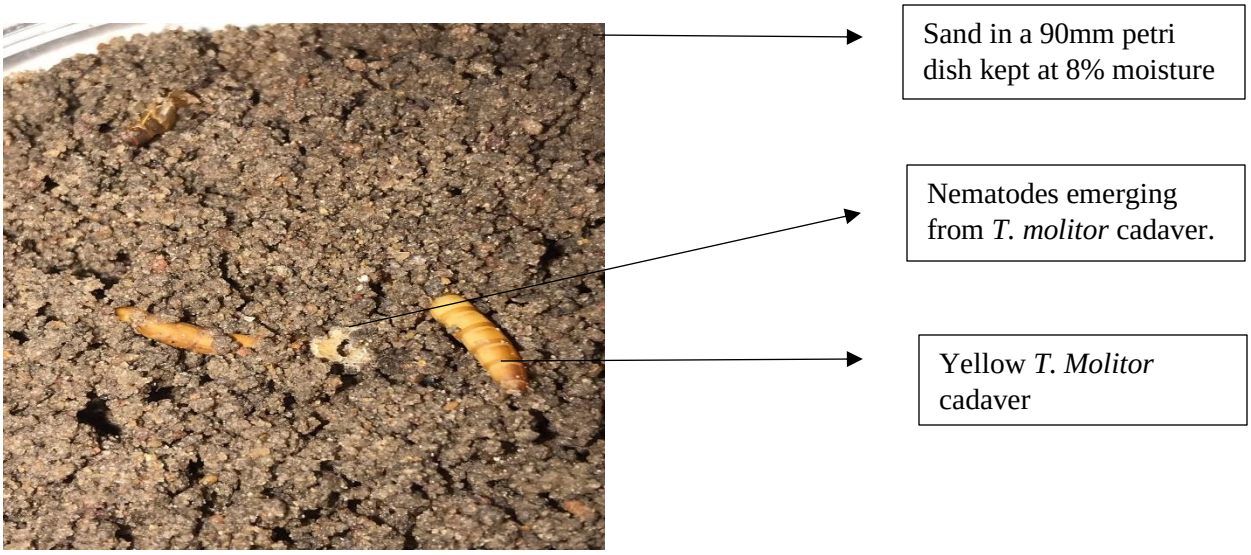

Figure 11 *T. molitor* larvae infected by nematodes in a petri dish filled with sand.

Molecular identification

Extraction of Genomic DNA & PCR amplification of the 18S rDNA

E.Z.N.A.® Insect DNA Kit was used to extract DNA from the nematodes following manufacturer's instructions (refer to Appendix A). The DNA products were sent to Inqaba Biotechnologies (PTY) LTD for PCR amplification and DNA sequencing of the 18S rDNA region. The following primers were used for the PCR amplification of the 18S rDNA region:

Table1: The nucleotide sequences of the universal primers used for the PCR amplification of the 18S rDNA region

Primers	**Sequences**
Forward	AB28 (ATATGCTTAAGTTCAGCGGGT)
Reverse	TW81 (GTTTCCGTAGGTGAACCTGC)

Construction of the phylogenetic tree

The sequences received from Inqaba Biotechnological Industries (PTY) LTD were edited using the FinchTV 1.4.0 software tool and saved as FASTA sequence files. The edited FASTA consensus sequences were uploaded onto the Basic Local Alignment Search Tool (BLAST) on the National Center for Biotechnology Information (NCBI) for identification of the unknown isolated nematodes (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The retrieved 18S rDNA sequences of the known and unknown sequences were used to construct the phylogenetic tree using Maximum likelihood method and the Tamura-Nei model in Molecular Evolutionary Genetics Analysis (MEGA) version 11 (Tamura et al., 2021). The sequences were aligned using MUSCLE (Multiple Sequence Alignment Method) on MEGA 11 (Edgar, 2004). The bootstrap tree was performed with 1000 replications.

The following known taxa obtained from the NCBI Genbank were used to construct the phylogenetic tree of *Cruznema* NTM-2021 : *Heterorhabditis indica* MOR14 (KU214822), *Heterorhabditis bacteriophora* H39 (KT598249), *Oscheius chongmingensis* Tumian (EU273597), *Oscheius onirici* GA3 (KX036758), *Oscheius tipulae* PS1131 (U81587), *Steinernema carpocapsae* SMF7 (KY914571), *Steinernema monticolum* Korea (HM778114), *Steinernema glaseri* Iran2 (EU048543), *Steinernema feltiae* Boj9 (KC675184), *Steinernema scapterisci* SE61 (HM140695), *Cruznema* sp. JN2018 (MK156051), *Cruznema* sp. GD-1 (ON191475), *Oscheius chongmingensis* Jorhat (OP077311), *Oscheius* sp. TEL-2014 T8 (KM492926), *Heterorhabditis safricana* SF670 (FJ473361), *Heterorhabditis megidis* AGC (AY321480), *Heterorhabditis pakistanense* Pak.S.S.302 (JX144740), *Caenorhabditis elegans* (NR 000054) was used as an outgroup. The taxa were selected based on their similarity to *Cruznema* NTM-2021.

Results

Isolation of nematodes

The cadavers infected by the nematodes isolated from the Lesotho soil samples were discolored with shades of brown as seen in figure 12. The larvae were soft and floppy. Brown color and floppiness is associated with symptoms of infection by *Steinernematid.*

Figure 12 An image showing emergence of infective juveniles from dead insects moving into the water. The infective juveniles emerged from the cadaver within 4 - 6 days.

Molecular identification of the nematodes

Genomic DNA extraction

The sequence of the 18S rDNA region was viewed and edited using FinchTV 1.4.0 software. The 18S rDNA consensus sequence of *Cruznema* NTM-2021 is shown below:

GAGAGGATGGCGATGAATCGATGCACGTTGCTTTTGCGACGATACTTAATCGC
TCTGCCTGAGAGTGGCCTCGGATTGGTTTGGTGGCTTGGAAGCCTTGTGCTTCT
AGTCTTAATGAGATAGCTTGCTATCCGCCATCCAATCACTTCTTTCATAACTGA
GTTGGTAATCGAAACGTGACAGGCTCACGCTAAAAAGCCTACTAACTAGCATT
AGCGATGGATCGGTTGATTTGGTCATCGATGAAAAACGCAGCCAGCTGCGTTA
TTTACCACGAATCGCAAATTTGTAGAGTGGTAAAATTTTGAATGCATAGCGCC
GAAGGGTATCATCCCTTCGGCACGCCTGGTTCAGGGTTGTTTAATCGAATTCC
GTCGACAGACAGTTGATGGATAGTCGAGGGGCTGCGCTTCGGCGTACCCTCTC
TGGTTTCGAGAACTATGTTCCTAGTGAACTGTGAATTCGCGTTTGCGTAGCCTC
CATG

Figure 13: The18S rDNA consensus sequence of *Cruznema* NTM-2021. The NCBI BLAST results showed a strong affinity to *Cruznema* sp. JN2018.

The trimmed consensus sequence was deposited on the NCBI Genbank submission portal. The data was processed, and an email was sent with accessions and the final processed records. The processed records were posted in the GeneBank Submission Portal. The 18S rDNA sequence was allocated the accession (0Q408141) by Genbank, National Centre of Bioinformatics Information (NCBI).

Phylogenetic analysis of *Cruznema* NTM-2021

The 18S rDNA sequence of *Cruznema* NTM-2021 was aligned using MUSCLE on MEGA 11. As shown in figure 14, the 18S rDNA sequence of *Cruznema* NTM-2021 was aligned with other nematode species.

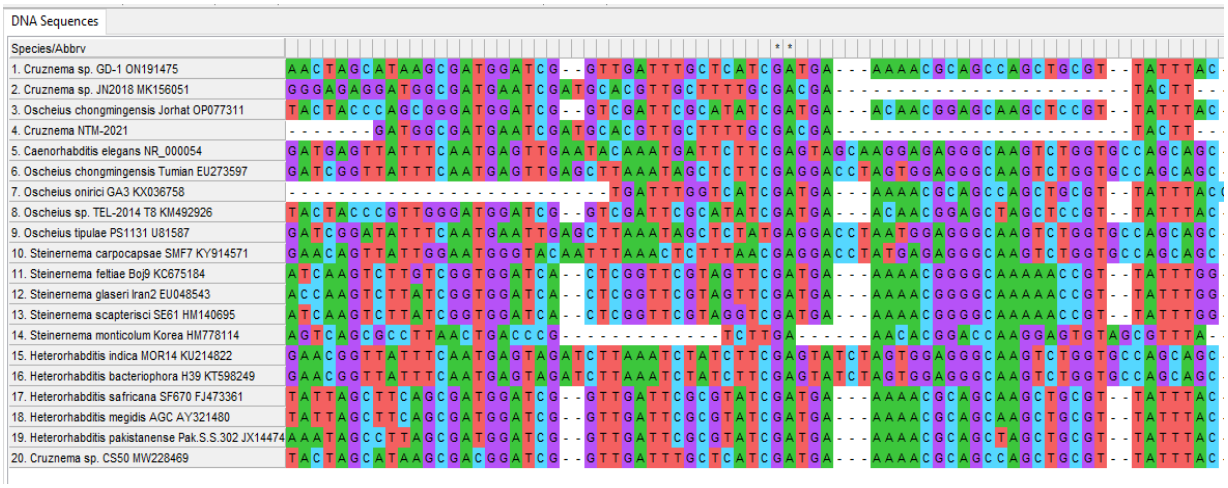

Figure 14 A segment of the alignment for the 18S rDNA sequence between *Cruznema* NTM-2021 and other species of nematodes using MUSCLE in MEGA 11. Red (Thymine), Green (Adinine), Blue (Cytosine), and Purple (Guanine).

The Maximum Likelihood Method in the MEGA 11 software application was used for the identification and relatedness of *Cruznema* NTM-2021. As seen in figure 15, The phylogenetic tree showed that the *Cruznema* NTM-2021 shared a clade with *Cruznema* sp. JN2018. *Cruznema* NTM-2021 and *Cruznema* sp. JN2018 share a more recent common ancestor.

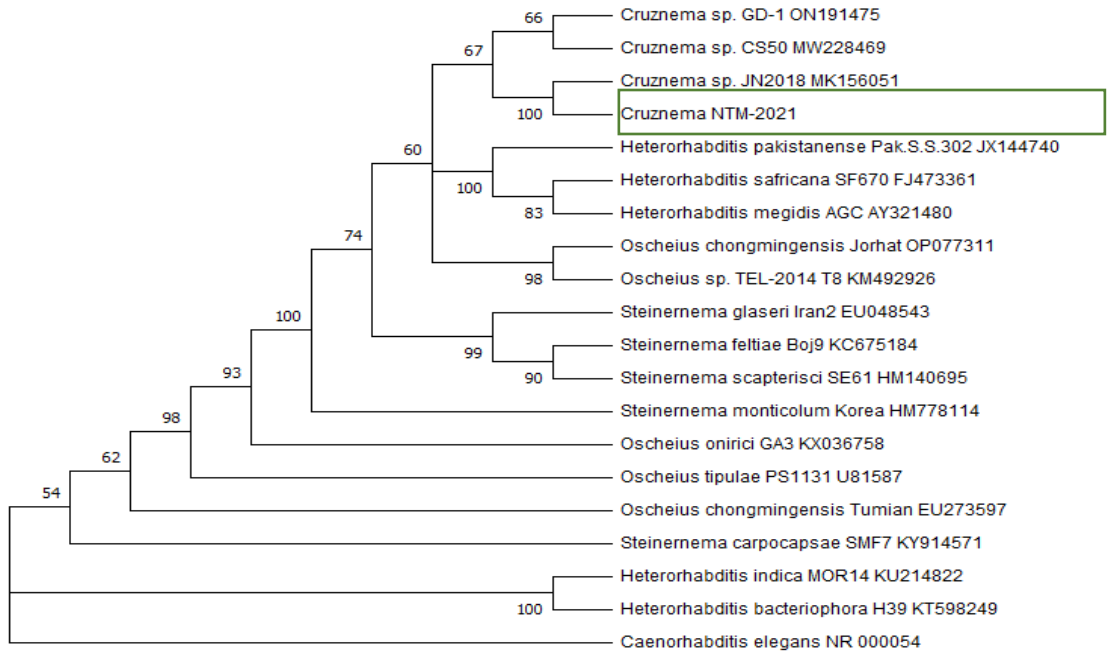

Figure 15: Phylogenetic tree of Unknown X. isolated from Lesotho soil with other related species based on the 18S rDNA region constructed using MEGA11 application software. The tree was

rooted on *Caenorhabditis elegans* (outgroup) and the bootstrap values are based on 1000 replications. The phylogenetic tree was drawn to a scale.

Discussions

The nematodes were identified based on their molecular characteristics. The symptoms of *T. molitor* cadaver following infection by the isolated nematodes matched that of infections caused by *Steinenerma* species (discoloration with brown shades) (Chaerani et al., 2018; Eilenberg et al., 2015). Brown coloration is a symptom also observed in infections caused by *Heterorhabditids* and it is due to low bacterial concentration or absence of pigment, and antibiotics known to be secreted by symbiotic bacteria of the nematodes (Salazar-Gutiérrez et al., 2017). Furthermore, a brown color was observed on *Galleria melonella* infected by *Oscheius chongmingensis* (Fu & Liu, 2019). The cadavers were soft and floppy after the infection by *Cruznema* NTM-2021. The floppy morphology is a result of degradation of the middle part of a digestive tract of the insect, endocytosis, and damage to the cells brought on by the numerous conserved domains existing in the Mcf toxin (Ullah et al., 2014). Such morphological characteristics are seen with infections caused by *Photorhabdus* sp. (Daborn et al., 2002). This indicates that *Cruznema* species may not be identified based on the color induced in their host insects.

The 18S rDNA sequence was aligned using MUSCLE on MEGA 11. Aligning sequences when constructing phylogenetic trees is of great importance as it plays a crucial role in accurately inferring evolutionary relationships between species or sequences (Wang et al., 2022). MUSCLE is a reliable software tool to use for alignment as it is deemed accurate, fast, and flexible (Edgar, 2004).

The phylogenetic tree elucidated the evolutionary relationships between *Cruznema* and other nematode species. The 18S rDNA sequence revealed that the isolate was an uncharacterized nematode species that had high affinity for *Cruznema* species. The uncharacterized species was named *Cruznema* NTM-2021, with accession number OQ408141. Furthermore, the phylogenetic tree demonstrated that *Cruznema* NTM-2021 was closely related to *Cruznema* sp. JN2018. The relationship between the two *Cruznema* species is supported by a bootstrap value of 100, which indicates that the same branch can be observed when generating a phylogenetic tree again using a

resampled dataset. It also indicates that the branch or node that joins *Cruznema* NTM-2021 and *Cruznema* sp. JN2018 has maximal support and is reliable. Although (Du et al., 2022) performed a study of the mitogenome of *Cruznema tripartitum* and revealed that the species share a clade with *Caenorhabditis elegans* and *Oscheius chongmingensis,* the present study shows that *Cruznema* NTM-2021 is not closely related to either *C. elegans* nor *Oscheuis* sp. Previous reports have described *Cruznema* nematodes as free-living parasitic nematodes that feed on bacteria and affect crickets and molluscs/snails (d'Ovidio et al., 2019; Du et al., 2022; Latheef & Seshadri, 1972). However, a more recent study has shown that *Cruznema* nematodes are parasitic to mealworms and darkling beetles (Shokoohi et al., 2022). The present study also revealed that *Cruznema* NTM-2021 can infect and kill *T. molitor* (mealworms) larvae.

Cruznema NTM-2021 was pathogenic to *T. molitor*, killing the larvae within 3-6 days at room temperature. (Dillman et al., 2012) reported that the killing of an insect host within five days is what distinguishes entomopathogenic nematodes from parasitic nematodes. (Dillman et al. 2012) further proposed that for nematodes to be classified as entomopathogenic, the following criteria must be met: 1) the nematodes exploit a symbiotic interaction with bacteria to promote infection, suggesting that the association is non-transient but not completely essential. 2) Insect mortality occurs quickly enough to be clearly distinguished from phoretic, necromenic, and other parasitic relationships (i.e., within 120 h), which also suggests that the nematode vector efficiently releases the symbiotic bacteria. Based on the second criteria proposed by (Dillman et al., 2012), *Cruznema* NTM-2021 may be hypothesized as an entomopathogen as it was able to cause insect mortality within 120h. What's of interest would be to find out which bacteria is associated with *Cruznema* NTM-2021 and this is to be uncovered in Chapter 3.

Cruznema tripartium, the most commonly isolated species of *Cruznema,* is known to be a fairly robust nematode with a body length ranging between 0.9 and 2.2 mm long (Kang et al., 2019; Tahseen et al., 2012). Another species of *Cruznema* with a very small body length of 0.05mm is *Cruznema lincolnensis* (Reboredo & Camino, 1998). Furthermore, *Cruznema brevicaudatum* n. sp was reported to have a body length of between 1.13 to 1.27mm (Latheef & Seshadri, 1972). On the other hand, *Oscheuis* species' body lengths seem to be a bit larger than those of *Cruznema*, with body lengths that range between 0.50 and 3.25 mm long (Abolafia & Peña-Santiago, 2019; Serepa-Dlamini & Gray, 2018; Serepa, 2015). *Cruznema* species differ from most *Oscheius* in that

the former are quite smaller in body length while the latter are mostly large. *Cruznema* are more similar to *Heterorhabditis* species in shorter body lengths than *Ocheius* (Malan et al., 2014; Nthenga et al., 2021). *Cruznema* species have shorter stoma whereas *Oscheius* species have long and norrow stoma (Latheef & Seshadri, 1972; Liu et al., 2012; Reboredo & Camino, 1998; Serepa, 2015). Although the body lengths of *Cruznema* and *Oscheius* differ, they have a similar body shape (C-shaped) when relaxed (Latheef & Seshadri, 1972; Serepa, 2015).

Conclusion

An uncharacterized species of nematodes from the genus *Cruznema* was isolated and identified. *Cruznema* NTM-2021 was described as a new nematode species based on its 18S rDNA sequence. It was pathogenic to *T. Molitor* larvae. Ongoing investigations are presently underway within our laboratory to further validate the potential application of *Cruznema* NTM-2021 as a viable biological control agent. To enhance our understanding and expand the scope of our research, it is recommended that future studies focus on assessing the pathogenicity of *Cruznema* NTM-2021 against a wider range of insect species.

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Chapter 3: Isolation of bacteria associated with *Cruznema* NTM-2021

Introduction

Nematodes are non-segmented organisms, cylindrical worms sometimes referred to as roundworms, eels, or threadworms (Koppenhofer, 2000). Nematodes are diverse, abundant and play a crucial role in the community they occupy (Wasmuth et al., 2008; J. Zhao & Neher, 2013). Nematodes are associated with a diverse range of bacteria, which play a fundamental role in their protection against harsh conditions or competitors, as well as assisting in their development and nutrition (Bouchet et al., 2022; Cao et al., 2015; Ogier et al., 2020; Samuel et al., 2016). Nematodes that are parasitic to insects, known as entomopathogenic nematodes, live symbiotically with bacteria, which has been reported to serve as a food source and aid in their pathogenicity and development (Fukruksa et al., 2017).

Bacteria that live in association with nematodes are referred to as endosymbionts as they are found to live within the nematodes (Castillo, 2016). Endosymbionts and their hosts have a variety of relationships or interactions, including commensal, parasitic, and mutualistic. A commensal relationship is one where the symbiont uses the host without affecting it (Leung et al., 2021). This type of interaction is exhibited between millepede and nematodes. The nematodes feed on the bacteria found in the gut of the millepede (Nagae et al., 2021; Phillips et al., 2019). A parasitic relationship is an association where the symbiont uses the host as a source of food and causes harm to it (Leung et al., 2021). A relationship of this nature is seen with root knot nematodes that cause damage to economically important crops such as cassava, potato, and others (Onkendi et al., 2014).

A mutualistic relationship is where both the host and symbiont equally benefit from the relationship (Leung et al., 2021). An example of a mutualistic relationship is one between filarial nematodes and endosymbiont *Wolbachia*, the bacterial symbiont provides the nematodes with essential metabolites such as glycolytic enzymes, riboflavin, glutathione (Palmieri et al., 2022; Slatko et al., 2010). Additionally, the endosymbiont aids in the development, fertility, and viability of the nematode host (Hise et al., 2004). Another example of mutualistic interaction that has been studied extensively is the endosymbiont of entomopathogenic nematodes, *Xenorhabdus*,

Photorhabdus and *Serratia* associated with *Steinernematidae*, *Heterorhabditidae* and *Osheius* respectively (Ghavamabad, 2021; Lephoto et al., 2015; de Waal et al., 2011; Taylor et al., 2011). These are gram-negative, rod shaped, non-spore forming bacteria belonging to Enterobacteriaceae (Yang et al., 2012). The endosymbiotic bacteria are carried within the nematodes' free-living form known as the third-stage infective juvenile – IJ (Murfin et al., 2013; Stock & Blair, 2008). Bacterial symbiont of *Steinenerma*, *Xenorhabdus* is found in the vesicles while *Photorhabdus* lives in the gut of its nematode (Stock & Blair, 2008). The bacterial symbiont produces antibiotics and also provides the host nematode with nutrition (Cardoso et al., 2015; Ngugi et al., 2021). The antibiotics and other toxins secreted assist the nematode to overcome the defence mechanism of the insect hosts (Eleftherianos et al., 2007; Eliáš et al., 2020).

Xenorhabdus and *Photorhabdus* secrete several extracellular products and a wide range of antibiotics (Forst & Nealson, 1996). Similarly, the symbiotic bacteria of *Oscheius*, *Serrattia marcescens*, synthesize prodigiosin, a bacterial secondary metabolite that is produced by several types of bacteria to compete and survive (Kimyon et al., 2016; Yip et al., 2021). In addition, *S. marcescen*s is reported to inhibit the growth of numerous mosquito microbiota isolates by producing prodigiosin and other secondary metabolites (Heu et al., 2021). *Morexella osloensis*, a symbiotic bacterium of the parasitic nematode *Phasmarhabditis hermaphrodita*, also kills insect hosts via secretion of endotoxins (Rae, Verdun, et al., 2008; Soenens et al., 2017).

The endosymbionts can be cultured independently in the laboratory, and this has led to numerous studies that explore the use of the endosymbionts or compounds they secrete in various fields, including agriculture (Murfin et al., 2013; Platt et al., 2020; Ruiu et al., 2022). In agriculture, numerous nematodes including EPNs and filarial nematodes and their endosymbionts are used as natural enemies of pests or rather as biological control agents against crop pests (Mahar et al., 2005; Vavre & Charlat 2012;Murfin, Dillman & Goodrich-Blair). Moreover, the bacterial symbionts have a potential to be used as bioinsecticides against mosquitos (Da Silva et al., 2020; Ofori et al., 2020). *Photorhabdus luminescens* have demonstrated inhibitory effect against plant pathogen such as *Erwinia carotovora* (Derzelle et al., 2002; Stock & Blair, 2008). The endosymbionts of nematodes have exhibited pathogenicity against insects' pests and have been recommended as potential biological control agent several insects.

Free-living parasitic nematodes such as *Cruznema* nematodes are reported to be associated with *Pseudomonas corrugata*, *P. fluorescens* and *Bacillus subtilis* (Dey et al., 2014; Knox et al., 2003). A study by (Du et al., 2022) suggested that, according to phylogenetic analyses based on amino acid data, *Caenorhabditis elegans, Oscheius chongmingensis, and C. tripartitum* belong to the same group. Given that *Cruznema* nematodes infect and kill insects and their relatedness to entomopathogenic nematodes in the genus *Oscheius,* can these nematodes be classified as entomopathogenic nematodes? Can their mutualistic bacteria induce pathogenesis in insects?

Aim

The aims of this study were to isolate and identify bacteria associated with *Cruznema* NTM-2021 nematodes, and to also investigate the pathogenicity of the bacterial isolates against *Tenebrio Molitor* insect larvae.

Objectives

The objectives of this study were to 1) Isolate the bacteria associated with *Cruznema* NTM-2021 from homogenised mixture of IJs. 2) Culture the bacterial isolates on selective media. 3) Extract the genomic DNA of bacterial isolates. 4) Sequence the internal transcribed spacer (ITS) region within the 16S rDNA. 5) Phylogenetically analyse the bacterial isolates based on 16S rDNA sequences. 6) Test the pathogenicity of the bacterial isolates against *Tenebrio Molitor* larvae.

Methods and materials

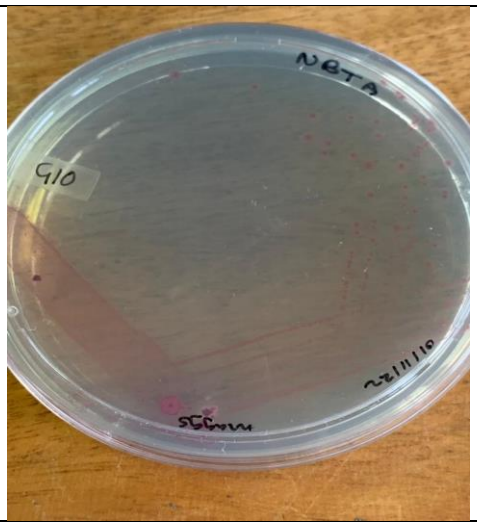
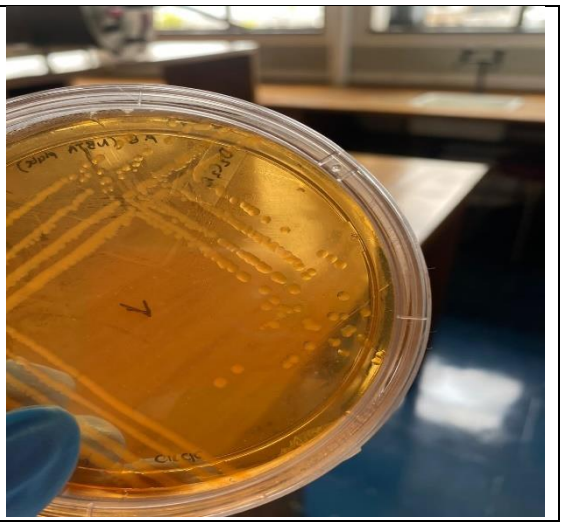
Isolation of bacteria from Infective Juveniles

Cruznema infective juveniles (IJs) were obtained from White traps. The IJs were collected from white traps and added into a 50ml sterile falcon tube. The IJs were left at room temperature to sediment, and water from the White traps was removed. 10ml of 0.1% of sodium hypochlorite (NaClO) solution was added to surface sterilise the IJs. This was to ensure that any bacteria or contaminant that may have adhered to the surface of the IJs is removed. The IJs were soaked in 10ml of 0.1% of NaClO for 3 hours and then rinsed with sterile 10ml of Ringer's solution. The IJs were transferred to a sterile 1.5ml Eppendorf tube and crashed with a sterile plastic pestle to obtain a homogenate. 100µl of sterilised Nutrient broth was added to the crashed IJs and the mixture was

incubated at 25°C for 24 hours. To obtain colonies of the homogenate, the 24-hour culture was streaked on NBTA (Nutrient bromothymol Triphenyl tetrazolium Agar) and MacConkey plates and incubated at 25°C for 48 hours. Pure cultures of the colonies were obtained by sub-culturing red colonies on fresh NBTA and MacConkey, and these were used for other experiments. Colonies obtained from the homogenized mixture of *Cruznema* NTM-2021 on NBTA and MacConkey selective media are shown in Table 2.

Table 2: Bacterial isolates of *Cruznema* NTM-2021 on NBTA and MacConkey selective media incubated at 25˚C for 24-96h.

Isolates	**Species name**	**NBTA**	**MacConkey**
Unknown isolate D6	*Alcaligenes* sp.		
Unknown isolate G2	*Enterobacte*r sp.		

Unknown isolate G10	*Elizabethkingia* sp.		

Molecular identification of the bacteria associated with *Cruznema* NTM-2021 by 16S rDNA sanger sequencing.

Genomic DNA extraction and PCR amplification of 16S rDNA region

Single colonies of the unknown bacterial isolates were sent to Inqaba Biotechnological Industries PTY (LTD), South Africa for colony PCR and sequencing of the 16S rDNA region. The universal primers pair used to amplify the 16S rDNA (ITS) are listed in table 3.

Table 3: Universal primers used for the amplification of the 16S rDNA region.

Name of Primer	**Sequence (5' to 3')**
16S-27F	AGAGTTTGATCMTGGCTCAG
16S-1492R	CGGTTACCTTGTTACGACTT

Table 4: PCR parameters

Reagents	volume
2X MasterMix	-
Genomic DNA	10-30ng/µl

Forward Primer	10μM
Reverse Primer	10μM
Nuclease Free water	-

Table 5 PCR conditions used for the amplification of the 16S rDNA sequence.

Steps	Temperature	Duration
Initial denaturation	94°C	5 minutes
Denaturation	94°C	30 seconds
Annealing	50°C	30 seconds
Extension	68°C	50 seconds
Final extension	68°C	10 minutes

The reaction was then held at 4°C until further processing.

To analyze the PCR products, agarose gel electrophoresis was conducted using a 1% agarose gel stained with EZ-vision® Bluelight DNA Dye. The NEB Fast Ladder was included as a size standard for comparison. Following gel analysis, the PCR amplicons were purified using the ExoSAP procedure (NEB M0293L; NEB M0371), which involved enzymatic purification of the fragments. The purified amplicons were then subjected to further processing using the Zymo Research ZR-96 DNA Sequencing Clean-up Kit™ (Catalogue No. D4050). Sequencing was performed in both the forward and reverse directions using the Nimagen BrilliantDye™ Terminator Cycle Sequencing Kit V3.1 (BRD3-100/1000) on the ABI 3730xl Genetic Analyzer (Applied Biosystems, Thermo Fisher Scientific).

The sequences received from Inqaba Biotechnological Industries PTY (LTD), were viewed, and edited using Finch TV 1.4.0 software (Wessner, 1995). The edited consensus sequences were uploaded into the Basic Local Alignment Search Tool (BLAST) on the National Centre for Biotechnology Information (NCBI) for the identification of the unknown bacterial isolates of *Cruznema* NTM-2021.

Phylogenetic Analysis via MEGA11

The retrieved 16S rDNA sequences were used to construct the phylogenetic trees using Maximum likelihood and Neighbour-joining methods, Tamura-Nei model on Molecular Evolutionary Genetics Analysis (MEGA) version 11 (Tamura et al., 2021). The sequences were aligned using MUSCLE (Multiple Sequence Alignment Method) in MEGA11 (Edgar, 2004). The bootstrap trees were inferred from 1000 replications.

The phylogenetic tree for *Alcaligenes* sp. was constructed using the following known bacteria isolates obtained from the NCBI GenBank database: *Alcaligenes aquatilis strain* RC43 *(*MT572474*), Alcaligenes faecalis* strain Go1 (OP001725), *Alcaligenes faecalis* strain MP2-40 (ON965295), *Alicaligenes faecalis subsp.* Phenolics strain YL3 (OP577956*), Alcaligenes faecalis subsp.* phenolicus strain BH011241 *(OL851585), Bacterium* 120 *(*MZ414116), *Alcaligenes faecalis subsp.* phenolicus strain AMR1B *(*OM977116), *Alcaligenes faecalis* strain CK15 *(*OP143727), *Alcaligenes sp.* strain E2 *(*OP050321) *Alcaligenes faecalis strain TB STinf3* (BC1ON974243) *Alcaligenes faecalis strain VITLJ3 (*ON626494), *Alcaligenes aquatilis strain O6 (*MZ424646) *Alcaligenes faecalis strain* ORGM4 (OM278626) *Alcaligenes aquatilis* strain TB SLinf1 *(*BC5ON989869) *Alcaligenes faecalis* strain CEMTC 1642 *(*OP604385), *Xenorhabdus stockiae* Pak.S.B.69 *(*MF521969), *Xenorhabdus griffiniae* ID10 *(*DQ211710), *Xenorhabdus nematophila* ATCC 19061 (D78009), *Xenorhabdus bovienii* ATCC 35271 (D78007), *Xenorhabdus cabanillasii* USTX62 (AY521244), *Photorhabdus temperata subsp. temperata (*X82249), *Photorhabdus luminescens* subsp. luminescens NR *(*037074) *Photorhabdus hindustanensis (*IARI-SGMG3), *Photorhabdus asymbiotica* UENP04 *(*ON490468) *Photorhabdus laumondii* subsp. laumondii *NR 028870 Serratia entomophila* DSM 12358 NR (025338) *Serratia surfactantfaciens* YD25 NR (169468), *Serratia marcescens* KRED DSM 17174 NR *(*036886), *Serratia nematodiphila* DZ0503SBS1 NR (044385), *Serratia nematodiphila* Ge2G3 (HE801236) and *E. coli* (01: Kl:H7 NR 024570) - outgroup.

The phylogenetic tree for *Enterobacter* sp. was constructed using the following known bacterial isolates obtained from the NCBI GenBank database: *Enterobacter ludwigii* Tula39 (MZ437037), *Enterobacter ludwigii* (OQ345655), *Enterobacter* sp. (OM164406), *Enterobacter ludwigii* OQ421654, *Enterobacter ludwigii* BR56 (OM103652), *Enterobacter ludwigii* (OQ726510), *Enterobacter ludwigii* (OQ727376), *Enterobacter* sp. (ON255853), *Enterobacter* sp. BT11MT (OL662831), *Enterobacter* sp. (ON545847), *Enterobacter kobei* (OQ255861), *Enterobacter* sp. (LC573961), *Enterobacter sichuanensis* (ON241832), Enterobacter sp. (OQ380832), E*nterobacter huaxiensis* (ON241839), and *Xenorhabdus khoisanae* (HQ142625) – outgroup.

The phylogenetic tree for *Elizabethkingia* sp. was constructed using the following known bacteria isolates obtained from the NCBI GenBank database: *Elizabethkingia miricola* fy2 *Elizabethkingia sp. (*PNW20200726012), *Elizabethkingia miricola (*FACW2012072501), *Elizabethkingia miricola MW26 Elizabethkingia miricola (*MHW2022053110), *Elizabethkingia miricola* KMUH27 *2, Elizabethkingia meningoseptica W3, Elizabethkingia miricola* R-54733, *Elizabethkingia occulta* G4070, *Elizabethkingia miricola YMC (*R1459), *Elizabethkingia meningoseptica* Mantella expectata *(*MF525912) *Elizabethkingia ursingii* G4122, *Elizabethkingia ursingii* KMUH31, *Elizabethkingia miricola* Adu42 *(*MN709334), *Elizabethkingia* sp. NB0903 *(*MN706626), *Xenorhabdus cabanillasii* USTX62, *Xenorhabdus nematophila ATCC* 19061, *Xenorhabdus stockiae* TH01, *Xenorhabdus bovienii* ATCC 35271 *Xenorhabdus griffiniae* ID10, *Photorhabdus luminescens subsp. luminescens* Hb, *Photorhabdus temperata subsp.* temperata DSM 3369, *Photorhabdus hindustanensis* IARI-SGMG3, *Photorhabdus noenieputensis* AM7, *Photorhabdus laumondii subsp. laumondii* TT01 DSM 15139 *Serratia entomophila (DSM* 12358) *Serratia surfactantfaciens YD25 Serratia marcescens KRED* (DSM 17174), *Serratia marcescens 2A2 Serratia nematodiphila* DZ0503SBS1 (NR 044385), *Serratia nematodiphila (*DZ0503SBS1) and *E. coli* (01: Kl:H7 NR 024570) - outgroup.

Morphological identification

Gram staining

A drop of sterile water was aseptically transferred onto a sterilised microscope slide and a single colony obtained from NBTA agar was mixed with the drop of water with a sterile inoculation loop. The smear was allowed to air dry, and heat fixed by passing the microscope slide over a flame

three times. The slide was flooded with crystal violet dye for 60 seconds and this was followed by rinsing with running tap water. Gram's iodine was poured onto the smear for 60 seconds and this was rinsed with tap water. Decolorizing agent - 95% alcohol was added to the slide for 30 seconds and washed with tap water. The slide was flooded with safranin for 30 seconds and washed with tap water and carefully blot dried with a paper towel. The slides were viewed under a light microscope at 100X objective with oil immersion.

Pathogenicity of bacterial isolate of *Cruznema* NTM-2021 against *Tenebrio molitor* larvae

To test for pathogenicity of the bacterial isolates of *Cruznema* nematodes, *Tenebrio Molitor* larvae were used. The preparation of nutrient agar (NA) followed the manufacturer's recommendations (Appendix A). The NA media were inoculated with 100µl of 48h of the bacterial isolates culture and spread with a sterilised glass rod across the plates. The NA media were sealed with a parafilm and then incubated overnight at 30˚C. *T. Molitor* larvae were surface sterilized with 70% (v/v) ethanol and gently patted with a paper towel to remove contaminants that may have adhered to the surface of the larvae. Five sterile larvae were placed in each petri dish and spread with the bacterial isolates. The plates were loosely covered with a Parafilm to allow aeration and prevent the larvae from escaping. This experiment consisted of five treatments in three replicates. *E. coli* was used as a positive control and NA not inoculated with bacteria was used as a negative control. Mortality of the larvae was monitored over a period of 3 days.

Symbiosis test

The symbiosis test was performed using lipid agar method (McMullen & Patricia Stock, 2014). The lipid agar was prepared by mixing nutrient broth, agar, and yeast extracts into a 2 L Erlenmeyer flask. Distilled H_2O and $MgCl_2 \bullet 6H_2O$ were added to the mixture and autoclaved for 15 min at 121 °C. This was followed by an addition of sterile mix of corn oil and corn syrup and swirling vigorously to ensure that the mixture was even. The mixture was poured into petri dishes and allowed to solidify under the laminar airflow. All three isolates were grown in Luria-Bertani broth (LB) for 16hr and 100 µl was suspended onto the solidified agar, spread, and incubated at 28 °C for 24-48 hr. Approximately 400 µl of sterilized nematode suspension (Infective juveniles) was added into the plates and incubated at 23 °C in the dark. The cultures were monitored daily for a period of 7 days.

Data analysis

Larval mortality data was analysed through two-way Analysis of variance (ANOVA), using GraphPad prism statistical software (McHugh, 2011; Motulsky, 2007). Two-way ANOVA was used to compare the mean of the mortality caused by the three bacteria isolates. The means were separated using Sadik's multiple comparisons test least significant difference test at a 5% significance level.

Results & Discussions

Bacterial isolates of *Cruznema* nematodes on selective media

Bacterial isolates were streaked on NBTA and MacConkey media and incubated at 25˚C for 3 days (Table 2). The NBTA and MacConkey media were used to select for gram-negative bacteria, and further differentiate the bacteria based on their lactose fermenting ability. Colonies of unknown isolates G2 and D6 appeared red in colour on NBTA medium, and pink on MacConkey medium. Unknown isolate G10 appeared white on MacConkey and red on NBTA. There were clear zones surrounding the colonies of all the isolates on NBTA. Unknown isolate D6 was slightly elevated. Unknown isolate G10 and G2 were elevated. Morphological analysis of the bacterial isolates is summarized in Table 6.

Table 6: Morphological characteristics of colonies of the unknown bacterial isolates.

Bacterial Isolates	Morphological characteristics of colony					Gram reaction	Cell shape
	Size	Colony shape	elevation	Colour on NBTA	Colour on MacConkey		
Unknown isolate D6	Small	Circular	Elevated	Red	Pink	-	rods
Unknown isolate G2	Small	Circular	Elevated	Red	Pink	-	rods
Unknown isolate G10	Small	Circular	Slightly elevated	Red	white	-	rods

In the present study bacterial isolates of *Cruznema* NTM-2021 were isolated and identified. The isolates were identified based on their morphological and molecular characteristics. Morphological identity was performed on selective and differential medium. MacConkey agar which is a selective and differential medium containing violet dye, bile salts, lactose, and neutral red – pH indicator was used for characterization of the bacterial isolates (Allen, 2005). This medium was ideal for the identification of the bacterial isolates because to it selects for growth of gram-negative bacteria and inhibits growth gram positive bacteria. Nematodes are usually associated with *Enterobacteriaceae* species which are known to play a role in the protection of the nematodes against competitors (Whittaker et al., 2016). MacConkey differentiates microorganisms based on their lactose fermenting ability, where lactose fermenting microorganisms produce organic acid and appear red/pink while those that do not ferment lactose remain white (Allen, 2005). As seen in Table 2, unknown isolates D6 and G2 appeared pink, circular with clear zones around them, and this confirms that they are lactose fermenters. The results conformed to previous studies that isolated *Alcaligenes* sp. and *Enterobacter* sp. colonies with similar morphological features (Chawla et al., 2015; Elazhary et al., 1973; Hoffmann et al., 2005). Unknown isolate G10, (*Elizabethkingia* sp.) appeared white on MacConkey, indicating that the isolate is a non-lactose fermenter. A previous study done on the isolation of the same species demonstrated that these species are non-lactose fermenters (Gupta et al., 2017). The red bacteria colonies surrounded by a clear zone on the NBTA indicate that the bacterial isolates reduced triphenyl triazolium chloride (TTC) in the medium, however, they were unable to absorb bromothymol blue dye as they appeared red instead of blue (AKHURST, 1980). *Cruznema tripartium* has been reported to be

associated with *Pseudomonas tolaasii*, a gram-negative bacteria that causes infections in mushrooms (Grewal, 1990; Lo Cantore et al., 2015).

Phylogenetic analysis of bacterial isolates of *Cruznema* NTM-2021

Phylogenetic analysis was performed using MEGA11 software (Tamura et al., 2021). The trees were constructed using Maximum likelihood and Neighbor joining methods based on Tamura-Nei model. The bootstrap consensus tree was drawn to scale and derived from 1000 replications. Bootstrap values indicating branch support, were assigned to each node in the trees. A threshold of 70% for well-supported branches was established. However, it is noteworthy that several branches in our phylogenetic trees exhibited bootstrap values below this threshold.

The sequence of the unknown isolate D6 was compared to known sequences and the isolate showed high affinity to the genus *Alcaligene*s. The unknown isolate D6 shares the same clade with *Alcaligenes faecalis* strain CEMTC 19061 (OP604385). These two species are closely related and have a bootstrap value of 82% , which indicates that the branch is well supported (Figure 16). *Alcaligenes faecalis* is a gram-negative bacterium typically found medical settings, water, and soil (Huang, 2020).

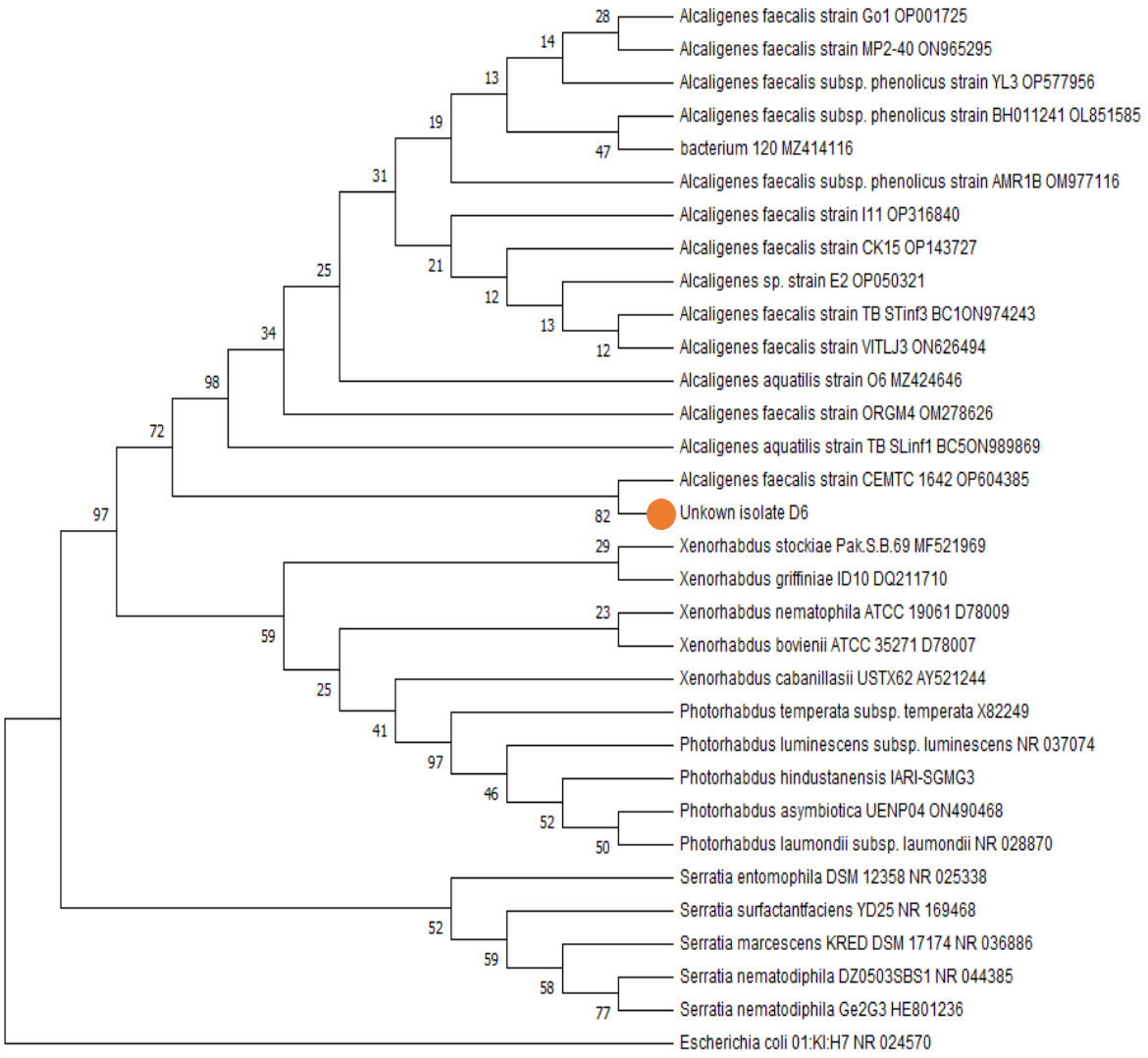


Figure 16 Phylogenetic tree of unknown isolate D6 with other related species based on the 16S rDNA region constructed using MEGA11 application software. The tree was rooted on *E. coli* (outgroup) and the bootstrap values were based on 1000 replications. The phylogenetic tree was drawn to a scale of 0.05.

Unknown isolate G2 is clustered with *Enterobacter ludwigii* Tula39 (MZ437037) in the same clade (Figure 17). The unknown isolate G2 shares a recent common ancestor with *Enterobacter ludwigii 6-a* Blue and have a bootstrap value of 84%. *Enterobacter ludwigii* strains are gram-negative rods, and fermentative (Hoffmann 2005). *Enterobacter sp.* form part of the family Enterobacteriaceae and some species from this family have emerged as nosocomial pathogens (Davin-Regli & Pagès, 2015).

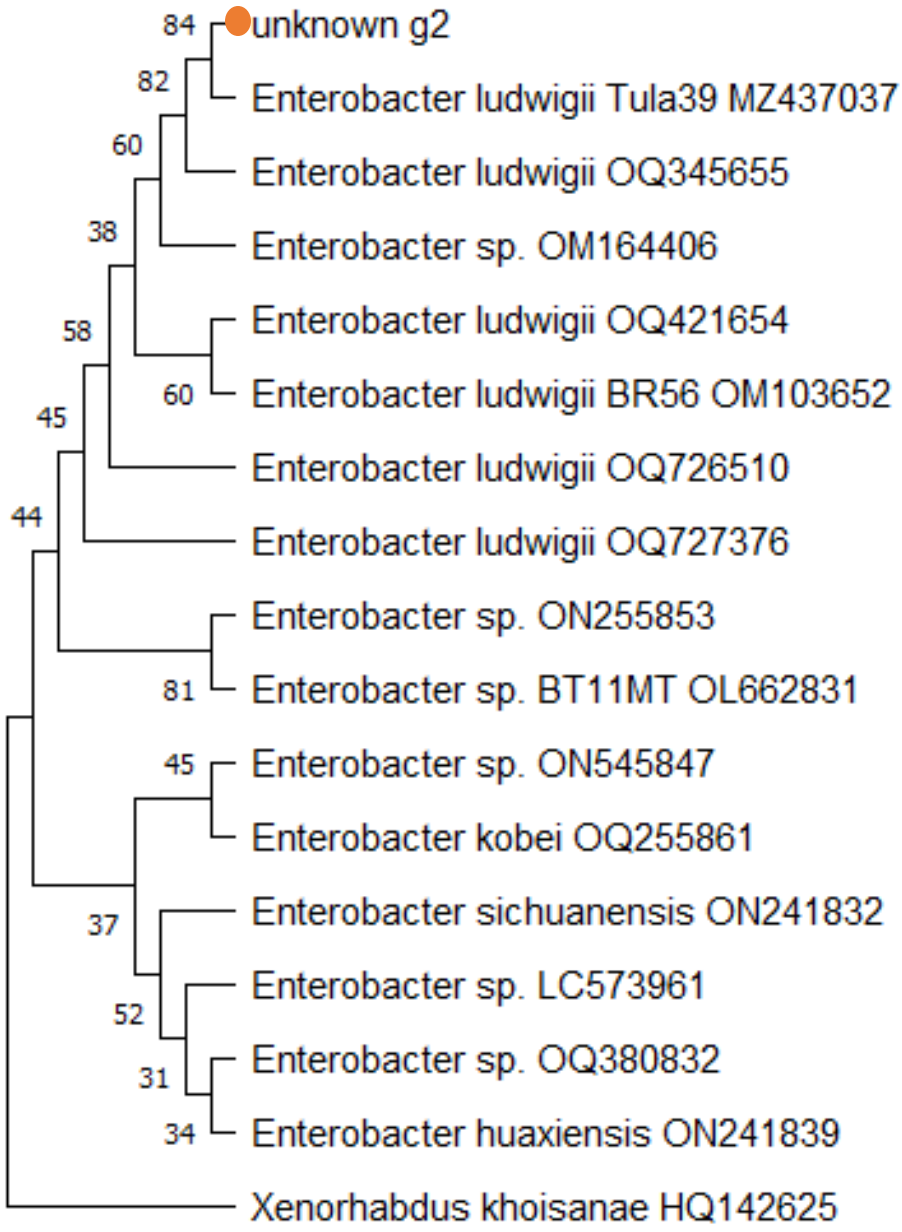


Figure 17: Phylogenetic tree of unknown isolate G2 with other related species based on the 16S rDNA region constructed using MEGA11 application software. The tree was rooted on *Xenorhabdus Khoisanae* (outgroup) and the bootstrap values were based on 1000 replications. The phylogenetic tree was drawn to a scale of 0.05.

As illustrated in figure 18, unknown isolate G10 shares the same clade with *Elizabethkingia miricola* strain Adu42 MN706626 and *Elizabethkingia* sp. NB0903 MN706626. They have a bootstrap value of 87%, indicating that their relationship is well supported. *Elizabethkingia miricola* is a gram-negative, bacillus, aerobic, non-fermenting, non-motile bacterium that causes infections in humans (Gao et al., 2021).

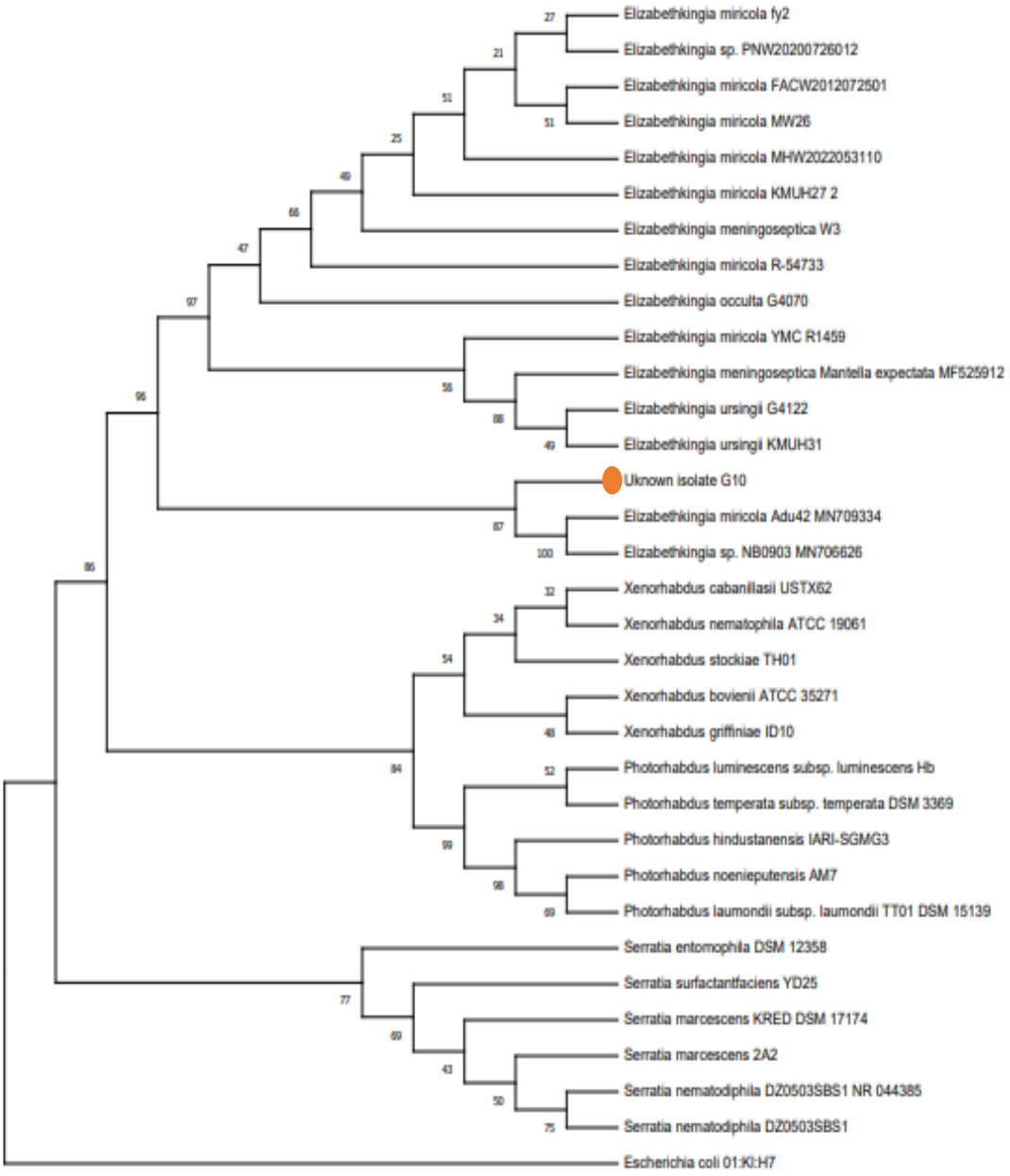


Figure 18: Unknown Isolate G10 with other related species based on the 16S rDNA region constructed using MEGA11 application software. The tree was rooted on *E. coli* (outgroup) and the bootstrap values were based on 1000 replications. The phylogenetic tree was drawn to a scale of 0.05.

Based on the analysis of 16S rDNA sequencing results, the bacterial isolates in this study were confidently identified as *Alcaligenes faecalis*., *Enterobacter lwudigii*., and *Elizabethkingia miricola*. The sequencing data was subjected to phylogenetic analysis, which further corroborated and supported the assigned identifications for these isolates. *Alcaligenes faecalis* exhibited a sequence similarity of 98% to *Alcaligenes faecalis* strain CEMTC 1642 (OP604385), indicating a close genetic relationship between these two species.This high similarity percentage suggests that they have a close evolutionary relationship. In our phylogenetic analysis, bootstrap values were used as an indicator of the reliability of inferred evolutionary relationships. Unknown isolate D6 and *Alcaligenes faecalis* strain CEMTC 1642 (OP604385) showed a bootstrap value of 82, indicating that the branching pattern has received 82% support from the resampled data, suggesting it is relatively well-supported and reliable. However, several branches in the tree (figure 18) exhibited extremely low bootstrap values. These low bootstrap values, particularly in the group that has *Alcaligenes faecalis* strain TB ST INF3 BC10N974243 and *Alcaligenes faecalis strain* VITLJ13 ON626494, raise important considerations. Such low bootstrap values may indicate a level of uncertainty in the relationship between the species.The low values may be as a result of an error in the alignment or limited taxon sampling the dataset (Wiesem, 2013). In addition to their phylogenetic results, the morphological characteristics of the colonies of the *Alcaligenes* sp. isolated in this study resemble those of *Alcaligenes faecalis*-like colonies on MacConkey agar (Elazhary et al., 1973).Unknown isolate G2 is closely related to *Enterobacter ludwigii* Tula39 (MZ437037) with a 99.80% similarity and a bootstrap value of 84%. The high bootstrap value suggests that the branch or the grouping is well supported and reliable. Low bootstrap values are also noted on the *Enterobacter* phylogenetic tree (figure 19), and this indicates that the branches are not well supported. The reason for the low bootstrap values observed in figure 19, may be that the dataset was too small (P. Soltis & D. Soltis, 2003). The clustering of our isolate with *Enterobacter ludwigii* further supports the relatedness of these two species, as previous studies have reported that *Enterobacter ludwigii* strains exhibit lactose fermentation capabilities. This aligns with our findings, as our isolate displayed a pink color on MacConkey, indicating lactose fermentation (Hoffmann et al., 2005). Based on the 16S rDNA sequencing results, *Elizabethkingia* sp. exhibited a striking 99% similarity to the *Elizabethkingia miricola* strain (MN706626), suggesting a close relationship between the two. Furthermore, both species share a common ancestor with *Elizabethkingia* sp. NB0903 and have a bootstrap value of 87%.The phylogenetic

tree for *Elizabethkingia* species also had branches with extremely low bootstrap values and factors that may have contributed to the low values may be sequence divergence and instability in certain linages (de Moraes Russo & Selvatti, 2018; Talavera & Castresana, 2007). The isolated species of *Elizabethkingia* in our study displayed a similar white appearance on MacConkey agar, indicating that, like *Elizabethkingia miricola*, it is a non-lactose fermenter (Chawla et al., 2015). This similarity in morphological characteristics provides further evidence supporting their relatedness and reinforces the notion of a shared evolutionary lineage among these *Elizabethkingia* species. (Gupta et al., 2017).

The isolation of diverse bacterial species from *Cruznema* sp. concurs with previous studies that suggest that nematodes are associated with a broad range of bacteria, either as part of their microbiota (gut) or cuticle (Bouchet et al., 2022; Donmez Ozkan et al., 2019; Lefoulon et al., 2022). (Sinnathamby et al., 2018) investigated the microbiota of *Haemonchus contortus*, a parasitic nematode that causes infections in sheep and isolated numerous bacteria from various stages of the nematode. Similarly, *Caenorhabditis elegans*, a nematode mostly used as a model organism, is reported to consist of a diverse microbiome (Zhang et al., 2017). Taken together, this confirms that nematodes are indeed associated with a diverse range of bacteria, and it is of no surprise that three different genera of bacteria were isolated from *Cruznema* NTM-2021.

Bacteria associated with nematodes play a crucial role in their survival/defence, development, reproduction, and acquisition of food (Murfin et al., 2013). A study carried out by (Whittaker et al., 2016) revealed that intestinal Enterobacteriaceae is involved in the protection of nematodes from the effects of benzimidazoles. Furthermore, (Babic et al., 2000) postulated that non symbiotic bacteria O*chrobactrum* spp associated with *Heterorhabditis Indica* plays a crucial role of providing the nematodes with nutrition. Previous studies have shown that symbiotic bacteria of entomopathogenic nematodes play a significant role in the fitness of nematodes. However, non-symbiotic bacteria have a negative effect on the development of nematodes. Additionally, (Roder & Stock, 2018) tested the effect of symbiotic and non-symbiotic bacteria on the development of the entomopathogenic nematodes and discovered that non-symbiotic bacteria *Serratia proteamaculans* supported the growth of *Steinenerma carpocapsea* and prohibited the development of *Steinenerma faltiae*. This indicates that associated bacteria may benefit one type

of species and be detrimental to the other within the same genera. Further symbiosis tests are required to confirm the interaction between the bacterial isolates and *Cruznema* NTM-2021.

Pathogenicity of the bacterial isolates

The *T. molitor* larvae infected by *Enterobacter* sp. and *Elizabethkingia* sp. appeared yellow in color. Yellow coloration is a symptom exhibited in insects infected by *Steinernema* nematodes (Guide et al., 2018). As depicted in figure 19, larvae treated with *Alcaligene*s sp. appeared discolored and yellow. Discoloration is a phenotypic trait observed in infection caused by bacteria associated with *Steinernematid* (Chaerani et al., 2018; Eilenberg et al., 2015). The reason for no color change or yellow color of infected *T. molitor* larvae may be that the concentration of the bacterial isolates were low in all the treatments or that the bacterial isolates do no produce pigments (Salazar-Gutiérrez et al., 2017; Sharma et al., 2016).

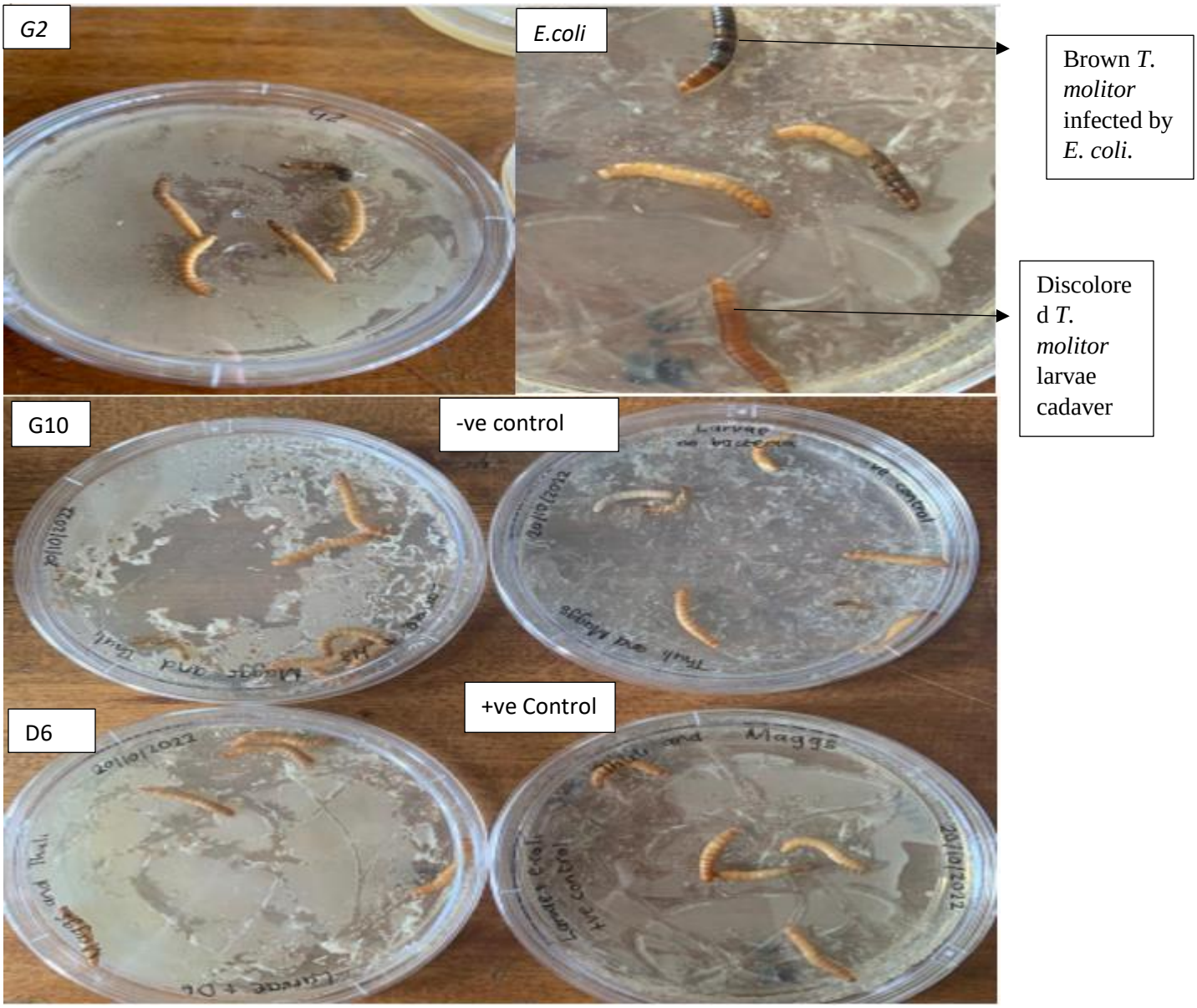


Figure 19: Pathogenicity test of *Cruznema* NTM-2021 bacterial isolates. The above image shows *T. molitor* larvae placed on NA streaked with bacterial isolates of *Cruznema* NTM-2021. D6: *Alcaligenes* sp. treatment, some larvae were discolored while others were yellow. G2: *Enterobacter* sp. treatment, the larvae retained their yellow color. G10: After *Elizabethkingia* sp. treatment, the larvae were yellow. Positive control: *E. coli* larvae appeared brown. Negative control: Larvae retained their natural color.

Larval mortality was observed on all the treatments. The negative error bars show that the standard deviation is larger than the mean, which indicates a huge amount of dispersion in the data. The high dispersion of data may be caused by the sample size or measurement errors. Although the mortality rate was below 50%, all the isolates were pathogenic to *T. Molitor* as they were able to

cause infection and death of the larvae. As shown in figure 20, on day 1, *Enterobacter* sp. exhibited a higher mortality rate compared to *Elizabethkingia* sp. and *Alcaligenes* sp. However, on day 2, both *Alcaligenes* and *Enterobacter* sp. induced similar mortality rates. On day three, *Enterobacter* and *Elizabethkingia* sp. caused higher mortality rate compared to *Alcaligenes* sp. It is logical to anticipate that *Enterobacter* species would exhibit high mortality rates on all three days, given the findings reported in a study conducted by (Loulou et al., 2022). This study demonstrated that *Enterobacter* sp. is pathogenic to *Galleria mellonella* even at low doses. Therefore, the observed higher mortality caused by *Enterobacter* sp. throughout the experimental period aligns with the prior understanding of its pathogenic nature. Furthermore, bacteria in the Enterobacteriaceae family interact with nematodes as they live in close proximity with each other (Whittaker et al., 2016). A study conducted by (Ogier et al., 2020) demonstrated that *S. carpocapsea* was associated with a variety of bacterial strains, and these strains were tested for pathogenicity against insects; two strains were pathogenic to larvae of the lepidopteran insect *Spodoptera littoralis* (Chaston et al., 2011). Additionally, two *Enterobacter* sp. associated with *Heterorhabditis* nematodes were pathogenic to *Galleria mellonella* (Liao et al., 2017). These previous studies corroborate with our results, which exhibited the pathogenicity of the bacterial isolates of *Cruznema* nematodes to *T. molitor* larvae. This suggests that the isolates may be playing a role in the pathogenesis of *Cruznema* NTM-2021.

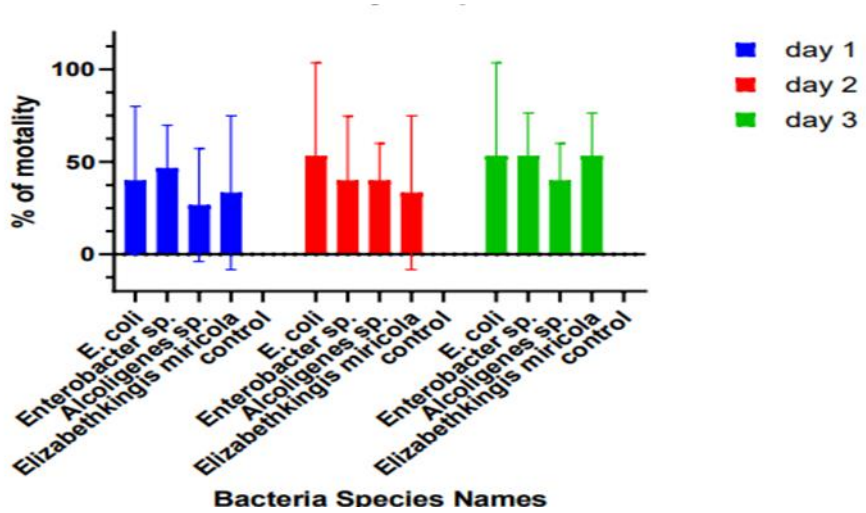


Figure 20 Mortality caused by the bacteria isolates of *Cruznema* NTM-2021 in *Tenebrio Molitor* over the course of three days (day 1, day 2, and day 3)

Symbiosis Assay

We performed a symbiosis test of the nematodes on *Alcaligenes, Enterobacter,* and *Elizabethkingia* species and discovered that the nematodes reproduced and developed in lipid agar inoculated with all three isolates. Abundant growth was observed on plates inoculated with *Alcaligenes sp*. As depicted in Figure 21, nematodes have reproduced on a lipid agar plate with bacterial growth. *Alcaligenes* sp, have been isolated from entomopathogenic nematodes and have established a symbiotic relationship with entomopathogenic nematodes from the genus *Oscheuis* (Shan et al., 2019; Prevez et al., 2020). However, (Ju et al., 2016) demonstrated that *Alcaligenes faecalis* ZD02 can damage the intestines of *C. elegans* and has nematicidal activity against root-knot nematodes. To the best of our knowledge, this is the first report of the isolation of *Alcaligenes* sp. from *Cruznema* nematodes, and we speculate that there is a mutualistic relationship between *Alcaligenes* sp. and *Cruznema* NTM-2021. Further studies are required to confirm the relationship between *Cruznema* nematodes and the bacterial isolates, or rather the role of each bacterial isolate.

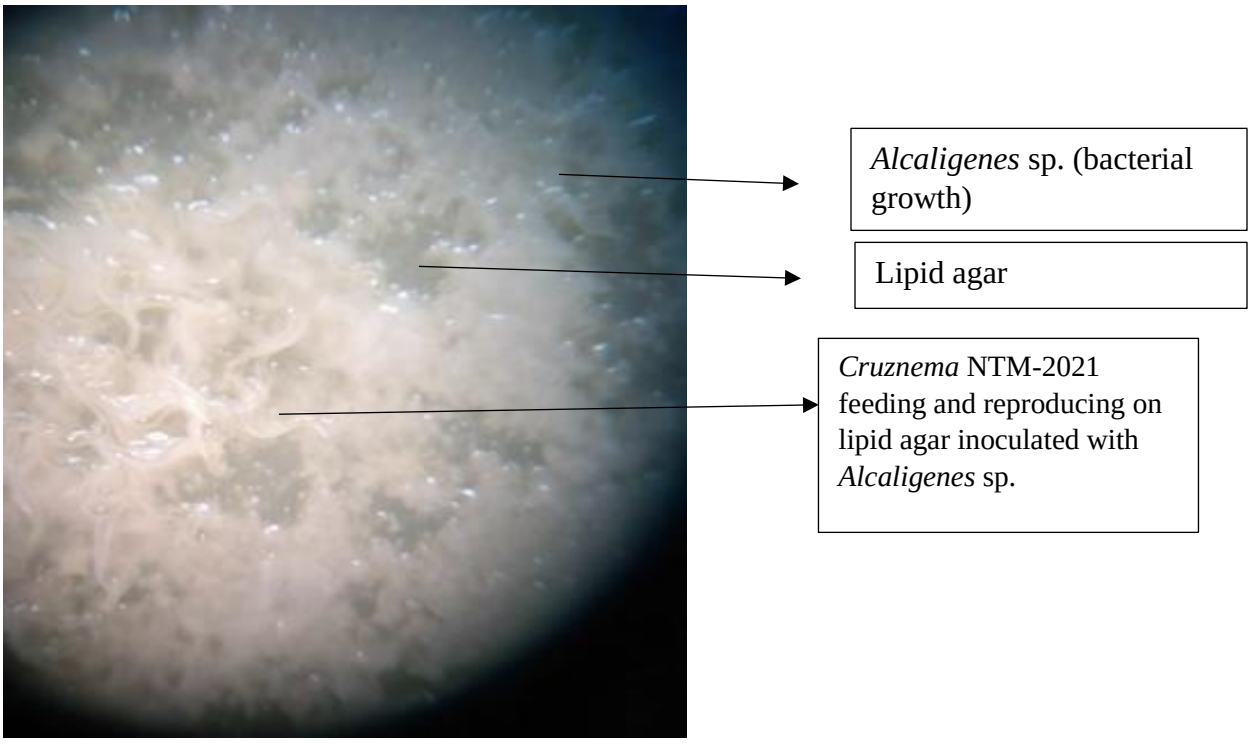


Figure 21: Abundant *Cruznema* NTM-2021 nematodes on lipid agar inoculated with *Alcaligenes* sp. isolated from the IJs.

Enterobacter cloacae isolated from *C. elegans* was found to offer protection from *Enterococcus faecalis* infection (Berg et al., 2016). (Zhao et al., 2022) reported that *Enterobacter ludwigii* has nematocidal activity against plant parasitic nematodes. We suggest that the *Enterobacter* species isolated in our study may play crucial a role in the protection of *Cruznema* NTM-2021.

A study by (Midha et al., 2017) reported that *Elizabethkingia* sp. causes damage to the cuticles of nematodes. However, a recent study demonstrated that pine wood nematodes use *Elizabethikingia* sp. to resist high oxidative stress and survive (Xue et al., 2019). In the present study, the symbiosis test revealed that *Cruznema* nematodes exhibited viability when exposed to *Elizabethkingia* culture. This finding suggests that *Cruznema* NTM-2021 potentially employs the *Elizabethkingia* sp. as a means of survival in harsh conditions. Additionally, the nematodes may have developed the ability to recognize and avoid the bacteria in an equivalent manner as *Pristionchus pacificus* avoids pathogenic species such as *Xenorhabdus nematophila* (Rae, Riebeseil, et al., 2008). Furthermore, *Cruznema* NTM-2021 may have developed a commensal or mutualistic relationship with *Elizabethkingia* sp. *Elizabethkingia* species isolated in this study may be aiding in the protection of the nematodes through the formation biofilm, a process associated with induction of resistance in *C. elegans* (Smolentseva et al., 2017).

Conclusion

From the results, it may be concluded that *Cruznema* NTM-2021 is mutually associated with *Alcaligenes*, *Enterobacter*, and *Elizabethkingia* species. The bacterial isolates showed activity against *T. Molitor* larvae, and they may be considered a possible biological control agent against *T. Molitor* larvae. To better explore the microbial diversity of the *Cruznema* nematodes, metagenomic studies are recommended.

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Chapter 4: Antimicrobial activity and antibiotic susceptibility of bacterial isolates of *Cruznema* NTM-2021

INTRODUCTION

Antibiotic resistance has become a major challenge in public health as most antibiotics are now becoming ineffective in treating infections caused by pathogens considered resistant to drugs (Khan et al., 2019; Muangpat et al., 2020b; Pidot et al., 2014; Viens & Littmann, 2015). Globally, according to data published by (Lancet, 2022), infections caused by antibiotic-resistant bacteria were responsible for an estimated 1.27 million deaths in the year 2019. Despite antibiotic resistance being considered as a phenomenon that occurs naturally or through horizontal gene transfer, it is sometimes as a result of "man-made" factors such as misuse of antibiotics in people, animals, and plants, and inadequate waste management systems (Khan et al., 2019; Levy, 2002; Michael et al., 2014; Ventola, 2015).

Recent developments in drug discovery have shown the need for new effective antibiotics against drug-resistant bacteria (Hutchings et al., 2020; Kwon & Powderly, 2021; Terreni et al., 2021). Gram-negative bacteria are ranked among the high-priority pathogens that require research and the development of novel antibiotics (Kanj et al., 2022). Additionally, the World Health Organization (WHO) reported that there was an urgent need for new antibiotics effective against carbapenem-resistant gram-negative bacterial infections (World Health Organization, 2021). Moreover, studies have reported that the few developed antibiotics are less effective against drug-resistant gram-negative bacteria (Dreyer et al., 2018; Iskandar et al., 2022; World Health Organization, 2021; Yu et al., 2022). This is why new antimicrobial compounds or sources are investigated to determine whether they might be effective against gram-negative organisms (De Mandal et al., 2021; Sibanda & Okoh, 2007; Taneja & Kaur, 2020).

Antimicrobial compounds derived from endosymbionts of nematodes are a promising alternative for antibiotics that could be used for treatment against pathogenic bacteria including antibiotic resistant bacteria (Adams et al., 2006; Awori et al., 2017). Furthermore, some of the antibiotics derived from endosymbionts of nematodes have proven to produce antimicrobial peptides with

novel modes of action (De Mandal et al., 2021; Gualtieri et al., 2009). A considerable amount of literature (Anju et al., 2015; Awori et al., 2017; Donmez Ozkan et al., 2019; Dreyer et al., 2018; Muangpat et al., 2017; Pidot et al., 2014) have shown the production of antimicrobial compounds from endosymbionts of entomopathogenic nematodes and their efficacy in inhibiting several pathogenic bacteria. However, inadequate literature exists on the potential use of antimicrobial compounds sourced from bacteria associated with different species of nematodes. In addition, susceptibility testing of bacteria isolates from soil borne nematodes remain infant. Therefore, the first aim of this study was to investigate the antimicrobial activity of bacteria isolates from *Cruznema* NTM-2021 against *Escherichia coli, Pseudomonas aeruginosa, Enterococcus faecalis,* and *Staphylococcus aureus*. The second aim was to further test for the susceptibility of bacteria isolates of *Cruznema* NTM-2021 to common antibiotics.

The objectives were to: (1) Determine the antimicrobial activity of the bacterial isolates of *Cruznema* NTM-2021 against *Escherichia coli, Pseudomonas aeruginosa, Enterococcus faecalis,* and *Staphylococcus aureus* using the Kirby-Bauer disk diffusion method. (2) Evaluate the susceptibility of *Alcaligenes* sp., *Enterobacter* sp., and *Elizabethkingia* sp. to common antibiotics using the Kirby-Bauer disk diffusion method.

Methodology

Determination of the antimicrobial activity of the bacterial isolates of *Cruznema NTM-2021* against *Escherichia coli, Pseudomonas aeruginosa, Enterococcus faecalis,* and *Staphylococcus aureus* using the Kirby-Bauer disk diffusion method

Preparation of test pathogens

Five strains of pathogens *Escherichia coli* ATCC1157, S*taphylococcus aureus ATCC, Pseudomonas aeruginosa* ATCC 27853, and *Enterococcus faecalis* non-ATCC were used as test pathogens for antimicrobial activity. The test pathogens were sourced from the Molecular and Cell biology stock culture at the University of the Witwatersrand, South Africa. For overnight culture, a single colony of each test pathogen was inoculated in 50 ml of Luria-Bertini (LB) medium and incubated at 30°C for 24 hours. The turbidity of the test pathogen was adjusted to 0.5 McFarland to ensure that the number of bacteria were within a given range to standardize microbial testing

(Hombach et al., 2013). 100µl of the test bacterial suspension was swabbed on Muller-Hinton agar (MHA) for disk diffusion method.

Antimicrobial activity of bacteria isolates of *Cruznema* NTM-2021: Kirby-Bauer Disk Diffusion method

Antimicrobial susceptibility testing was performed by standard disk diffusion assay. The method was adapted from (Muangpat et al., 2020) with some modifications. Some of the modifications included using supernatants as antibacterial agents rather than crude compounds. Secondary metabolites are produced in the medium following depletion of nutrient sources during the late phase of bacterial growth, hence the use of supernatants as a representative of antimicrobial agents (Eom et al., 2014). To evaluate the antimicrobial activity of bacteria isolates from *Cruznema* sp. nematodes (*Alcaligenes* sp. *Enterobacter* sp., and *Elizabethkingia* sp.) against test pathogens, the bacteria isolates were cultured on Nutrient bromothymol Triphenyl tetrazolium Agar (NBTA) for 3 days at 25˚C. A single colony of each bacteria isolate was inoculated in 100ml of Tryptic Soy Broth (TSB) in 250ml conical flasks and incubated for 72 hours on a shaker (120rmp) at 25°C. The bacterial cultures were collected and centrifuged for 20minutes at 13000rpm, 4°C. The supernatants were separated from the pellets by transferring the supernatants into new sterile tubes. To remove any unwanted material or cell debris the supernatants were filtered using 0.22µm filter (Deepa et al., 2015). 20µl of the filtered supernatants were suspended on 6mm blank discs and left to dry for 5minutes under laminar air flow. The discs were placed on MHA plates swabbed with the test pathogens. Positive control were antibiotic disks infused with 10µg of Ampicillin. The plates were incubated at 37°C for 24 hours and the diameter of the inhibition zone were measured. For each bacteria test, the experiment was done in triplicates.

Evaluating the susceptibility of *Alcaligenes sp., Enterobacter sp.,* and *Elizabethkingia sp*. to common antibiotics using the Kirby-Bauer disk diffusion method.

Preparation of bacteria isolates & Susceptibility testing.

To test for antibiotic susceptibility of the bacterial isolates of C*ruznema* NTM-2021 Kirby Bauer disk diffusion method was used. The Clinical & Laboratory Standards Institute interpretive (CLSI) criteria M100–25 was used to determine susceptibility of the isolates. The method was adapted from (Jorgensen & Ferraro, 2009) with some modifications. A single colony from NBTA plate was transferred to 50ml of nutrient broth in a 100ml Erlenmeyer flask and incubated overnight at

25°C. The broth culture was adjusted to 0.5MacFarland to ensure that the number of bacteria were within a given range to standardize microbial testing (Hombach et al., 2013). 100µl of the broth culture from each isolate were pipetted onto Muller-Hinton Agar plates and spread evenly using a sterile hockey stick. Disks infused with different antibiotics were placed on the surface of the MHA plates and gently pressed with a sterile forceps. (Jorgensen & Ferraro, 2009) incubated the MHA plates at 35°C for 16-24 hours, however, in this study the plates were incubated in the dark for 24 hours at 37°C. The diameter of the zone of inhibition for each antibiotic discs were measured in millimetres. For each bacteria test, the experiment was done in triplicates.

Statistical Analysis

The data was analysed through two-way Analysis of variance (ANOVA), using GraphPad prisms statistical software (Manalo et al., 2017). Two-way ANOVA was used to compare:

1. The mean zone of inhibition values (mm) of the antimicrobial agents of the bacterial isolates
2. The mean zone of inhibition values (mm) of different antibiotics used against the bacterial isolates of *Cruznema* NTM-2021.

The means were separated using Geisser-Greemhouse's epsilon test least significant difference test at a 5% significance level ($P<0.05$).

RESULTS

Antimicrobial activity of bacterial isolates of *Cruznema NTM-2021*

Disk diffusion method was used to evaluate the antimicrobial activity of the isolates against four different test pathogens (Figure 22).Supernatants (antimicrobial agents) of the isolates were tested against *E. coli, S. aureus, E. faecalis*, and *P. aeruginosa. Alcaligenes* sp. A moderate zone of inhibition against *E. coli* was observed around the disk infused with *Alcaligenes* supernatant. *Alcaligenes* supernatants showed a weak inhibition zone against *P. aeruginosa*. A weak inhibition zone was observed on the disk infused with *Elizabethkingia* supernatant against *E. faecalis*. Supernatants from all three isolates had low inhibitory activity against *S. aureus* and *P. aeruginosa*. Disks infused with *Enterobacter* sp. supernatant had a weak inhibitory effect against *E. coli*. The ampicillin (control) was effective against all the isolates.

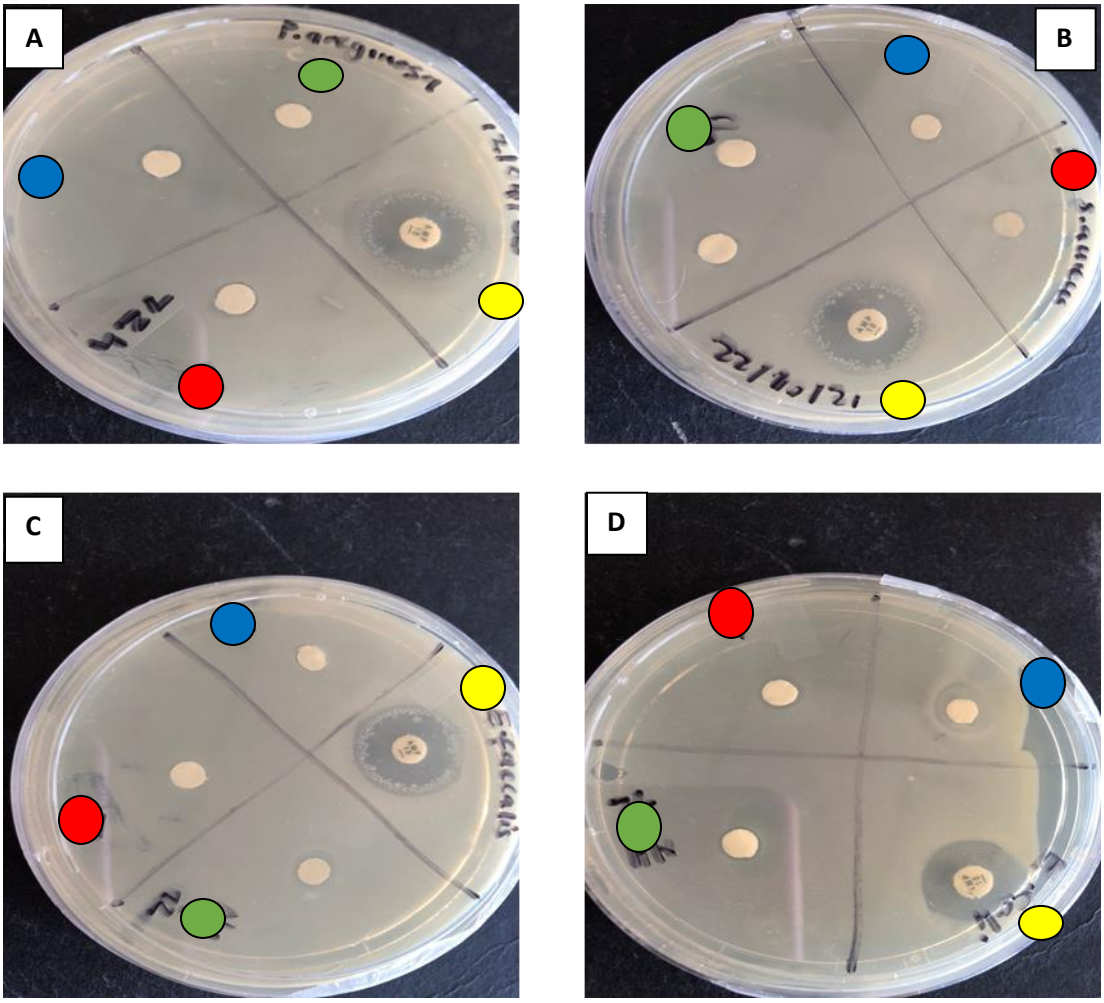

Figure 22: Kirby Bauer disk diffusion plates showing antimicrobial activity. A, B, C, and D are MHA plates streaked with overnight cultures of *P. aeruginosa*, *S.aureus*, *E. faecalis,* and *E. coli* respectively. The disks on the streaked plates are infused with supernatants of bacterial isolates of *Cruznema* NTM-2021. The isolates are represented by the letters and numbers in the brackets on the plates denoted as *Alcaligenes* sp. (Blue circle), *Enterobacter* sp. (Red circle), and *Elizabethkingia* sp. (Green circle). The control is represented by (yellow circle). The clear zones around the disks are zones of inhibition.

Alcaligenes and *Enterobacter* species displayed greater inhibitory activity against *E. coli* and a weak inhibitory effect against *P. aeruginosa, S. aureus,* and *E. faecalis*. There were no significant differences between the mean zone of inhibition values (mm) of the antimicrobial agents against the test pathogens (P>0.05). Although there was no inhibition zone on *Enterobacter* sp. against *P.*

aeruginosa, *Enterobacter* sp. had the most inhibitory activity against the other three test pathogens than *Elizabethkingia* sp. *P. aeruginosa* had the least zones of inhibition while *E. coli* had the most zones of inhibition. *E. feacalis* had moderate zones of inhibition.

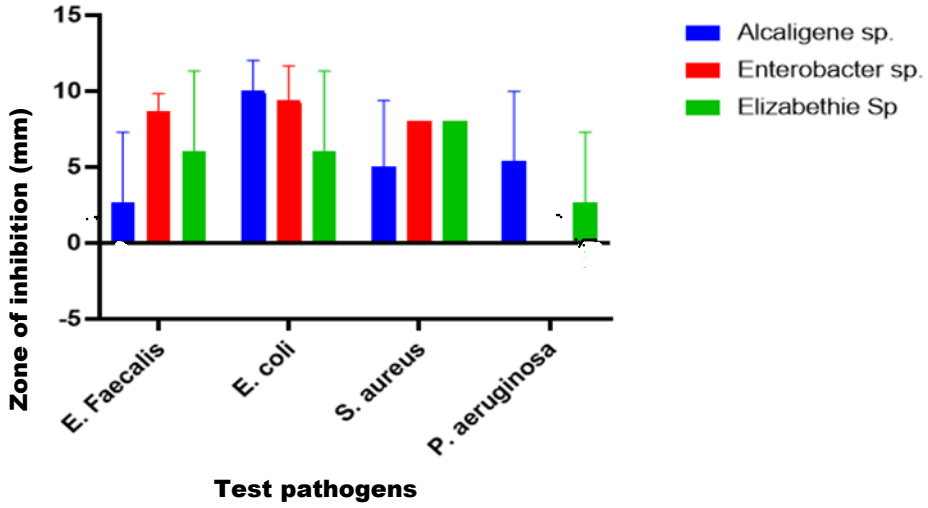

Figure 23: A bar graph showing average zones of inhibition (measured in millimetres) of each isolate against *E. coli, S. aureus, P. aeruginosa* and *E. faecalis*. The error bars represent standard deviation of the mean of antimicrobial activity of the isolates.

Antibiotic susceptibility of bacterial isolates of *Cruznema* NTM-2021

Figure (24) shows zones of inhibition of the antibiotics against *Alcaligenes, Enterobacter* and *Elizabethkingia* species on MHA plates. Gentamicin showed a strong inhibition activity (greater zone of inhibition) compared to all the other antibiotics. Ampicillin, cefuroxime, cephalothin and amoxicillin showed weak to no inhibition activity against the isolates. Vancomycin showed moderate to weak inhibition zones.

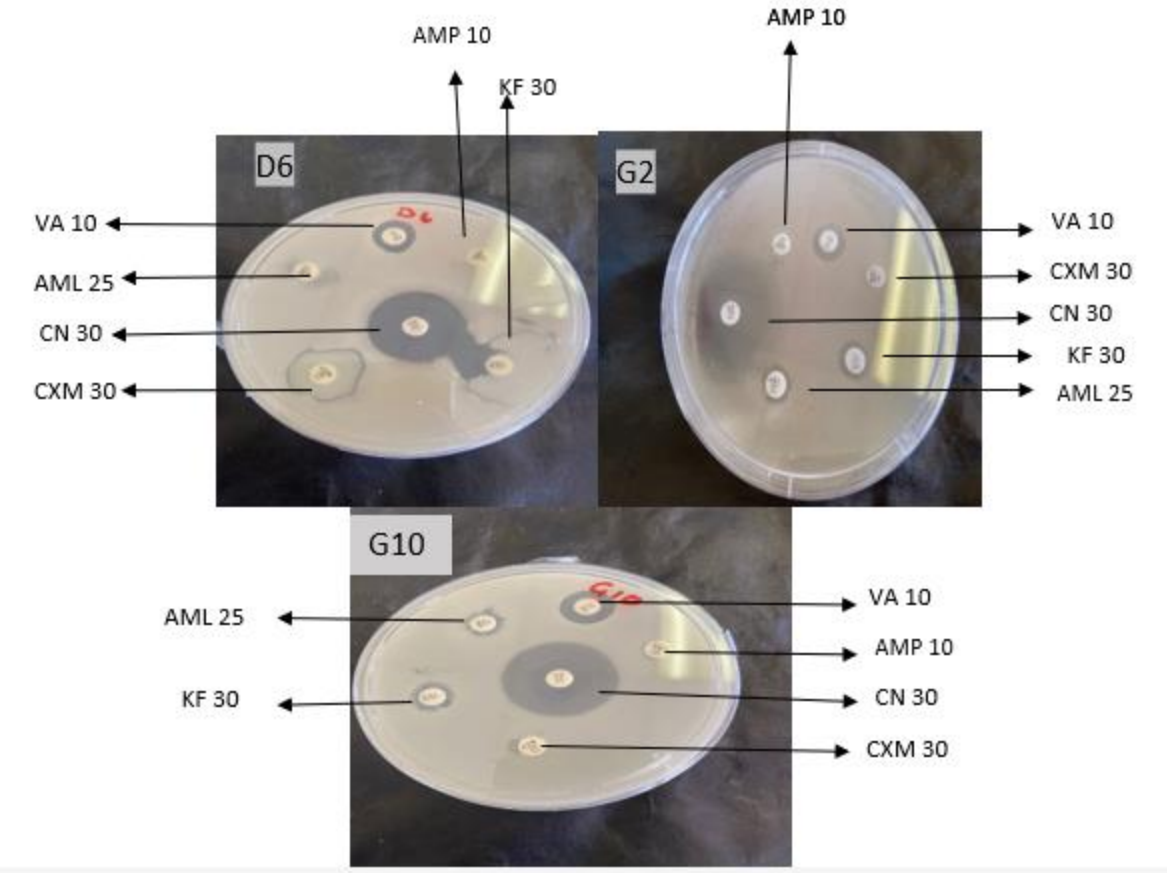

Figure 24: Zones of inhibition of the antibiotics against bacterial isolates of *Cruznema* NTM-2021 on MHA plates. The antibiotics used were: ampicillin 10 µg (**AMP**), gentamicin 30 µg (**CN**), cephalothin 30 µg (**KF**), cefuroxime/sodium 30 µg (**CXM**), vancomycin 10 µg (**VA**) and amoxicillin /clavulanic acid 25 µg (**AML**).

Interpretation of the results for antibiotics used was according to the criteria provided by the CLSI susceptibility breakpoints for Enterobacteriaceae. For *Elizabethkingia* isolates, we used the CLSI "non-Enterobacteriaceae" breakpoints. The breakpoint values of the antibiotics are shown in Table 7.

Table 7: Breakpoint values of the antibiotics used measured in millimeters. R: resistant, I: Intermediate, S: susceptible

Breakpoint values (mm)			
Antibiotics	Susceptible (S)	Intermediate (I)	Resistant (R)
Ampicillin	≥17	14-16	≤13
Gentamicin	≥15	13-14	≤12
Cephalothin	≥15	-	≤14
Cefuroxime	≥23	15-22	≤14
Amoxicillin	≥18	14-17	≤13

Vancomycin	≥17	15-16	≤14

Antibiotics used in this study were: ampicillin 10 µg (AMP), gentamicin 30 µg (CN), cephalothin 30 µg (KF), cefuroxime/sodium 30 µg (CXM), vancomycin 10 µg (VA) and amoxicillin /clavulanic acid 25 µg (AML). G10: *Elizabethkingia* Sp. G2: *Enterobacter* sp. D6: *Alcaligenes* sp.

A displayed in table 8, CN shows the largest zone of inhibition against all three isolates compared to the rest of the antibiotics, while VA showed moderate zones of inhibition. AMP and AML showed low zones of inhibitions. CXM and KF showed extremely small zones of inhibitions against all three isolates.

Table 8: Shows the averaged zones of inhibition measured in millimetres of the antibiotics against the *Alcaligenes*, *Enterobacter* and *Elizabethkingia* species.

Zones of inhibition (mm)						
Isolates	Antibiotics					
	VA	AML	AMP	CN	CXM	KF
G10	7	3	3	23	0	0
G2	6	9	0	22	0	0
D6	14	0	11	23	0	0

There was no significant difference among the means of the isolates. However, there was a significant difference between the mean of the antibiotics used. Gentamicin's mean value differed significantly from all other antibiotics against all isolates. As represented in figure 25, gentamicin was the most active antibiotic against all three isolates, while CXM and KF were the least active antibiotics.

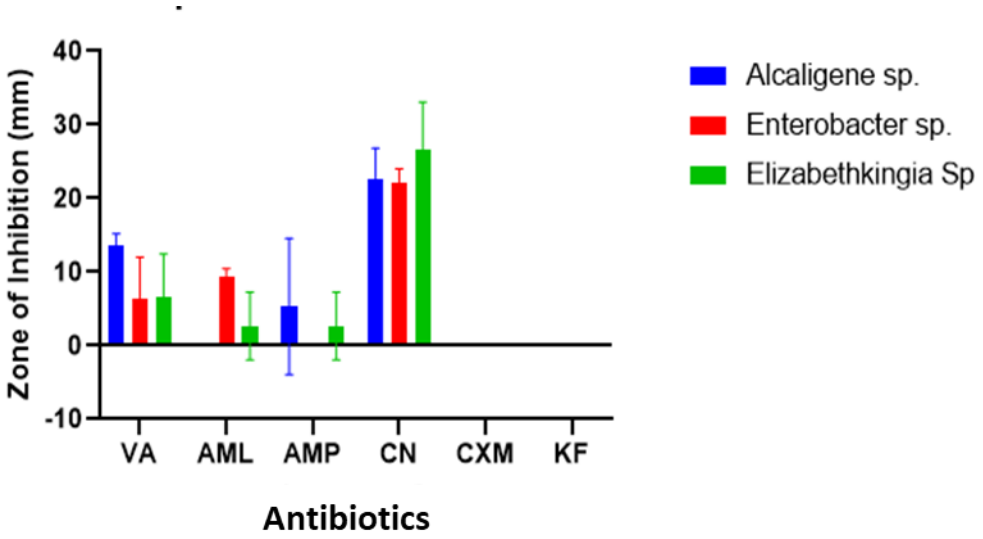

Figure 25: shows a bar graph of antibiotics against bacterial isolates. Error bars represent the standard deviation of the mean. Antibiotics: ampicillin 10 µg (AMP), gentamicin 30 µg (CN), cephalothin 30 µg (KF), cefuroxime/sodium 30 µg (CXM), vancomycin 10 µg (VA) and amoxicillin /clavulanic acid 25 µg (AML).

Discussions

Antibiotic resistance has become an emerging public health issue (Lancet, 2022). There are currently a limited number of effective antibiotics against antibiotic resistant bacteria (Nwobodo et al., 2022). Therefore, alternative or novel sources of antibiotics are required to tackle the issue of antibiotic resistance (Frieri et al., 2017). In the present study, the antimicrobial activity of bacteria isolates from *Cruznema* NTM-2021 was investigated. *Alcaligenes* sp. showed inhibitory activity against test pathogens than *Elizabethkingia* sp. and *Enterobacter* sp. (Hasan et al., 2019) reported that *Alcaligenes* sp. produce important compounds used as antimicrobial agents. Moreover, (Sharad et al., 2016) demonstrated that crude extracts of *A. faecalis* inhibited sulfate-reducing bacteria, indicating that *Alcaligenes* sp. have antimicrobial activity against other microorganisms. According to (Ilham et al., 2013) compounds extracted from *Alcaligenes* sp. have antagonistic effects against *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Mycobacterium smegmatis*. (Eltokhy et al., 2021) isolated an *A. faecalis* isolate from the soil and reported that the isolate produced secondary metabolites such as bacteriocins, bacillibactin, β-lactone, terpene that showed activity against *Klebsiella pneumoniae*, vancomycin-resistant *S.*

aureus, multidrug-resistant *Escherichia coli*, and moderate activity against *Candida albicans.* Additionally, another strain of *A. faecalis* also isolated from the soil demonstrated the ability to produce two antimicrobial compounds, Kalimantacin and tunicamycin, which exhibited inhibitory effects against *S. aureus*, *Bacillus subtilis*, *E. coli*, *K. pneumoniae*, and *P. aeruginosa* (Onajobi et al., 2020). Although the activity of our *Alcaligenes sp*. against *E. coli* was moderate and weak against *P. aeruginosa*, it shows that the isolate has the potential to produce antimicrobial compounds. Resistance was observed against *S. aureus* and *Enterococcus faecalis,* and this contradicts with previous studies that have demonstrated the inhibitory effect of *Alcaligenes sp.* against *S. aureus* (Eltokhy et al., 2021; Ilham et al., 2013; Onajobi et al., 2020). Given that *E. faecalis* also produces bacteriocin, it may be possible that it inactivates the function of bacteriocin via the production of bacteriocin-like inhibitory substances (Chen et al., 2007; Lajis, 2020).

Elizabethkingia sp. and *Enterobacter* sp. showed weak inhibition against *E. coli,* while there was no activity observed against *P. aeruginosa*. Furthermore, a weak zone of inhibition was observed around disks infused with *Elizabethkingia* sp. against *E. faecalis*. (Makuwa and Serepa-Dlamini., 2021) identified an *Enterobacter* species that produced benzyl benzoate, a compound known to have antibacterial activity against other pathogenic bacteria, but found that the compound had no effect on *E. coli, P. aeruginosa, S. aureus,* or *Klebsiella oxytoca*.. Although some inhibitory activity was observed on *Elizabethkingia* sp. against *E. coli*, we also report resistance of the above-mentioned pathogens to *Enterobacter* sp. compounds. To the best of our knowledge, there are no reports on the antimicrobial activity of compounds extracted from *Elizabethkingia* sp. against any of the test pathogens used in our study. Taken together, these indicate that bacterial isolates from *Cruznema* NTM-2021 have the potential to produce antimicrobial compounds that are active against *E. coli* and *E. faecalis*.

All three isolates showed susceptibility to gentamicin and resistance to all other antibiotics used in the study. Data obtained from this study is consistent with that of (Filipe et al., 2017; Mordi et al., 2013; Tena et al., 2015; ÜSTÜNTÜRK-ONAN, 2020), which showed that *Alcaligenes* sp. were susceptible to gentamicin but resistant to ampicillin, cefuroxime, and vancomycin. However, (Hasan et al., 2019) reported gentamicin resistance in *A. faecalis*. (Huang, 2020) reported that, in 2019, the susceptibility rate of *A. faecalis* to antibiotics was declining. The ability of the species to alter its genome to counteract the effects of an antibiotic or class of antibiotics that the bacteria

were previously sensitive to may be the cause of resistance (Deyno & Toma, 2015). (Lang et al., 2022) revealed that horizontal gene transfer and chromosomal gene mutations are two ways *A. faecalis* use to develop antibiotic resistance. Moreover, the inability of antibiotics such as vancomycin to penetrate the outer membrane barrier is the cause of antibiotic resistance in gram-negative bacteria (Arzanlou et al., 2017).

In the present study, *Enterobacter* species isolated from *Cruznema* NTM-2021 showed resistance to most antibiotics used and susceptibility to gentamicin. Findings of the present study are consistent with the findings of (Davin-Regli & Pagès, 2015; Hoffmann et al., 2005) which demonstrated the resistance of *Enterobacter ludwigii* and other *Enterobacter* species to ampicillin, amoxicillin and cefoxitin. Additionally, *Enterobacter ludwigii* strains were reported to be susceptible to gentamicin (Davin-Regli & Pagès, 2015; Hoffmann et al., 2005). Nevertheless, (Lykov & Volodkin, 2021) investigated the antibiotic sensitivity of various antibiotics against *Enterobacter aerogenes* and *A. faecalis* and discovered that the two species were resistant to both ampicillin and gentamicin. This contradicts our results, where susceptibility to gentamicin was observed for both bacterial species. The resistance of the *Enterobacter* sp. in our study may be due to the species' ability to produce beta-lactamase and form biofilms (Wagner et al., 2020). In addition, some *Enterobacter* sp. are reported to naturally produce insufficient amounts of the Bush group 1 natural inducible cephalosporinase, making it resistant to ampicillin, amoxicillin-clavulanic acid, cephalothin, and cefoxitin (class C) (Davin-Regli & Pagès, 2015). Furthermore, some members of the genus *Enterobacter* such as *Enterobacter Cloacae Complex* possess a special capacity to acquire genes encoding resistance to numerous types of antibiotics, including a range of carbapenemase genes (Annavajhala et al., 2019; Izdebski et al., 2015). Carbapenem-encoding genes are known for hydrolyzing nearly all β-lactam antibiotics, including carbapenems (Diene & Rolain, 2014). The results obtained in this study were expected as *Enterobacter sp*. is part of the ESKAPE group (*Enterococcus faecalis, Staphylococcus aureus, Klebsiella pneumonae, Acinetobacter baumannii, Pseudomonas aeruginosa, and Enterobacter* species), which is a leading cause of resistant infections across the globe (Annavajhala et al., 2019; Santajit & Indrawattana, 2016).

(Zdziarski et al., 2017) isolated a strain of *Elizabethkingia miricola* that was resistant to gentamicin. In contrast, (Frank et al., 2013) reported *Elizabethkingia* strain that was susceptible to

gentamicin. The findings correlated with our results which showed susceptibility of *Elizabethkingia* sp. to gentamicin. Resistance in *Elizabethkingia* sp. isolated in our study may be due to production of metallo-β-lactamases that inactivate the action of β-lactams as some multidrug resistant *E. miricola* strain was reported to produce β-lactamases (Colapietro et al., 2016). Species in the genus *Elizabethkingia* have shown to form biofilms, and this is the mechanism they use to resist antibiotics (Fritch et al., 2018; Jacobs & Chenia, 2011). Additionally, (Hu et al., 2022) demonstrated a positive correlation between biofilm formation and antibiotic resistance. We suggest that in addition to the production of beta-lactmase, our isolate be resisting antibiotic through the formation of biofilm. Previous studies have reported the susceptibility of *Elizabethkingia* species to vancomycin (Chawla et al., 2015; Lau et al., 2016; Perrin et al., 2017; Zajmi et al., 2022). However, we report resistance of *Elizabethkingia* sp. to vancomycin. Similar findings were made in a more recent investigation by (Burnard et al., 2020), where it was discovered that the isolated *Elizabethkingia* strain was vancomycin resistant. Resistance to vancomycin may be due to the ability of *Elizabethkingia* species to express vancomycin resistance gene (Liang et al., 2019). Moreover, resistance to vancomycin may be conferred by expression of efflux pumps in addition to production of β-lactmases and resistant genes (Chen et al., 2019; Liang et al., 2019). Microorganisms in the genus *Elizabethkingia* are susceptible to antibiotics from minocycline class but resistant to most β-lactams, β-lactam/β-lactam inhibitors, carbapenems, and aminoglycosides (Lin et al., 2018). *Elizabethkingia miricola* were reported to be susceptible to levofloxacin, moxifloxacin, and gatifloxacin (Han et al., 2016). Furthermore, (Burnard et al., 2020) suggested minocycline, levofloxacin and trimethoprim/sulfamethoxazole as the effective therapy against human infections caused by *Elizabethkingia* sp. It is worth noting that species of *Elizabethkingia* respond differently to various antibiotics, susceptibility is strain specific as some species of *Elizabethkingia* have shown to be susceptible to vancomycin (Hashmi et al., 2023).

Conclusion

The purpose of the present research was to investigate the antimicrobial activity of *Alcaligenes*, *Elizabethkingia*, and *Enterobacter* species against pathogenic bacteria and further test their antibiotic susceptibility to common antibiotics from different classes. *Alcaligenes sp*. showed some antimicrobial activity against *E. coli*, while *Enterobacter and Elizabethkingia* supernatant showed the least activity against all four test bacteria. Genetic studies, such as gene knockout experiments, are recommended to identify the genes that produce antimicrobial compounds in *Alcaligenes* sp.

All isolates were resistant to most antibiotics and susceptible to gentamicin. This indicates that *Cruznema* NTM-2021 harbors drug-resistant bacteria, and users such as farmers should handle the nematodes cautiously when applying them in the field to avoid exposure to the bacterial isolates. Furthermore, gentamicin is the recommended therapy. Avenues for future research include the extraction and identification of antimicrobial compounds produced by the isolated *Alcaligenes* sp. Microdilution assays are recommended for susceptibility determination. More investigations are required to better understand the mechanism of antimicrobial compounds produced by the *Alcaligenes* sp. isolated from *Cruznema* NTM-2021. Furthermore, antimicrobial compounds derived from this isolate should be tested against a wide range of pathogenic bacteria. Antibiotics from different classes should be used synergistically with other classes of antibiotics against *Alcaligenes, Elizabethkingia*, and *Enterobacter* species.

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Chapter 4: Conclusion and Recommendations

In this study, nematodes and their associated bacteria were isolated and identified. Identification was performed using molecular identification techniques, including genomic DNA extraction, PCR amplification, and DNA sequencing. A new species of *Cruznema* nematodes was identified based on its 18S rDNA gene and named *Cruznema* NTM-2021 (Accession number: 0Q408141). *Cruznema* NTM-2021 is hypothesized as an entomopathogenic nematode. Further research examining the pathogenicity of *Cruznema* against other insects, may shed light on its ability to be considered as a potential biological control agent. Genomic comparisons of *Cruznema* NTM-2021 with other EPNS and their symbiotic bacteria is also recommended.

Alcaligenes sp., *Enterobacter* sp., and *Elizabethkingia* sp. were isolated from *Cruznema* NTM and identified based on their 16S rDNA gene originality. Bacterial isolates of nematodes have been shown to be lethal to insects at very low concentrations. Bacterial isolates of *Cruznema* NTM-2021 are pathogenic to *Tenebrio Molitor*. However, it should be noted that the 72-hour period limits the accuracy of the data, and further tests should be done to confirm the pathogenicity of the isolates to *T. Molitor* and other insects.

The discovery of new antibiotics is an important factor in addressing the issue of antibiotic resistance, which is currently an emerging challenge in public health. To combat antibiotic resistance, new antimicrobial agents with novel modes of action are required. Different sources of antimicrobial agents have been explored, and of interest are sources of these antibiotics from natural sources such as nematodes and/or their symbionts. We investigated the potential of bacterial isolates from *Cruznema* NTM-2021 to produce antimicrobial agents active against a few antibiotic-resistant bacteria. Some inhibitory activity was observed around disks infused with supernatants of all three isolates; however, the most effective was *Alcaligenes* sp. One of the limitations of this study was using supernatants as antimicrobial agents, which may have contributed to low metabolite production. Further studies are needed to extract and identify antimicrobial metabolites produced by the bacterial isolates.

Given that the *Alcaligenes, Enterobacter,* and *Elizabethkingia* species cause infections in humans, we investigated their susceptibility to commonly used antibiotics. All three isolates were resistant

to most antibiotics but susceptible to gentamicin. Different concentrations of the antibiotics used in this study and other antibiotics reported to inhibit these bacterial isolates are recommended.

Appendix A: Chapter 2

Preparation of 0.1% sodium hypochlorite (NaClO) for sterilizing the infective juvenile

34ml distilled water.

1ml 3.5% NaClO.

Autoclave distilled water.

Mix NaClO and distilled water in a bottle.

Autoclave for 15 mins at 121˚C.

Extraction of Genomic DNA of *Cruznema* nematodes

E.Z.N.A.® Insect DNA Kit was used to extract DNA from the nematodes. Briefly, approximately 30 mg of the nematodes were pulverized using a sterile plastic pestle in a clean microcentrifuge tube. 350 µl of CTL buffer and 25 µl of proteinase K solution were added and vortexed. This was followed by overnight incubation at 37°C. The overnight incubation was to allow the sample to solubilize completely. 350µl of chloroform: isoamyl alcohol were added and vortexed to mix. The sample was centrifuged at 10,000 x g for 2 minutes at room temperature. The upper aqueous phase was transferred into a sterile 1.5-ml microcentrifuge tube; this was done with vigilance to avoid transferring contaminants and inhibitors from the milky phase. One volume of BL buffer and 2 µl RNase A were added and vortexed for 15 seconds. Following 10 minutes of incubation at 70°C, one volume of 100% ethanol was added and vortexed for 15 seconds. Approximately 750 µl of clear lysate was aspirated into a HiBind® DNA column that was inserted in a 2 ml collection tube. The sample was centrifuged at maximum speed for 1 minute. The HiBind® DNA column was transferred into a clean 2 ml collection tube. 500 µl HBC buffer was added, followed by 30 seconds of centrifugation at maximum speed. The filtrate was discarded, and 700 µl of DNA wash buffer was added. The sample was centrifuged at maximum speed for 1 minute, and the filtrate was discarded. A second DNA wash buffer was added, and the filtrate was discarded. The empty HiBind® DNA column was dried by centrifugation at maximum speed for 2 minutes. The HiBind® DNA mini column was transferred to a sterile 1.5 ml microcentrifuge tube. The quality or concentration of the DNA was measured using a spectrophotometer. Following extraction, the DNA products were sent for sequencing of the 18S rDNA region at Inqaba Biotechnological Industries (PTY) LTD.

Bacterial sequence of *Cruznema* NTM-2021

18S rDNA consensus sequence of *Cruznema* NTM-2021.

AGAGGATGGCGATGAATCGATGCACGTTGCTTTTGCGACGATACTTAATC
GCTCTGCCTGAGAGTGGCCTCGGATTGGTTTGGTGGCTTGGAAGCCTTGT
GCTTCTAGTCTTAATGAGATAGCTTGCTATCCGCCATCCAATCACTTCTTT
CATAACTGAGTTGGTAATCGAAACGTGACAGGCTCACGCTAAAAAGCCTA
CTAACTAGCATTAGCGATGGATCGGTTGATTTGGTCATCGATGAAAAACG
CAGCCAGCTGCGTTATTTACCACGAATCGCAAATTTGTAGAGTGGTAAAA
TTTTGAATGCATAGCGCCGAAGGGTATCATCCCTTCGGCACGCCTGGTTC
AGGGTTGTTTAATCGAATTCCGTCGACAGACAGTTGATGGATAGTCGAGG
GGCTGCGCTTCGGCGTACCCTCTCTGGTTTCGAGAACTATGTTCCTAGTGA
ACTGTGAATTCGCGTTTGCGTAGCCTCCATG

Appendix B: Chapter 3

Luria-Bertini (LB)(adapted from Macwilliams & Liao, 2016)

Tryptone	10g
Yeast extracts	5 g
NaCl	5 g
Glucose	1 g

Mix with 1L of distilled water and autoclave at 121˚C for 15 minutes.

Nutrient bromothymol Triphenyl tetrazolium Agar (NBTA) adapted from (AKHURST, 1980)

1 litre nutrient agar

Triphenyltetrazolium chloride (TTC)	0.04g
bromothymol blue (BTB)	0.025g

Mix the nutrient agar with BTB and autoclave at 121˚C for 15 minutes.

Add TTC to the mixture when the medium temperature is below 50˚C, just before dispensing into petri dishes. This is done to ensure that TTC does not break, since it breaks when the medium is too hot.

Gently swirl to mix the TTC.

Dispense into sterile petri dishes and leave to solidify under laminar flow.

MacConkey Agar (Commercially available)

Peptone	20 g
Bile salts	5 g
Lactose	10 g
Neutral red	0.075 g
Sodium chloride	15 g
Agar	12 g

Suspend 52 g of MacConkey powder in 1 litre of distilled water.

Bring to the boil to dissolve completely.

Sterilize by autoclaving at 121˚C for 15 minutes.

Let it cool, dispense into sterile petri dishes and leave it solidify.

Bacterial sequences of the *Alcaligenes* sp., *Enterobacter* sp., and *Elizabethkingia* sp.

GGTGAGCCCTGGGATTTCACATCTTTCTTTCCGAACCGCCTACACACGCTTT
ACGCCCAGTAATTCCGATTAACGCTTGCACCCTACGTATTACCGCGGCTGCT
GGCACGTAGTTAGCCGGTGCTTATTCTGSAGATACCGTCAGCAGTATCCCGT
ATTAGGGGATACCTTTTCTTCTCTGCCAAAAGTACTTTACAACCCGAAGGCC
TTCATCATACACGCGGGATGGCTGGATCAGGGTTTCCCCCATTGTCCAAAAT
TCCCCACTGCTGCCTCCCGTAGGAGTCTGGGCCGTGTCTCAGTCCCAGTGTG
GCTGGTCGTCCTCTCAAACCAGCTACGGATCGTTGCCTTGGTGAGCCTTTAC
CCCACCAACTAGCTAATCCGATATCGGCCGCTCCAATAGTGAGAGGTCTTG
CGATCCCCCCCTTTCCCCCGTAGGGCGTATGCGGTATTAGCCACTCTTTCGA
G

Figure 26 16S rDNA bacterial sequence of unknown isolate D6. The BLAST results revealed high similarity to *Alcaligenes* species.

TACTTCTTTTGCAACCCACTCCCATGGTGTGACGGGCGGTGTGTACAAGGCCC
GGGAACGTATTCACCGTAGCATTCTGATCTACGATTACTAGCGATTCCGACTT
CATGGAGTCGAGTTGCAGACTCCAATCCGGACTACGACGCACTTTATGAGGTC
CGCTTGCTCTCGCGAGTTCGCTTCTCTTTGTATGCGCCATTGTAGCACGTGTGT
AGCCCTACTCGTAAGGGCCATGATGACTTGACGTCATCCCCACCTTCCTCCAG
TTTATCACTGGCAGTCTCCTTTGAGTTCCCGGCCGGACCGCTGGCAACAAAGG
ATAAGGGTTGCGCTCGTTGCGGGACTTAACCCAACATTTCACAACACGAGCTG
ACGACAGCCATGCAGCACCTGTCTCAGAGTTCCCGAAGGCACCAATCCATCTC
TGGAAGTTCTCTGGATGTCAAGAGTAGGTAAGGTTCTTCGCGTTGCATCGAAT
TAAACCACATGCTCCACCGCTTGTGC

Figure 27 16S rDNA bacterial sequence of unknown isolate G2. The BLAST results revealed high similarity to *Enterobacter* species.

TACCGGCCATTGTAGCACGTGTGTGGCCCAAGACGTAAGGGCCGTGATGATTT
GACGTCATCCCCACCTTCCTCTCTACTTGCGTAGGSAGTCTCACTAGAGTCCTC
AACTTAATGTTAGCAACTAGTGACAGGGGTTGCGCTCGTTGCAGGACTTAACC
TAACACCTCACGGCACGAGCTGACGACAACCATGCAGCACCTTGAAAAATGT
CCGAAGAAGGATCTATTTCTAAATCTGTATTTCCCATTTAAGTCTTGGTAAGG
TTCCTCGCGTATCATCGAATTAAACCACATGCTCCACCGCTTGTGCGGGCCCC
CGTCAATTCCTTTGAGTTTCATTCTTGCGAACGTACTCCCCAGGTGGATTACTT
ATCACTTTCGCTTAGTCTCTGAATCCTAAAACCCAAAAACGAGTAATCATCGT
TACGGCGTGGACTACCAGGGTATCTAATCCTGGTTCGCTCCCCACGCTTTCGT
CCATCAGCGTCAG

Figure 28 Bacterial sequence of unknown isolate G10. The BLAST results revealed high similarity to *Elizabethkingia* species.

Appendix C: Chapter 4

Muller-Hinton Agar (g/L) (Commercially available)

Beef dehydrated infusion	0.3g
Starch	1.5g
Casein hydrolysate	17.5g
Agar	17g

Suspend 38g in 1 litre of distilled water.

Bring to the boil to dissolve completely.

Sterilize by autoclaving at 121 °C for 15 minutes.

Allow it to cool to 45-50 °C before dispensing into petri dishes and leave it solidify.

Tryptone Soy Broth (TSB)

Soybean-Casein Digest medium

Tryptone	17g
Soy peptone	3g
Sodium chloride	5g
di-Potassium hydrogen phosphate	2.5g
Dextrose	2.5g

Suspend 30 g of TSB powder in 1L of deionized water and mix well.

Sterilizer by autoclaving at 121˚C for 15 minutes.

Preparation of 0.5 MacFarland solution

1.175% of barium chloride ($BaCl_2$)
Weigh out 1.175 g of $BaCl_2$ and add it in 100ml volumetric flask.

Add 50 ml of deionized water and mix well to dissolve.

Bring to 100 ml with deionized water and mix well.

1% of Sulfuric acid (H_2SO_4)

Measure 100 ml of water in a flask and add 6.9 ml of sulfuric acid.

Fill the flask with 150 ml of water to make 250 ml of H_2SO_4.

Solution of 0.5 MacFarland standard

Measure 0.05 ml of 1.175% $BaCl_2$ and mix with 9.95 ml of 1% of sulfuric acid.

ANOVA results

Statistical analysis: ANOVA results for antimicrobial activity and antibiotic susceptibility

Antimicrobial activity

Table 9: Antimicrobial activity of bacterial isolates of *Cruznema* NTM-2021

Table Analyzed	Data 2
Two-way RM ANOVA	Matching: Both factors
Assume sphericity?	No
Alpha	0,05

Source of Variation	% of total variation	P value	P value summary	Significant?	Geisser-Greenhouse's epsilon
Row Factor	26,98	0,2956	ns	No	0,3577
Column Factor	0,8357	0,5850	ns	No	0,6781
Row Factor x Column Factor	22,71	0,1884	ns	No	0,3140
Subject x Row Factor	27,89				
Subject x Column Factor	3,269				
Subject	1,387				

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Row Factor	163,2	3	54,40	F (1,073, 2,146) = 1,934	P=0,2956
Column Factor	5,056	2	2,528	F (1,356, 2,712) = 0,5112	P=0,5850
Row Factor x Column Factor	137,4	6	22,90	F (1,884, 3,768) = 2,682	P=0,1884
Subject x Row Factor	168,7	6	28,12		
Subject x Column Factor	19,78	4	4,944		
Subject	8,389	2	4,194		
Residual	102,4	12	8,537		

Data summary	
Number of columns (Column Factor)	3
Number of rows (Row Factor)	4
Number of subjects (Subject)	3
Number of missing values	0

Antibiotic Susceptibility

Table 10: Statistical analysis for antibiotic susceptibility of bacterial isolates of *Cruznema* NTM-2021

Number of families	1				
Number of comparisons per family	153				
Alpha	0,05				
Tukey's multiple comparisons test	Mean Diff,	95,00% CI of diff,	Significant?	Summary	Adjusted P Value
VA:Alcaligene sp. vs. VA:Enterobacter sp.	7,333	-5,727 to 20,39	No	ns	0,7185
VA:Alcaligene sp. vs. VA:Elizabethkingia Sp	7,000	-6,060 to 20,06	No	ns	0,7752
VA:Alcaligene sp. vs. AML:Alcaligene sp.	13,67	0,6063 to 26,73	Yes	*	0,0345
VA:Alcaligene sp. vs. AML:Enterobacter sp.	4,333	-8,727 to 17,39	No	ns	0,9943
VA:Alcaligene sp. vs. AML:Elizabethkingia Sp	11,00	-2,060 to 24,06	No	ns	0,1623
VA:Alcaligene sp. vs. AMP:Alcaligene sp.	8,333	-4,727 to 21,39	No	ns	0,5347
VA:Alcaligene sp. vs. AMP:Enterobacter sp.	13,67	0,6063 to 26,73	Yes	*	0,0345
VA:Alcaligene sp. vs. AMP:Elizabethkingia Sp	11,00	-2,060 to 24,06	No	ns	0,1623
VA:Alcaligene sp. vs. CN:Alcaligene sp.	-9,000	-22,06 to 4,060	No	ns	0,4169
VA:Alcaligene sp. vs. CN:Enterobacter sp.	-8,333	-21,39 to 4,727	No	ns	0,5347
VA:Alcaligene sp. vs. CN:Elizabethkingia Sp	-13,00	-26,06 to 0,06039	No	ns	0,0519
VA:Alcaligene sp. vs. CXM:Alcaligene sp.	13,67	0,6063 to 26,73	Yes	*	0,0345
VA:Alcaligene sp. vs. CXM:Enterobacter sp.	13,67	0,6063 to 26,73	Yes	*	0,0345
VA:Alcaligene sp. vs. CXM:Elizabethkingia Sp	13,67	0,6063 to 26,73	Yes	*	0,0345
VA:Alcaligene sp. vs. KF:Alcaligene sp.	13,67	0,6063 to 26,73	Yes	*	0,0345
VA:Alcaligene sp. vs. KF:Enterobacter sp.	13,67	0,6063 to 26,73	Yes	*	0,0345
VA:Alcaligene sp. vs. KF:Elizabethkingia Sp	13,67	0,6063 to 26,73	Yes	*	0,0345
VA:Enterobacter sp. vs. VA:Elizabethkingia Sp	-0,3333	-13,39 to 12,73	No	ns	>0,9999
VA:Enterobacter sp. vs. AML:Alcaligene sp.	6,333	-6,727 to 19,39	No	ns	0,8721

VA:Enterobacter sp. vs. AML:Enterobacter sp.	-3,000	-16,06 to 10,06	No	ns	>0,9999
VA:Enterobacter sp. vs. AML:Elizabethkingia Sp	3,667	-9,394 to 16,73	No	ns	0,9991
VA:Enterobacter sp. vs. AMP:Alcaligene sp.	1,000	-12,06 to 14,06	No	ns	>0,9999
VA:Enterobacter sp. vs. AMP:Enterobacter sp.	6,333	-6,727 to 19,39	No	ns	0,8721
VA:Enterobacter sp. vs. AMP:Elizabethkingia Sp	3,667	-9,394 to 16,73	No	ns	0,9991
VA:Enterobacter sp. vs. CN:Alcaligene sp.	-16,33	-29,39 to -3,273	Yes	**	0,0064
VA:Enterobacter sp. vs. CN:Enterobacter sp.	-15,67	-28,73 to -2,606	Yes	**	0,0098
VA:Enterobacter sp. vs. CN:Elizabethkingia Sp	-20,33	-33,39 to -7,273	Yes	***	0,0005
VA:Enterobacter sp. vs. CXM:Alcaligene sp.	6,333	-6,727 to 19,39	No	ns	0,8721
VA:Enterobacter sp. vs. CXM:Enterobacter sp.	6,333	-6,727 to 19,39	No	ns	0,8721
VA:Enterobacter sp. vs. CXM:Elizabethkingia Sp	6,333	-6,727 to 19,39	No	ns	0,8721
VA:Enterobacter sp. vs. KF:Alcaligene sp.	6,333	-6,727 to 19,39	No	ns	0,8721
VA:Enterobacter sp. vs. KF:Enterobacter sp.	6,333	-6,727 to 19,39	No	ns	0,8721
VA:Enterobacter sp. vs. KF:Elizabethkingia Sp	6,333	-6,727 to 19,39	No	ns	0,8721
VA:Elizabethkingia Sp vs. AML:Alcaligene sp.	6,667	-6,394 to 19,73	No	ns	0,8269
VA:Elizabethkingia Sp vs. AML:Enterobacter sp.	-2,667	-15,73 to 10,39	No	ns	>0,9999
VA:Elizabethkingia Sp vs. AML:Elizabethkingia Sp	4,000	-9,060 to 17,06	No	ns	0,9976
VA:Elizabethkingia Sp vs. AMP:Alcaligene sp.	1,333	-11,73 to 14,39	No	ns	>0,9999

VA:Elizabethkingia Sp vs. AMP:Enterobacter sp.	6,667	-6,394 to 19,73	No	ns	0,8269
VA:Elizabethkingia Sp vs. AMP:Elizabethkingia Sp	4,000	-9,060 to 17,06	No	ns	0,9976
VA:Elizabethkingia Sp vs. CN:Alcaligene sp.	-16,00	-29,06 to -2,940	Yes	**	0,0079
VA:Elizabethkingia Sp vs. CN:Enterobacter sp.	-15,33	-28,39 to -2,273	Yes	*	0,0121
VA:Elizabethkingia Sp vs. CN:Elizabethkingia Sp	-20,00	-33,06 to -6,940	Yes	***	0,0006
VA:Elizabethkingia Sp vs. CXM:Alcaligene sp.	6,667	-6,394 to 19,73	No	ns	0,8269
VA:Elizabethkingia Sp vs. CXM:Enterobacter sp.	6,667	-6,394 to 19,73	No	ns	0,8269
VA:Elizabethkingia Sp vs. CXM:Elizabethkingia Sp	6,667	-6,394 to 19,73	No	ns	0,8269
VA:Elizabethkingia Sp vs. KF:Alcaligene sp.	6,667	-6,394 to 19,73	No	ns	0,8269
VA:Elizabethkingia Sp vs. KF:Enterobacter sp.	6,667	-6,394 to 19,73	No	ns	0,8269
VA:Elizabethkingia Sp vs. KF:Elizabethkingia Sp	6,667	-6,394 to 19,73	No	ns	