

https://dspace.org/

Genomic characterisation of possible symbiotic pathogenic bacteria species isolated from undescribed Cruznema nematodes bacterivore

Sekgobela, Pusang King

2024-08

University of the Witwatersrand, Johannesburg

https://hdl.handle.net/10539/46609

Sekgobela, Pusang King. (2024). Genomic characterisation of possible symbiotic pathogenic bacteria species isolated from undescribed Cruznema nematodes bacterivore. [Master's dissertation, University of the Witwatersrand, Johannesburg]. WIReDSpace. https://hdl.handle.net/10539/46609

Downloaded from a DSpace repository. https://dspace.org/



Genomic characterisation of possible symbiotic pathogenic bacteria species isolated from undescribed *Cruznema* nematodes bacterivore

By

Pusang King Sekgobela

Student no. 2620888

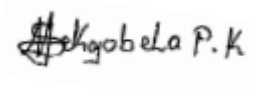
Supervisor: Dr Tiisetso Elizabeth Lephoto

University of Witwatersrand

A dissertation submitted to the faculty of science under the School of Molecular and Cell Biology in fulfilment of the requirements for the degree of Master of Science

Declarations

I, Pusang King Sekgobela, declare that this dissertation is my own work. Therefore, this dissertation is submitted in fulfilment of Master of Science degree at the University of Witwatersrand, Johannesburg. Furthermore, it has not been submitted before for any degree or examination at any university.



Mr Pusang King Sekgobela

Student no. 2620888

21 August 2024

Dedications

I dedicate this dissertation to my parents (Mr Joseph Thomas Sekgobela and Mrs Vulani Betty Sekgobela) and my siblings (Mr Pusiso Respect Sekgobela, Miss Puso Patience Sekgobela and Miss Pusile Peaceful Sekgobela).

Abstract

Pest infestation has devastating effects towards food security and gross domestic product (GDP) of the country. The use of synthetic chemical pesticides is common throughout the world, and it believe to be an ultimate solution for pest attacks on farms. However, the long use of these synthetic pesticides has led to environmental and human health hazard. Entomopathogenic nematodes (EPNs) are microscopic insect parasitic worms that are found in different ecological niche and classified as biological agent. EPNs have symbiotic relationship with pathogenic bacteria from a phylum of *Pseudomonadota,* a class *of Gammaproteobacteriar.* These bacteria synthesize bio-active toxic compounds which are responsible for killing the insect host. *Cruznema* nematodes belong to the phylum order *Rhabditida* as EPNs. This study explores the possible symbionts bacteria of *Cruznema sp.* NTM 2021 and their genomic make-up. Molecular identification of *Cruznema* nematodes was performed, which involves DNA extraction, PCR, DNA purification and 18S rDNA sanger sequencing. Furthermore, *Cruznema sp.* NTM 2021 gut microbiome was explored through metagenomics sequencing using PacBio sequencer and the results showed that *Pseudomonadales* dominated with 40 %, *Hyphomicrobiales* 23 % and *Bacteroidetes* 22 %. Moreover, two novel bacteria (namely *Enterobacter sp.* PTB and *Pseudomonas sp.* PKS) were isolated from *Cruznema sp.* NTM 2021 and pass a symbiosis test for *Cruznema sp.* NTM 2021. Both *Enterobacter sp.* PTB and *Pseudomonas sp.* PKS *Cruznema* bacteria symbionts were assessed for their pathogenic against *Tenebrio Molitor* (*T. molitor*), and both bacteria were found to be pathogenic. Furthermore, both bacteria were found to produce metabolites.The metabolites were checked for their effectiveness as antimicrobial agents against common antibiotic resistant bacteria (namely *Enterococcus faecalis, Pseudomonas aeruginosa, Escherichia coli, Staphylococcus aureus*). The metabolites produce by both bacteria were found to be bacteriostatic against all four antibiotic resistant bacteria. The metabolites identification of both *Cruznema* bacteria symbionts was performed using high pressure liquid chromatography (HPLC). Further five metabolites were found to be produced by *Enterobacter sp.* PTB meanwhile 15 metabolites were identified to be produced by *Pseudomonas sp.* PKS. Whole genome sequencing and annotation of both *Enterobacter sp.* PTB and *Pseudomonas sp.* PKS was achieved through sequencing the genomic DNA of each bacterium separately by Illumina Miseq. The quality control was conducted with the aid of FastQC and Trimmomatric. The assembling of genomic reads was conducted using *de novo* method on SPADES tool and annotation was conducted using RAST tool. *Enterobacter sp.* PTB genome is comprise of 5 609 562 bp, 54.2 % GC content, 76 RNAs, 572 subsystems whereas *Pseudomonas sp.* PKS

genome is comprise of 5 660 896 bp, 54.2 % GC content, 75 RNAs, 569 subsystems. However, Antismash tool reveals that both bacteria species consist of 5 secondary metabolites gene cluster which produce compound such as NI-siderophore, Thiopeptide, Arylpolyne, NRP-metallophore, Hserlactone.

Keywords: Chemical pesticides, *Gammaproteobacteriar,* Metabolites, *Cruznema* nematodes, *Pseudomonadales.*

Acknowledgment

I thank God for the strength He gave me to further my studies I wish to express my sincere gratitude to my supervisor Dr Tiisetso Elizabeth Lephoto for her supervision, mentorship, patience, and guidance throughout the project.

I also acknowledge:

- The University of Witwatersrand Research Committee for my research funding
- The University of Witwatersrand work study program for tuition and accommodation funding

I also want to thank Professor Timothy Sibanda, Dr Tshimangadzo Ndiitwani and Mr Oboikanyo Austin Mokoka for their assistances and guidance. I want to extend my gratitude to the Nematology research team at the University of Witwatersrand for their support.

Table of Contents

Declarations i

Dedications ii

Abstract iii

Acknowledgment v

List of Figures xi

List of Tables xv

List of Abbreviation xvi

Chapter 1 1

Literature review 1

1.1 Introduction 1

1.1.1 Nematodes 1

1.1.2 Entomopathogenic nematodes 2

1.1.3 EPNs Life cycle 2

1.1.4 Mutualistic benefits among EPNs and endopathogenic bacteria association 3

1.1.5 Metabolites production by EPNs bacteria symbionts 4

1.1.6 Application of EPNs as biological control agent 5

1.1.7 Possible strategies to upgrade EPNs efficacy 6

1.1.8 Importance of decrypting the individual gene of EPNs and their bacteria symbiont to upgrade EPNs efficacy 7

1.1.9 Research motivation 9

1.1.10 Aim and objectives 9

1.1.10.1 Aim(s) 9

1.1.10.2 Objectives 10

1.1.11 References 11

Chapter 2 21

Pathogenicity and molecular identification of *Cruznema sp*. NTM 2021 21

Abstract 21

2.1 Introduction 22

2..1.1 Pest infestation in agriculture 22

2.1.2 The effects of chemical pesticides practise in horticulture 22

2.1.3 Biological control 22

2.1.5 Entomopathogenic nematodes biological control agent 23

2.2 Method and Materials 24

2.2.1 Surface sterilization of *Cruznema* nematodes 24

2.2.3 Koch's Postulate 24

2.2.4 White trap technique 25
2.2.5 Genomic DNA extraction and Polymerase chain reaction 26
2.2.6 Molecular identification and Phylogenetic Construction of *Cruznema sp.* NTM 2021 27
2.2.6.1 Genomic sequencing using Sanger sequencing technique 27
2.2.6.2 Quality control 27
2.2.6.3 Multiple sequence alignment using CLUSTALW 27
2.2.6.4 Evolutionary divergence estimation 27
2.2.6.5 Phylogenetic tree construction 27
2.3 Results 28
2.4 Discussions 29
2.5 Conclusions and recommendations 31
2.6 References 32
Chapter 3 35
16S RDNA metagenomic amplicon-based profiling of endobacteria community of *Cruznema sp*. NTM 2021 35
Abstract 35
3.1 Introduction 36
3.1.1 Application of entomopathogenic nematode in agriculture 36
3.1.2 Entomopathogenic nematodes overview 36
3.1.3 Next generation sequencing 37
3.1.4 *Cruznema* nematodes overview 38
3.2 Methods and materials 38
3.2.1 Culturing of *Cruznema sp.* NTM 2021 38
3.2.2 Preparation of *Cruznema sp*. NTM metagenomic samples 38
3.2.3 16S RNA amplicon metagenomics analysis for *Cruznema sp.* NTM 2021 39
3.3 Results 39
3.4 Discussion 49
3.5 Conclusion 52
3.6 References 53
Chapter 4 58
Isolation and Molecular characterisation of *Pseudomonas* sp. PKS and *Enterobacter sp.* PTB gut *Cruznema sp.* NTM 2021 bacteria 58
Abstract 58
4.1 Introduction 59
4.1.1 Entomopathogenic nematodes 59
4.1.2 Overview of entomopathogenic nematodes bacteria symbionts taxonomy 59

4.1.3 Entomopathogenic nematodes bacterial symbionts life cycle 59
4.1.4 Bacteria pathogenicity within the insect host 60
4.1.5 Evolutionary relationship entomopathogenic nematodes bacteria symbionts 61
4.1.6 The use of 16S RDNA to identify bacteria species 61
4.2 Method and materials 62
4.2.1 Isolation of *Cruznema* sp. NTM 2021 gut bacteria 62
4.2.2 Bacterial Gram Staining 62
4.2.3 16S RNA sequencing of *Cruznema sp.* NTM 2021 nematode bacteria isolates 62
4.2.4 Molecular identification and Phylogenetic Construction 63
4.2.4.1 Quality control 63
4.2.4.2 Multiple sequence alignment using CLUSTALW 63
4.2.4.3 Phylogenetic tree construction 63
4.3 Results 64
4.3.1 Examining the cell wall of *Cruznema* bacteria isolates 64
4.3.2 Isolation of endobacteria from *Cruznema sp.* NTM 2021 64
4.5 Conclusion 72
4.6 Reference 73
Chapter 5 78
Pathogenicity and symbiosis assessment of gut *Cruznema sp.* NTM 2021 bacteria isolate (*Pseudomonas sp.* PKS and *Enterobater sp.* PTB) 78
Abstract 78
5.1 Introduction 79
5.1.1 Overview of Phylum *Nematoda* 79
5.1.2 Classification of nematodes into the feeding trophic groups 79
5.1.3 Entomopathogenic nematodes overview 81
5.2 Methodology 83
5.2.1 *Cruznema* endobacteria symbionts pathogenicity test against *Tenebrio molitor* (*T. molitor*) larvae 83
5.2.2 *Cruznema sp.* NTM 2021 gut endobacteria symbiosis test 83
5.3 Results 83
5.3 Discussion 85
5.4 Conclusion and future recommendations 87
5.5 References 88
CHAPTER 6 92
Investigating the effects of *Cruznema sp*. NTM 2021 endopathogenic bacteria symbiotic (*Enterobacter sp.* PTB and *Pseudomonas sp.* PKS) intracellular metabolites against known ATCC antibiotic resistant bacteria 92

Abstract 92
6.1 Introduction 94
6.1.1 Entomopathogenic nematodes 94
6.1.2 Entomopathogenic nematodes infection mechanisms against insect host 94
6.1.3 Metabolites 96
6.1.4 Screening of Bioactive compounds 96
6.2 Methodology 97
6.2.1 Metabolites production of *Cruznema sp.* NTM 2021 endopathogenic bacteria isolates 97
6.2.2 High pressure liquid chromatography to identify metabolites produced by *Cruznema sp.* NTM 2021 endopathogenic bacteria isolates 97
6.2.3 Screening antimicrobial activity using minimal inhibition assay 98
6.3 Results 99
6.4 Discussion 104
6.5 Conclusion 107
6.6 References 108
Chapter 7 113
Whole Genome sequencing and annotation of *Pseudomonas* sp. PKS isolated from *Rhabditida: Cruznema* entomopathogenic nematodes 113
Abstract 113
7.1 Introduction 114
7.1.1 Entomopathogenic nematode overview 114
7.1.2 Entomopathogenic nematodes bacteria associates 115
7.1.3 *Cruznema* nematodes 115
7.1.4 *Pseudomonas* bacteria 115
7.2 Methods and materials 116
7.2.1 DNA extraction and purification of *Pseudomonas sp.* PKS 116
7.2.2 Whole genome sequencing of *Pseudomonas sp.* PKS 116
7.2.3 Quality assessment and control 116
7.2.4 Genome assembly 116
7.2.5 Whole genome annotation 116
7.2.6 Identification of genes producing secondary metabolites 117
7.3 Results 117
7.4 Discussions 130
7.5 Conclusion and recommendations 131
7.6 Reference 132
Chapter 8 134

Whole genome sequencing and annotation of *Enterobacter sp*. PTB isolated from *Cruznema* nematode (*Rhabditida*) 134
Abstract 134
8.1 Introduction 135
8.1.1 Entomopathogenic overview 135
8.1.2 Enterobacteriaceae family overview 135
8.1.3 Next generation sequencing 135
8.2 Methodology 136
8.2.1 DNA extraction and purification of *Enterobacter sp*. PTB 136
8.2.2 Whole genome sequencing of *Pseudomonas sp.* PKS 136
8.2.3 Quality assessment and control 136
8.2.4 Genome assembly 136
8.2.5 Whole genome annotation 137
8.2.6 Identification of genes producing secondary metabolites 137
8.3 Results 137
8.4 Discussions 149
8.5 Conclusion and recommendation 150
8.6 Reference 151
Chapter 9 153
Structural and functional characterization of 16 kDa heat shock protein A and B 153
Abstract 153
Introduction 154
9.1.1 Entomopathogenic nematodes applications and efficacy 154
9.1.2 Genetic manipulation to improve EPNs field efficacy 154
9.1.3 Molecular chaperones 155
9.1.4 16 kDa heat shock protein A and B isolated from EPNs bacteria symbionts 155
9.2 Methodology 155
9.3 Results 156
9.4 Discussions 160
9.5 Conclusions and recommendations 163
9.6 References 165
Appendix A: Supplementary data 167

List of Figures

Figure 1.1: Schematic diagram elucidating EPNs life cycle……...…….……...3

Figure 1.1.7: Schematic diagram elucidating various routes toward enhancement of EPNs field efficacy……6

Figure 2.1: Schematic diagram showing entomopathogenic nematodes life cycle. Infective juveniles search for the host insect…….……24

Figure 2.2: Image showing a prepared Petri dish during Koch's postulate……...…….25

Figure 2.3: Elucidating a white trap technique of isolating nematodes infective juveniles…...……..26

Figure 2.4: Image showing symptoms of *Cruznema sp.* NTM 2021 infection on a) a healthy *T. molitor* B) an infected *T. molitor*……..…….…..……28

Figure 2.5: Elucidating 18S RDNA evolutionary relationship of *Cruznema sp.* NTM 2021. There phylogenetic tree has a scale of 0.2 evolutionary lineage over time……..…..29

Figure 3.1: A schematic diagram demonstrating natural life cycle of entomopathogenic nematodes (EPNs)……37

Figure 3.2: Elucidating taxonomic information and relative frequency of untreated *Cruznema* 16S RNA metagenomic sample……..…...40-43

Figure 3.3: Showing endobacteria community of *Cruznema* nematode taxonomic information with the relative frequency, 16S RNA amplicon metagenomic based (Treated sample)…….…….……..44-47

Figure 3.4: Depicting taxonomic relative abundance of *Cruznema* gut 16S RNA amplicon-based metagenomics. This data was obtained through the utilization of Krona version 1.9.0 with the aid of National canter for biotechnology information reference database…….…...48

Figure 4.1: Life cycle of entomopathogenic nematodes symbionts bacteria……...……...…..60

Figure 4.2: Image depicting gram staining of A) *Pseudomonas sp*. PKS and *Enterobacter sp.* PTB……64

Figure 4.3: Elucidating morphology of *Cruznema sp.* NTM 2021 bacteria isolates a) Phase I of *Pseudomonas sp.* PKS vs *Enterobacter sp.* PTB and and B) Phase II of *Pseudomonas sp.* PKS vs *Enterobacter sp.* PTB……....66-67

Figure 4.4: Elucidating 16S RDNA evolutionary relationship of A) *Pseudomonas sp.* PKS and B) *Enterobater sp.* PTB endopathogenic *Cruznema sp.* NTM 2021 bacteria symbionts……..…...69

Figure 5.1: Displaying nematodes structural mouthparts based on their feeding preference……..…....81

Figure 5.2: Elucidating the transmission of symbiotic bacteria inside nematodes……...…..82

Figure 5.3: depicting of *Cruznema* endobacteria symbiosis test conducted in lipid agar plates…..…...84

Figure 5.4: Elucidating mortality rate of *Cruznema* endosymbionts infections in *Tenebrio molitor* larvae. The *T. molitor* larvae was either infected with *Enterobacteria sp*. PTB, *Pseudomona sp.* PKS or *Enterobacteria sp*. PTB-*Pseudomona sp.* PKS……...85

Figure 6.1 Schematic diagram depicting life cycle of entomopathogenic nematodes…….....95

Figure 6.2: Represent distribution of metabolites concentration of either *Pseudomonas sp.* PKS or *Enterobacter sp.* PTB during the MIC (Minimum inhibition concentration) assay……....99

Figure 6.3: A chromatogram elucidating metabolites detection peaks of *Enterobacter sp.* PTB by high pressure liquid chromatograph (HPLC) at the absorbance of 254 nm for 15 minutes……..……...……100

Figure 6.4 A chromatogram elucidating metabolites detection peaks of *Pseudomonas sp.* PKS by high pressure liquid chromatograph (HPLC) at the absorbance of 254 nm for 15 minutes…...……...…101

Figure 6.5: Elucidating the effects of either (A) *Pseudomonas sp.* PKS and (B) *Enterobacter sp.* PTB metabolites against common antibiotic resistant bacteria…….....103

Figure 7.1: Schematic diagram of entomopathogenic nematodes life cycle……..……..114

Figure 7.2: showing the command used to assembly the whole genome sequences….……..116

Figure 7.3: An overview diagram of *Pseudomonas sp.* PKS subsystem categories into their correspondent genes annotated by RAST server…….....118

Figure 7.4: A) Genome alignment of *Pseudomonas sp.* PKS against *Pseudomonas aeruginosa* PAO1 (*Pseudomonas aeruginosa* PAO1 was used as a reference genome)…….129

Figure 7.5: Blast dot plot of *Pseudomonas sp.* PKS genome aligned against *Pseudomonas aeruginosa* PAO1 genome……..130

Figure 8.1: showing the command used to assembly the whole genome sequences……..137

Figure 8.2: An overview diagram of *Enterobacter sp*. PTB subsystem categories into their correspondent genes annotated by RAST server……...139

Figure 8.3: Genome alignment of *Enterobacter sp*. PTB against *Enterobacter sp*. 638 (*Enterobacter sp*. 638 was used as a reference genome)……..148

Figure 8.4: Blast dot plot of *Enterobacter sp*. PTB genome aligned against *Enterobacter sp*. 638 genome…….149

Figure 9.1: 3D secondary structure of 16 kDa A) heat shock protein A and B) heat shock protein B)……..156

Figure 9.2: Ramachandran plot of secondary structure of 16 kDa A) heat shock protein A and B) heat shock protein B…….157

Figure 9.3: Functional characterisation of 16 kDa A) heat shock protein A and B) heat shock protein B……...…158

Figure 9.4: A schematic diagram of 16 kDa heat shock protein A……...159

Figure 9.5: A schematic diagram of 16 kDa heat shock protein B……...160

Figure A1: Graphical representation of per base sequence quality of *Pseudomonas sp*. PKS A) Prio to trimming by Trimmomatic tool B) Post trimming with Trimmomatic……..169

Figure A2: Graphical representation of per tile sequence quality of *Pseudomonas sp*. PKS A) Prio to trimming by Trimmomatic tool B) Post trimming with Trimmomatic……..…169

Figure A3: Graphical representation of quality score distribution over all sequences of *Pseudomonas sp*. PKS A) Prio to trimming by Trimmomatic tool B) Post trimming with Trimmomatic……170

Figure A4: Graphical representation of per base sequence content of of *Pseudomonas sp*. PKS. PKS A) Prio to trimming by Trimmomatic tool B) Post trimming by Trimmomatic ……170

Figure A5: Graphical representation of per base sequence content of *Pseudomonas sp*. PKS A) Prio to trimming by Trimmomatic tool B) Post trimming by Trimmomatic…….171

Figure A6: Graphical representation of per base N content A) Prio to trimming by Trimmomatic tool B) Post trimming by Trimmomatic……...171

Figure A7: Elucidating the *Pseudomonas sp.* PKS distribution of sequence lengths over all sequences A) Prio to trimming by Trimmomatic tool B) Post trimming by Trimmomatic……..……..172

Figure A8: Elucidating the overrepresented sequence(s) A) Prio to trimming by Trimmomatic tool B) Post trimming by Trimmomatic……172

Figure A9: Graphical representation of sequence duplication levels of *Pseudomonas sp.* PKS A) Prio to trimming by Trimmomatic tool B) Post trimming by Trimmomatic…….172

Figure A10: Graphical representation of sequence adapter content A) Prio to trimming by Trimmomatic tool B) Post trimming with Trimmomatic…….173

Figure A11: Graphical representation of per base sequence quality of *Enterobacter sp.* PTB A) Prio to trimming by Trimmomatic tool B) Post trimming with Trimmomatic……..173

Figure A12: Graphical representation of per tile sequence quality of *Enterobacter sp.* PTB A) Prio to trimming by Trimmomatic tool B) Post trimming with Trimmomatic……..174

Figure A13: Graphical representation of quality score distribution over all sequences of *Enterobacter sp.* PTB A) Prio to trimming by Trimmomatic tool B) Post trimming with Trimmomatic……174

Figure A14: Graphical representation of per base sequence content of *Enterobacter sp.* PTB A) Prio to trimming by Trimmomatic tool B) Post trimming by Trimmomatic…….175

Figure A15: Graphical representation of per base sequence content of *Enterobacter sp.* PTB A) Prio to trimming by Trimmomatic tool B) Post trimming by Trimmomatic…….175

Figure A16: Graphical representation of per base N content of *Enterobacter sp.* PTB A) Prio to trimming by Trimmomatic tool B) Post trimming by Trimmomatic…….176

Figure A17: Elucidating the *Enterobacter sp.* PTB distribution of sequence lengths over all sequences A) Prio to trimming by Trimmomatic tool B) Post trimming by Trimmomatic ……...…….176

Figure A18: Elucidating the overrepresented sequence(s) of *Enterobacter sp.* PTB A) Prio to trimming by Trimmomatic tool B) Post trimming by Trimmomatic……176

Figure A19: Graphical representation of sequence duplication levels of *Enterobacter sp.* PTB A) Prio to trimming by Trimmomatic tool B) Post trimming by Trimmomatic…….177

Figure A20: Graphical representation of sequence adapter content of *Enterobacter sp.* PTB A) Prio to trimming by Trimmomatic tool B) Post trimming with Trimmomatic……..177

Figure A21: Elucidating the contig length in Quast report for assembly for A) *Enterobacter sp.* PTB B) *Pseudomona sp.* PKS……...178

Figure A22: Elucidating the contig cumulative length in Quast report for assembly for A) *Enterobacter sp.* PTB B) *Pseudomona sp.* PKS…….178

Figure A23: Line graph showing the GC content in Quast report for assembly for A) *Enterobacter sp.* PTB B) *Pseudomona sp.* PKS……...179

Figure A24: Histogram graph showing the GC content in Quast report for assembly for A) *Enterobacter sp.* PTB B) *Pseudomona sp.* PKS…….179

Figure A25: Line graph showing the contigs coverage in Quast report for assembly for A) *Enterobacter sp.* PTB B) *Pseudomona sp.* PKS…….....180

Figure A26: histogram graph showing the contigs coverage in Quast report for assembly for A) *Enterobacter sp.* PTB B) *Pseudomona sp.* PKS……180

Figure A27: Secondary structure showing disordered sequence, alpha helix and beta sheets of A) 16 kDa heat shock protein A B) 16 kDA heat shock protein B……...181

Figure A28: Tamplete alignment used to model secondary structure of A) 16 kDa heat shock protein A B) 16 kDA heat shock protein B……182

Figure A29: Elucidating the 16 kDa heat shock protein A functional interaction partners……...183

Figure A30: Elucidating the 16 kDa heat shock protein B functional interaction partners……...183

List of Tables

Table 2.1: Matrix table elucidating evolutionary divergence prediction by CLUSTALW…….....29

Table 4.1: Entomopathogenic nematodes bacteria symbiont morphology at different phase arcording to Kaya and Stock, (1997)……..……65

Table 4.2: Matrix table elucidating evolutionary divergence prediction by Pairwise distance A) *Pseudomonas sp*. PKS and B) *Enterobacter sp.* PTB……..…68

Table 6.1: Showing the names of *Enterobacter sp.* PTB detected metabolites with their respective retention time……100

Table 6.2: Showing the names of *Pseudomonas sp.* PKS detected metabolites with their respective retention time……101-102

Table 6.3: Examining the bacteria recovery status of common antibiotic resistant bacteria after being exposed to minimal inhibition concentration (MIC) of either *Enterobacter sp*. PTB, or *Pseudomonas sp.* PKS intracellular metabolites……104

Table 7.1: Quality assessment of the assembly of *Pseudomona sp.* PKS. (Please note the supplementary data is available in Figure A21-26)……...117-118

Table 7.2: Overview of *Pseudomonas sp.* PKS whole genome annotation by RAST…….118

Table 7.3: Elucidating genes found in the subsystem (Virulence, disease and defence) *Pseudomonas sp.* PKS……..119-127

Table 7.4: Identification of genes involve in secondary metabolites production using Antismash software……128

Table 8.1: Quality assessment of the assembly of *Enterobacter sp.* PTB. (Please note the supplementary data is available in Figure A 21-26)……..……...137-138

Table 8.2: Overview of *Enterobacter sp*. PTB whole genome annotation by RAST…….138

Table 8.3: Elucidating *Enterobacter sp*. PTB genes that are clustered in subsystem (Virulence, disease, and defence)…….…….139-147

Table 8.4: Identification of *Enterobacter sp.* PTB genes producing secondary metabolites using Antismash program……...147-148

Table A1: 2-way ANOVA statistical analysis of endo-pathogenic bacteria symbionts isolates from *Cruznema sp*. NTM 2021……..167-169

List of Abbreviation

BLAST – Basic local Alignment Search Tool

DNA – Deoxyribonucleic Acid

EPNs – Entomopathogenic nematodes

HPLC – High Pressure Liquid Chromatography

IJ – Infective Juveniles

INT dye – p-Iodonitrotetrazolium violet dye

MEGA – Molecular Evolutionary Genetics Analysis

NBT - Nitro-blue tetrazolium

NBTA – Nutrient Bromothymol Blue Agar

PCR – Polymerase Chain Reaction

Qiime – Quantitative Insights Into Microbial Ecology

Quast – Quality Assessment Tool

RNA – Ribonucleic Acid

TTC - 2,3,5-Triphenyl tetrazolium chloride

Chapter 1

Literature review

1.1 Introduction

1.1.1 Nematodes

Nematodes are small, microscopic organisms originating from phylum *Nematoda* that are commonly found in soil (Moosavi and Zare, 2020). Nematodes are one of the ubiquitous species in the ecological niche, that has peculiar features considering their small size (Huang *et al.,* 2022). Nematodes are multicellular organisms with the following features: non-segmented, triploblastic, bilaterally symmetrical and with pseudocoelomic cavity (Decraemer *et al.,* 2013). Nematodes has tubular body with two openings at the anterior and sub-terminal in the posterior (Huang *et al.,* 2022). Furthermore, both the head and mouth of the nematodes are radial shape (Decraemer *et al.,* 2013; Schafer *et al.,* 2016). The nematodes mouth consists of four to six lips that has papillae, which is used as taxonomic attribute (Schafer *et al.,* 2016). There are three nematode functional groups namely predaceous, saprophytic, and parasitic nematodes (Moosavi and Zare, 2020; Yadav *et al.,* 2023). Predaceous nematodes belong to phylum order *Mononchida* and *Dorylaimida* (Jairajpuri, 2002). Predaceous nematodes are well known for their ability to feeds on wide range of insects including other nematodes, whereas saprophytic nematodes are renowned for acting as decomposers (they break down soil compost) belonging to phylum order *Aphelenchida* and *Rhabditida*. (Khan and Kim 2007; Hamza *et al.,* 2020) Furthermore, soil compost consists of high relative biomass of microorganisms (including fungi and bacteria), hence these nematodes are called fungivores and bacteriovores (Yadav *et al.,* 2023). Both, predaceous and saprophytic nematodes are natural soil inhabitants and are involve in mineral/ nutrients cycling crucial for horticulture practices (Shokoohi, 2023). During the cropping season, horticulture practitioners observed the presence of specific functional group of nematodes in the soil and use them as biological indicators of soil fertility and quality, since they are reported to be involved in nutrients cycling (Lu *et al.,* 2020). Segregation of nematodes into functional groups and types, provides insights into their functional relationships with other microorganisms.

Parasite nematodes feed on both animal (includes humans) and plants. The plant parasitic nematodes are often referred to as herbivorous, and they belong to *Solonaceae* and *Heteroderidae* family (Hauri and Szendrei, 2022). Plant parasitic nematodes they are considered as a threat to crop production by horticulture practitioners due to their feeding

preference. Meanwhile. other types of parasitic nematodes (excluding helminth the genus Ascaris) are beneficial to horticulture practices, since they feed on a wide range of agriculture problematic pest, they are regarded as biological control agents namely entomopathogenic nematodes (EPNs) (Askary and Abd-Elgawad, 2017; Wilschut and Geisen, 2021). Furthermore, EPNs are not pathogenic to humans, whereas helminths are pathogenic to humans (Brindley *et al.,* 2009).

1.1.2 Entomopathogenic nematodes

Entomopathogenic nematodes (EPNs) are microscopic worms which are found in soil and well known for their ability to infect and kill agriculture problematic insects (Salame and Glazer, 2015). EPNs have a symbiotic relationship with endopathogenenic bacteria (Lephoto and Gray, 2020; Khumalo *et al.,* 2021). EPNs belong to three genera namely *Heterorhabdits*, *Steinernema* and *Oscheus* (Lephoto and Gray, 2019). Furthermore, EPNs bacteria associate are classified under *Xenorhabdes*, *Photorhabdus* and *Seratia* genus (Didiza *et al.,* 2021; Singha *et al.,* 2022). Moreover, EPNs bacteria associates are the source of EPNs ability to kill insects. EPNs are reported to have high tolerance of the environmental factors such as: violet radiation tolerance, desiccation, osmotic, ability to survive in aerobic condition and extreme temperature (Abdolmaleki *et al.,* 2016; Ardpairin *et al.,* 2020).

1.1.3 EPNs Life cycle

The EPNs infective juveniles (IJs) possesses biological functions to infect and kill specific insects (Fu *et al.,* 2021). IJs use biological openings such as anus, mouth, etc, to enter the host body Figure 1 (Khumalo *et al.,* 2021; Singha *et al.,* 2022). Furthermore, after a successful invasion of IJs into the insect host body, they release symbiotic bacterial into hemocoel (Shapiro *et al.,* 2000; Liu *et al.,* 2020). *Steinernema* IJs are known to develop into amphimictic males or amphimictic females, whereas *Oscheius* and *Heterorhabditis* matures into hermaphrodites and subsequently there is production of males, amphimictic females and hermaphroditic females in the 2nd generation (Lephoto *et al.,* 2016; Didiza *et al.,* 2021; Matlhabe *et al.,* 2022). Pathogenic bacteria are known to generate toxic chemicals that weaken the host immune system, which subsequently causing death (Khumalo *et al.,* 2021; Heinzelmann *et al.,* 2002). Moreover, nematodes resume proliferation within the host cadaver and once the nutrients are depleted, nematodes are release in the soil to hunt for their new hosts (Lephoto *et al.,* 2016; Ogier *et al.,* 2020).

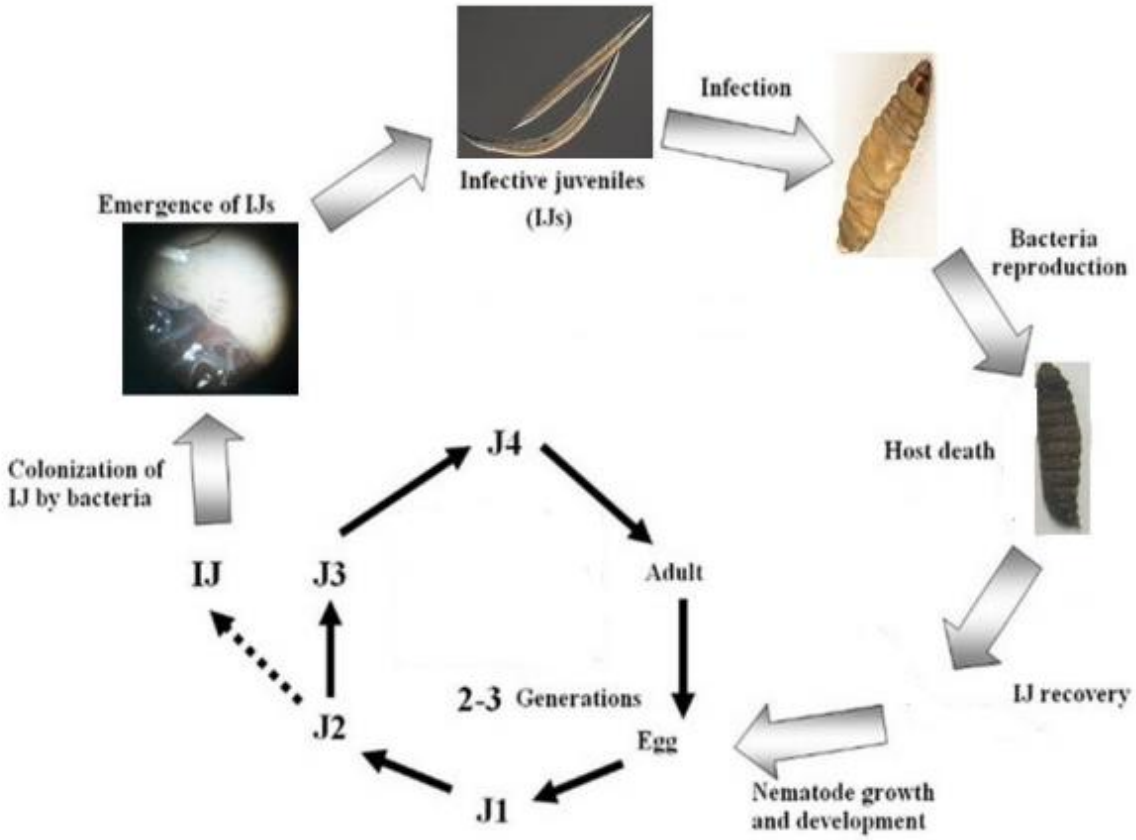


Figure 1.1 Schematic diagram elucidating EPNs life cycle. The infective juvenile's (IJs) searches for host and once the host is located, the IJs use an external opening to enter inside the host body (Schematic diagram adapted from Lephoto and Gray, 2019). Endosymbionts released within the gut of the host, to infect and kill the host within few hours. Furthermore, the IJs proliferation and reproduction continues while the nutrients are being depleted within the host. Moreover, emergency of IJs within host cadaver take place and they exist the host body seeking for new prey.

1.1.4 Mutualistic benefits among EPNs and endopathogenic bacteria association

Nematodes serve as vector for pathogenic bacterial symbionts. Furthermore, nematodes are responsible for shielding the bacterial symbionts from extrinsic environmental conditions and transportation of the bacteria to the nutrient-rich haemolymph (Fukruksa *et al.,* 2017; Deka *et al.,* 2021). Meanwhile, the bacteria symbionts are responsible for infecting and killing of the insect host through synthesis of toxic bioactive chemicals (Yooyangket *et al.,* 2018). The bacterial symbionts are reported to produce hydrolytic enzymes that are key towards bio conversion of insect haemolymph condition to suit/ favour the nematodes reproduction and development (Hussaini *et al.,* 2010; Pervez *et al.,* 2020). The carbohydrates and lipids found in insect cadaver serve as nutrients for both nematodes and bacteria symbionts (Blackburn *et al.,* 2016). The bacteria symbionts are tasked with deprivation of the insect host immune defence (Aiswarya *et al.,* 2017). Hence the bacteria symbionts produce the metabolites that

have antimicrobial activity, which are used to abrogate saprophytic microorganisms and scavenging insects, while killing the insect host (Inman *et al.,* 2012; Aiswarya *et al.,* 2017; Zhang *et al.,* 2023).

Nematodes that do not have a symbiotic relationship with pathogenic bacteria are not regarded as EPNs (Dillman *et al.,* 2012). Therefore, they also lack the ability to kill the insect host (Dillman *et al.,* 2012). The EPNs bacteria associates can be cultured separately from EPNs and they attain their pathogenicity activity against insect host (Shan *et al*., 2019). The relationship among EPNs and their pathogenic association is precise. Nematodes belonging to a particular genus may only be associated with a single specific bacteria species, meanwhile that specific bacteria may be associated with more than a single nematode species of same genus, therefore, the association between nematode species and the bacteria symbionts is horizontal transfer (Clarke, 2008).

The ability of bacteria to possess insecticidal activity is a fundamental requirement for initiating and maintaining the symbiosis relationship between nematode and bacteria (Clarke, 2008; Dillman *et al.,* 2012). Furthermore, for nematodes to be regarded as EPNs, it must evolve a symbiotic relationship with a pathogenic bacterium (Clarke, 2008). Moreover, for nematode species to be classified as EPNs, it should be able to seek and infect various insects associated with soil (Clarke, 2008; Dillman *et al.,* 2012). In contrast, EPNs should possess the ability to retain the pathogenic bacteria within their gut during non-feeding stage in soil, until such period it re-infects the insect host (Dillman *et al.,* 2012).

1.1.5 Metabolites production by EPNs bacteria symbionts

Screening of metabolites synthesized by EPNs bacteria symbionts have provide newly insights about their chemical structure, biosynthetic pathways, and their involvement in symbiosis. Furthermore, previous studies have reported that these metabolites possess diverse spectrum of antibiotic activity that may be used against fungi and bacteria including multi-drug resistant pathogens that affect humans (Bode, 2009; Booysen and Dicks, 2020; Fodor *et al.,* 2022). These metabolites have wide use in both agroforestry and pharmaceutical industries (Wyatt and Greathouse, 2021). There have been approximately 50 metabolites identified from all three EPNs bacteria associates, these metabolites include: xenorxides, nematophin, cyclolipopeptide (PAX), xenocoumacins, hydroxystilbenes, xenematide, xenorhabdins, indoles and indole derivatives (Lang *et al.,* 2008; Shan *et al.,* 2020). Metabolites production varies among bacteria genus, strains and among different growth phase of the bacteria (Lang *et al.,* 2008; Bode, 2009;

Challinor and Bode, 2015; Wyatt and Greathouse, 2021). Furthermore, Phase I is reported to have high metabolites production meanwhile Phase II have less production (Wang *et al.,* 2011; Booysen and Dicks, 2020). It is reported that metabolites are produced in high concentrations with the insect host cadaver compere in bacteria culture during *in vitro* experiments (Chen *et al.,* 1996; Wang *et al.,* 2011). Therefore, this may be affected by different physiological conditions between host cadaver and synthetic bacteria culture (Booysen and Dicks, 2020). Both aeration and temperature also have effects in metabolites production (Chen *et al.,* 1996).

1.1.6 Application of EPNs as biological control agent

Over 2 past centuries the agriculture communities depended on synthetic chemical pesticides practise (Lephoto *et al.,* 2016). However, the use of synthetic chemical pesticides has adverse negative impacts, which includes non-specific extermination of beneficial insects, crop contamination, development of chemical resistance in insects and human health related cases (Koch *et al.,* 2015). There is a need to search for biological control agents of insect, to help reduce application of chemical control agents (Dunn *et al.,* 2020). EPNs are reported to be satisfactory solution towards controlling problematic insects (Lephoto and Gray, 2020; Platt *et al.,* 2020). Hence, they do not cause any threat to humans and other related vertebrate species (Helmberger *et al.,* 2017; Matlhabe *et al.,* 2022). EPNs are known to be applicable in both large-scale and small-scale farming, diverse agriculture industries, forestry including home gardens (Coupland *et al.,* 2017). There are several insect pests that have been deemed problematic by the Agriculture Research Council (ARC), furthermore, these problematic insect pests dismantle the stem and leaves of the plants (Lephoto *et al.,* 2016; Oguh *et al.,* 2019).

Aphid species are known to colonise the plants and increase their population size while consuming the plant nutrients, leading to withering and death (Alyokhin *et al.,* 2020; Singh and Singh, 2021). Furthermore, aphid honeydew excrement is known to serve as a growth medium for sooty molds for fungal diseases (Saha *et al.,* 2016). There are various types of insect pets considered to be problematic by ARC maize aphid, aphid, etc (Velasco *et al.,* 2020). These insect pets they are known to feed on plant leaves, disrupting the photosynthesis process of plants, thus effect the plant (Huang *et al.,* 2011; Lephoto *et al.,* 2016). Which it is likely to occur during winter rainfall season and summer season (Lephoto and Gray, 2020). Chemical insecticides remain the preferable method to most farm industries to control these pests (Carvalho, 2017). Despite numerous studies proving EPNs have ability to kill pests (Steyn *et al.,* 2021), for example: *Heterorhabditis bacteriophora* have been elucidated to kill *Ceratitis*

capitata and *Heterorhabditis zealandica* has been repoeted to kill both *Planococcus citri* and *citrus mealybug* (Shaurub *et al.*, 2015; Mohamedova *et al.*, 2017).

1.1.7 Possible strategies to upgrade EPNs efficacy

Both *Heterorhabditis* and *Steinernema* genera of EPNs species are most common studied and highly applied as biological control agents of various insect pest (Lu *et al.*, 2016). EPNs are commercialized in few countries and proven to be applicable in both large-scale Agriculture industries and in individual gardens (Abate *et al.*, 2017). However, regardless of the various successful studies conducted on EPNs as biological control agents, they are still not widely used horticulture sector (Lu *et al.*, 2016; Abate *et al.*, 2017). This is due to the lack of consistent efficacy in the field (Baiocchi *et al.*, 2017). Researchers have been working effortlessly to upgrade efficacy of EPNs against arthropod pests under general field conditions (Kergunteuil *et al.*, 2016). There are two techniques (namely genetic upgrade through mutagenesis and artificial selection) which were used to upgrade EPNs efficacy elucidated in Figure 1.2.

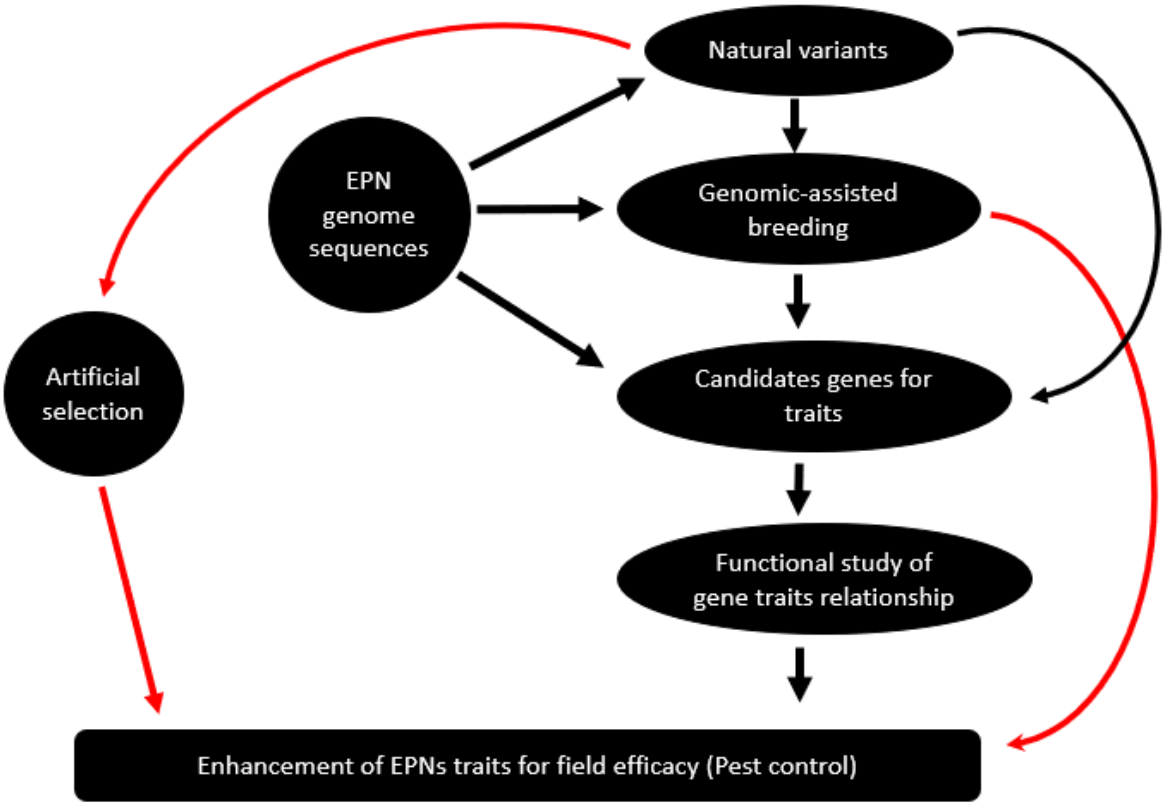


Figure 1.2 Schematic diagram elucidating various routes toward enhancement of EPNs field efficacy (*Adapted* from Lu *et al.*, 2016 with minor modifications). There are 3 factors that are identified namely natural gene variants, genes influencing desirable trait and potential genomes for breeding.

There two major ways used to enhance EPNs efficacy; firstly, by performing artificial selection with an aid of diversity of natural gene variants. Secondly, through selective breeding of identified EPNs genomes which can either improve EPNs field efficacy or not. However, the first method does not require any knowledge about genomes or gene functions. Whilst the second method also do not require any knowledge about specific gene-trait relationships. Both methods lack the knowledge about the gene functions in desirable trait and gene-trait relationships.

The fundamental principle of artificial selection technique is dependent on genetic differences between EPNs species and their uniqueness towards adaptation to certain environmental conditions and their effectiveness against pests (Kergunteuil *et al.,* 2016; Lu *et al.,* 2016). Furthermore, there are numerous new EPNs that have been discovered, thus raises the genetic difference, and enhances the chances of development of new EPNs strains (Zhang *et al.,* 2019; Machado *et al.,* 2020). Although, through Breeding of different EPNs species allows manipulating specific traits, thus improve EPNs biological control activity upon insect pests (Singha *et al.,* 2022). Manipulation of these specific traits retains high chances towards improving temperature tolerance, ultraviolet rays, and desiccation (Bhat *et al.,* 2021). Thus, enhances virulence and resistance to nematicides (Meher *et al.,* 2009; Sparks *et al.,* 2020).

Genetic selection or breeding techniques have been used to improve EPNs specific traits (Santhi *et al.,* 2016; Baiocchi *et al.,* 2017). However, enhancement of these traits in these methods have undesirable drawbacks, such as some specific traits are lost due to absences of selective pressure, selection of certain traits may have a negative impact as it may reduce other traits or overall fitness unintentional (Stuart *et al.,* 2015; Lu *et al.,* 2016; Stelinski *et al.,* 2019). Moreover, during mass production of EPNs via repeatedly laboratory culturing, some fitness traits may be lost (Anbesse *et al.,* 2013; Blackburn *et al.,* 2016). The 2nd upgrading technique of EPNs field efficacy is via modern genetic and other molecular tools (Saha *et al.,* 2019). Most recently studies on EPNs are based on their behaviour, host-parasite interactions, and neurobiology (Baiocchi *et al.,* 2017; Morris *et al*., 2017; Chang *et al.,* 2019). However, there is gap between understanding the individual gene functions, as this knowledge will serve as fundamental base towards improvement of EPNs field efficacy.

1.1.8 Importance of decrypting the individual gene of EPNs and their bacteria symbiont to upgrade EPNs efficacy

Improving the EPNs field efficacy through molecular techniques by focusing on 3 major traits categories namely persistence, infectivity, and storage stability (Banu, 2017; Thakur *et al.,*

2020). The persistence traits are associated with enhanced survival rate of EPNs in the field (including desiccation, UV rays and temperature tolerance) (Nxitywa and Malan, 2021). The infectivity traits are associated with behaviour characters such as hunting, infecting, and killing of the specific insects (Leclercq *et al.,* 2017). Storage stability traits are associated with enhanced shelf life which is also vital for distribution (Hiltpold, 2015; Lu *et al.,* 2016). Therefore, these traits are recognised as potential targets toward upgrading the EPNs efficacy.

EPNs are expected to possess the ability to hunt and kill the insect host (Grunseich *et al.,* 2021). Furthermore, there are various studies which were conducted to modify EPNs host-seeking behaviour (Li *et al.,* 2019; Helmberger *et al.,* 2017). It was discovered that the host -seeking trait is highly heritable (Valverde *et al.,* 2020). Hence, it can be improved via a selective breeding technique, for example, by breeding *Steinernema feltiae* and *Steinernema carpocapsae* (Laleh *et al.,* 2016). Previously studies on host-seeking ability improvement of EPNs was only based on selective breeding (Santhi *et al.,* 2016). However, the specific genes involved were not identified (Booysen *et al.,* 2022). EPNs host hunting performance is linked to an increase in fitness (Grunseich *et al.,* 2021). Furthermore, it was found that through improving the host hunting ability, had led to decrease in desiccation tolerance ability, thus have impact towards storage stability of EPNs (Lu *et al.,* 2016).

Persistence plays a vital role in EPNs infectivity efficiency (Vashisth *et al.,* 2019; Khumalo *et al.,* 2021). EPNs persistence and production abilities are dependent to the desiccation tolerance ability (Lephoto and Gray, 2020). Furthermore, the higher level of desiccation tolerance by EPNs, enhances the shelf life, thus also enhances their longevity in the soil (Kagimu *et al.,* 2017; Kary *et al.,* 2021). The storage stability is correlated to EPNs longevity within the soil, marketable production, and distribution (Lu *et al.,* 2016; Mwaniki, 2016). Desiccation tolerance of EPNs can be altered via artificial selection and hybridization (Saleh *et al.,* 2020). However, if suppression of selection pressure may result in the loss of some vital traits (Helmberger *et al.,* 2017). Therefore, there is a need to study the genetic make-up of EPNs and further understand the genetic regulator network. Hence, this is key towards identifying and improving the stability among these enhanced traits. Furthermore, deciphering the individual genes of EPNs and manipulating the bacterial partners may leads into enhancement of EPNs field efficacy correlated traits.

1.1.9 Research motivation

Many developing countries depends on agriculture to maintain food security and gross domestic product (GDP). However, the report from previous years shows drastic decline in horticulture sector which is due to pest infestation (Grunseich *et al.,* 2021). Therefore, farmers have introduced synthetic chemical pesticides to mitigate pest attacks. However, the use of synthetic chemical pesticides has negative effects towards ecological function. Furthermore, overuse of chemical pesticides leads to accumulation of heavy metal in soil that initiate oligodynamic processes. Hence, some of the metals are washed off into nearby water system, endangering animals (including humans). There is need to find alternative ways to abrogate pest attacks such as biological control.

Entomopathogenic nematodes (EPNs) are soil dwelling insect parasitic microorganisms used in both small-scale and large-scale farming, as biological control agent. EPNs feeds on various agriculture insect pest and involved in nutrient cycling in soil. EPNs have evolve a symbiotic relationship with pathogenic bacteria from *Enterobacteriaceae* family (Khumalo *et al.,* 2021). These bacterial symbionts are crucial for pathogenicity and survival of EPNs. There are several topological factors which makes it challenging to implement EPNs in different countries. South Africa is divided into nine biomes. Further each biome experiences its own climate system. Therefore, this make it challenging for farmers to replace chemical pesticides with EPNs, since it is reported that environmental factors (temperature, desiccation, UV light) affects EPNs efficacy (Didiza *et al.,* 2021). Therefore, it is crucial to explore the genome of their symbionts to obtain the insights and knowledge to be able to genomic manipulate EPNs to improve EPNs field efficacy and ability to adapt different ecological region.

1.1.10 Aim and objectives

1.1.10.1 Aim(s)

I) To isolate and identify possible pathogenic *Cruznema* bacteria symbionts.

II) To identify secondary metabolites produced *Cruznema* bacteria symbionts.

III) To explore the effects of *Cruznema* bacteria symbionts secondary metabolites against ATCC antibiotic resistant bacteria species.

IV) To explore the whole genome sequencing and annotation of *Cruznema* bacteria symbionts.

V) To structural and functional characterised the Heat shock proteins found in virulence, disease, and defence subsystem of bacterial symbionts.

1.1.10.2 Objectives

I) Molecular identification of *Cruznema* nematodes.

II) 16S amplicon RNA based Metagenomics for *Cruznema* nematodes.

III) Isolation and morphological characterisation *Cruznema* nematodes possible symbiotic pathogenic bacteria.

IV) Molecular identification and phylogenetic characterization of *Cruznema* nematodes possible symbiotic pathogenic bacteria.

V) Screening of bacteria metabolites isolated from *Cruznema* nematodes.

VI) Identification of metabolic compounds synthesis by bacteria isolated from *Cruznema* nematodes.

VII) Whole genome sequencing and annotation of bacteria isolated from *Cruznema* nematodes.

VIII) Structural and functional characterisation of heat shock proteins found in virulence, disease, and defence subsystem.

1.1.11 References

Abate, B.A., Wingfield, M.J., Slippers, B. and Hurley, B.P., 2017. Commercialisation of entomopathogenic nematodes: should import regulations be revised. *Biocontrol Science And Technology*, *27*(2), pp.149-168.

Abdolmaleki, A., Tanha Maafi, Z., Rafie Dastjerdi, H., Naseri, B. and Ghasemi, A., 2016. Isolation and identification of entomopathogenic nematodes and their symbiotic bacteria from Kurdistan province in Iran. *Journal Of Crop Protection*, *5*(2), pp.259-271.

Aiswarya, D., Karthik Raja, R., Gowthaman, G., Deepak, P., Balasubramani, G. and Perumal, P., 2017. Antibacterial activities of extracellular metabolites of symbiotic bacteria, Xenorhabdus and Photorhabdus isolated from entomopathogenic nematodes. *International Biological And Biomedical Journal*, *3*(2), pp.80-88.

Alyokhin, A., Nault, B. and Brown, B., 2020. Soil conservation practices for insect pest management in highly disturbed agroecosystems–a review. *Entomologia Experimentalis Et Applicata*, *168*(1), pp.7-27.

Ardpairin, J., Muangpat, P., Sonpom, S., Dumidae, A., Subkrasae, C., Tandhavanant, S., Thanwisai, A. and Vitta, A., 2020. A survey of entomopathogenic nematodes and their symbiotic bacteria in agricultural areas of northern Thailand. *Journal Of Helminthology*, *94*.

Askary, T.H. and Abd-Elgawad, M.M.M., 2017. Beneficial nematodes in agroecosystems: a global perspective. In *Biocontrol Agents: Entomopathogenic And Slug Parasitic nematodes*, pp. 3-25. Wallingford UK: CABI.

Baiocchi, T.I.F.F.A.N.Y., Abd-Elgawad, M.M., Dillman, A.R., Abd-Elgawad, M.M.M., Askary, T.H. and Coupland, J., 2017. Genetic improvement of entomopathogenic nematodes for enhanced biological control. *Biocontrol Agents: Entomopathogenic And Slug Parasitic nematodes. CAB International, Wallingford*, pp.505-517.

Banu, J.G., 2017. Efficacy of entomopathogenic nematodes against coleopteran pests. *Biocontrol Agents: Entomopathogenic And Slug Parasitic Nematodes*, pp.174-192.

Bhat, M.A., Mir, R.A., Kumar, V., Shah, A.A., Zargar, S.M., Rahman, S. and Jan, A.T., 2021. Mechanistic insights of CRISPR/Cas-mediated genome editing towards enhancing abiotic stress tolerance in plants. *Physiologia Plantarum*, *172*(2), pp.1255-1268.

Blackburn, D., Shapiro-Ilan, D.I. and Adams, B.J., 2016. Biological control and nutrition: Food for thought. *Biological control*, *97*, pp.131-138.

Bode, H.B., 2009. Entomopathogenic bacteria as a source of secondary metabolites. *Current opinion in chemical biology*, *13*(2), pp.224-230.

Booysen, E. and Dicks, L.M.T., 2020. Does the future of antibiotics lie in secondary metabolites produced by Xenorhabdus spp.? A review. *Probiotics And Antimicrobial Proteins*, *12*, pp.1310-1320.

Booysen, E., Malan, A.P. and Dicks, L.M., 2022. Colour of heterorhabditis zealandica-infected-Galleria mellonella dependent on the Photorhabdus symbiont, with two new nematode-symbiotic associations reported. *Journal Of Invertebrate Pathology*, *189*, pp.107729.

Brindley, P.J., Mitreva, M., Ghedin, E. and Lustigman, S., 2009. Helminth genomics: The implications for human health. *PLoS neglected tropical diseases*, *3*(10), p.e538.

Carvalho, F.P., 2017. Pesticides, environment, and food safety. *Food And Energy security*, *6*(2), pp.48-60.

Challinor, V.L. and Bode, H.B., 2015. Bioactive natural products from novel microbial sources. *Annals Of The New York Academy Of Sciences*, *1354*(1), pp.82-97.

Chang, D.Z., Serra, L., Lu, D., Mortazavi, A. and Dillman, A.R., 2019. A core set of venom proteins is released by entomopathogenic nematodes in the genus Steinernema. *PLoS Pathogens*, *15*(5), p.e1007626.

Chen, G., Maxwell, P., Dunyhy, G.B. and Webster, J.M., 1996. Culture conditions for Xenorhabdus and Photorhabdus symbionts of entomopathogenic nematodes. *Nematologica*, *42*(1), pp.124-130.

Clarke, D.J., 2008. Photorhabdus: a model for the analysis of pathogenicity and mutualism. *Cellular Microbiology*, *10*(11), pp.2159-2167.

Coupland, J.A.M.E.S., Abd-Elgawad, M.M. and Askary, T.H., 2017. Beneficial nematodes and the changing scope of crop protection. *Biocontrol Agents: Entomopathogenic and Slug Parasitic Nematodes; Abd-Elgawad, MMM, Askary, TH, Coupland, J., Eds*, pp.26-42.

Decraemer, W., Coomans, A. and Baldwin, J., 2013. Morphology of nematoda. *Nematoda*, *2*, pp.1-60.

Deka, B., Baruah, C. and Babu, A., 2021. Entomopathogenic microorganisms: their role in insect pest management. *Egyptian Journal Of Biological Pest Control*, *31*(1), pp.1-8.

Didiza, L., Lephoto, T.E. and Gray, V.M., 2021. Morphological and molecular phylogenetic description of Steinernema batswanae n. sp.(Rhabditida: Steinernematidae): a new species of

an entomopathogenic nematode from South Africa. *Archives Of Phytopathology And Plant Protection*, *54*(19-20), pp.1603-1616.

Dillman, A.R., Chaston, J.M., Adams, B.J., Ciche, T.A., Goodrich-Blair, H., Stock, S.P. and Sternberg, P.W., 2012. An entomopathogenic nematode by any other name. *PLoS Pathogens*, *8*(3), p.e1002527.
Dillman, A.R., Chaston, J.M., Adams, B.J., Ciche, T.A., Goodrich-Blair, H., Stock, S.P. and Sternberg, P.W., 2012. An entomopathogenic nematode by any other name. *PLoS Pathogens*, *8*(3), p.e1002527.
Dunn, L., Lequerica, M., Reid, C.R. and Latty, T., 2020. Dual ecosystem services of syrphid flies (Diptera: Syrphidae): pollinators and biological control agents. *Pest Management Science*, *76*(6), pp.1973-1979.

Fodor, A., Gualtieri, M., Zeller, M., Tarasco, E., Klein, M.G., Fodor, A.M., Haynes, L., Lengyel, K., Forst, S.A., Furgani, G.M. and Karaffa, L., 2022. Type strains of entomopathogenic nematode-symbiotic bacterium species, *Xenorhabdus szentirmaii* (EMC) and *X. budapestensis* (EMA), are exceptional sources of non-ribosomal templated, large-target-spectral, thermotolerant-antimicrobial peptides (by both), and iodinin (by EMC). *Pathogens*, *11*(3), pp.342.

Fu, Y., Wang, W., Chen, C., Shan, S., Wei, X., Liu, Y., Shapiro-Ilan, D., Gu, X., Hu, B., Yoshiga, T. and Ruan, W., 2021. Chemotaxis behaviour of *Steinernema carpocapsae* in response to *Galleria mellonella* (L.) larvae infected by con-or hetero-specific entomopathogenic nematodes. *Biocontrol Science And Technology*, *31*(3), pp.299-313.

Fukruksa, C., Yimthin, T., Suwannaroj, M., Muangpat, P., Tandhavanant, S., Thanwisai, A. and Vitta, A., 2017. Isolation and identification of *Xenorhabdus* and *Photorhabdus* bacteria associated with entomopathogenic nematodes and their larvicidal activity against Aedes aegypti. *Parasites & Vectors*, *10*(1), pp.1-10.

Grunseich, J.M., Aguirre, N.M., Thompson, M.N., Ali, J.G. and Helms, A.M., 2021. Chemical Cues from Entomopathogenic Nematodes Vary Across Three Species with Different Foraging Strategies, Triggering Different Behavioral Responses in Prey and Competitors. *Journal Of chemical ecology*, *47*(10), pp.822-833.

Hamza, M.A., Tazi, H., Fossati-Gaschignard, O., Moukhli, A., Lakhtar, H., Ferji, Z., Roussos, S., El Mousadik, A., Boubaker, H. and Mateille, T., 2020. May passive dispersal of fungal enemies with native substrates in olive nurseries help to control phytonematodes?.

Biotechnologie, Agronomie, Société et Environnement/Biotechnology, Agronomy, Society And Environment, *24*(1), pp.37-45.
Hauri, K.C. and Szendrei, Z., 2022. A meta-analysis of interactions between insect herbivores and plant-parasitic nematodes. *Environmental Entomology*, *51*(1), pp.1-10.
Heinzelmann, M., Scott, M. and Lam, T., 2002. Factors predisposing to bacterial invasion and infection. *The American Journal Of Surgery*, *183*(2), pp.179-190.

Helmberger, M.S., Shields, E.J. and Wickings, K.G., 2017. Ecology of belowground biological control: Entomopathogenic nematode interactions with soil biota. *Applied Soil Ecology*, *121*, pp.201-213.

Hiltpold, I., 2015. Prospects in the application technology and formulation of entomopathogenic nematodes for biological control of insect pests. In *Nematode pathogenesis Of insects And other Pests*, pp. 187-205. Springer, Cham.

Huang, T., Jander, G. and de Vos, M., 2011. Non-protein amino acids in plant defense against insect herbivores: representative cases and opportunities for further functional analysis. *Phytochemistry*, *72*(13), pp.1531-1537.

Huang, Y., Guo, Y., Huang, Y. and Guo, Y., 2022. Morphology of Free-Living Nematode. *Free-living Marine Nematodes From The East China Sea*, pp.7-15.
Hussaini, S.S., Nagesh, M., Walia, R.K., Vyas, R.V., Kamra, A. and Mohan, S., 2010. Beneficial role of nematodes in soil and plant health. *Indian Journal Of Nematology*, *40*(1), pp.12-22.
Inman, F.L., Singh, S. and Holmes, L.D., 2012. Mass production of the beneficial nematode *Heterorhabditis bacteriophora* and its bacterial symbiont *Photorhabdus luminescens*. *Indian Journal Of Microbiology*, *52*, pp.316-324.
Jairajpuri, M.S., 2002. Nematode structure: with special reference to plant parasitic, predatory, soil-inhabiting nematodes of the orders Dorylaimida, Mononchida & Tylenchida. *Nematode Structure: With Special Reference To Plant Parasitic, Predatory, Soil-Inhabiting Nematodes Of The Orders Dorylaimida, Mononchida & Tylenchida.*
Kagimu, N., Ferreira, T. and Malan, A.P., 2017. The attributes of survival in the formulation of entomopathogenic nematodes utilised as insect biocontrol agents. *African Entomology*, *25*(2), pp.275-291.

Kary, N.E., Chahardoli, S., Mohammadi, D. and Dillon, A.B., 2021. Efficacy of carboxymethyl cellulose as an inert water-soluble carrier for formulation of entomopathogenic nematodes,

Heterorhabditis bacteriophora and *Steinernema carpocapsae*. *Biological Control*, *160*, p.104690.

Kergunteuil, A., Bakhtiari, M., Formenti, L., Xiao, Z., Defossez, E. and Rasmann, S., 2016. Biological control beneath the feet: a review of crop protection against insect root herbivores. *Insects*, *7*(4), pp.70.

Khan, Z. and Kim, Y.H., 2007. A review on the role of predatory soil nematodes in the biological control of plant parasitic nematodes. *Applied Soil Ecology*, *35*(2), pp.370-379.

Khumalo, N.N., Lephoto, T.E. and Gray, V.M., 2021. The effect of organic compost and soil texture on the survival and infectivity of entomopathogenic nematode species. *Archives Of Phytopathology And Plant Protection*, *54*(17-18), pp.1443-1455.

Koch, M.S., Ward, J.M., Levine, S.L., Baum, J.A., Vicini, J.L. and Hammond, B.G., 2015. The food and environmental safety of Bt crops. *Frontiers In Plant Science*, *6*, pp.283.

Laleh, E., Shiri, M.R. and Dunphy, G.B., 2016. Efficacy of the Entomopathogenic Nematode, *Steinernema feltiae* Against the Vegetable Leaf Miner, *Liriomyza sativae* Blanchard (Diptera: Agromyzidae). *Egyptian Journal Of Biological Pest Control*, *26*(3), pp.583.

Lang, G., Kalvelage, T., Peters, A., Wiese, J. and Imhoff, J.F., 2008. Linear and cyclic peptides from the entomopathogenic bacterium *Xenorhabdus nematophilus*. *Journal Of Natural Products*, *71*(6), pp.1074-1077.

Leclercq, G., Pannebakker, B., Gengler, N., Nguyen, B.K. and Francis, F., 2017. Drawbacks and benefits of hygienic behavior in honeybees (Apis mellifera L.): a review. *Journal Of Apicultural Research*, *56*(4), pp.366-375.

Lephoto, T.E. and Gray, V.M., 2019. *Oscheius basothovii* n. sp.(Nematoda: *Rhabditidae*), a new entomopathogenic nematode isolated from an uncultivated grassland in South Africa. *Archives of Phytopathology And Plant Protection*, *52*(1-2), pp.125-140.

Lephoto, T.E. and Gray, V.M., 2020. Desiccation stress tolerance of Steinernema australe and Heterorhabditis bacteriophora. *Archives Of Phytopathology And Plant Protection*, *54*(11-12), pp.611-624.

Lephoto, T.E., Mpangase, P.T., Aron, S. and Gray, V.M., 2016. Whole genome sequence of Oscheius sp. TEL-2014 entomopathogenic nematodes isolated from South Africa. *Genomics Data*, *7*, pp.259-261.

Li, C., Zhou, X., Lewis, E.E., Yu, Y. and Wang, C., 2019. Study on host-seeking behavior and chemotaxis of entomopathogenic nematodes using Pluronic F-127 gel. *Journal Of Invertebrate Pathology*, *161*, pp.54-60.

Liu, W.T., Chen, T.L., Hou, R.F., Chen, C.C. and Tu, W.C., 2020. The invasion and encapsulation of the entomopathogenic nematode, *Steinernema abbasi*, in Aedes albopictus (Diptera: Culicidae) Larvae. *Insects*, *11*(12), p.832.

Lu, D., Baiocchi, T. and Dillman, A.R., 2016. Genomics of entomopathogenic nematodes and implications for pest control. *Trends In Parasitology*, *32*(8), pp.588-598.

Lu, Q., Liu, T., Wang, N., Dou, Z., Wang, K. and Zuo, Y., 2020. A review of soil nematodes as biological indicators for the assessment of soil health. *Front. Agric. Sci. Eng*, *7*, pp.275-281.

Machado, R.A., Thönen, L., Arce, C., Theepan, V., Prada, F., Wüthrich, D., Robert, C.A., Vogiatzaki, E., Shi, Y.M., Schaeren, O.P. and Notter, M., 2020. Engineering bacterial symbionts of nematodes improves their biocontrol potential to counter the western corn rootworm. *Nature Biotechnology*, *38*(5), pp.600-608.

Matlhabe, O.D., Lephoto, T.E. and Gray, V.M., 2022. Monitoring life cycle stages of axenic cultures of in vitro produced *Oscheius* L8 MCB in semi-solid state and liquid state and comparative efficacy studies between in vitro and in vivo nematode production against *Galleria mellonella*. *Biological Control*, pp.104904.

Meher, H.C., Gajbhiye, V.T., Chawla, G. and Singh, G., 2009. Virulence development and genetic polymorphism in Meloidogyne incognita (Kofoid & White) Chitwood after prolonged exposure to sublethal concentrations of nematicides and continuous growing of resistant tomato cultivars. *Pest Management Science: Formerly Pesticide Science*, *65*(11), pp.1201-1207.

Mohamedova, M.S., Valcheva, I.S., Draganova, D.G., Naydenov, M.K. and Borisov, Y.B., 2017. Effect of Bacillus amyloliquefaciens A1, Paenibacillus polymyxa AB3 and Providencia rettgeri K10 on the Citrus Mealybug, Planococcus citri (Risso) (Hemiptera: Pseudococcidae). *Egyptian Journal of Biological Pest Control*, *27*(1).

Moosavi, M.R. and Zare, R., 2020. Fungi as biological control agents of plant-parasitic nematodes. *Plant defence: Biological Control*, pp.333-384.

Morris, R., Wilson, L., Sturrock, M., Warnock, N.D., Carrizo, D., Cox, D., Maule, A.G. and Dalzell, J.J., 2017. A neuropeptide modulates sensory perception in the entomopathogenic nematode *Steinernema carpocapsae*. *PLoS Pathogens*, *13*(3), p.e1006185.

Mwaniki, N., 2016. *Potential of Entomopathogenic Nematodes as a Biological Control and Management Tool for Banana Weevil (Cosmopolites Sordidus) in Banana Orchards* (Doctoral dissertation, University Of Nairobi).

Nxitywa, A. and Malan, A.P., 2021. Formulation of Entomopathogenic Nematodes for the Control of Key Pests of Grapevine: A Review. *South African Journal Of Enology And Viticulture*, *42*(2), pp.123-135.

Ogier, J.C., Frayssinet, M. and Gaudriault, S., 2020. Entomopathogenic nematode-associated microbiota: from monoxenic paradigm to pathobiome. *Microbiome*, *8*(1), pp.1-17.

Oguh, C.E., Okpaka, C.O., Ubani, C.S., Okekeaji, U., Joseph, P.S. and Amadi, E.U., 2019. Natural pesticides (biopesticides) and uses in pest management-a critical review. *Asian Journal Of Biotechnology And Genetic Engineering*, *2*(3), pp.1-18.

Pervez, R., Lone, S.A. and Pattnaik, S., 2020. Characterization of symbiotic and associated bacteria from entomopathogenic nematode Heterorhabditis sp.(nematode: Heterorhabditidae) isolated from India. *Egyptian Journal Of Biological Pest Control*, *30*(1), pp.1-9.

Platt, T., Stokwe, N.F. and Malan, A.P., 2020. A review of the potential use of entomopathogenic nematodes to control above-ground insect pests in South Africa. *South African Journal of Enology And Viticulture*, *41*(1), pp.1-16.

Saha, S., Pfeufer, E., Bessin, R., Wright, S. and Kaiser, C.A., 2016. An IPM Scouting Guide for Common Problems of High Tunnel and Greenhouse Vegetable Crops in Kentucky.

Saha, S.K., Saikot, F.K., Rahman, M.S., Jamal, M.A.H.M., Rahman, S.K., Islam, S.R. and Kim, K.H., 2019. Programmable molecular scissors: applications of a new tool for genome editing in biotech. *Molecular Therapy-Nucleic Acids*, *14*, pp.212-238.

Salame, L. and Glazer, I., 2015. Stress avoidance: vertical movement of entomopathogenic nematodes in response to soil moisture gradient. *Phytoparasitica*, *43*(5), pp.647-655.

Saleh, M.M., Metwally, H. and Abonaem, M., 2020. Commercialization of biopesticides based on entomopathogenic nematodes. In *Cottage Industry of Biocontrol Agents and Their Applications*, pp. 253-275. Springer, Cham.

Santhi, V.S., Ment, D., Salame, L., Soroker, V. and Glazer, I., 2016. Genetic improvement of host-seeking ability in the entomopathogenic nematodes *Steinernema carpocapsae* and *Heterorhabditis bacteriophora* toward the Red Palm Weevil *Rhynchophorus ferrugineus*. *Biological Control*, *100*, pp.29-36.

Schafer, W., 2016. Nematode nervous systems. *Current Biology*, *26*(20), pp.R955-R959.

Shan, S., Ma, H., Li, Y., Huang, C., Gu, X., Jiang, Z., Sun, B., Chen, C., Wei, X., Shen, G. and Shapiro-Ilan, D., 2020. Metabolites from symbiotic bacteria of entomopathogenic nematodes have antimicrobial effects against Pythium myriotylum. *European Journal Of Plant Pathology*, *158*, pp.35-44.

Shan, S., Wang, W., Song, C., Wang, M., Sun, B., Li, Y., Fu, Y., Gu, X., Ruan, W. and Rasmann, S., 2019. The symbiotic bacteria Alcaligenes faecalis of the entomopathogenic nematodes Oscheius spp. exhibit potential biocontrol of plant-and entomopathogenic fungi. *Microbial Biotechnology*, *12*(3), pp.459-471.

Shapiro, D.I., Lewis, E.E., Paramasivam, S. and McCoy, C.W., 2000. Nitrogen partitioning in Heterorhabditis bacteriophora-infected hosts and the effects of nitrogen on attraction/repulsion. *Journal Of Invertebrate Pathology*, *76*(1), pp.43-48.

Shaurub, E.H., Soliman, N.A., Hashem, A.G. and Abdel-Rahman, A.M., 2015. Infectivity of four entomopathogenic nematodes in relation to environmental factors and their effects on the biochemistry of the Medfly Ceratitis capitata (Wied.)(Diptera: Tephritidae). *Neotropical Entomology*, *44*(6), pp.610-618.

Shokoohi, E., 2023. Impact of agricultural land use on nematode diversity and soil quality in Dalmada, South Africa. *Horticulturae*, *9*(7), pp.749.

Singh, R. and Singh, G., 2021. Aphids. In *Polyphagous Pests of Crops*, pp. 105-182. Springer, Singapore.

Singha, A.K., Kumarb, M., Ahujab, A., Vinayb, B.K., Kommuc, K.K., Thakurd, S., Paschapura, A.U., Jeevana, B., Mishraa, K.K., Meenae, R.P. and Parihare, M., 2022. Entomopathogenic nematodes: a sustainable option for insect pest management.

Sparks, T.C., Crossthwaite, A.J., Nauen, R., Banba, S., Cordova, D., Earley, F., Ebbinghaus-Kintscher, U., Fujioka, S., Hirao, A., Karmon, D. and Kennedy, R., 2020. Insecticides, biologics and nematicides: Updates to IRAC's mode of action classification-a tool for resistance management. *Pesticide Biochemistry And Physiology*, *167*, pp.104587.

Steyn, V.M., Malan, A.P. and Addison, P., 2021. Efficacy of entomopathogens against Thaumatotibia leucotreta under laboratory conditions. *Entomologia Experimentalis et Applicata*, *169*(5), pp.449-461.

Thakur, N., Kaur, S., Tomar, P., Thakur, S. and Yadav, A.N., 2020. Microbial biopesticides: current status and advancement for sustainable agriculture and environment. In *New and future developments in microbial biotechnology and bioengineering*, pp. 243-282. Elsevier.

Valverde, S., Vidiella, B., Montanez, R., Fraile, A., Sacristán, S. and Garcia-Arenal, F., 2020. Coexistence of nestedness and modularity in host–pathogen infection networks. *Nature Ecology & Evolution*, *4*(4), pp.568-577.

Vashisth, S., Chandel, Y.S. and Chandel, R.S., 2019. Comparative efficacy of indigenous *Heterorhabditid* nematodes from north western Himalaya and *Heterorhabditis indica* (Poinar, Karunakar & David) against the larvae of Helicoverpa armigera (Hubner). *International Journal Of Pest Management*, *65*(1), pp.16-22.

Velasco, L., Ruiz, L., Galipienso, L., Rubio, L. and Janssen, D., 2020. A historical account of viruses in intensive horticultural crops in the Spanish mediterranean arc: New challenges for a sustainable agriculture. *Agronomy*, *10*(6), pp.860.

Wang, Y., Fang, X., An, F., Wang, G. and Zhang, X., 2011. Improvement of antibiotic activity of Xenorhabdus bovienii by medium optimization using response surface methodology. *Microbial Cell Factories*, *10*(1), pp.1-15.

Wilschut, R.A. and Geisen, S., 2021. Nematodes as drivers of plant performance in natural systems. *Trends In Plant Science*, *26*(3), pp.237-247.

Wyatt, M. and Greathouse, K.L., 2021. Targeting dietary and microbial tryptophan-indole metabolism as therapeutic approaches to colon cancer. *Nutrients*, *13*(4), pp.1189.

Yadav, B., Singh, U.B., Malviya, D., Vishwakarma, S.K., Ilyas, T., Shafi, Z., Shahid, M. and Singh, H.V., 2023. Nematophagous Fungi: Biology, Ecology and Potential Application. In *Detection, Diagnosis And Management Of Soil-borne Phytopathogens,* pp.309-328. Singapore: Springer Nature Singapore.

Yooyangket, T., Muangpat, P., Polseela, R., Tandhavanant, S., Thanwisai, A. and Vitta, A., 2018. Identification of entomopathogenic nematodes and symbiotic bacteria from Nam Nao National Park in Thailand and larvicidal activity of symbiotic bacteria against *Aedes aegypti* and *Aedes albopictus*. *PloS One*, *13*(4), p.e0195681.

Zhang, X., Machado, R.A., Van Doan, C., Arce, C.C., Hu, L. and Robert, C.A., 2019. Entomopathogenic nematodes increase predation success by inducing cadaver volatiles that attract healthy herbivores. *Elife*, *8*, pp.46668.

Zhang, Y., Li, H., Wang, F., Liu, C., Reddy, G.V., Li, H., Li, Z., Sun, Y. and Zhao, Z., 2023. Discovery of a new highly pathogenic toxin involved in insect sepsis. *Microbiology Spectrum*, *11*(6), pp.01422-2.

Chapter 2

Pathogenicity and molecular identification of *Cruznema sp*. NTM 2021

Abstract

Pest infestation in agriculture horticulture sector pose a significant threat to food security, especially in most developing countries. Many horticulture practitioners across the globe uses integrated chemical pesticides as the ultimate solution for pest infestations. However, the long use of chemical pesticides has resulted in detrimental effects to the ecosystem and human health. Mostly of synthetic chemical pesticides consists of large amounts of heavy metals. The accumulation of these heavy metals on soil leads to oligodynamic process. Some of these metals are insoluble and runoff to nearby waterbodies causing ground water contamination. Conservation biological control is the use of indigenous predatory species to prevent pest infestation. Entomopathogenic nematodes (EPNs) are biological control agents of agriculture problematic pests. These species have symbiotic relationship with pathogenic bacteria from *Entobacteriaceae* family (namely *Photorhabdus*, *Serratia* and *Xenorhabdus*). The pathogenetic bacteria is located within EPNs gut and released upon host infection. EPNs bacteria symbionts are important for infection and depression of host immune defence. The Koch's Postulate was performed assess *Cruznema sp.* NTM 2021 pathogenicity on Petri dish consisting of 8 % of loam soil and *Tenebrio molitor* (*T. molitor*) was used as host model. DNA of *Cruznema sp.* NTM 2021 was extracted using E.Z.N.A.® Insect DNA kit and subjected to PCR before sent for sanger sequencing at Inqaba biotech (PTY) (South Africa). The sequencing was subjected to quality control to remove base ambiguity and low-quality base(s) using Finch TV (version 1.4.0) and assembled using CLC Bio Main Benchwork. Consensus sequence was subjected to BLASTn algorithm to find homologous sequence species. All homologous sequences and query sequence were subjected to pairwise alignment using CLUSTALW. The alignment data was used to calculate evolutionary divergence using Tajima-Nei model. The phylogenetic tree was constructed using Maximum likelihood using Tajima-Nei nucleotide substitution model and 1000 bootstrap. *Cruznema sp.* NTM 2021 was able to infect and kill *T. molitor*. *Cruznema sp.* NTM 2021 is closely related to *Cruznema sp*. isolate 734CI by 97 % and has no evolutionary distance divergence with *Cruznema sp.* JN2018. *Cruznema sp.* NTM 2021 exhibits EPNs characteristics and therefore it is a pontetial biological control agent.

Keywords: *Entobacteriaceae*, Homologous, Koch's Postulate, Symbiosis, *Tenebrio molitor*.

2.1 Introduction

2..1.1 Pest infestation in agriculture

Agriculture is crucial in maintaining food security in most developing countries including South Africa. However, pest infestation in horticulture has led into low food production which consequently result in food scarcity within the country (Chatterjee, 2022). Therefore, the horticulture practitioners have resorted into the use of chemical pesticides and fertilizers to sustain their annually food production (Wan *et al.,* 2013). Furthermore, the long use of the chemical integrated pesticides has detrimental effects to the environment and human health (Alengebawy *et al.,* 2021; Kannan *et al.,* 2023). Hence, there is an urgently need to develop alternative means to pest control that do not have negative impact towards environment and human health.

2.1.2 The effects of chemical pesticides practise in horticulture

Chemical integrated pesticides application in horticulture sector has pivotal role towards defending crops against problematic insect pest. Recent studies shows that the use of synthetic chemical pesticides triggers oligodynamic processes in soil (Kannan *et al.,* 2023; Yang *et al.,* 2023). Some synthetic chemical pesticides comprise of heavy metals such as Zn (Zinc), Pb (lead), Cu (copper) and Cd (cadmium) which are deposited on soil initiating oligodynamic processes (Alengebawy *et al.,* 2021). Accumulation of these heavy metals alters the soil pH level rendering the soil inhabitable for beneficial microorganisms and inadequate for crop farming (Wołejko *et al.,* 2020). Furthermore, some of these synthetic chemical pesticide compounds are insoluble in water and therefore during precipitation of these metals runoff from the surface and become deposited into nearby waterbodies (Srivastav, 2020; Alengebawy *et al.,* 2021). Moreover, the contaminated waterbodies pose a significant health threat all organisms that uses that waterbody as source of water (Srivastav, 2020).

2.1.3 Biological control

Biological control refers to regulatory process of pest population through the implementation of natural agents (pathogens or parasites) (Clausen, 1958). Pest management is essential towards successful crop production however, pest management method should be eco-friendly and not have negative impact towards human health. There are three types of biological control methods (namely conservation, augmentation and inoculative) (Stiling and Cornelissen, 2005; Hajek and Eilenberg, 2018). The aim of this study base on conservation method. Conservation method uses the natural indigenous parasite that has adaptability to the environment to control the population of pests (Wyckhuys *et al.,* 2013; Begg *et al.,* 2017; Shields *et al.,* 2019).

Therefore, this is the more desirable way towards mitigating pest infestation in horticulture rather, using a foreign parasite as it may decline its efficacy (Hajek *et al.,* 2016). Therefore, EPNs are adequate biological control agent, due to the widely distribution in ecosystem.

2.1.5 Entomopathogenic nematodes biological control agent

Entomopathogenic nematodes (EPNs) are micro-organisms that are natural soil habitants. These micro-organisms house a symbiotic relationship with bacteria that is a main agent of causing insect death. There are only three (*Oscheius, Heterorhabditidae* and *Steinernematidae*) genera in nematodes known to consists of EPNs (Nthenga *et al.,* 2021). Furthermore, not all nematodes are EPNs (Lewis *et al.*, 2015). This is the results of the absence of symbiotic bacteria. EPNs are biological control agents against various problematic insects (Hiltpold, 2015). The EPNs bacteria symbionts belong to *Entobacteriaceae* family (namely *Photorhabdus, Serratia* and *Xenorhabdus*) (Lephoto and Gray, 2019; Nthenga *et al.,* 2021; Toopaang *et al.,* 2022). The pathogenic symbiotic bacteria harboured within the nematodes important for survival and virulence against other organisms, field efficacy and bacterial infections (Toopaang *et al.,* 2022).

EPNs search for host and uses any external openings to gain an entry into the host body (Lephoto and Gray, 2019; Matlhabe *et al.,* 2022). Furthermore, the symbiotic pathogenic bacteria are released within the host gut witch results into the host death elucidated in Figure 2.1. Moreover, since there is enough food in the host body and the conditions are ideal for reproduction of the EPNs infective juveniles (IJs) (Khan *et al.,* 2020). When the nutrients or food within the host declines, emergence of IJs outside of the host body into the soil take place searching for the host (Lephoto and Gray, 2022). IJs possess the ability to survive for long time in the soil without feeding or within the host body (Lephoto and Gray, 2022).

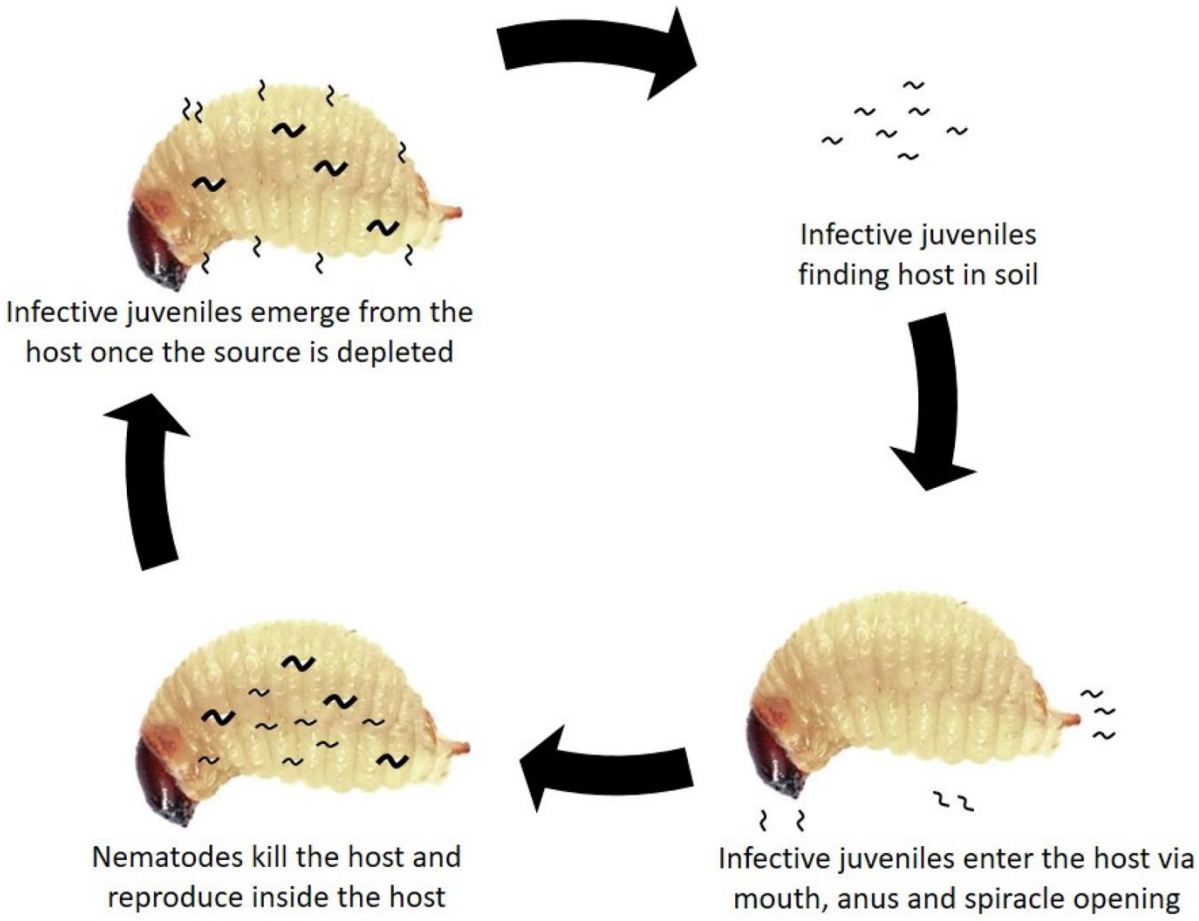


Figure 2.1: Schematic diagram showing entomopathogenic nematodes life cycle. Infective juveniles search for the host insect. Once the IJs penetrate the host system the release the symbiotic causing infection resulting in the death of insect hots. The IJs reproduce within the host and emerger out searching for new host (food). (Image adapted from Nurashikin-Khairuddin *et al.*, 2022)

2.2 Method and Materials

2.2.1 Surface sterilization of *Cruznema* nematodes

The nematodes were supplied by Nematology laboratory stationed at University of Witwatersrand. Ten ml of 0.1 % sodium hypochlorite (NaOCl) was used to suspend the IJs in 50 mL falcon tubes and tube was gently inverted three times and incubated at room temperature for three hours. The NaOCl was withdrawn from the tube and subsequently the IJs were rinsed three times using 10 mL sterile deionised water (Lephoto and Gray, 2019).

2.2.3 Koch's Postulate

A loam soil was collected from the ground and filtered from plant debris using 0.2 cm sieve into glass jars. The sieved soil was autoclaved at 121 ˚C at 15 psi atmospheric pressure for 20 minutes to sterilised it from possible biological species. Then 20 g of sterilised soil was added into a sterilised transparent petri dish. Sterilised deionised water was used to hydrate the soil

to 8 % moisture. Subsequently, three thousand surface sterilised nematodes infective juveniles (IJs) were dispensed into the soil followed by addition of five *Tenebrio molitor* (*T. molitor*) larvae that were surface sterilised using 70 % ethanol (Figure 2.2). The plates were closed and incubated at 25 ˚C and constantly monitored. The dead larvae were removed from the plate and used in white trap technique to isolate the IJs as described in Section 2.2.4. This experiment was repeated three times.



Figure 2.2 Image showing a prepared Petri dish during Koch's postulate

2.2.4 White trap technique

A small Petri dish cap with as size of 50 mm was at the centre of a larger Petri dish with a size of 90 mm. Furthermore, a 54 mm Whatman filter paper is then placed was placed on the cap of small Petri dish that is inside a large Petri dish. Subsequently, sterilised deionised water was poured until the edges of the Whatman filter paper touches the water. A dead larva is then sterilised with 70 % ethanol and placed on the top of the Whitman filter paper Figure 2.3. The plate is closed and incubated in the room temperature, allowing the emergence of nematodes form the larva into the water.



Figure 2.3 Elucidating a white trap technique of isolating nematodes infective juveniles

2.2.5 Genomic DNA extraction and Polymerase chain reaction

The infective juveniles (IJs) of *Cruznema sp*. NTM 2021 were collected from the White traps and surface sterilised as previously described in Section 2.2.1. The IJs were transferred to sterile 1.5 ml Eppendorf tube and sterile plastic pestle was used crush the IJs. Furthermore, the DNA of the IJs was extracted using E.Z.N.A.® Insect DNA kit following the manufactures instructions. Furthermore, Polymerase chain reaction (PCR) protocol was carried out using both forward primer TW8 (5'-GCGGATCCGTTTCC GTAGGTGAACCTGC-3') and reverse primers AB28 (3'- GCGGATCCATATGCTTAA GTTCAGCGGGT-5') to amplify ITS region located between 18S and 28S rDNA. The PCR was caried using the following conditions as previously described in Lephoto and Gray, (2019). The initial denaturing was conducted at 94 ℃ for 5 minutes, denaturing at 95 ℃ for 60 seconds, annealing at 64 ℃ for a minute, extension at 72 ℃ for 2 minutes and final extension was carried at 72 ℃for 10 minutes. Furthermore, the total number of circles conducted were 55.

2.2.6 Molecular identification and Phylogenetic Construction of *Cruznema sp.* NTM 2021

2.2.6.1 Genomic sequencing using Sanger sequencing technique

The amplicon product was purified using Zymo Research, ZR-96 DNA Sequencing Clean-up Kit™ and sequenced in both (forward and reverse) directions utilizing (Nimagen, BrilliantDye™ Terminator Cycle Sequencing Kit V3.1, BRD3-100/1000) with the aid of ABI 3730*xl* Genetic Analyzer (Applied Biosystems, Thermo Fisher Scientific) in sanger sequencing machine at Inqaba Biotech PTY (South Africa).

2.2.6.2 Quality control

Both forward and reverse sequences raw reads for 18S RNA *Cruznema sp.* NTM 2021 aquired from sanger sequencing were filtered and trimmed manual using Finch TV (version 1.4.0) separately (Butler *et al.,* 2014). Furthermore, both forward and reverse reads of each species were respectively assembled using CLC Bio Main Benchwork to produce a consensus/ contig sequences (Zeden and Gründling, 2023).

2.2.6.3 Multiple sequence alignment using CLUSTALW

Each contig sequences of the respective species was subjected to online BLASTn search tool in NCBI database using default parameters. The query sequence and sequence with high similarty from NCBI BLASTn results were subjected to CLUSTALW for sequence alignment in MEGA 11 software (Choi *et al.,* 2000). *C. isolate 734C-1* accession number: OR606776.1; *C. GD1* accession number: ON191475.1; *C. JN2018* accession number: ML156051.1; *C. JH-2004*; *C. tripartitum* accession number: EU196012.1 and *C. elegans* accession number: MG551717.1 was used to construct phylogenetic tree of *Cruznema sp.* NTM 2021, note this were the only homologous species in the data base, due to that these species are hardly studied)

2.2.6.4 Evolutionary divergence estimation

The evolutionary divergence estimation among the speed was archive by subjecting the pairwise alignment obtained from Section 2.2.6.3 to distance estimation program embedded in MEGA 11 software using Tajima-Nei nucleotide substitution method (Tamura *et al.,* 2021). The bootstrap value was adjusted to 1000 and both gaps and missing data was complete removed. Furthermore, Caenorhabditis elegans of MG551717.1 accession number was used as an outgroup.

2.2.6.5 Phylogenetic tree construction

The phylogenetic tree for each species was constructed using Maximum likelihood on MEGA version 11 tool, using the data obtain from CLUSTALW multiple alignments (Section 2.2.6.3).

The parameters were set to Kimura Nei parameter sequence substitution model with bootstrap value of one thousand (Tamura *et al.*, 2021).

2.3 Results



Figure 2.4: Image showing symptoms of *Cruznema sp.* NTM 2021 infection on a) a healthy *T. molitor* B) an infected *T. molitor*

Table 2.1: Matrix table elucidating evolutionary divergence prediction by CLUSTALW. The evolutionary divergence was estimated through Tajima-Nei model as previously described in Tamura *et al.* (2021). Base sequence substitution per site values among the species are below the clear diagonal line, whereas the above values indicate the standard error estimates (SEE) which were obtain by a 1000 bootstrap. The sequence position that consists of gaps and missing data were eliminated.

	1	2	3	4	5	6	7
1. AY284658.1 Cruznema sp. JH-2004		0.0482610141	0.0194925997	0.0546358631	0.0554175240	0.0532640210	0.0554175240
2. MG551717.1 Caenorhabditis elegans	0.7045454545		0.0490138018	0.0506983878	0.0522230790	0.0536023464	0.0522230790
3. EU196012.1 Cruznema tripartitum strain SB202	0.0340909091	0.6818181818		0.0543641947	0.0550809352	0.0527551067	0.0550809352
4. OR606776.1 Cruznema sp. isolate 734C-1	0.5568181818	0.6136363636	0.5568181818		0.0256312039	0.0497128093	0.0256312039
5. Cruznema sp. NTM 2021	0.5454545455	0.5909090909	0.5454545455	0.0568181818		0.0513983868	0.0000000000
6. ON191475.1 Cruznema sp. GD-1	0.6250000000	0.6250000000	0.6250000000	0.3295454545	0.3750000000		0.0513983868
7. MK156051.1 Cruznema sp. JN2018	0.5454545455	0.5909090909	0.5454545455	0.0568181818	0.0000000000	0.3750000000	

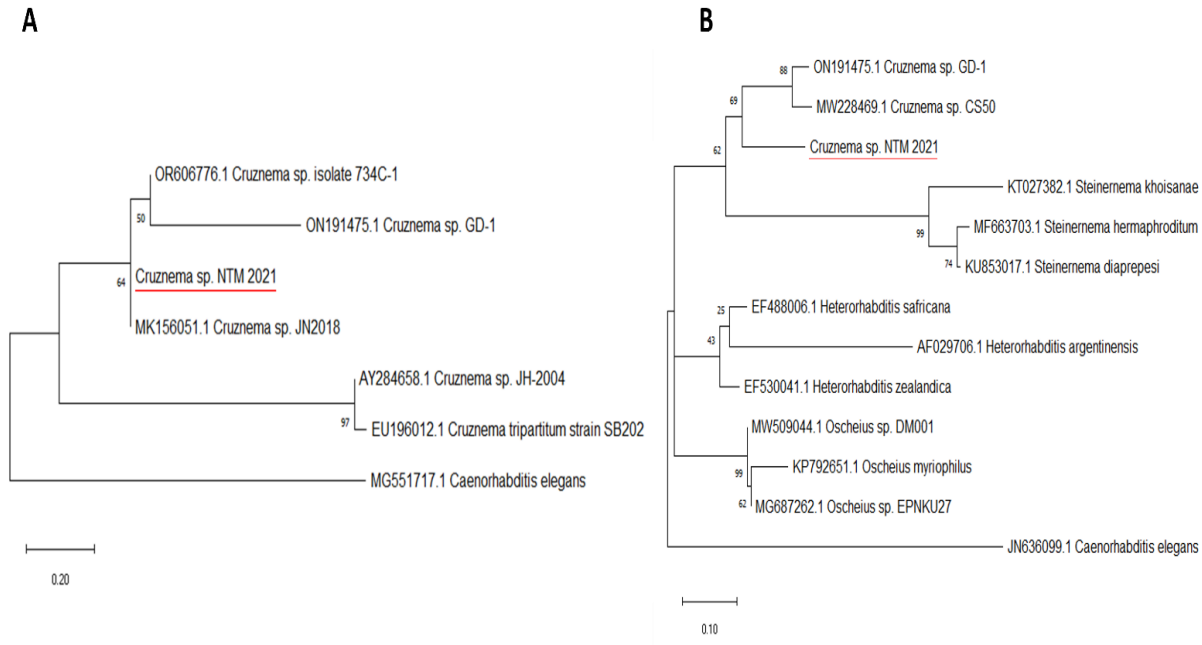


Figure 2.5: Elucidating 18S RDNA evolutionary relationship of *Cruznema sp.* NTM 2021. There phylogenetic tree has a scale of 0.2 evolutionary lineage over time. A) *Cruznema* sp. NTM among *Cruznema* nematodes species B) *Cruznema sp*. NTM among EPNs species. The phylogenetic tree was constructed using statistical analysis Maximum likelihood and Tamura-Nei nucleotide substitution method (Tamura *et al*., 2021).

2.4 Discussions

Genomic identification is a very important phenomenon towards understanding evolution and geographical distribution among organisms. Furthermore, this forms a fundamental role of

population genetics and identifying a potential biological control agent species (Valadas *et al*, 2011). Newly identify species should be clustered into their respectively taxonomic groups, hence in this study we implement 18S rDNA genomic molecular to identify undescribed nematode.

Koch's postulate experiment shows that *Cruznema sp.* NTM 2021 has ability to infect and kill *Tenebrio molitor* (*T. molitor*) larvae (Figure 2.4). Therefore, this is intriguing considering the previous studies stating that *Cruznema* nematodes are bacterivores. Hence, they are classified in bacterivore feeding trophic group. Moreover, it is believed that the morphological feature of the mouth of nematodes is designed to be adaptable to type of foods it consumes. However, this belief is questionable when it comes to entomopathogenic nematodes (EPNs), since the symbiosis test nematodes thus feeds on their bacteria symbionts, which also facilitates their growth. The *T. molitor* larvae used in this study were healthy with no mechanical words around their body (Figure 2.4), *T. molitor* skin membrane is rigid making it hard for possible pathogens to gain access to its internal body. Therefore, *Crunema sp.* NTM 2021 infective juveniles (IJs) may had gain access to *T. molitor* system through biological openings (mouth, eyes, and anus). However, the host seeking behaviour of the IJs it is unknown at this stage. The infection symptoms on *T. molitor*, the thorax section changes colour to black which eventually progresses to the abdomen section (Figure 2.4B).

The consensus 18S RDNA sequence was subjected to BLASTn algorithm from NCBI database (Section 2.2.6.3). the query sequence was closely identical with *Cruznema sp*. isolate 734CI (accension number: OR606776.1) by 97.11 % with an E value of 0.0, *Cruznema sp.* GD1 (accension number: ON1914575.1) by 88.44 % with an E value of $3e^{-100}$ and *Cruznema sp.* JN2018 (accension number: MK156051.1) by 100 % with an E value of $2e^{-38}$. E value refers to the expected number of times that a score appears in a random data base of the given size. Furthermore, the E value decreases exponentially when the score of the match it increases. Moreover, when the E value is 0.0 as the case with *Cruznema sp*. isolate 734CI means it exact match when it is aligned with our query sequence. However, it is important to note that BLASTn can perform the search by alignment despite the difference in sequence length. Therefore, despite the E value being 0.0 the sequence may not be 100 % identical. Furthermore, in case of query sequence with *Cruznema sp.* JN2018, it is crucial to note that *Cruznema sp.* JN2018 is 329 base pairs long whereases our query sequence is 654 base pairs long. Therefore, *Cruznema sp.* JN2018 matches to our sequence during nucleotide substitution by BLASTN alignment due to its small size however, there are gaps during the alignment with increases its

E value. Furthermore, despite *Cruznema sp.* JN2018 having 100 % identity to our query sequence do not mean it the same species with our query sequence since the E value is above 0.0.

Evolutionary divergence distance was calculated using. Furthermore, prior to evolutionary distance calculation, a pairwise alignment was performed using CLUSTALW algorithm and it shows that *Cruznema sp*. NTM 2021 has 0.0568 divergence distance with its closely related species *Cruznema sp*. isolate 734CI, 0.375 divergence distance with *Cruznema sp*. GD1 and no divergence with *Cruznema sp.* JN2018 (Table 2.1). Furthermore, more analyses were performed through using Tajima-Nei model with 1000 bootstrap value to produces standard error estimate (SSE) value(s) while removing all the gaps and missing bases and it reveals *Cruznema sp.* NTM 2021 has a 0.02563 SEE value with *Cruznema sp*. isolate 734CI, 0.05134 SEE value with *Cruznema sp*. GD1 and the SSE value was 0.0 with *Cruznema sp.* JN2018 substantiating the distant divergence generated by pairwise alignment. Further validations were conducted through construction of phylogenetic tree using Maximum likelihood method and Tajima-Nei nucleotide substitution method with bootstrap set to 1000 (Figure 2.5 A). *Caernorhabditis elegans* (accension number: MG551717.1) was used as outer group (Figure 2.5 A). *Cruznema sp.* NTM 2021 forms a clade with all three closely reported species (namely *Cruznema sp*. isolate 734CI, *Cruznema sp*. GD1 and *Cruznema sp.* JN2018) however, *Cruznema sp.* NTM 2021 do not form any divergence with *Cruznema sp.* JN2018 (Figure 2.5 A) this is supported with evolutionary distant divergence in Table 2.1. *Cruznema sp*. NTM 2021 phylogenetic tree has a scale of 0.2 representing number of nucleotides substitution per site (Figure 2.5 A). Therefore, considering the research gap in studying *Cruznema* nematodes and few available data in the database, it is not surprising that these species have undergone a huge change. Therefore, there is a need to recharacterize this species. Furthermore, *Cruznema* sp. NTM 2021 forms a clade with other *Cruznema* nematodes and brench off with EPNs species expected since EPNs and *Cruznema* originated from same phylum order *Rhabditida* in Figure 2.5 B. thus support the taxonomic classification in study conducted by Marcaida *et al.* (2022).

2.5 Conclusions and recommendations

Cruznema sp. NTM 2021 possess the ability to infect and kill insects and there it may be considered as a potential biological control agent. *Cruznema sp.* NTM 2021 mimic the entomopathogenic nematodes (EPNs) behaviour. Therefore, more studies such as metagenomics should be carried to profile its possible gut microbial community, to understand its pathogenicity mechanism.

2.6 References

Alengebawy, A., Abdelkhalek, S.T., Qureshi, S.R. and Wang, M.Q., 2021. Heavy metals and pesticides toxicity in agricultural soil and plants: Ecological risks and human health implications. *Toxics*, *9*(3), pp.42.

Begg, G.S., Cook, S.M., Dye, R., Ferrante, M., Franck, P., Lavigne, C., Lövei, G.L., Mansion-Vaquie, A., Pell, J.K., Petit, S. and Quesada, N., 2017. A functional overview of conservation biological control. *Crop Protection*, *97*, pp.145-158.

Butler, M., Henter, H. and Mel, S., 2014. From bugs to barcodes: Using molecular tools to study biodiversity. *Tested Studies For Laboratory Teaching*, *35*, pp.41-55.

Chatterjee, S., 2022. How hard did that sting? Estimating the economic costs of locust attacks on agricultural production. *Applied Economic Perspectives And Policy*, *44*(1), pp.434-459.

Choi, J.H., Jung, H.Y., Kim, H.S. and Cho, H.G., 2000. PhyloDraw: a phylogenetic tree drawing system. *Bioinformatics*, *16*(11), pp.1056-1058.

Clausen, C.P., 1958. Biological control of insect pests. *Annual Review Of Entomology*, *3*(1), pp.291-310.

Hajek, A.E. and Eilenberg, J., 2018. *Natural enemies: an introduction to biological control*. Cambridge University Press.

Hajek, A.E., Hurley, B.P., Kenis, M., Garnas, J.R., Bush, S.J., Wingfield, M.J., Van Lenteren, J.C. and Cock, M.J., 2016. Exotic biological control agents: a solution or contribution to arthropod invasions?. *Biological Invasions*, *18*, pp.953-969.

Hiltpold, I., 2015. Prospects in the application technology and formulation of entomopathogenic nematodes for biological control of insect pests. *Nematode Pathogenesis Of Insects And Other Pests*, pp.187-205.

Kannan, S., Perumal, V., Yuvaraj, A., Pittarate, S., Kim, J.S. and Krutmuang, P., 2023. Biodegradation of pesticide in agricultural soil employing entomopathogenic fungi: Current state of the art and future perspectives. *Heliyon*.

Khan, B., Javed, N., Khan, S.A., Rajput, N.A., Atiq, M., Jabbar, A., Rehman, A., Moosa, A. and Ali, M.A., 2020. Potential of entomopathogenic nematode (*Steinernema kraussei*) against last instar larvae of different *lepidopteran* insect pests. *Pakistan Journal Of Zoology*, *52*(4), pp.1275.

Lephoto, T.E. and Gray, V.M., 2019. *Oscheius basothovii* n. sp. (Nematoda: *Rhabditidae*), a new entomopathogenic nematode isolated from an uncultivated grassland in South Africa. *Archives Of Phytopathology And Plant Protection*, *52*(1-2), pp.125-140.

Lephoto, T.E. and Gray, V.M., 2022. Inspecting the pathogenicity of the integument of *Oscheius basothovii* following sterilization with sodium hypochlorite. *Biological Control*, *171*, pp.104924.

Lewis, E.E., Hazir, S., Hodson, A. and Gulcu, B., 2015. Trophic relationships of entomopathogenic nematodes in agricultural habitats. In *Nematode pathogenesis of insects and other pests*, pp.139-163. Springer, Cham.

Marcaida, A.J.B., Nakao, M., Fukutani, K., Nishikawa, K. and Urabe, M., 2022. Phylogeography of *Rhabdias* spp.(Nematoda: *Rhabdiasidae*) collected from Bufo species in Honshu, Shikoku, and Kyushu, Japan including possible cryptic species. *Parasitology International*, *90*, pp.102612.

Matlhabe, O.D., Lephoto, T.E. and Gray, V.M., 2022. Monitoring life cycle stages of axenic cultures of in vitro produced *Oscheius* L8 MCB in semi-solid state and liquid state and comparative efficacy studies between in vitro and in vivo nematode production against *Galleria mellonella. Biological Control*, *170*, pp.104904.

Nthenga, I., Knoetze, R. and Malan, A.P., 2021. Distribution and diversity of entomopathogenic nematodes (*Steinernematidae* and *Heterorhabditidae*) in a South African nature reserve. *Koedoe*, *63*(1), pp.7.

Nurashikin-Khairuddin, W., Abdul-Hamid, S.N.A., Mansor, M.S., Bharudin, I., Othman, Z. and Jalinas, J., 2022. A review of entomopathogenic nematodes as a biological control agent for red palm weevil, *Rhynchophorus ferrugineus* (Coleoptera: *Curculionidae*). *Insects*, *13*(3), pp.245.

Shields, M.W., Johnson, A.C., Pandey, S., Cullen, R., González-Chang, M., Wratten, S.D. and Gurr, G.M., 2019. History, current situation and challenges for conservation biological control. *Biological Control*, *131*, pp.25-35.

Srivastav, A.L., 2020. Chemical fertilizers and pesticides: role in groundwater contamination. In *Agrochemicals Detection, Treatment And Remediation*, pp.143-159. Butterworth-Heinemann.

Stiling, P. and Cornelissen, T., 2005. What makes a successful biocontrol agent? A meta-analysis of biological control agent performance. *Biological Control*, *34*(3), pp.236-246.

Tamura, K., Stecher, G. and Kumar, S., 2021. MEGA11: molecular evolutionary genetics analysis version 11. *Molecular biology and evolution*, *38*(7), pp.3022-3027.

Toopaang, W., Bunnak, W., Srisuksam, C., Wattananukit, W., Tanticharoen, M., Yang, Y.L. and Amnuaykanjanasin, A., 2022. Microbial polyketides and their roles in insect virulence: from genomics to biological functions. *Natural Product Reports*.

Valadas, V., Mrácek, Z., Oliveira, S. and Mota, M., 2011. Three species of entomopathogenic nematodes of the family *Steinernematidae* (Nematoda: *Rhabditida*) new to Continental Portugal.

Wan, N., Ji, X., Jiang, J., Qiao, H. and Huang, K., 2013. A methodological approach to assess the combined reduction of chemical pesticides and chemical fertilizers for low-carbon agriculture. *Ecological Indicators*, *24*, pp.344-352.

Wołejko, E., Jabłońska-Trypuć, A., Wydro, U., Butarewicz, A. and Łozowicka, B., 2020. Soil biological activity as an indicator of soil pollution with pesticides–a review. *Applied Soil Ecology*, *147*, pp.103356.

Wyckhuys, K.A., Lu, Y., Morales, H., Vazquez, L.L., Legaspi, J.C., Eliopoulos, P.A. and Hernandez, L.M., 2013. Current status and potential of conservation biological control for agriculture in the developing world. *Biological Control*, *65*(1), pp.152-167.

Yang, Q., Li, G., Jin, N. and Zhang, D., 2023. Synergistic/antagonistic toxicity characterization and source-apportionment of heavy metals and organophosphorus pesticides by the biospectroscopy-bioreporter-coupling approach. *Science Of The Total Environment*, *905*, pp.167057.

Zeden, M.S. and Gründling, A., 2023. Bacterial whole-genome-resequencing analysis: basic steps using the CLC Genomics Workbench software. *Cold Spring Harbor Protocols*, *2023*(11), pp.107901.

Chapter 3

16S RDNA metagenomic amplicon-based profiling of endobacteria community of *Cruznema sp*. NTM 2021

Abstract

The use of chemical pesticides is one of the common practices in horticulture sector throughout the globe. However, this practice has negative drawbacks towards ecosystem and human health. Therefore, the horticulture practitioners have initiated conservation biological control practise with entomopathogenic nematodes (EPNs) as their biological control agent, to mitigate the detrimental effects of the use of chemical pesticides. EPNs are insect parasite microscopic worms that widely found throughout the ecological niche. They are renowned for their ability to seek and kill the insect pest. EPNs have symbiotic relationship with pathogenic bacteria from *Photorhabdus, Serratia* and *Xenorhabdus* genus, that they use to kill the insect host. These pathogenic bacteria they are not only known for important role of EPNs pathogenicity. However, they also serve an important role in immune defence of EPNs. *Cruznema* nematodes belong to phylum order *Rhabditida* as EPNs. However, they are known as bacterivores. Cruznema nematodes have ability to kill insect (Crickets and slugs). There is no evidence of *Cruznema* nematodes having a symbiosis relationship with pathogenic bacteria. This study seeks to profile possible *Cruznema sp.* NTM 2021 gut bacteria community. Mass reproduction of *Cruznema sp.* NTM 2021 infective juveniles (IJs) was archive by a surface sterilised IJs on Petri dish consisting of loam soil with 8 % moisture level and six *Tenebrio molitor* (*T. molitor*) larvae. White trap technique was performed to harvest the IJs emerging out from dead *T. molitor* larvae. One set of IJs were surface sterilised using 0.1 % sodium hypochlorite solution whereas the other set no sterilization was performed on them. Both samples were sent to Inqaba Biotech (PTY) (South Africa) for 16S RDNA amplicon metagenomics sequencing. The sequences data was subjected to quality control using SMRTlink Version 11.0. The data was subsequently analysed using Qiime 2 with the aid of silva version 138.1 reference database. The results were further processed in Krona version 1.9.0 pipeline. This study reveals the gut microbial community of *Cruznema sp.* NTM 2021 with predominantly phylum order *Pseudomonadales* at 40 %, *Hyphomicrobiales* at 23 % and *Bacteroidetes* at 22 %. Furthermore, the least dominant phylum order *Bacilli* at 4 % and both *Xanthomonadales* and *Burkholderiales* at 3 %.

Keywords: *Bacilli, Hyphomicrobiales*, pathogenicity, *Rhabditida*, *Xanthomonada.*

3.1 Introduction

3.1.1 Application of entomopathogenic nematode in agriculture

Entomopathogenic nematodes (EPNs) are soil dwelling insect parasite microscopic worms. These parasitic worms have been reported to have a wide feeding range (Hein *et al.,* 2023). Insect pest infestation is considered one of the major threats in food security in horticulture sector (Khan *et al.,* 2023). Furthermore, the horticulture practitioners have introduced the use of chemical pesticides for over two centuries. The use of chemical pesticides has negative drawbacks towards ecosystem and human health (Khan *et al.,* 2023). Therefore, the implementation of conservation biological control method requires a biological control with an ability to adapt and strive through different environmental factors (namely UV light, desiccation, temperature, pH) (Li *et al.,* 2020). Hence, EPNs are considered as potential biological control agent since they are well distributed throughout the ecosystem (Abate *et al.,* 2023). Furthermore, EPNs have unique abilities that enables them to survive throughout different ecological factors, making them a suitable candidates of conservation biological control agent (Mhatre *et al.,* 2017; Lephoto and Gray, 2019). EPNs has symbiosis with the pathogenic bacteria, which are reported to be responsible for causing the death of insect host death (Abate *et al.,* 2018; Doremus and Hunter, 2020; Lephoto and Gray, 2022). EPNs are eco-friendly and does not pose a threat towards human health (Kumar and Yadav, 2020).

3.1.2 Entomopathogenic nematodes overview

There are only three known genera (*Heterorhabditis, Oscheius and Steinernema*) so far, that EPNs originated from. All these EPNs belonging to these three genera has symbiosis relationship with endo-pathogenic bacteria belonging to *Photorhabdus, Serratia* and *Xenorhabdus* genus respectively (Lephoto and Gray, 2019; Katumanyane *et al.,* 2020; Ogier *et al.,* 2023). Therefore, any nematodes that do not have a symbiosis insect pathogenic bacterium are not EPNs (Yooyangket *et al*., 2018). EPNs use the natural external opening such as eyes, anus, or mouth to invade the host (Matlhabe *et al.,* 2022). Once the EPNs infective juveniles (IJs) have invaded the host, they travel to host hemocoel, where they release the endo-pathogenic bacteria symbionts (Atwa, 2014). Endo-pathogenic bacteria symbionts they synthesis toxins, that are used to depress the host immune system as previously described by Lephoto and Gray, 2022. Therefore, IJs proliferation take place exponentially due to the abundance nutrients from the host cadaver (Atwa, 2014; Yooyangket *et al.,* 2018). Furthermore, depletions of nutrients within the host cadaver, forces the emergence of IJs into the soil (Matlhabe *et al.,* 2022). Subsequently, the IJs searches for new host Figure 3.1.

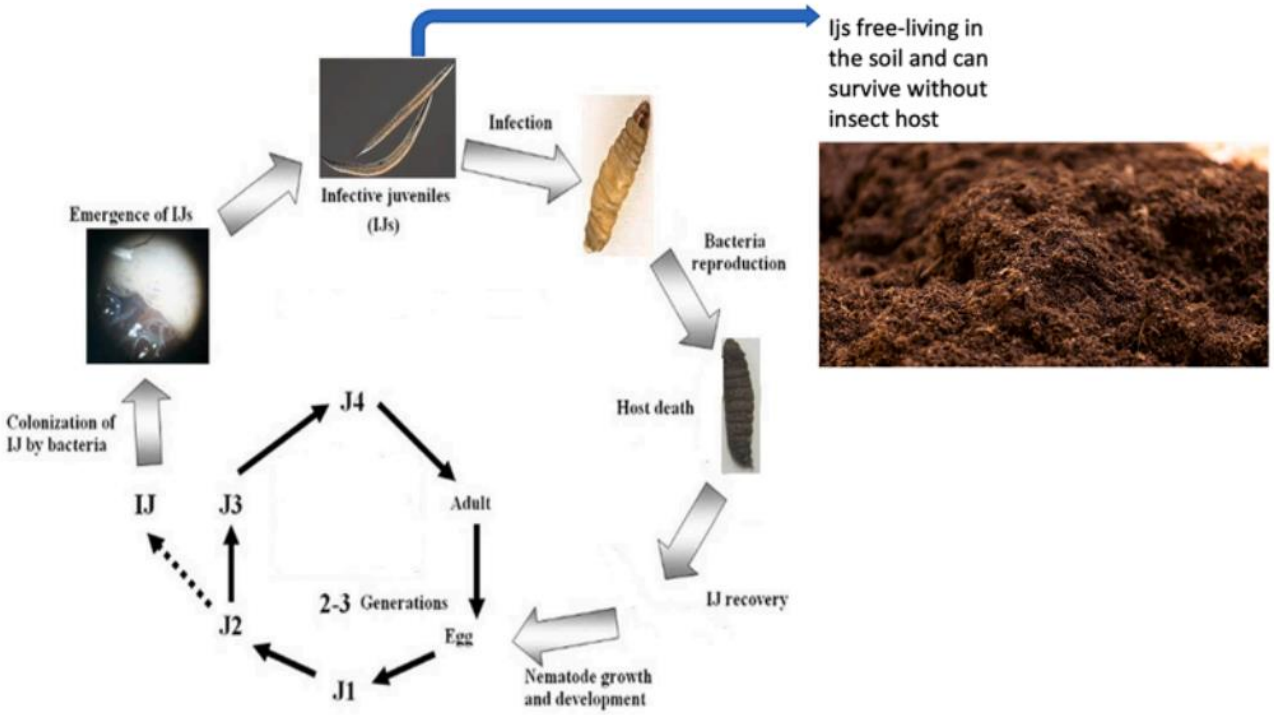


Figure 3.1: A schematic diagram demonstrating natural life cycle of entomopathogenic nematodes (EPNs). EPNs are soil free living organisms that invade the host through natural openings. Nematodes release bacteria that cause infections results in host immunity depression result in host death. The nematodes feed on the host, reproduce and upon depletion of food. the IJs exit the host into the soil on the bases of scout for new host as source of food. Diagram adapted from Lephoto *et al.,* 2022.

3.1.3 Next generation sequencing

Next generation sequencing (NGS) is also known as high throughput sequencing, that use sophisticated machines to study biological genome(s). Furthermore, there are various sequencing technology platforms that are involve in NGS, which do not form a part of the scope of this study. Nonetheless, every NGS platform sequences millions of chunks of fragmented DNA in parallel (Behjati and Tarpey, 2013). Furthermore, computational bioinformatic tools are utilized to rearrange the DNA fragment, mapping out the reads to the reference genome. The scope of this study focusses on the metagenomics sequencing. Metagenomics is classified under high throughput sequencing, however, it is commonly used to study microbial communities. Furthermore, to conduct a metagenomics study there is two distinct routes namely whole genome shotgun (WGS) metagenomics and gene marker method (Pérez-Cobas *et al.*, 2020). WGS metagenomics sequencies all genomes present in a particular sample and provides the insights about biodiversity and functional capabilities of the microbial community in question. Therefore, WGS metagenomics thus enables you to characterize habitat diversity for eukaryotes, archaea, viruses, bacteria, and plasmids including the insights

of the gene content (Pérez-Cobas *et al.,* 2020). However, gene marker method targets a specific region on the DNA to provide the insights about the diversity and composition of taxonomic groups (Pérez-Cobas *et al.,* 2020). Furthermore, to profile microbial community using gene marker method we target 16S RDNA gene to detect the presence of archaea and bacteria.

3.1.4 *Cruznema* nematodes overview

Cruznema nematodes genus are from the family Rhabditida (Reboredo and Camino, 1988; Du *et al.,* 2022). Furthermore, from past centuries *Cruznema* nematodes are labelled as either parasite or bacterivore nematodes (Du *et al.,* 2022). However, there is no evidence that indicate that *Cruznema* have symbiosis relationship with bacteria. Despite numerous publication that suggest that *Cruznema* nematodes that may be used as a biological control agent of agricultural pest Slugs (Mollusca: Gastropoda) and Crickets (d'Ovidio *et al.,* 2019; Du *et al.,* 2022). Therefore, the aim of this study is to explore possible bacteria communities present within the undescribed *Cruznema* nematodes using 16S amplicon-based metagenomics.

3.2 Methods and materials

3.2.1 Culturing of *Cruznema sp.* NTM 2021

Cruznema sp. NTM 2021 Infective juveniles (IJs) were surface sterilised using 0.1 % of sodium hypochlorite as previously described in Section 3.2.2. subsequently 2000 IJs were dispense in Petri dish consisting of 55 g sterilized loam soil. Prior to the additional of IJs in the soil, sterile deionised water was used to hydrate the soil to 8 % moisture level to allow the movement of IJs. Furthermore, six *Tenebrio molitor* (*T. molitor*) larvae were surface sterilised using 70 % ethanol and added into the Petri dish. The Petri dish was kept in 25 ℃ incubator and constantly monitored. The dead larvae were surface sterilised and used to perform White trap techniques as previously described in Section 2.2.4 to facilitate the emergence of IJs.

3.2.2 Preparation of *Cruznema sp.* NTM metagenomic samples

The freshly emerged IJs from *T. molitor* larvae were collected from white trap plate into two 50 mL falcon tubes. The IJs in both tubes were allowed to settle at the bottom of the tube to allow the removal of the excess water. The IJs in one of the two tubes was transferred into a sterile 1.5 mL Eppendorf tube and crushed with pestle whereas, the IJs in another tube were surfaced sterilised with 10 mL (0.1 % sodium hypochlorite) by suspending the IJs in sodium hypochlorite and gently inverted three times and incubated at room temperature for three hours. Subsequently, the IJs were rinsed three times using 10 ml sterile deionised water. Later, the treated IJs were transferred into a new 1.5 mL sterile tube and subsequently crushed with pestle.

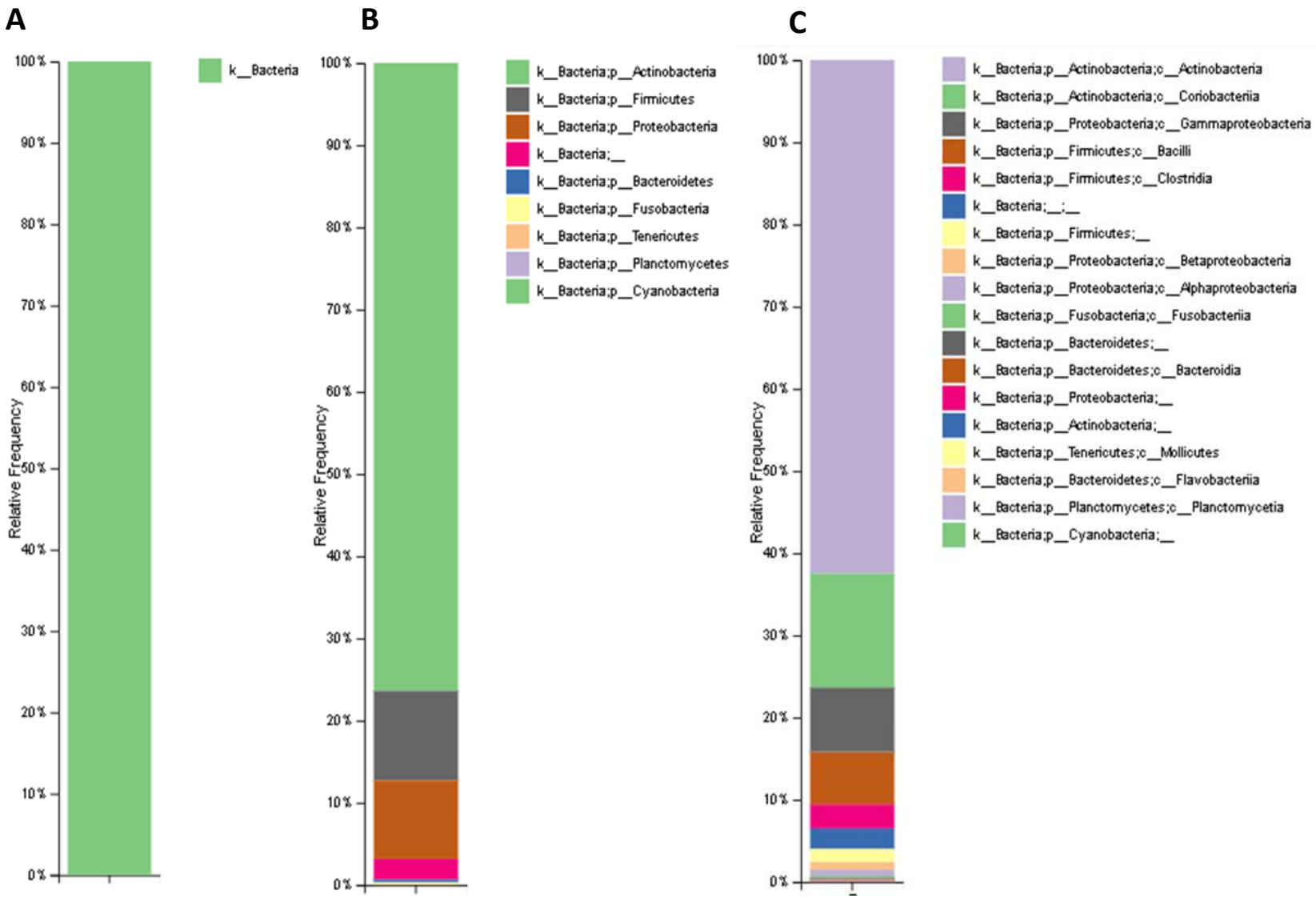
Both treated and untreated sample were sent to Inqaba Biotech PTY (South Africa) for metagenomics sequencing.

3.2.3 16S RNA amplicon metagenomics analysis for *Cruznema sp.* NTM 2021

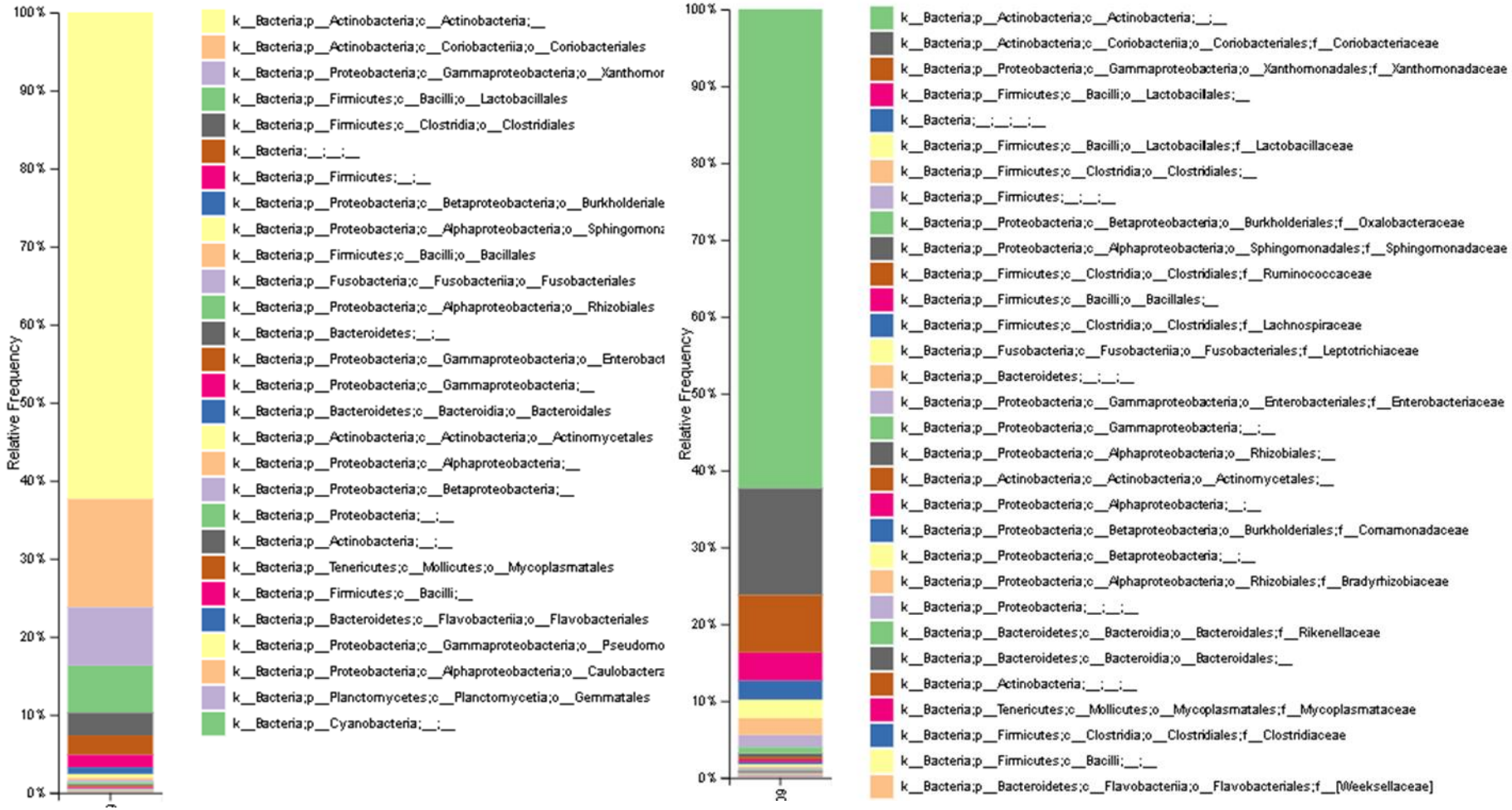
Both non treated and treated sample were sequenced using PacBio sequencing machine at to Inqaba Biotech PTY (South Africa) as previously stipulated in Kingan *et al.* (2019). Quality trimming was performed using SMRTlink (Version 11.0) This resulted into Circular Consensus sequences (CCS) and produce (Hifi reads) High accurate reads with great quality above 40 (>QV40). The Vsearch plugins tools in qiime2 were used to dereplicated and clustered the reads into operation taxonomic units (OTUs) against latest silva version 138.1 reference database with 0.97 % identity threshold cut off. Furthermore, the taxonomy information assignment to the generate OTUs was conducted using feature-classifier classify-sklearn tool (Lima *et al.*, 2021). Moreover, to visualize the taxonomy composition of the sample(s) a taxa bar-plot file was generated and visualize in qiim2 viewer site (https://view.qiime2.org). The denoised processed metagenomic reads of either treated or untreated *Cruznema* nematode were also subjected to Krona version 1.9.0 pipeline in online platform KBase (https://www.kbase.us) bioinformatics site according to Colagiero *et al.* (2020) with minor modification. Furthermore, the reads were clustered and assigned against National centre for biotechnology information (NCBI) database.

3.3 Results

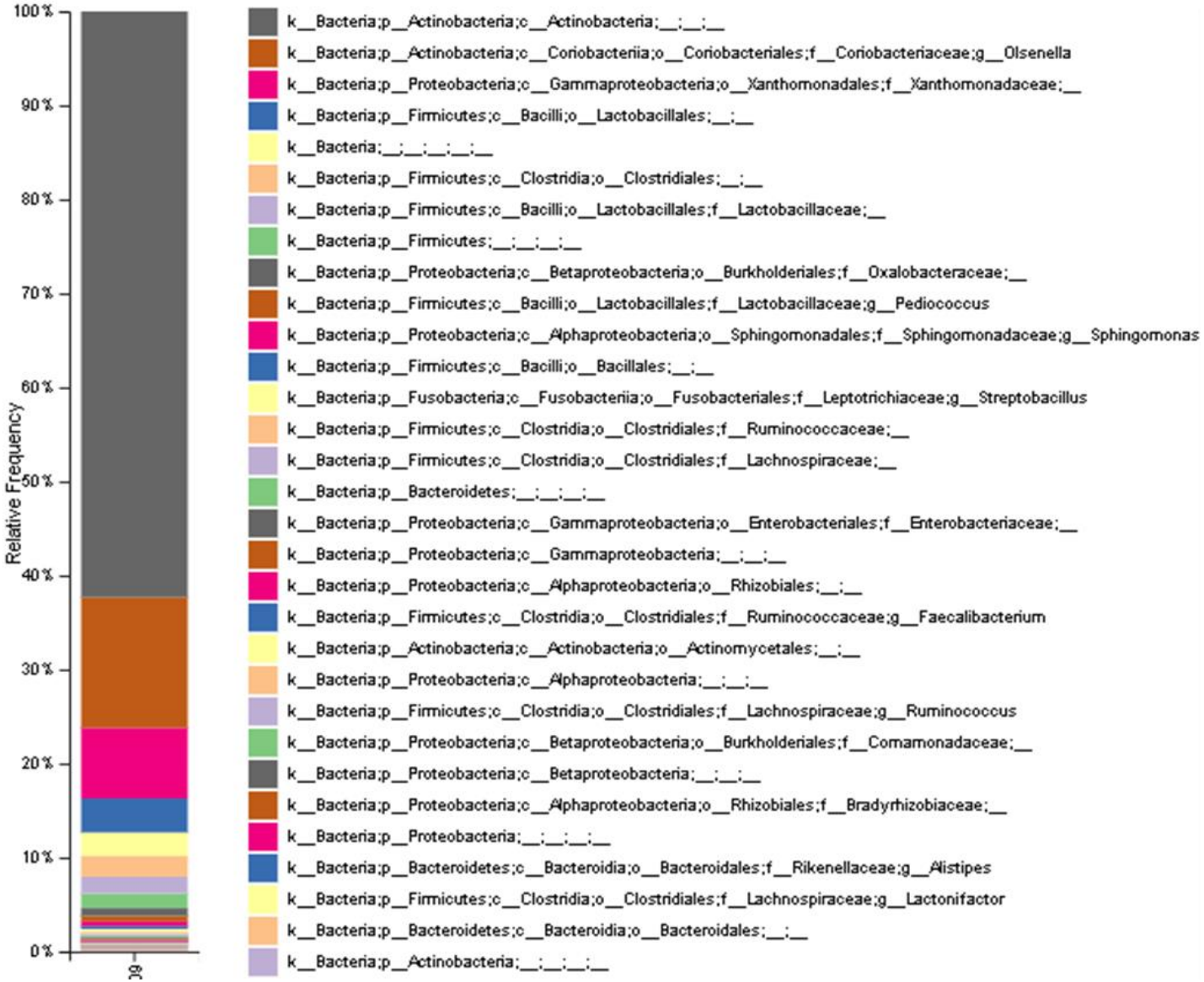
The endo-microbiome community profiling of undescribed *Cruznema* species was achieved through sequencing the 16S RNA metagenomics obtain from freshly emerged IJs gut (from *T. molitor*). Prior from 16S RNA metagenome DNA extraction, the IJs were surfaced sterilised by 0.1 % sodium hypochlorite) (Figure 3.2). Whilst in the other sample the IJs were not surface sterilised (Figure 3.3). The 16S RNA metagenomic sequenced data sequenced and analysed using bioinformatics tools previously described in Section 3.2.3.


A
B
C
Relative Frequency
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
k__Bacteria
k__Bacteria;p__Actinobacteria
k__Bacteria;p__Firmicutes
k__Bacteria;p__Proteobacteria
k__Bacteria;__
k__Bacteria;p__Bacteroidetes
k__Bacteria;p__Fusobacteria
k__Bacteria;p__Tenericutes
k__Bacteria;p__Planctomycetes
k__Bacteria;p__Cyanobacteria
k__Bacteria;p__Actinobacteria;c__Actinobacteria
k__Bacteria;p__Actinobacteria;c__Coriobacteriia
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria
k__Bacteria;p__Firmicutes;c__Bacilli
k__Bacteria;p__Firmicutes;c__Clostridia
k__Bacteria;__;__
k__Bacteria;p__Firmicutes;__
k__Bacteria;p__Proteobacteria;c__Betaproteobacteria
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria
k__Bacteria;p__Fusobacteria;c__Fusobacteriia
k__Bacteria;p__Bacteroidetes;__
k__Bacteria;p__Bacteroidetes;c__Bacteroidia
k__Bacteria;p__Proteobacteria;__
k__Bacteria;p__Actinobacteria;__
k__Bacteria;p__Tenericutes;c__Mollicutes
k__Bacteria;p__Bacteroidetes;c__Flavobacteriia
k__Bacteria;p__Planctomycetes;c__Planctomycetia
k__Bacteria;p__Cyanobacteria;__

E


100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
Relative Frequency
k__Bacteria;p__Actinobacteria;c__Actinobacteria;__
k__Bacteria;p__Actinobacteria;c__Coriobacteriia;o__Coriobacteriales
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Xanthomor
k__Bacteria;p__Firmicutes;c__Bacilli;o__Lactobacillales
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales
k__Bacteria;__;__;__
k__Bacteria;p__Firmicutes;__;__
k__Bacteria;p__Proteobacteria;c__Betaproteobacteria;o__Burkholderiale
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Sphingomona
k__Bacteria;p__Firmicutes;c__Bacilli;o__Bacillales
k__Bacteria;p__Fusobacteria;c__Fusobacteriia;o__Fusobacteriales
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhizobiales
k__Bacteria;p__Bacteroidetes;__;__
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Enterobact
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;__
k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales
k__Bacteria;p__Actinobacteria;c__Actinobacteria;o__Actinomycetales
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;__
k__Bacteria;p__Proteobacteria;c__Betaproteobacteria;__
k__Bacteria;p__Proteobacteria;__;__
k__Bacteria;p__Actinobacteria;__;__
k__Bacteria;p__Tenericutes;c__Mollicutes;o__Mycoplasmatales
k__Bacteria;p__Firmicutes;c__Bacilli;__
k__Bacteria;p__Bacteroidetes;c__Flavobacteriia;o__Flavobacteriales
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Pseudomo
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Caulobacter
k__Bacteria;p__Planctomycetes;c__Planctomycetia;o__Gemmatales
k__Bacteria;p__Cyanobacteria;__;__
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
Relative Frequency
k__Bacteria;p__Actinobacteria;c__Actinobacteria;__;__
k__Bacteria;p__Actinobacteria;c__Coriobacteriia;o__Coriobacteriales;f__Coriobacteriaceae
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Xanthomonadales;f__Xanthomonadaceae
k__Bacteria;p__Firmicutes;c__Bacilli;o__Lactobacillales;__
k__Bacteria;__;__;__;__
k__Bacteria;p__Firmicutes;c__Bacilli;o__Lactobacillales;f__Lactobacillaceae
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;__
k__Bacteria;p__Firmicutes;__;__;__
k__Bacteria;p__Proteobacteria;c__Betaproteobacteria;o__Burkholderiales;f__Oxalobacteraceae
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Sphingomonadales;f__Sphingomonadaceae
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Ruminococcaceae
k__Bacteria;p__Firmicutes;c__Bacilli;o__Bacillales;__
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae
k__Bacteria;p__Fusobacteria;c__Fusobacteriia;o__Fusobacteriales;f__Leptotrichiaceae
k__Bacteria;p__Bacteroidetes;__;__;__
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Enterobacteriales;f__Enterobacteriaceae
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;__;__
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhizobiales;__
k__Bacteria;p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;__
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;__;__
k__Bacteria;p__Proteobacteria;c__Betaproteobacteria;o__Burkholderiales;f__Comamonadaceae
k__Bacteria;p__Proteobacteria;c__Betaproteobacteria;__;__
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhizobiales;f__Bradyrhizobiaceae
k__Bacteria;p__Proteobacteria;__;__;__
k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Rikenellaceae
k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;__
k__Bacteria;p__Actinobacteria;__;__;__
k__Bacteria;p__Tenericutes;c__Mollicutes;o__Mycoplasmatales;f__Mycoplasmataceae
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Clostridiaceae
k__Bacteria;p__Firmicutes;c__Bacilli;__;__
k__Bacteria;p__Bacteroidetes;c__Flavobacteriia;o__Flavobacteriales;f__[Weeksellaceae]

F


100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
Relative Frequency
k__Bacteria;p__Actinobacteria;c__Actinobacteria;__;__;__
k__Bacteria;p__Actinobacteria;c__Coriobacteriia;o__Coriobacteriales;f__Coriobacteriaceae;g__Olsenella
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Xanthomonadales;f__Xanthomonadaceae;__
k__Bacteria;p__Firmicutes;c__Bacilli;o__Lactobacillales;__;__
k__Bacteria;__;__;__;__;__
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;__;__
k__Bacteria;p__Firmicutes;c__Bacilli;o__Lactobacillales;f__Lactobacillaceae;__
k__Bacteria;p__Firmicutes;__;__;__;__
k__Bacteria;p__Proteobacteria;c__Betaproteobacteria;o__Burkholderiales;f__Oxalobacteraceae;__
k__Bacteria;p__Firmicutes;c__Bacilli;o__Lactobacillales;f__Lactobacillaceae;g__Pediococcus
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Sphingomonadales;f__Sphingomonadaceae;g__Sphingomonas
k__Bacteria;p__Firmicutes;c__Bacilli;o__Bacillales;__;__
k__Bacteria;p__Fusobacteria;c__Fusobacteriia;o__Fusobacteriales;f__Leptotrichiaceae;g__Streptobacillus
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Ruminococcaceae;__
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae;__
k__Bacteria;p__Bacteroidetes;__;__;__;__
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Enterobacteriales;f__Enterobacteriaceae;__
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;__;__;__
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhizobiales;__;__
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Ruminococcaceae;g__Faecalibacterium
k__Bacteria;p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;__;__
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;__;__;__
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae;g__Ruminococcus
k__Bacteria;p__Proteobacteria;c__Betaproteobacteria;o__Burkholderiales;f__Comamonadaceae;__
k__Bacteria;p__Proteobacteria;c__Betaproteobacteria;__;__;__
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhizobiales;f__Bradyrhizobiaceae;__
k__Bacteria;p__Proteobacteria;__;__;__;__
k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Rikenellaceae;g__Alistipes
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae;g__Lactonifactor
k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;__;__
k__Bacteria;p__Actinobacteria;__;__;__;__

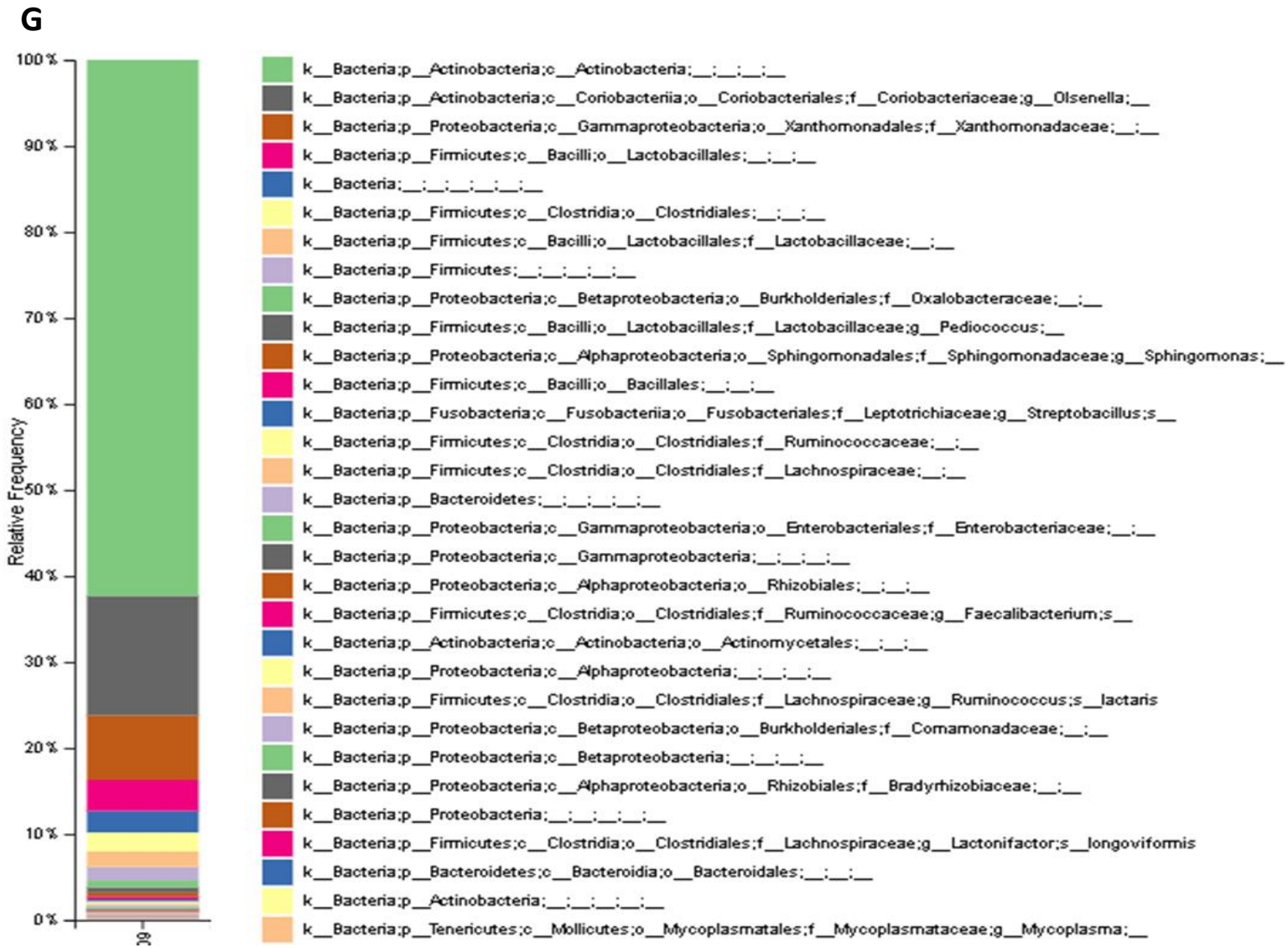
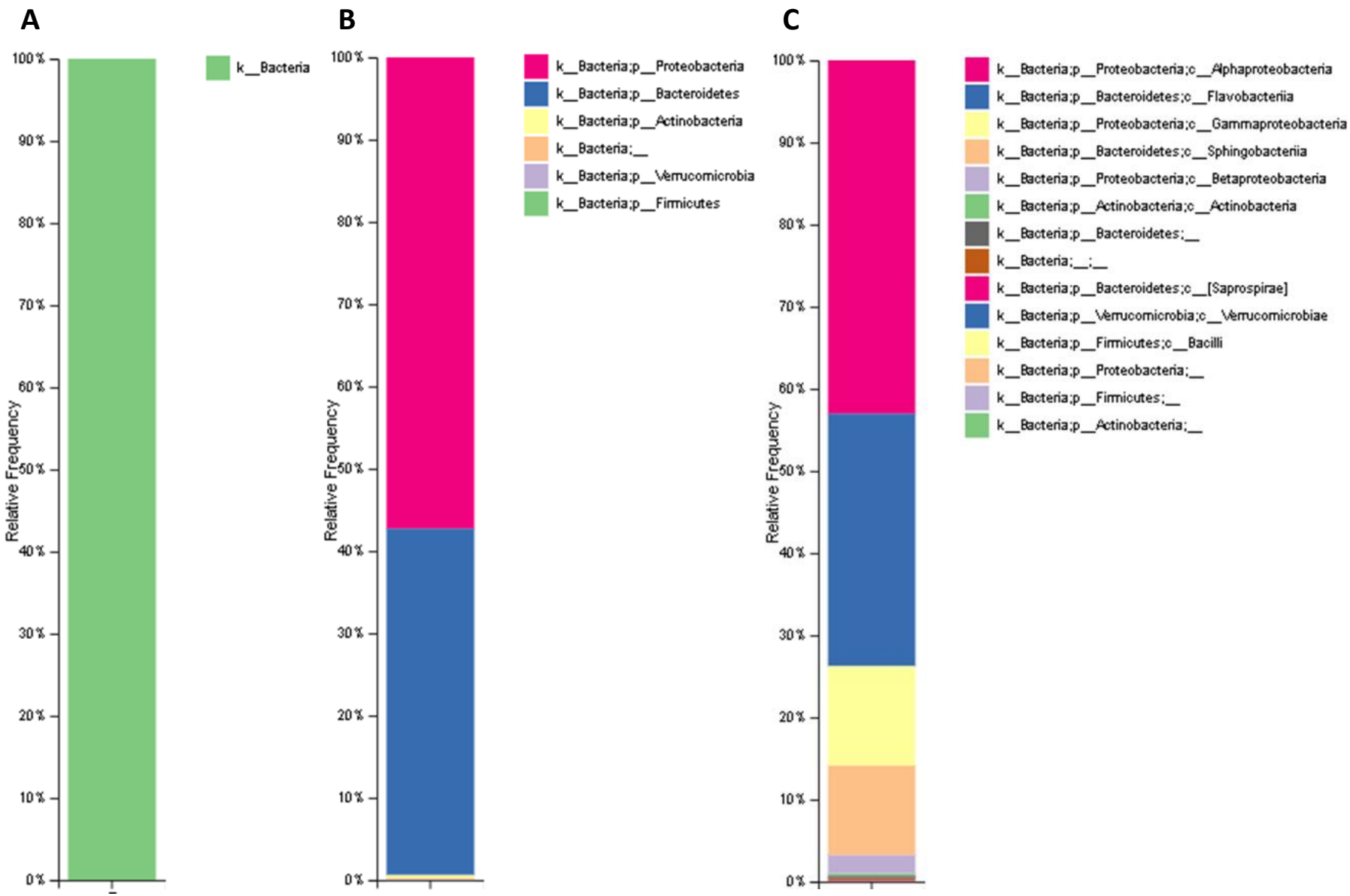
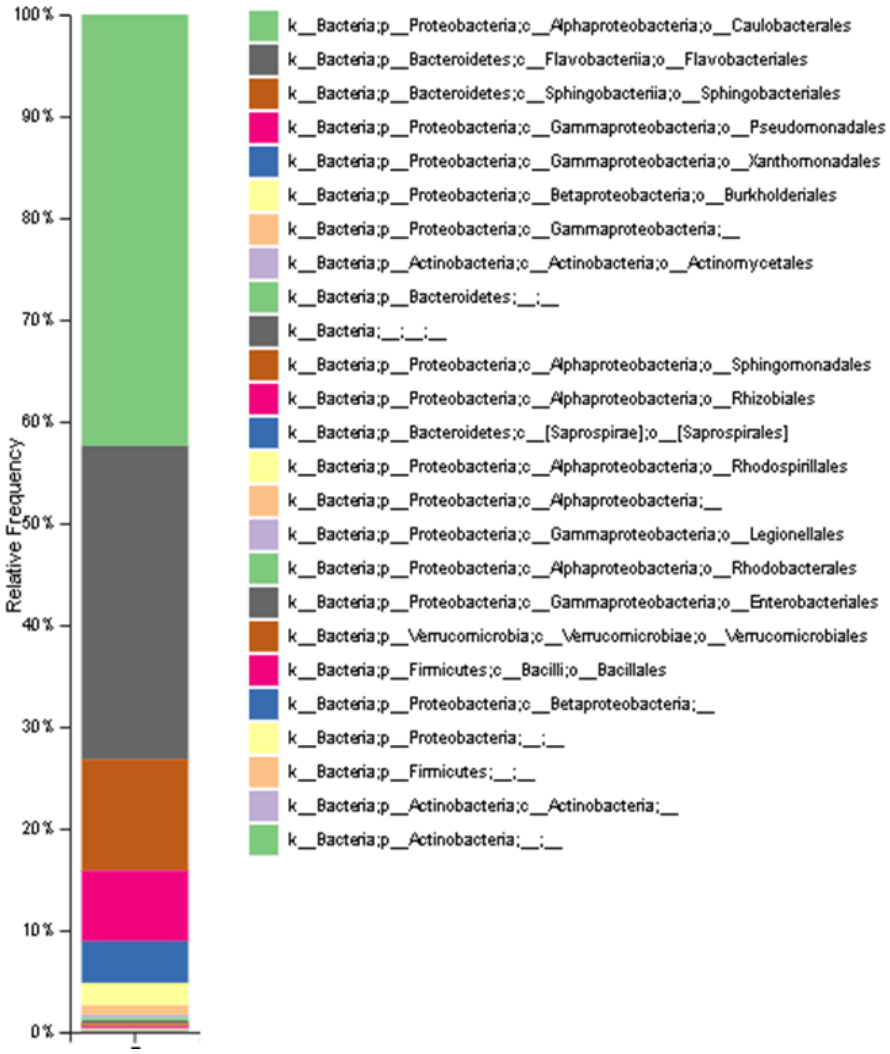


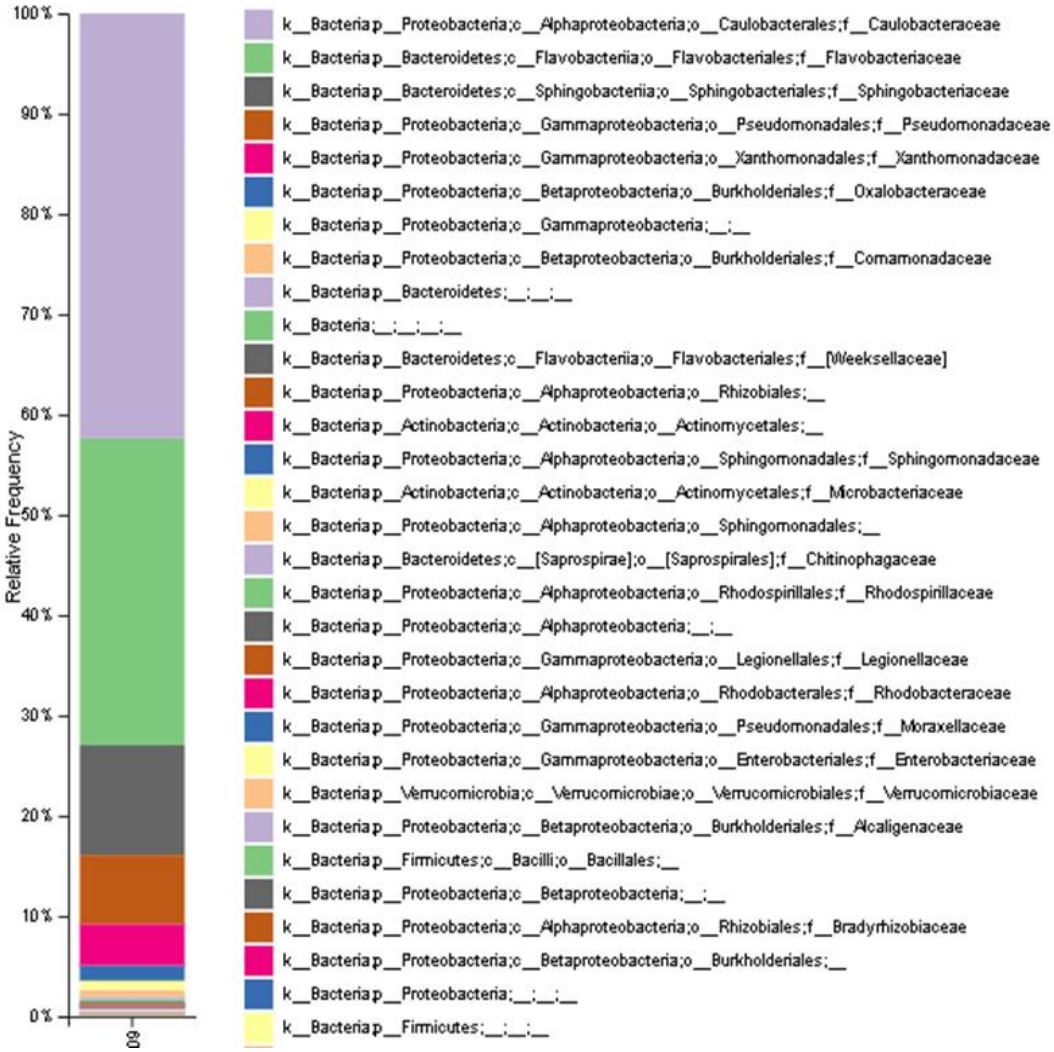
Figure 3.2 Elucidating taxonomic information and relative frequency of untreated *Cruznema* 16S RNA metagenomic sample. The figures are labelled using hierarchy system A) representing Kingdom B) phylum C) Class D) Order E) Family F) Genus G) Species.


A
B
C
Relative Frequency
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
k__Bacteria
k__Bacteria;p__Proteobacteria
k__Bacteria;p__Bacteroidetes
k__Bacteria;p__Actinobacteria
k__Bacteria;__
k__Bacteria;p__Verrucomicrobia
k__Bacteria;p__Firmicutes
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria
k__Bacteria;p__Bacteroidetes;c__Flavobacteriia
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria
k__Bacteria;p__Bacteroidetes;c__Sphingobacteriia
k__Bacteria;p__Proteobacteria;c__Betaproteobacteria
k__Bacteria;p__Actinobacteria;c__Actinobacteria
k__Bacteria;p__Bacteroidetes;__
k__Bacteria;__;__
k__Bacteria;p__Bacteroidetes;c__[Saprospirae]
k__Bacteria;p__Verrucomicrobia;c__Verrucomicrobiae
k__Bacteria;p__Firmicutes;c__Bacilli
k__Bacteria;p__Proteobacteria;__
k__Bacteria;p__Firmicutes;__
k__Bacteria;p__Actinobacteria;__

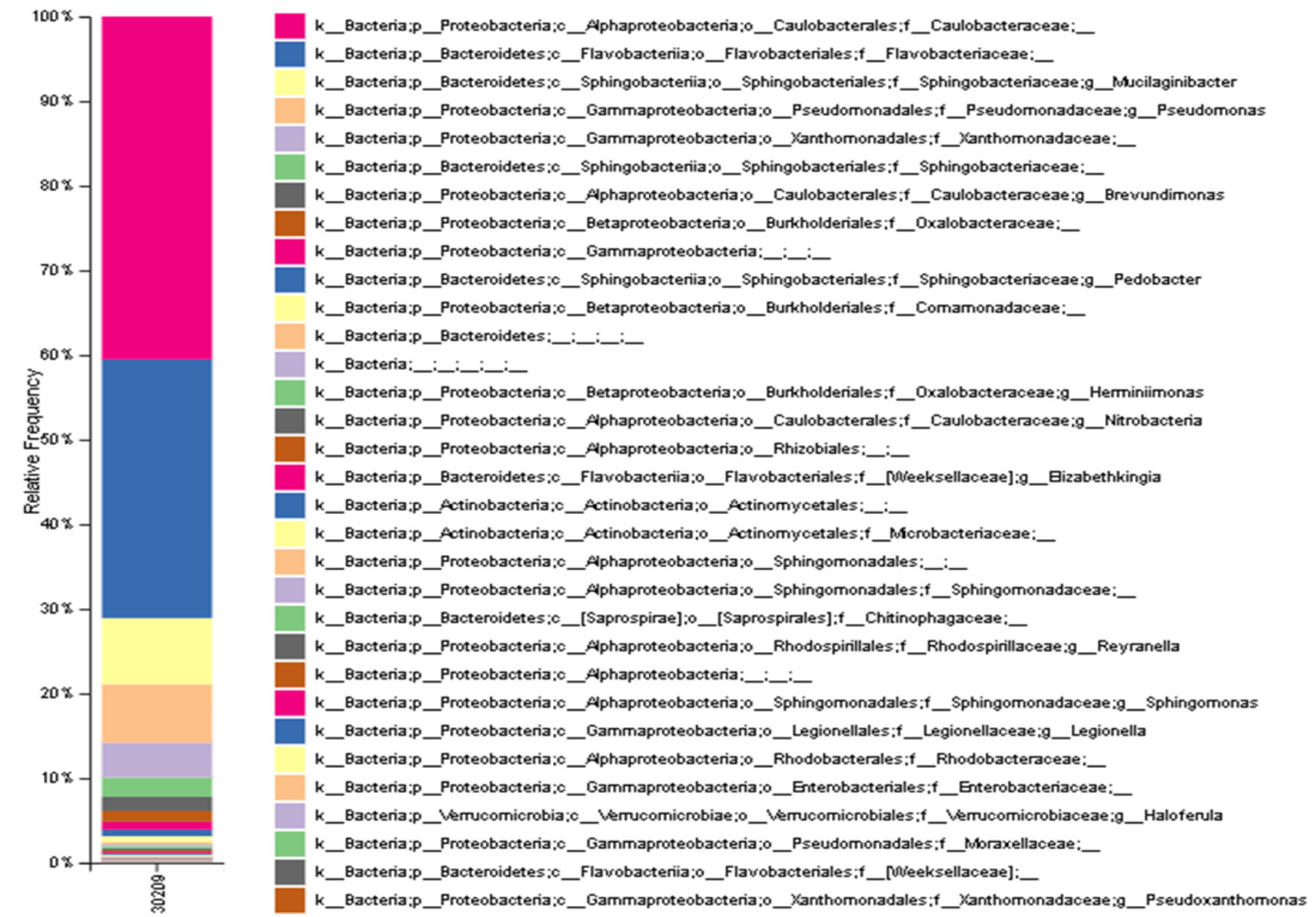
D


100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
Relative Frequency
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Caulobacterales
k__Bacteria;p__Bacteroidetes;c__Flavobacteriia;o__Flavobacteriales
k__Bacteria;p__Bacteroidetes;c__Sphingobacteriia;o__Sphingobacteriales
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Pseudomonadales
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Xanthomonadales
k__Bacteria;p__Proteobacteria;c__Betaproteobacteria;o__Burkholderiales
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;__
k__Bacteria;p__Actinobacteria;c__Actinobacteria;o__Actinomycetales
k__Bacteria;p__Bacteroidetes;__;__
k__Bacteria;__;__;__
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Sphingomonadales
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhizobiales
k__Bacteria;p__Bacteroidetes;c__[Saprospirae];o__[Saprospirales]
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhodospirillales
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;__
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Legionellales
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhodobacterales
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Enterobacteriales
k__Bacteria;p__Verrucomicrobia;c__Verrucomicrobiae;o__Verrucomicrobiales
k__Bacteria;p__Firmicutes;c__Bacilli;o__Bacillales
k__Bacteria;p__Proteobacteria;c__Betaproteobacteria;__
k__Bacteria;p__Proteobacteria;__;__
k__Bacteria;p__Firmicutes;__;__
k__Bacteria;p__Actinobacteria;c__Actinobacteria;__
k__Bacteria;p__Actinobacteria;__;__

E


100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
Relative Frequency
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Caulobacterales;f__Caulobacteraceae
k__Bacteria;p__Bacteroidetes;c__Flavobacteriia;o__Flavobacteriales;f__Flavobacteriaceae
k__Bacteria;p__Bacteroidetes;c__Sphingobacteriia;o__Sphingobacteriales;f__Sphingobacteriaceae
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Pseudomonadales;f__Pseudomonadaceae
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Xanthomonadales;f__Xanthomonadaceae
k__Bacteria;p__Proteobacteria;c__Betaproteobacteria;o__Burkholderiales;f__Oxalobacteraceae
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;__;__
k__Bacteria;p__Proteobacteria;c__Betaproteobacteria;o__Burkholderiales;f__Comamonadaceae
k__Bacteria;p__Bacteroidetes;__;__;__
k__Bacteria;__;__;__;__
k__Bacteria;p__Bacteroidetes;c__Flavobacteriia;o__Flavobacteriales;f__[Weeksellaceae]
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhizobiales;__
k__Bacteria;p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;__
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Sphingomonadales;f__Sphingomonadaceae
k__Bacteria;p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;f__Microbacteriaceae
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Sphingomonadales;__
k__Bacteria;p__Bacteroidetes;c__[Saprospirae];o__[Saprospirales];f__Chitinophagaceae
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhodospirillales;f__Rhodospirillaceae
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;__;__
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Legionellales;f__Legionellaceae
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhodobacterales;f__Rhodobacteraceae
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Pseudomonadales;f__Moraxellaceae
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Enterobacteriales;f__Enterobacteriaceae
k__Bacteria;p__Verrucomicrobia;c__Verrucomicrobiae;o__Verrucomicrobiales;f__Verrucomicrobiaceae
k__Bacteria;p__Proteobacteria;c__Betaproteobacteria;o__Burkholderiales;f__Alcaligenaceae
k__Bacteria;p__Firmicutes;c__Bacilli;o__Bacillales;__
k__Bacteria;p__Proteobacteria;c__Betaproteobacteria;__;__
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhizobiales;f__Bradyrhizobiaceae
k__Bacteria;p__Proteobacteria;c__Betaproteobacteria;o__Burkholderiales;__
k__Bacteria;p__Proteobacteria;__;__;__
k__Bacteria;p__Firmicutes;__;__;__

F


100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
Relative Frequency
30209
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Caulobacterales;f__Caulobacteraceae;__
k__Bacteria;p__Bacteroidetes;c__Flavobacteriia;o__Flavobacteriales;f__Flavobacteriaceae;__
k__Bacteria;p__Bacteroidetes;c__Sphingobacteriia;o__Sphingobacteriales;f__Sphingobacteriaceae;g__Mucilaginibacter
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Pseudomonadales;f__Pseudomonadaceae;g__Pseudomonas
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Xanthomonadales;f__Xanthomonadaceae;__
k__Bacteria;p__Bacteroidetes;c__Sphingobacteriia;o__Sphingobacteriales;f__Sphingobacteriaceae;__
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Caulobacterales;f__Caulobacteraceae;g__Brevundimonas
k__Bacteria;p__Proteobacteria;c__Betaproteobacteria;o__Burkholderiales;f__Oxalobacteraceae;__
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;__;__;__
k__Bacteria;p__Bacteroidetes;c__Sphingobacteriia;o__Sphingobacteriales;f__Sphingobacteriaceae;g__Pedobacter
k__Bacteria;p__Proteobacteria;c__Betaproteobacteria;o__Burkholderiales;f__Comamonadaceae;__
k__Bacteria;p__Bacteroidetes;__;__;__;__
k__Bacteria;__;__;__;__;__
k__Bacteria;p__Proteobacteria;c__Betaproteobacteria;o__Burkholderiales;f__Oxalobacteraceae;g__Herminiimonas
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Caulobacterales;f__Caulobacteraceae;g__Nitrobacteria
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhizobiales;__;__
k__Bacteria;p__Bacteroidetes;c__Flavobacteriia;o__Flavobacteriales;f__[Weeksellaceae];g__Elizabethkingia
k__Bacteria;p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;__;__
k__Bacteria;p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;f__Microbacteriaceae;__
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Sphingomonadales;__;__
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Sphingomonadales;f__Sphingomonadaceae;__
k__Bacteria;p__Bacteroidetes;c__[Saprospirae];o__[Saprospirales];f__Chitinophagaceae;__
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhodospirillales;f__Rhodospirillaceae;g__Reyranella
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;__;__;__
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Sphingomonadales;f__Sphingomonadaceae;g__Sphingomonas
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Legionellales;f__Legionellaceae;g__Legionella
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhodobacterales;f__Rhodobacteraceae;__
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Enterobacteriales;f__Enterobacteriaceae;__
k__Bacteria;p__Verrucomicrobia;c__Verrucomicrobiae;o__Verrucomicrobiales;f__Verrucomicrobiaceae;g__Haloferula
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Pseudomonadales;f__Moraxellaceae;__
k__Bacteria;p__Bacteroidetes;c__Flavobacteriia;o__Flavobacteriales;f__[Weeksellaceae];__
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Xanthomonadales;f__Xanthomonadaceae;g__Pseudoxanthomonas

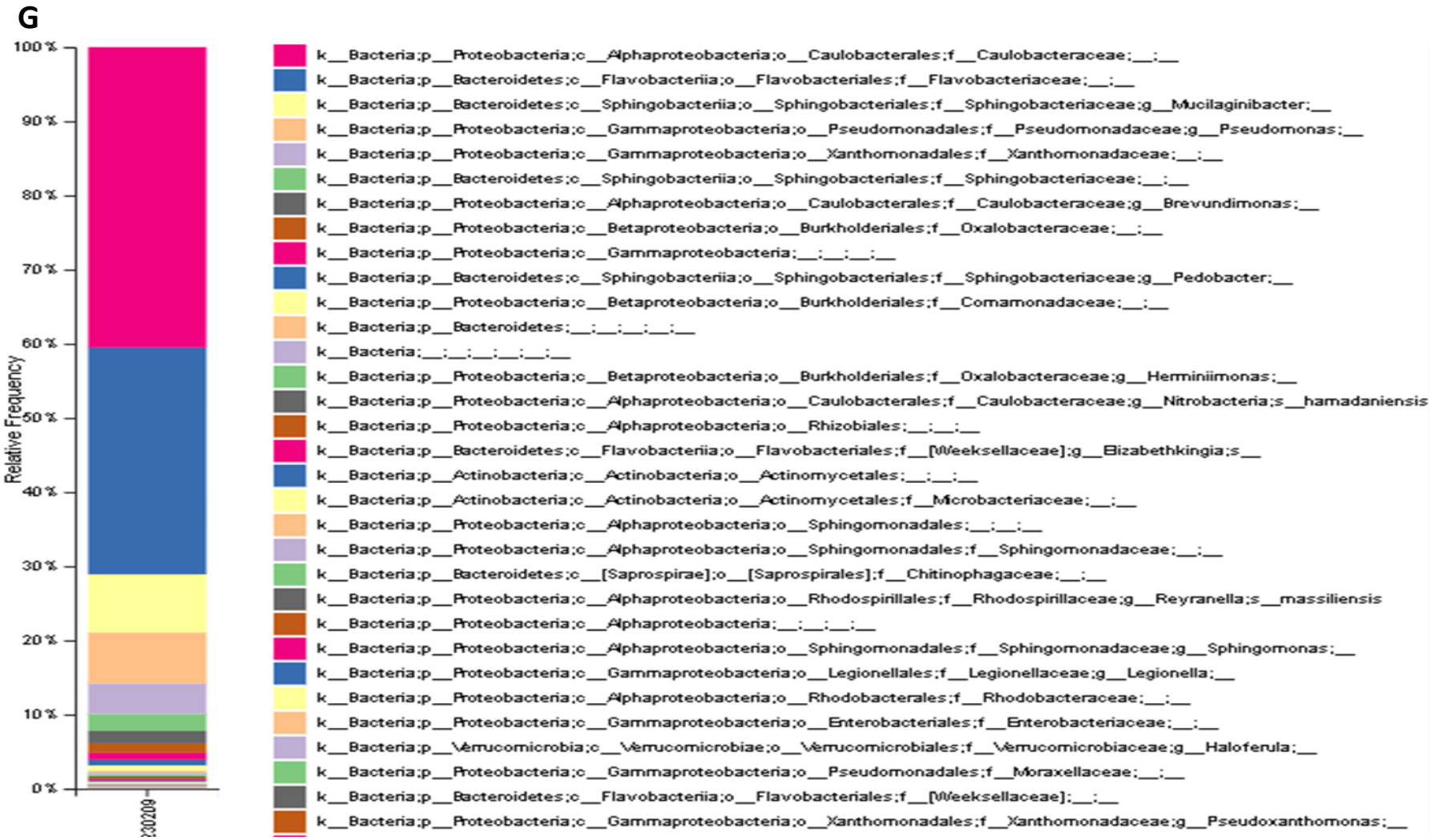


Figure 3.3: Showing endobacteria community of *Cruznema* nematode taxonomic information with the relative frequency, 16S RNA amplicon metagenomic based (Treated sample). The figures are labelled using hierarchy system A) representing Kingdom B) phylum C) Class D) Order E) Family F) Genus G) Species.

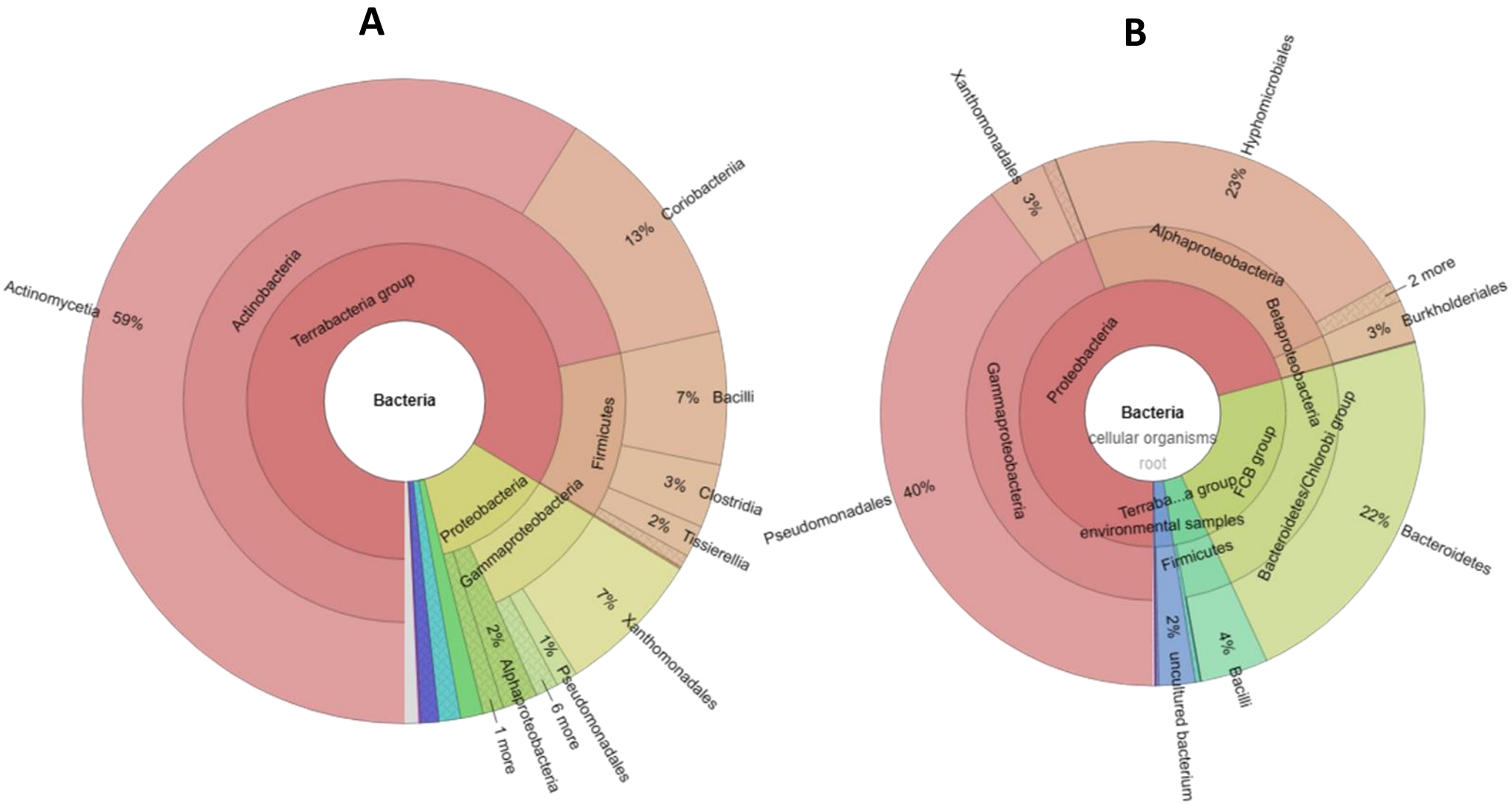


Figure 3.4: Depicting taxonomic relative abundance of *Cruznema* gut 16S RNA amplicon-based metagenomics. This data was obtained through the utilization of Krona version 1.9.0 with the aid of National canter for biotechnology information reference database. A) represent untreated sample and B) represent treated sample.

3.4 Discussion

The 16S rRNA metagenomics for both treated and untreated samples were sequenced using PacBio. The sequenced data for both samples were analysed using Qiime2 tools to explore the taxonomic information of each sample (Figure 3.2 and 3.3). Both the samples shows that they only have one kingdom namely bacteria (Figure 3.2A and 3.3A). However, the untreated sample had nine phyla (namely *Actinobacteria, Firmicutes, Proteobacteria, Bacteroidetes, Fusobacteria, Tenericutes, Planctomycetes, Cyanobacteria* and unidentified phyla) (Figure 3.2B). Whereas the treated sample had only six phyla (namely *Proteobacteria, Bacteroidetes, Actinobacteria, Verrucomicrobia, Firmicutes* and unidentified phyla) (Figure 3.3B). Furthermore, the top 3 phyla with highest relative frequency (*Actinobacteria* at 76.39 %, *Firmicutes* at 10.89 % and *Proteobacteria* at 9.51 %) in Figure 3.2B. However, the top 3 highest frequency phyla in Figure 3.3B consists of *Bacteroidetes* at 42.11 %, *Proteobacteria* at 57.26 % and *Actinobacteria* at 0.32 %. Therefore, the decrease in the number of phyla in Figure 3.4B and massive increase in relative frequency of *Proteobacteria* phylum by 47.75 % from Figure 3.21B to Figure 3.3B, as a result of surface sterilization of *Crunema* IJs sample with sodium hypochlorite prior to the extraction of 16S amplicon rRNA. Moreover, the number of phyla that were present in Figure 3.2B and absent in Figure 3.3B, they are not part of the gut endobacteria community of *Cruznema* nematode. Furthermore, the bacteria taxonomic information elucidated from Figure 3.2C to 3.2G respectively, are associated with phyla showed at Figure 3.2B, are not present in Figure 3.3B, may be from *T. molito*r larvae haemocoel.

The study conducted by Brandon *et al.* (2018) and Urbanek *et al.* (2020) indicated the gut microbe community of *T. molitor* larvae consists of *Enterococcus, Streptococcus, Enterobacter, Lactococus, Paenibacillus, Klebsiella* and *Erwina* genus. Furthermore, various studies shows that *Proteobacteria, Tenericutes* and *Firmicutes* phyla are more likely to be found in high abundance (Jung *et al.,* 2014; Urbanek *et al.,* 2020; Przemieniecki *et al.,* 2022). However, *Actinobacteria* and *Bacteroides* phyla are reported to be occurring in low abundant within the EPNs gut (Jung *et al.,* 2014; Przemieniecki *et al.,* 2022). Therefore, these findings support our claims that those phyla identified in Figure 3.2B to 3.2G will not be present in Figure 3.3B to 3.3G. Furthermore, the sightly changes in the values may be due to the presence of the gut endobacteria community of *Cruznema* nematode in the sample. Despite the evidence that endobacteria communities of among individuals belonging to the same group of species growing in the same environment may vary (Jung *et al.,* 2014; Urbanek *et al.,* 2020).

The data depicted in Figure 3.3B indicates that *Cruznema* nematode share a common endobacteria community phyla groups (namely *Proteobacteria, firmicute, Actinobacteria* and *Bacteroidetes*) with *T. molitor* larvae. However, there is a variation in relative frequency of these phyla between *Cruznema* nematode and *T. molitor* larvae, *Proteobacteria* and *Bacteroidetes* phyla had a higher abundance in *Cruznema* nematode (Figure 3.3B). Whilst *Bacteroidetes* and *Actinobacteria* phyla are in low abundant in *T. molitor* larvae (Urbanek *et al.,* 2020). Therefore, this poses a question in relation to the ability of *Cruznema* nematode to infect and kill the *T. molitor* larvae during culturing of IJs (Section 3.2.1). Furthermore, if *Cruznema* nematode could be assumed to be entomopathogenic nematode because of the evidence that *Cruaznema* nematodes is harbouring endobacteria species in the gut (Figure 3.3). Moreover, *Cruznema* nematodes has bacteria species from phyla *Verrucomicrobia* that can be suggested to be potential cause of death of *T. molitor* (Figure 3.3B to 3.3G). Since phyla *Verrucomicrobia* have not been reported to be associated with *T. molitor*.

Figure 3.4 was generated through Krona version 1.9.0 metagenomic pipeline embedded in KBase platform. Furthermore, the taxonomic assignment of the reads was conducted with the utilization of NCBI reference database. Moreover, despite that the data presented in Figure 3.2 and 3.3, were analysed with different bioinformatic tools that uses different algorithms compared to data depicted in Figure 3.4. Furthermore, despite the utilization of different reference database, the data presented in Figure 3.2 and 3.3, closely resembles the data elucidated in Figure 3.4. There are two high relative abundance bacteria phylum order namely *Actinomycetia* with 59 % and *Coriobacteriia* with 13 % (Figure 3.4A). Whilst in figure 3.3.3B there are only three bacteria phylum order (namely *Pseudomonadales* with 40 %, *Hyphomicrobiales* with 23 % and *Bacteroidetes* with 22 %) with high relative abundance. However, *Pseudomonadales* order has a relative abundance of 1 % in Figure 3.4B, and the reason emanates around the different sample treatment of sodium hypochlorite. Therefore, the results depicted in Figure 3.4B provide a gut endobacteria community profile of *Cruznema* nematode, Since the nematodes were surfaced sterilised against any possible bacteria attachment from at the nematodes surface.

This study highlights that *Cruznema* nematodes is associated with *Pseudomonadaies, Hyphomicrobiaies, Bacteroidetes, Bacilli, Burkholderiaies* and uncultured Bacterium species. Regardless, that only four of the bacteria order (namely *Bacilli, Burkholderiaies, Xanthomonadeles* and unculture Bacterium) have lower relative abundance (Figure 3.4B). Bacilli species have been reported to be use as control measurement against root-knot

nematodes (Mostafa *et al.,* 2018). However, since there is a low concentration of *Bacilli* species, does this suggest that *Cruznema* nematodes have immunity over the bacillus species. Furthermore, *Cruznema* nematodes have been reported to feed on bacteria. There have been countless reports made about *Cruznema* feeds on the bacteria (d'Ovidio *et al*. 2019; Salas and Achinelly, 2020; Shokoohi *et al*., 2022;). Therefore, this feeding habit deviate from the EPNs feeding behaviour. However, there is no denial that there is a gap in either feeding habit or food/host seeking behaviour of *Cruznema* nematodes. Hence, this *Cruznema* specie demonstrate EPNs feeding habit (Section 3.2.1). Therefore, this study is set to investigate the possibilities of *Cruznema* species being an EPNs, by unravelling the gut endobacteria community profile of *Cruznema* nematode. The finding in the study shows *Pseudomonadales* order to have the highest relative abundance at 40 % (Figure 3.4B). However, *Pseudomonas aeruginosa* PAO1 *(P. aeruginosa* PAO1) which belong to *Pseudomonadales* genus has been reported to kill *Caenorhabditis elegans* (*C. elegans*) through the production of cyanide (Gallagher and Manoil 2001; Orozco *et al.,* 2016). *P. aeruginosa is* known to kill various organisms such as bacteria, mammals, plants, and insects, thus includes nematodes (Gallagher and Manoil 2001; Sifri *et al.,* 2005; Gamalero and Glick, 2020; Loulou *et al.,* 2023). Therefore, *Pseudomonadales* species can be suggested to be potential *Cruznema* endo-pathogenic symbiotic bacteria since it retain the highest relative abundance (Figure 3.4B). Furthermore, they can produce hydrogen cyanide which can be one of the contribution factors in killing *T. molitor* larvae and used to kill other bacteria within *T. molitor*, which serves as defence mechanism against invaders. However, this suggestion does not dismiss the possibility that there may be other endobacteria community members that may serves as the counter parts towards infecting and killing of the host or *T. molitor* larvae. Therefore, more studies should be conducted to test *Cruznema* potential endosymbiotic pathogenic bacteria.

All EPNs species are traced back to the evolutionary order namely Rhabditida (Laznik and Trdan, 2012; Lephoto *et al.,* 2016; Canhilal *et al.,* 2016; Lalitha *et al.,* 2023). Furthermore, *Cruznema* species also belong the same evolutionary order as EPNs (Sommer *et al.,* 1994; Canhilal *et al.,* 2016; Du *et al.,* 2022). Therefore, due reason that *Cruznema* nematodes are found feeding on bacteria in the study conducted by Bongers and Ferris, 1999, does not mean they must be classified as bacterivores. Since, all EPNs species feeds on their own symbiotic bacteria during symbiosis test respectively (Chaston *et al.,* 2011; Ramakuwela *et al.,* 2016). Therefore, this does not label EPNs species as bacterivores. Moreover, this *Cruznema* species

demonstrated the unique ability to feed on *T. molitor* larvae which resembles EPNs behaviour (Figure 3.1). Therefore, *Cruznema* are potential EPNs species.

3.5 Conclusion

Cruznema nematodes have been known to be known to be bacterivore species for centuries. There is lack of knowledge about the ability of *Cruznema* nematodes able to infect and kill the insects. Thus includes the possibility of *Cruznema* having bacteria symbionts. EPNs are praised of their abilities to hunt and infect the insect host with aid of bacteria symbionts. However, this identified *Cruznema* nematodes possess the ability to seek and infect insect host, resembling the EPNs life cycle (Chapter 2). Moreover, the data presented in this study show the endobacteria community found in the *Cruznema* nematodes. Thus changes the previously knowledge about the *Cruznema* species group, making it a potential an EPN. However, more studies should be conducted in relation to *Cruznema* bacteria symbiosis tests, bacteria symbionts characterization and host seeking behaviour.

3.6 References

Abate, B.A., Mamo, F.T., Tesfaye, K., Yuankai, C., Qi, R. and Malan, A.P., 2023. Entomopathogenic nematodes and their symbiotic bacteria from Africa: diversity and use as biological control agents. *Biocontrol Science And Technology*, pp.1-24.

Abate, B.A., Slippers, B., Wingfield, M.J., Malan, A.P. and Hurley, B.P., 2018. Diversity of entomopathogenic nematodes and their symbiotic bacteria in South African plantations and indigenous forests. *Nematology*, *20*(4), pp.355-371.

Atwa, A.A., 2014. Entomopathogenic nematodes as biopesticides. *Basic And Applied Aspects Of Biopesticides*, pp.69-98.

Behjati, S. and Tarpey, P.S., 2013. What is next generation sequencing?. *Archives of Disease in Childhood-Education and Practice*.

Bongers, T. and Ferris, H., 1999. Nematode community structure as a bioindicator in environmental monitoring. *Trends In Ecology And Evolution*, *14*(6), pp.224-228.

Brandon, A.M., Gao, S.H., Tian, R., Ning, D., Yang, S.S., Zhou, J., Wu, W.M. and Criddle, C.S., 2018. Biodegradation of polyethylene and plastic mixtures in mealworms (larvae of *Tenebrio molitor*) and effects on the gut microbiome. *Environmental Science And Technology*, *52*(11), pp.6526-6533.

Canhilal, R., Waeyenberge, L., Toktay, H., Bozbuga, R., Çerintas, R. and Imren, M., 2016. Distribution of *Steinernematids* and *Heterorhabditids* (*Rhabditida*: *Steinernematidae* and *Heterorhabditidae*) in the Southern Anatolia Region of Turkey. *Egyptian Journal of Biological Pest Control*, *26*(4).

Chaston, J.M., Dillman, A.R., Shapiro-Ilan, D.I., Bilgrami, A.L., Gaugler, R., Hopper, K.R. and Adams, B.J., 2011. Outcrossing and crossbreeding recovers deteriorated traits in laboratory cultured Steinernema carpocapsae nematodes. *International Journal For Parasitology*, *41*(7), pp.801-809.

Colagiero, M., Rosso, L.C., Catalano, D., Schena, L. and Ciancio, A., 2020. Response of tomato rhizosphere bacteria to root-knot nematodes, fenamiphos and sampling time shows differential effects on low level taxa. *Frontiers In Microbiology*, *11*, pp.390.

Doremus, M.R. and Hunter, M.S., 2020. The saboteur's tools: Common mechanistic themes across manipulative symbioses. *In Advances In Insect Physiology* 58, pp.317-353. Academic Press.

d'Ovidio, D., Nermut, J., Adami, C. and Santoro, M., 2019. Occurrence of *Rhabditid* nematodes in the pet giant African land snails (Achatina fulica). *Frontiers In Veterinary Science*, *6*, pp.88.

Du, H., Guo, F., Gao, Y., Wang, X., Qing, X. and Li, H., 2022. Complete Mitogenome of Confirms Highly Conserved Gene Arrangement within Family *Rhabditidae*. *Journal Of Nematology*, *54*(1), pp.1-10.

Gallagher, L.A. and Manoil, C., 2001. *Pseudomonas aeruginosa* PAO1 kills *Caenorhabditis elegans* by cyanide poisoning. *Journal Of Bacteriology*, *183*(21), pp.6207-6214.

Gamalero, E. and Glick, B.R., 2020. The use of plant growth-promoting bacteria to prevent nematode damage to plants. *Biology*, *9*(11), pp.381.

Hein, N., Hainz, A.A., Wehren, T. and Fischer, K., 2023. Effects of an entomopathogenic nematode applied against Thaumetopoea processionea on non-target arthropods under field conditions. *Biological Control*, *179*, pp.105177.

Jung, J., Heo, A., Park, Y.W., Kim, Y.J., Koh, H. and Park, W., 2014. Gut microbiota of Tenebrio molitor and their response to environmental change. *Journal Of Microbiology And Biotechnology*, *24*(7), pp.888-897.

Katumanyane, A., Malan, A.P., Tiedt, L.R. and Hurley, B.P., 2020. *Steinernema bertusi* n. sp.(*Rhabditida*: *Steinernematidae*), a new entomopathogenic nematode from South Africa. *Nematology*, *22*(3), pp.343-360.

Khan, B.A., Nadeem, M.A., Nawaz, H., Amin, M.M., Abbasi, G.H., Nadeem, M., Ali, M., Ameen, M., Javaid, M.M., Maqbool, R. and Ikram, M., 2023. Pesticides: impacts on agriculture productivity, environment, and management strategies. In *Emerging Contaminants and Plants: Interactions, Adaptations And Remediation Technologies*, pp.109-134. Cham: Springer International Publishing.

Kingan, S.B., Heaton, H., Cudini, J., Lambert, C.C., Baybayan, P., Galvin, B.D., Durbin, R., Korlach, J. and Lawniczak, M.K., 2019. A high-quality de novo genome assembly from a single mosquito using PacBio sequencing. *Genes*, *10*(1), p.62.

Kumar, Y. and Yadav, B.C., 2020. Plant-parasitic nematodes: Nature's most successful plant parasite. *Int. J. Res. Rev*, *7*(3), pp.379-386.

Lalitha, K., Nithya, K., Bharathi, B.G., Venkatesan, S. and Shivakumar, M.S., 2023. Long-term storage does not affect the infectivity of entomopathogenic nematodes on insect hosts. *Applied Microbiology And Biotechnology*, *107*(1), pp.419-431.

Laznik, Z. and Trdan, S., 2012. Entomopathogenic nematodes (Nematoda: *Rhabditida*) in Slovenia: from tabula rasa to implementation into crop production systems. In *Insecticides-advances In Integrated Pest Management*. IntechOpen.

Lephoto, T.E. and Gray, V.M., 2019. *Oscheius basothovii* n. sp.(Nematoda: *Rhabditidae*), a new entomopathogenic nematode isolated from an uncultivated grassland in South Africa. *Archives Of Phytopathology And Plant Protection*, *52*(1-2), pp.125-140.

Lephoto, T.E. and Gray, V.M., 2022. Inspecting the pathogenicity of the integument of *Oscheius basothovii* following sterilization with sodium hypochlorite. *Biological Control*, *171*, pp.104924.

Lephoto, T.E., Mpangase, P.T., Aron, S. and Gray, V.M., 2016. Whole genome sequence of *Oscheius sp*. TEL-2014 entomopathogenic nematodes isolated from South Africa. *Genomics Data*, *7*, pp.259-261.

Li, J., Peng, P. and Zhao, J., 2020. Assessment of soil nematode diversity based on different taxonomic levels and functional groups. *Soil Ecology Letters*, *2*, pp.33-39.

Lima, J., Manning, T., Rutherford, K.M., Baima, E.T., Dewhurst, R.J., Walsh, P. and Roehe, R., 2021. Taxonomic annotation of 16S rRNA sequences of pig intestinal samples using MG-RAST and QIIME2 generated different microbiota compositions. *Journal Of Microbiological Methods*, *186*, p.106235.

Loulou, A., Mastore, M., Caramella, S., Bhat, A.H., Brivio, M.F., Machado, R.A. and Kallel, S., 2023. Entomopathogenic potential of bacteria associated with soil-borne nematodes and insect immune responses to their infection. *PloS One*, *18*(1), p.e0280675.

Matlhabe, O.D., Lephoto, T.E. and Gray, V.M., 2022. Monitoring life cycle stages of axenic cultures of in vitro produced *Oscheius* L8 MCB in semi-solid state and liquid state and comparative efficacy studies between in vitro and in vivo nematode production against *Galleria mellonella*. *Biological Control*, *170*, pp.104904.

Mhatre, P.H., Patil, J., Vijayakumar, R., Venkatasalam, E.P., Divya, K.L., Jenifer, J. and Chavan, S.N., 2017. The first report of *Steinernema cholashanense* (*Rhabditida*: *Steinernematidae*) from India. *Indian Journal Of Nematology*, *47*(2), pp.254-254.

Mostafa, F.A., Khalil, A.E., Nour El-Deen, A.H. and Ibrahim, D.S., 2018. The role of *Bacillus megaterium* and other bio-agents in controlling root-knot nematodes infecting sugar beet under field conditions. *Egyptian Journal Of Biological Pest Control*, *28*(1), pp.1-6.

Ogier, J.C., Akhurst, R., Boemare, N. and Gaudriault, S., 2023. The endosymbiont and the second bacterial circle of entomopathogenic nematodes. *Trends In Microbiology*.

Orozco, R.A., Molnár, I., Bode, H. and Stock, S.P., 2016. Bioprospecting for secondary metabolites in the entomopathogenic bacterium *Photorhabdus luminescens* subsp. *Sonorensis*. *Journal Of Invertebrate Pathology*, *141*, pp.45-52.

Pérez-Cobas, A.E., Gomez-Valero, L. and Buchrieser, C., 2020. Metagenomic approaches in microbial ecology: an update on whole-genome and marker gene sequencing analyses. *Microbial Genomics*, *6*(8).

Przemieniecki, S.W., Kosewska, A., Kosewska, O., Purwin, C., Lipiński, K. and Ciesielski, S., 2022. Polyethylene, polystyrene and lignocellulose wastes as mealworm (*Tenebrio molitor* L.) diets and their impact on the breeding condition, biometric parameters, metabolism, and digestive microbiome. *Science Of The Total Environment*, *832*, pp.154758.

Ramakuwela, T., Hatting, J., Laing, M.D., Hazir, S. and Thiebaut, N., 2016. In vitro solid-state production of *Steinernema innovationi* with cost analysis. *Biocontrol Science And Technology*, *26*(6), pp.792-808.

Reboredo, G.R. and Camino, N.B., 1998. Two new species of nematodes (*Rhabditida*: *Diplogasteridae* and *Rhabditidae*) parasites of *Gryllodes laplatae* (*Orthoptera*: *Gryllidae*) in Argentina. *Memórias Do Instituto Oswaldo Cruz*, *93*, pp.763-766.

Salas, A. and Achinelly, M.F., 2020. Community structure of soil nematodes associated with the rhizosphere of *Solanum lycopersicum* in a major production area in Argentina: a case study among agroecosystem types. *Journal Of Soil Science And Plant Nutrition*, *20*(1), pp.43-54.

Shokoohi, E., Swart, A., Marais, M., Mashela, P.W., Tiedt, L. and Abolafia, J., 2022. Observations on *Cruznema tripartitum* (von Linstow, 1906) Zullini, 1982 (*Rhabditida*,

Rhabditidae) from South Africa with key to species of *Cruznema*. *Russian Journal Of Nematology*, *30*(2), pp.131-146.

Sifri, C.D., Begun, J. and Ausubel, F.M., 2005. The worm has turned–microbial virulence modeled in *Caenorhabditis elegans*. *Trends In Microbiology*, *13*(3), pp.119-127.

Sommer, R.J., Carta, L.K. and Sternberg, P.W., 1994. The evolution of cell lineage in nematodes. *Development*, *1994*(Supplement), pp.85-95.

Urbanek, A.K., Rybak, J., Wróbel, M., Leluk, K. and Mirończuk, A.M., 2020. A comprehensive assessment of microbiome diversity in *Tenebrio molitor* fed with polystyrene waste. *Environmental Pollution*, *262*, pp.114281.

Yooyangket, T., Muangpat, P., Polseela, R., Tandhavanant, S., Thanwisai, A. and Vitta, A., 2018. Identification of entomopathogenic nematodes and symbiotic bacteria from Nam Nao National Park in Thailand and larvicidal activity of symbiotic bacteria against *Aedes aegypti* and *Aedes albopictus*. *PloS One*, *13*(4), p.e0195681.

Chapter 4

Isolation and Molecular characterisation of *Pseudomonas* sp. PKS and *Enterobacter sp.* PTB gut *Cruznema sp*. NTM 2021 bacteria

Abstract

Entomopathogenic nematodes (EPNs) are insect parasites that are regarded as biological control agent in horticulture sector. EPNs are known to be associated with endopathogenic bacteria. EPNs are used in both small-scale and large-scale farming. These pathogenic bacteria possess both antimicrobial and insecticidal agents which they use to depress the host immune defence. All EPNs bacteria symbionts belong to *Enterobacteriaceae* family. Furthermore, the EPNs bacteria symbionts are known to undergoes two Phases during their life cycle. Phase I is reported to be the most important, as it involve secretion of lethal compounds that kill the host. Both bacteria Phases are reported to be crucial for growth and development of the nematodes during the feeding and reproduction stage of the nematodes. Two distinct medium agars (NBTA and McConkey) were used to isolate and study the two bacteria Phases. Furthermore, 16S rDNA molecular sequencing was performed to identify the bacteria isolates (*Pseudomonas sp.* PKS and *Enterobacter sp.* PTB). Phase I of *Enterobacter sp.* PTB on NBTA the bacteria colonies were 1.1-2 mm in diameter, blue-green like circular colonies meanwhile *Pseudomonas sp.* PKS circular, green concave colonies that are surrounded by white colour, ranging from 1.2-2.3 mm in diameter. Phase II of *Pseudomonas sp.* PKS colonies are opaque white with diameter range of 2.2-2.7 mm meanwhile *Enterobacter sp.* PTB has maroon colonies that has irregular shape with approximately 2.3-2.7 mm diameter. 16S rDNA for each bacteria isolate was sequenced using Sanger Sequencing machine. The sequence data was subjected to quality control to remove ambiguous bases and bases with low quality using Finch TV version 1.4.0. Furthermore, the trimmed data was used in CLC Bio Main Benchwork to generated contig was used as query sequence in the NCBI BLASTn tool to identify the homologous sequence. Both homologous sequences that showed high affinity to the query sequences were used to construct a phylogenetic tree respectively. *Cruznmea* NTM 2021 is associated with two endobacteria namely *Pseudomonas sp.* PKS and *Enterobacter sp.* PTB. Both the bacteria isolate share family *Enterobacteriaceae,* despite them to be divergent from one another.

Keywords: *Enterobacteriaceae, Pseudomonas* sp. PKS, *Enterobacter sp.* PTB, Horticulture, Biological control agent.

4.1 Introduction

4.1.1 Entomopathogenic nematodes

Entomopathogenic nematodes (EPNs) are specialised insect parasite that have evolved a commensal relationship with pathogenic bacteria. EPNs are praised for their function as biological control agent in horticulture sector (Kenney and Eleftherianos, 2016). EPNs are deemed a suitable pest management candidate due to the solely reason that they do not have negative impact to the environment (Shakeel *et al.,* 2022; Tomar *et al.,* 2022). EPNs can be used in both large and small-scale environment (Kenney and Eleftherianos, 2016; Skrzecz *et al.,* 2023). EPNs relies on the pathogenic bacteria which they harboured within the gut, to cause infection that will ultimately results into death of the host (Raja *et al.,* 2021). The bacteria symbionts are known to synthesis toxins which are responsible for causing infection (Raja *et al.,* 2021; Sheehy *et al.,*2022). Furthermore, these bacteria are vector dependent on the nematodes to be transported into insect host target (Raja *et al.,* 2021). Moreover, the bacteria symbionts form part of the immune defence of the nematodes (Alotaibi *et al.,* 2022).

4.1.2 Overview of entomopathogenic nematodes bacteria symbionts taxonomy

There are only three known EPNs symbionts bacteria genera namely *Xenorhabdus*, *Serratia* and *Photorhabdus* (Lephoto and Gray, 2022). The symbionts bacteria *Serratia*, *Photorhabdus* and *Xenorhabdus* are classified under Proteobacteria phylum and *Enterobacteriaceae* family (Lephoto and Gray, 2022; Sheehy *et al.,*2022). However, *Serratia* genera has unique features that make it to be easily differentiated from other *Enterobacteriaceae* (Rafii, 2014). Furthermore, all the EPNs bacteria symbionts are facultative anaerobic, gram negative, (Smith *et al.,* 2009; Akhurst and Boemare, 2018) having rod shape (Shahina *et al.* 2004; Lephoto and Gray, 2015). Moreover, none of the EPNs bacteria symbionts have been reported to be able to sporulate. There have been reports that suggest that other bacteria species belong to *Enterobacteriaceae and Pseudomonas* family that may have developed commensal relationship with EPNs (Boemare *et al.,* 1996; Alotaibi *et al.,* 2022; Ruiu *et al.,* 2022). It is important to highlight that free living form of pathogenic bacteria symbionts have never been isolated in external environment except in nematodes gut (Forst *et al,* 1997).

4.1.3 Entomopathogenic nematodes bacterial symbionts life cycle

Endopathogenic symbionts bacteria of EPNs are categorised into two phases (Kaya and Stock,1997; Owuama, 2001). Upon host invasion into host haemolymph by the infective juveniles (IJs), both Phase I and Phase II of endopathogenic bacteria symbionts are released by IJs (Owuama, 2001). Phase I dispense lethal intracellular metabolites that deprive the host

immune response (Kalia *et al.,* 2014). Previous studies have claimed that only Phase I bacteria are solely responsible for synthesizing the toxic biomolecule compounds that are both insecticidal and antimicrobial activity (Owuama, 2001; Krin *et al.,* 2006; Ogier *et al.,* 2023). Further, these compounds generate and sustain monoxetic physiological condition within the host cadaver, which it is crucial for growth and development of nematodes (Stella *et al.,* 2008; Ahmed *et al.,* 2022). The IJs are then developed immediately when nematodes enter into commensal relationship with the pathogenic bacteria (Owuama, 2001). This process only take place after 3 to 4 reproduction cycles. The IJs can now emerge out of host cadaver into the soil to seek new host upon depletion of food source (Figure 4.1).

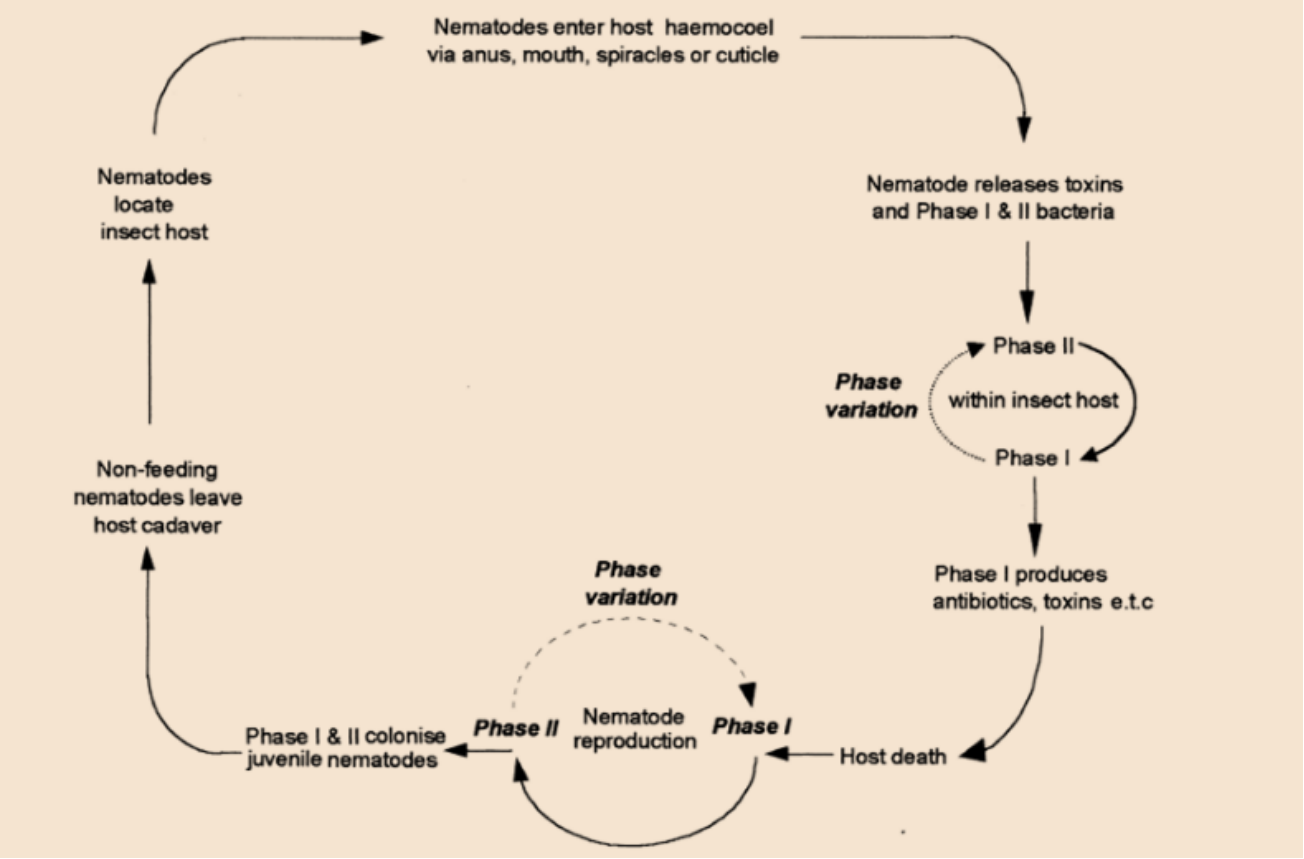


Figure 4.1 Life cycle of entomopathogenic nematodes symbionts bacteria. Both Phase I and II of the pathogenic bacteria symbionts are release within the host upon invasion, Phase I release toxins causing infection. Both Phase I and II pathogenic bacteria recolonise the nematode to form IJs. IJs emerge out seeking new host (Schematic diagram was adapted from Owuama, 2001).

4.1.4 Bacteria pathogenicity within the insect host

The insect host has innate immune response as other cellular living organisms, the host has humoral components that acts as sensors that detects foreign antigens (Kaaya, 1993; Strachecka *et al.,* 2021). Among humoral components available in the host are haemocytes, their function

is to detect the foreign antigens and encapsulate them as part of defence mechanism (Lavine and Strand, 2002; Eleftherianos *et al.,* 2021). Furthermore, they also synthesize cationic antimicrobial compounds that deprives the invading parasites (Eleftherianos *et al.,* 2021). However, EPNs bacteria symbionts has complex mechanism that they use as to evade host haemocytes detection and deprive the host immune system (Sajjadian and Kim, 2020). According to study conducted by Krin *et al.* (2006), *Phototorhabdus* generates a signalling molecule (namely Al-2) which blocks the first host immune response called reactive oxygen species (ROS). Furthermore, *Photorhabdus* modifies host lipopolysaccharides (LPS) as one of the other activities in deprivation of the host immune response (Easom *et al.,* 2010; Clarke, 2014). Previously studies have shown that all three *Serratia*, *Xenorhabdus* and *Photorhabdus* endobacteria symbionts are involved in suppression of CAMPs production (Chalabaev *et al.,* 2007; Stella *et al.,* 2008; Sajjadian and Kim, 2020; Ahmed *et al.,* 2022).

4.1.5 Evolutionary relationship entomopathogenic nematodes bacteria symbionts

Entomopathogenic nematode pathogenic bacteria symbionts are evolutionary divergent however, they convergently have similar behaviour/ lifestyle. They produces insecticidal and antimicrobial compounds (Shahina *et al.* 2004; Lephoto and Gray, 2015). Further, it is important to note that their mode of pathogenicity varies. Despite that *Xenorhabdus* is associated with *Steinernema* (Sajnaga *et al.,* 2018), *Serratia* is associated with *Oscheius* (Lephoto and Gray 2015), and *Photorhabdus* is associated *Heterorhabdus* nematodes (Kaur and Jindal, 2015), all the bacteria symbionts are located adjacent posterior to pharynx in all to their respectively nematodes associates (Ciche *et al.,* 2006). *Xenorhabdus* colonies the vesicles or pockets meanwhile *Photorhabdus* and *Serratia* colonies the lumen gut of the nematodes (Martens and Goodrich-Blair, 2005; Gouge *et al.,* 2006; Lephoto and Gray, 2019).

4.1.6 The use of 16S RDNA to identify bacteria species

The use of DNA to study evolutionary relationships of organisms have been practiced for over ten decades. The molecular identification of bacteria species relies on the conserved ribosomal (rDNA) regions called hypervariable region (Fuks *et al.,* 2018). Bacteria has nine hypervariable regions (Ghyselinck *et al.,* 2013; Bukin *et al.,* 2019). According to the study conducted by Bukin *et al*. (2019) amplification of 16 and 23 ribosomal DNA subunit provides accurate identification of species and data can be used for phylogenetic construction. The aim of this study is to isolate and identify *Cruznme sp.* NTM 2021 gut bacteria.

4.2 Method and materials

4.2.1 Isolation of *Cruznema* sp. NTM 2021 gut bacteria

The freshly IJs where collected from the white traps into 50 mL falcon tube. The excess water was withdrawn from the tube immediately after the settling of the IJs. The IJs were surface sterilised with 5 mL (0.1 % sodium hypochlorite). The IJs were grinded using the pestle to expose the gut bacteria and 1.5 mL of nutrient broth was added to the tube. The Eppendorf tube stored at the 120 rpm shaking incubator with the optimum temperature of 25 ℃ for 2 days. The bacteria culture was sticked at two distinct agar plate media namely McConkey and NBTA. The sticked plates were incubated at 25 ℃. The colonies were subcultured multiple times to obtain pure culture. The bacteria DNA of the pure culture colonies was extracted using E.Z.N.A.® bacteria DNA kit, following manufactures protocol and was stored in negative 20 ℃.

4.2.2 Bacterial Gram Staining

The gram staining of either *Pseudomonas sp.* PKS or *Enterobacter sp*. PTB was conducted by obtaining 1-2 bacteria colonies from NBTA pure culture plate using inoculating lob. The inoculum was smeared on clear microscopic slide consisting of 2 drops of sterile deionised water (ddH_2O) and the bacterial smear was air dried at room temperature. The slide was subsequently heat fixed using Bunsen burner. Furthermore, crystal violet stain was used to flood the smear on the slide and sat for 60 seconds before the slide was rinsed using ddH_2O. Few drops of gram iodine solution were added to the slide, followed by 60 seconds of room incubation. The slide was rinse with ddH_2O, after it was rinsed by decolorizing agent for 11 seconds. The smear was then stained using Safranin solution and room incubated for one minute before rinse with ddH_2O. The slide was gently blotted using bibulous paper before viewed using 100X objective lens of light microscope with an aid of oil-immersion. The images were captured using a cell phone device.

4.2.3 16S RNA sequencing of *Cruznema sp.* NTM 2021 nematode bacteria isolates

A PCR technique was performed through utilization of universal forward prime 16S-27F (AGAGTTTGATCMTGGCTCAG) and universal reverse primer 16S-142R (CGGTTACCTTGTTACGACTT). The reaction conditions were as follows: initial denaturation triggered at 94 ˚C for 5 mins, secondary denaturation maintained at 94 ˚C for 30 sec, annealing sustained at 50 ˚C for 45 sec, extension conducted at 72 ˚C for 1min, final extension was kept at for 72 ˚C 10 min. The total number of cycles performed are 25. The amplicon product was purified using Zymo Research, ZR-96 DNA Sequencing Clean-up Kit™

and sequenced in both (forward and reverse) directions utilizing (Nimagen, BrilliantDye™ Terminator Cycle Sequencing Kit V3.1, BRD3-100/1000) with the aid of ABI 3730*xl* Genetic Analyzer (Applied Biosystems, Thermo Fisher Scientific) in sanger sequencing machine.

4.2.4 Molecular identification and Phylogenetic Construction

4.2.4.1 Quality control

Molecular identification and phylogenetic tree construction was performed following protocol describe at Lephoto and Gray, (2022) with minor modifications. Both forward and reverse sequences raw reads for both 16S RNA *Cruznema sp.* NTM 2021 gut endobacteria filtered and trimmed manual using Finch TV (version 1.4.0) separately (Butler *et al.,* 2014). Furthermore, both forward and reverse reads of each species were respectively assembled using CLC Bio Main Benchwork to produce a consensus/ contig sequences (Zeden and Gründling, 2023).

4.2.4.2 Multiple sequence alignment using CLUSTALW

Each contig sequences of the respective species was subjected to online BLASTn search tool in NCBI database using default parameters. The query sequence and sequence with high similarty from NCBI BLASTn results were subjected to CLUSTALW for sequence alignment (Newman *et al.,* 2016). (Please note *Xenrhabdus ponarii* was used as out group of *Pseudomonas sp*. PKS and *Photorhabdus sp.* Strain R-66823 was used as out group of *Enterobacter sp.* PTB). The outgroups used were selected since they are known to form symbiosis with the EPNs. (*P. glycinae* accession number: MG592779; *P.alcaliphila* accession number: MN818709.1; *P. koreensi* accession number: ON738618.1; *P. punonensis* accession number: NR109583.1; *P. putida* accession number: KJ53590; *P.montelii* accession number:KM349495.1; *P. mendocina* accession number: MK844592.1 and *X. ponarii* accession number: KM371273.1 was used in creating *Pseudomonas sp*. PKS phylogenetic tree) and (*E. kobei* accession number: NR028993.1; *E. timonensis* accession number: NR179439.1; *E. sichuanensis* accession number: NR179946.1; *E. bugandensis* accession number: NR149649.1; *E. cancerogenus* accession number: NR116756.1; E. quasihormaechei; *E. hormaechei* accession number: NR180451.1; *E. soli* accession number: NR117547.1 and *P. starin* R-66823 accession number: MF353506.1 was used in creating *Enterobacter sp.* PTB phylogenetic tree).

4.2.4.3 Phylogenetic tree construction

The phylogenetic tree for each species was constructed using Maximum likely hood on MEGA version 11 tool, using the data obtain from multiple alignments. The parameters were set to

Kimura Nei parameter sequence substitution model with bootstrap value of 1 000 (Tamura *et al*., 2021).

4.3 Results

4.3.1 Examining the cell wall of *Cruznema* bacteria isolates

Gram stain was conducted as previously described in Section 4.2.2. The *Cruznema* gut bacteria isolates were found to have high lipid content, since they were found to be gram negative.

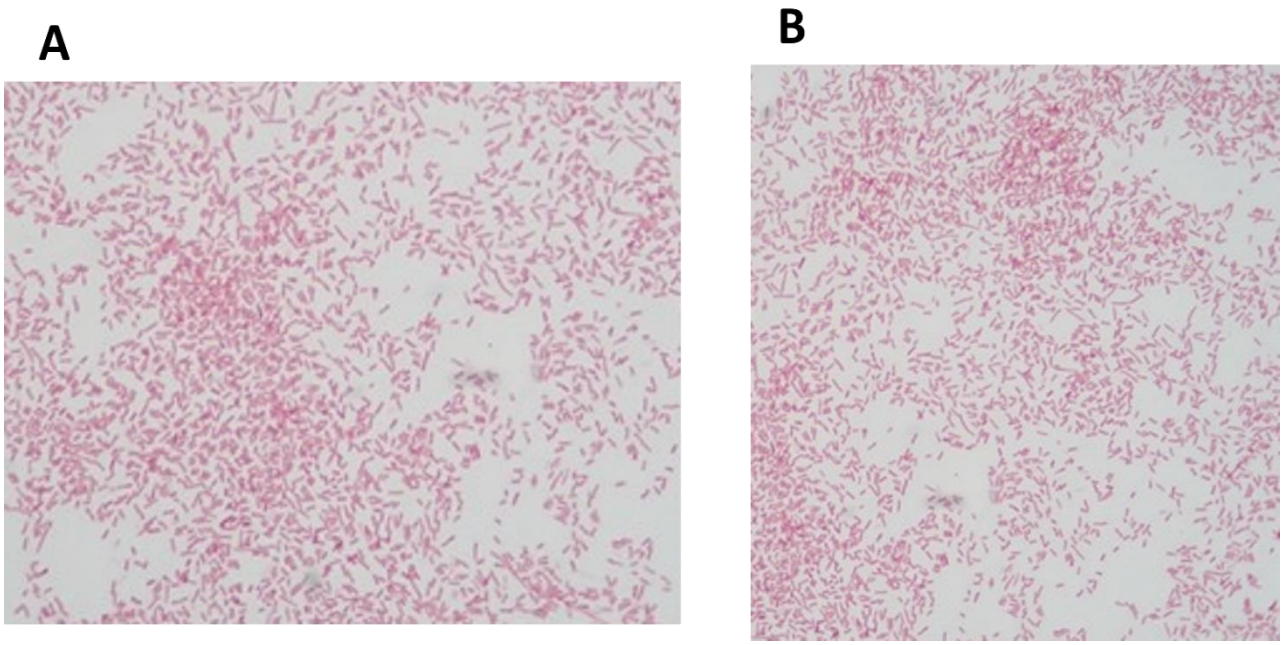


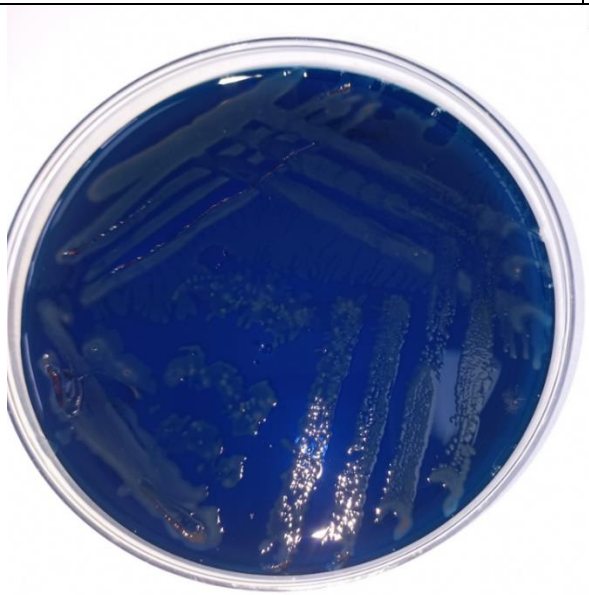
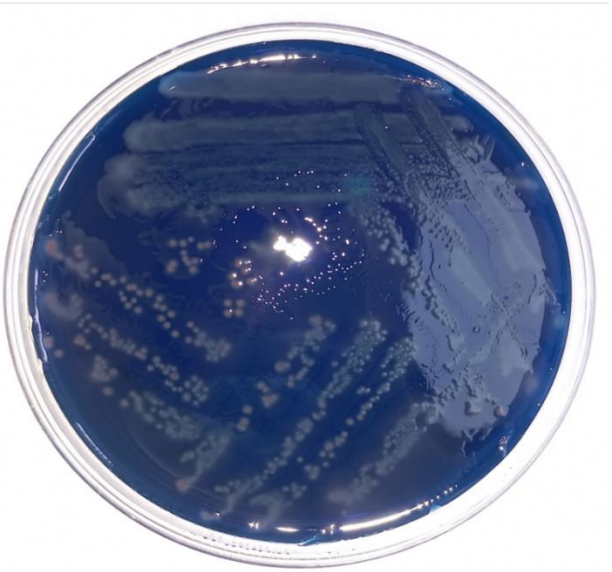
Figure 4.2 Image depicting gram staining of A) *Pseudomonas sp*. PKS and *Enterobacter sp.* PTB. Both *Cruznema sp.* NTM 2021 bacteria isolate are gram negative with rod shape (Image taken under light microscope Total magnification (ocular lens x objective lens): 1000X).

4.3.2 Isolation of endobacteria from *Cruznema sp.* NTM 2021

To accurate study phase I concentration of bromothymol blue was adjusted to 0.025g/L and for Phase II was set to 0.06g/L (Figure 4.8.1.2). *Pseudomonas sp.* PKS colonies size was approximately 1-2.1 mm in diameter for Phase I, and 2.2-2.7-mm in diameter for Phase II meanwhile *Enterobacter sp.* PTB has Phase II 2.3-2.7 mm in diameter and 1.1-2 mm in diameter for Phase I.

Table 4.1 Elucidating entomopathogenic nematodes bacterial symbionts morphology, at different phase according to Kaya and Stock, (1997).

			Organisms				
			Xenorhabdus	*Serratia*	*Photorhabdus*	*Pseudomonas* In this study	*Enterobacter* In this study
NBTA medium	Phase I	Morphology	Granulated, convex, opaque and circular with irregular margins	Colonies surrounded by a clear zone	Colonies surrounded by a clear zone	Circular, concave with white zone surrounding the colonies	Circular colonies
		Colour	Blue-green	Green	Dark green	Green	Blue-green
	Phase II	Morphology	Flat, translucent with irregular margins	Irregular colonies and some circular	colonies surrounded by a clear zone	Circular, concave with white zone surrounding the colonies	Irregular and some circular colonies
		Colour	Shaded from red to rust	Red	Reddish-brown	Opaque white	maroon
Mckonky Medium	Phase I	Morphology	Irregular and some circular colonies	Colonies surrounded by a clear zone	Circular colonies	Circular colonies	Circular colonies
		Colour	pink		Pink and red	Red	Maroon
	Phase II	Morphology	Flat and irregular colonies	Irregular colonies	Irregular colonies	Circular colonies and some are irregular	Flat Irregular colonies
		Colour	Yellow/brown		Yellow/brown	Pink	Pink
References			Kaya and Stock,1997	Lephoto, 2016	Kaya and Stock, 1997		

A	NBTA agar plates	
	Pseudonomas sp. PKS	***Enterobacter sp.* PTB**
Phase I		
Phase II		

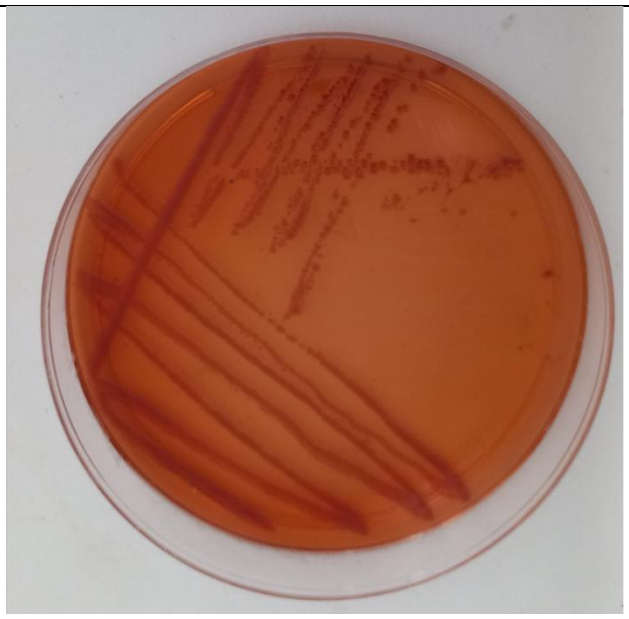
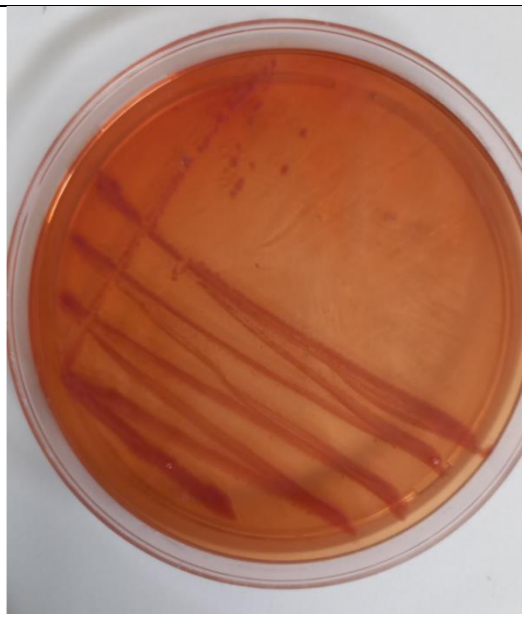
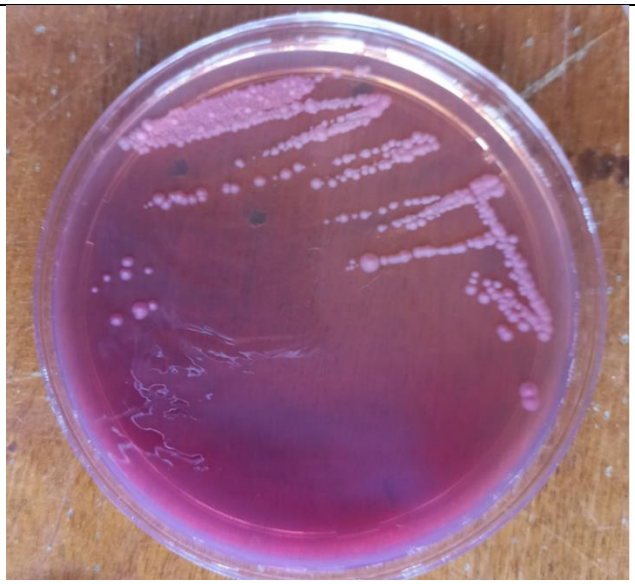
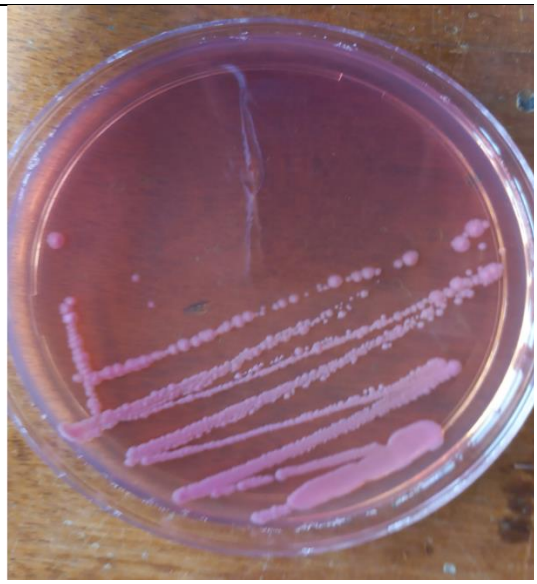
B	Mckonkey agar plates	
	***Pseudonomas sp.* PKS**	***Enterobacter sp.* PTB**
Phase I		
Phase II		

Figure 4.3 Elucidating morphology of *Cruznema sp.* NTM 2021 bacteria isolates a) Phase I of *Pseudomonas sp.* PKS vs *Enterobacter sp.* PTB and and B) Phase II of *Pseudomonas sp.* PKS vs *Enterobacter sp.* PTB. Cruznema *sp*. NTM bacteria isolates morphology was assessed in two distinct agar plates medium as previously describe in Section 4.2.1.

4.3 Phylogenetic evolutionary relationship

The 16S rDNA hyperlink variable was used to identify the two bacteria species, furthermore, the 16S rDNA amplicons sequences were further used to contrast maximum likelihood phylogenetic tree using closely related species.

Table 4.2 Matrix table elucidating evolutionary divergence prediction by Pairwise distance A) *Pseudomonas sp*. PKS and B) *Enterobacter sp.* PTB. The evolutionary divergence was estimated through Tajima-Nei model as previously described in Tamura *et al.* (2021). Base sequence substitution per site values among the species are below the clear diagonal line, whereas the above values indicate the standard error estimates (SEE) which were obtain by a 1000 bootstrap. The sequence position that consists of gaps and missing data were eliminated.

A

	1	2	3	4	5	6	7	8	9	10
1. MG692779.1 Pseudomonas glycinae strain MS586 16S ribosomal RNA gene partial sequence		0.0169	0.0018	0.0065	0.0013	0.0066	0.0033	0.0045	0.0013	0.0013
2. KM371273.1 Xenorhabdus poinarii strain SI-hyd-3B 16S ribosomal RNA gene partial sequence	0.1729		0.0167	0.0175	0.0168	0.0175	0.0169	0.0172	0.0168	0.0168
3. Pseudomonas sp. PKS	0.0027	0.1694		0.0064	0.0013	0.0066	0.0034	0.0041	0.0013	0.0013
4. MN818709.1 Pseudomonas alcaliphila strain AA19 16S ribosomal RNA gene partial sequence	0.0316	0.1784	0.0317		0.0063	0.0013	0.0065	0.0069	0.0063	0.0063
5. ON738618.1 Pseudomonas koreensis strain HW317 16S ribosomal RNA gene partial sequence	0.0013	0.1713	0.0013	0.0303		0.0065	0.0031	0.0043	0.0000	0.0000
6. MK844592.1 Pseudomonas mendocina strain NPRT1 16S ribosomal RNA gene partial sequence	0.0331	0.1803	0.0332	0.0013	0.0317		0.0067	0.0071	0.0065	0.0065
7. NR 104278.1 Pseudomonas parafulva NBRC 16636 = DSM 17004 strain AJ 2129 16S ribosomal RNA partial sequence	0.0081	0.1713	0.0081	0.0316	0.0067	0.0331		0.0035	0.0031	0.0031
8. NR 109583.1 Pseudomonas punonensis strain LMT03 16S ribosomal RNA partial sequence	0.0149	0.1763	0.0122	0.0359	0.0136	0.0374	0.0095		0.0043	0.0043
9. AY014805.1 Pseudomonas sp. NZ017 16S ribosomal RNA gene partial sequence	0.0013	0.1713	0.0013	0.0303	0.0000	0.0317	0.0067	0.0136		0.0000
10. MW850380.1 Pseudomonas vancouverensis strain N1 16S ribosomal RNA gene partial sequence	0.0013	0.1713	0.0013	0.0303	0.0000	0.0317	0.0067	0.0136	0.0000	

B

	1	2	3	4	5	6	7	8	9	10
1. NR 180450.1 Enterobacter wuhouensis strain WCHEs120002 16S ribosomal RNA partial sequence		0.0020	0.0033	0.0033	0.0076	0.0020	0.0023	0.0023	0.0031	0.0017
2. Enterobacter sp. PTB	0.0053		0.0031	0.0031	0.0076	0.0023	0.0026	0.0026	0.0032	0.0011
3. NR 028993.1 Enterobacter kobei strain CIP 105566 16S ribosomal RNA partial sequence	0.0129	0.0121		0.0033	0.0079	0.0034	0.0035	0.0035	0.0037	0.0031
4. NR 179439.1 Enterobacter timonensis strain mt20 16S ribosomal RNA partial sequence	0.0137	0.0129	0.0145		0.0079	0.0031	0.0031	0.0031	0.0041	0.0032
5. MF353506.1 Photorhabdus sp. strain R-66823 16S ribosomal RNA gene partial sequence	0.0703	0.0695	0.0736	0.0753		0.0074	0.0074	0.0074	0.0073	0.0075
6. NR 116756.1 Enterobacter cancerogenus strain LMG 2693 16S ribosomal RNA partial sequence	0.0053	0.0076	0.0152	0.0129	0.0662		0.0011	0.0011	0.0030	0.0021
7. NR 180451.1 Enterobacter quasihormaechei strain WCHEs120003 16S ribosomal RNA partial sequence	0.0068	0.0091	0.0152	0.0129	0.0662	0.0015		0.0000	0.0032	0.0023
8. NR 126208.1 Enterobacter hormaechei subsp. xiangfangensis strain 10-17 16S ribosomal RNA partial sequence	0.0068	0.0091	0.0152	0.0129	0.0662	0.0015	0.0000		0.0032	0.0023
9. NR 117547.1 Enterobacter soli ATCC BAA-2102 strain LF7 16S ribosomal RNA partial sequence	0.0129	0.0137	0.0183	0.0222	0.0678	0.0121	0.0137	0.0137		0.0030
10. NR 179946.1 Enterobacter sichuanensis strain WCHECL1597 16S ribosomal RNA partial sequence	0.0038	0.0015	0.0121	0.0129	0.0687	0.0060	0.0076	0.0076	0.0122	

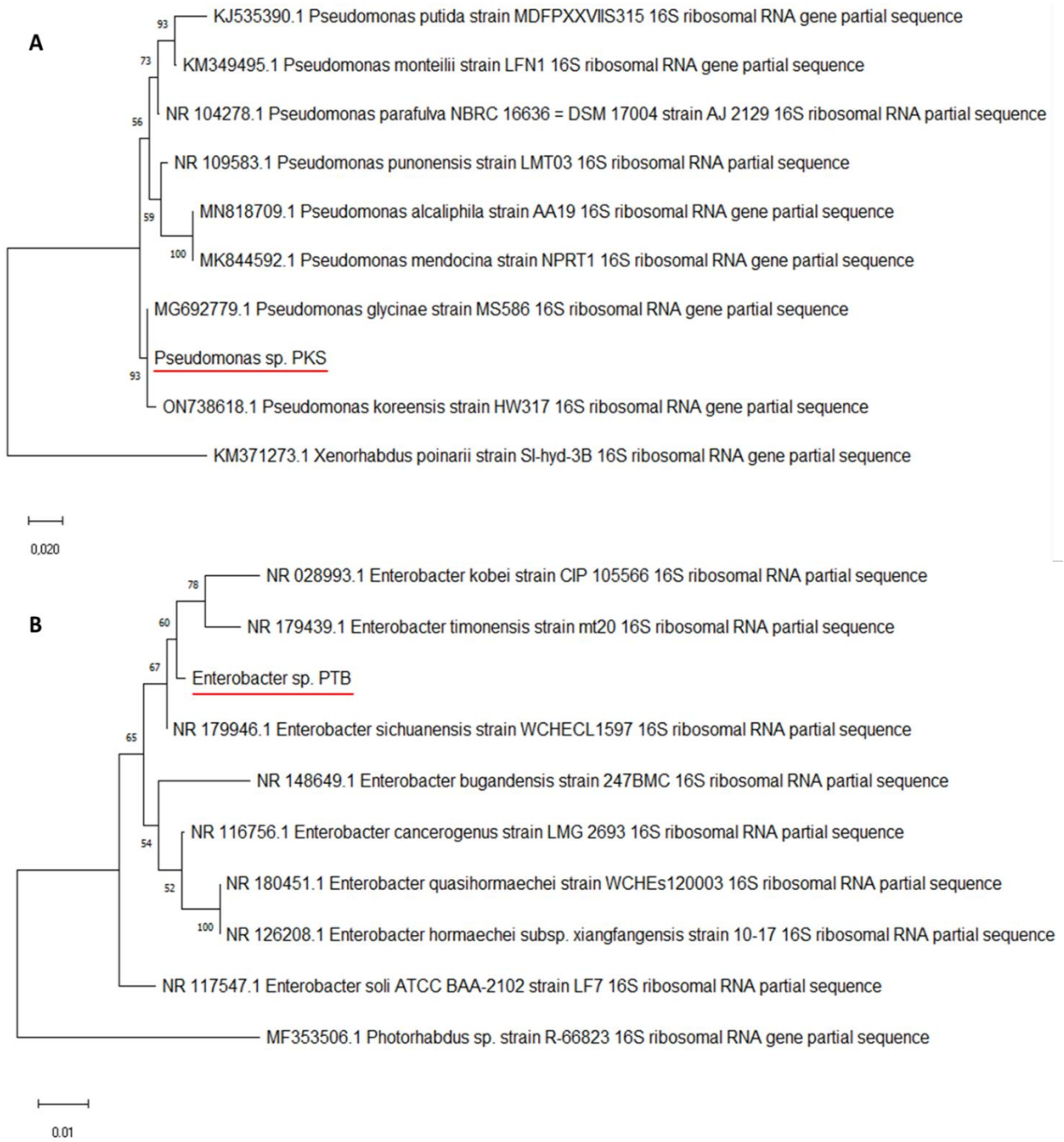


Figure 4.4 Elucidating 16S RDNA evolutionary relationship of A) *Pseudomonas sp.* PKS and B) *Enterobater sp.* PTB endopathogenic *Cruznema sp.* NTM 2021 bacteria symbionts. The *Pseudomonas sp.* PKS phylogenetic tree has a scale of 0.02 evolutionary lineage over time whereas *Enterobater sp.* PTB has a scale of 0.01. The phylogenetic tree(s) was constructed using statistical analysis Maximum likelihood and Tamura-Nei nucleotide substitution method, using 1 000 as a bootstrap value (Tamura *et al*., 2021).

4.4 Discussion

The *Cruznema sp.* NTM 2021 gut bacteria was successful isolated (Section 4.2.1) and pure bacteria colonies were archived through multiple subculturing on both NBTA and McConkey agar plates. Gram staining is a molecular technique performed to examine the bacteria cell wall (Tripathi and Sapra, 2020). The bacteria cell is made of peptidoglycan which serves as barrier that protects the bacteria from any external environmental factors for their entire life span (Vollmer, 2015; Garde *et al.*, 2021). Therefore, during gram staining test the thicker the bacteria cell wall supresses the diffusion of primary stain (namely crystal violet), out from the cell during decolorising step (Section 4.2.2).

Furthermore, it is significant to note that crystal violet dissolves the lipids layer in the bacteria cell wall, making it easy for the primary stain to wash off during discoloration despite the stain being previously fixed with iodine solution (Moyes, *et al.*, 2009; Tripathi and Sapra, 2020). Hence, the bacteria with high level of peptidoglycan retains the stain whereas the one with lower-level peptidoglycan loses the stain since, the lipid layer have been dissolved by primary stain. Both gut *Cruznema sp*. NTM 2021 bacteria isolates (namely *Pseudomonas sp.* PKS and *Enterobater sp.* PTB) were found to be gram negative due to the loss of the stain (Figure 4.2).

The entomopathogenic nematodes bacteria associates are reported to have two different Phase (Brunel *et al.,* 1997). According to Brunel *et al.,* (1997) during Phase II, the bacteria have irregular morphological traits thus affects its physiological and biochemical functioning. However, despite these irregularities there have no fundamental genetic molecular variation in the bacteria between the two Phase. Entomopathogenic nematodes bacteria symbionts undergoes Phase II during the initial development of nematodes or, in a stationary phase of *invitro* culture experiment (Brunel *et al.,* 1997).

Xenorhabdus bacteria species have been reported to absorb bromomethyl blue and they appear blue or blue-green on nutrient bromothymol blue-triphenyltetrazolium chloride agar (NBTA) medium. However, only one species of *Xenorhabdus* (namely *Xenorhabdus pionar*) have been reported to has red colonies on NBTA throughout both Phases, due to its inability to absorb bromomethyl blue. According to the study conducted by Fukruksa *et al.* (2017) *Photorhabdus* appears to have dark green colonies on NBTA. The variation in bromomethyl blue concentrations and both physiological and morphological of the bacteria, influence the colony phenotypical appearance on the NBTA agar plate. *Pseudomonas sp.* PKS has 1.1-2.1 diameter circular green colonies on NBTA in Phase I whereas in Phase II, it has 2.2-2.7 diameter opaque colonies surrounded by white colour, as results of bromomethyl blue absorption (Table 4.2 A

and Figure 4.3). Phase I of *Enterobater sp.* PTB on NBTA has circular blue colonies that are 1.1-2 in diameter whilst in Phase II has maroon colonies with circular shape that are 2.3-2.7 in dimeter (Table 4.1 A and Figure 4.2). according to Kaya and Stock, (1997) redish or maroon colour on NBTA may not necessary mean not able to absorb bromomethyl blue however, it may be due to absorption of triphenyltetrazolium (TTC).

Phase I and Phase II of *Cruznema sp*. NTM 2021 gut bacteria was further assessed on McConkey agar through streaking colonies of from NBTA medium into freshly prepared McConkey agar plates respectively to each Phase (Table 4.1 B and Figure 4.2). Both *Pseudomonas sp.* PKS and *Enterobater sp.* PTB has circular convex shape colonies with red colour in Phase I. However, in Phase II *Enterobater sp.* PTB has flat irregular colonies with pink colour whearas *Pseudomonas sp.* PKS has both irregular and circular elevated colonies with pink colour.

DNA-DNA hybridization have been reported to be a game changer in identification of either species or strains of the same species. The concept behind DNA-DNA hybridization uses computational power to calculate the genetic similarities of population pool to measure evolutionary distance amongst species, and cluster them into their respective taxa. The identity of *Cruznema sp.* NTM 2021 gut bacteria isolate was archived by performing a sanger sequencing (Section 4.2.3). The two bacteria species genomic sample data were subjected to NCBI BLASTn, one species was identified as *Pseudomonas sp.* PKS whereas other was identified to be *Enterobacter sp.* PTB. Maximum likelihood statistical analysis and Tamura-Nei nucleotide substitution method was used to study the evolutionary relationship amongst the species that showed high affinity with the query species from the NCBI BLASTn results (Figure 4.3.3) as previously described by Tamura *et al*. (2021). *Pseudomonas sp.* PKS forms a phylogenetic clade with *P. glaycinae* and *P. Koreensis* despite their evolutionary diverging distance (Figure 4.4 A). Furthermore, *Enterobacter sp.* PTB shows a huge evolutionary distance with *E. timonensis* and *E. kobei* (Figure 4.4 B). Furthermore, despite these species forming the same clade they have little evolutionary divergence among each other, *Pseudomonas sp.* PKS is 0.0018 divergent to *P. glaycinae*, 0.0013 divergent to *P. Koreensis* and both, *P. glaycinae* and *P. Koreensis* are 0.0013 divergent to each other (Table 4.2 A). *Enterobacter sp.* PTB shows a huge evolutionary distance with *E. timonensis* and *E. kobei* (Figure 4.4 B). This is supported by the evolutionary distance in Table 4.4 B, showing 0.0031 evolutionary distance among *Enterobacter sp.* PTB with both *E. timonensis* and *E. kobei.*

However, 0.0033 evolutionary distance is observed between *E. timonensis* and *E. kobei*, which is higher to the one between *Enterobacter sp.* PTB (Table 4.3.2 B).

4.5 Conclusion

Cruznema sp. NTM 2021 is associated with two endobacteria (namely *Pseudomonas sp.* PKS and *Enterobater sp.* PTB) which are suggested to have a commensal relationship with the *Cruznema* nematode. There has no report in the past stating that *Cruznema* nematodes is associated with any bacteria. Therefore, this bacteria isolate may be involved in cyto-protection and pathogenicity of the *Cruznema* nematodes. However, further studies should be conducted to validate the pathogenicity and symbionts relationship between *Cruznema* nematodes and the bacteria isolates.

4.6 Reference

Ahmed, S., Hrithik, M.T.H., Roy, M.C., Bode, H. and Kim, Y., 2022. Phurealipids, produced by the entomopathogenic bacteria, *Photorhabdus*, mimic juvenile hormone to suppress insect immunity and immature development. *Journal Of Invertebrate Pathology*, *193*, pp.107799.

Akhurst, R.J. and Boemare, N.E., 2018. Biology and taxonomy of *Xenorhabdus*. In *Entomopathogenic Nematodes In Biological Control*, pp.75-90. CRC press.

Alotaibi, S.S., Darwish, H., Zaynab, M., Alharthi, S., Alghamdi, A., Al-Barty, A., Asif, M., Wahdan, R.H., Baazeem, A. and Noureldeen, A., 2022. Isolation, identification, and biocontrol potential of entomopathogenic nematodes and associated bacteria against *Virachola livia* (*Lepidoptera*: *Lycaenidae*) and *Ectomyelois ceratoniae* (*Lepidoptera*: *Pyralidae*). *Biology*, *11*(2), pp.295.

Boemare, N., Laumond, C. and Mauleon, H., 1996. The entomopathogenic nematode-bacterium complex: biology, life cycle and vertebrate safety. *Biocontrol Science And Technology*, *6*(3), pp.333-346.

Bukin, Y.S., Galachyants, Y.P., Morozov, I.V., Bukin, S.V., Zakharenko, A.S. and Zemskaya, T.I., 2019. The effect of 16S rRNA region choice on bacterial community metabarcoding results. *Scientific Data*, *6*(1), pp.1-14.

Butler, M., Henter, H. and Mel, S., 2014. From bugs to barcodes: Using molecular tools to study biodiversity. *Tested Studies For Laboratory Teaching*, *35*, pp.41-55.

Chalabaev, S., Turlin, E., Charles, J.F., Namane, A., Pagès, S., Givaudan, A., Brito-Fravallo, E., Danchin, A. and Biville, F., 2007. The HcaR regulatory protein of *Photorhabdus luminescens* affects the production of proteins involved in oxidative stress and toxemia. *Proteomics*, *7*(24), pp.4499-4510.

Ciche, T.A., Darby, C., Ehlers, R.U., Forst, S. and Goodrich-Blair, H., 2006. Dangerous liaisons: the symbiosis of entomopathogenic nematodes and bacteria. *Biological Control*, *38*(1), pp.22-46.

Clarke, D.J., 2014. The genetic basis of the symbiosis between *Photorhabdus* and its invertebrate hosts. *Advances in applied microbiology*, *88*, pp.1-29.

Easom, C.A., Joyce, S.A. and Clarke, D.J., 2010. Identification of genes involved in the mutualistic colonization of the nematode *Heterorhabditis bacteriophora* by the bacterium *Photorhabdus luminescens*. *BMC Microbiology*, *10*(1), pp.1-10.

Eleftherianos, I., Heryanto, C., Bassal, T., Zhang, W., Tettamanti, G. and Mohamed, A., 2021. Haemocyte-mediated immunity in insects: Cells, processes and associated components in the fight against pathogens and parasites. *Immunology*, *164*(3), pp.401-432.

Forst, S., Dowds, B., Boemare, N. and Stackebrandt, E., 1997. *Xenorhabdus* and *Photorhabdus* spp.: bugs that kill bugs. *Annual Review Of Microbiology*, *51*(1), pp.47-72.

Fukruksa, C., Yimthin, T., Suwannaroj, M., Muangpat, P., Tandhavanant, S., Thanwisai, A. and Vitta, A., 2017. Isolation and identification of *Xenorhabdus* and *Photorhabdus* bacteria associated with entomopathogenic nematodes and their larvicidal activity against *Aedes aegypti*. *Parasites & vectors*, *10*(1), pp.1-10.

Fuks, G., Elgart, M., Amir, A., Zeisel, A., Turnbaugh, P.J., Soen, Y. and Shental, N., 2018. Combining 16S rRNA gene variable regions enables high-resolution microbial community profiling. *Microbiome*, *6*(1), pp.1-13.

Garde, S., Chodisetti, P.K. and Reddy, M., 2021. Peptidoglycan: structure, synthesis, and regulation. *EcoSal Plus*, *9*(2).

Ghyselinck, J., Pfeiffer, S., Heylen, K., Sessitsch, A. and De Vos, P., 2013. The effect of primer choice and short read sequences on the outcome of 16S rRNA gene based diversity studies. *PloS One*, *8*(8), p.e71360.

Gouge, D.H. and Snyder, J.L., 2006. Temporal association of entomopathogenic nematodes (*Rhabditida*: *Steinernematidae* and *Heterorhabditidae*) and bacteria. *Journal Of Invertebrate Pathology*, *91*(3), pp.147-157.

Kaaya, G.P., 1993. Inducible humoral antibacterial immunity in insects. In *Insect immunity*, pp. 69-89. Dordrecht: Springer Netherlands.

Kalia, V., Sharma, G., Shapiro-Ilan, D.I. and Ganguly, S., 2014. Biocontrol potential of *Steinernema thermophilum* and its symbiont *Xenorhabdus indica* against *lepidopteran* pests: virulence to egg and larval stages. *Journal Of Nematology*, *46*(1), pp.18.

Kaur, R. and Jindal, V., 2015. Genetic Relatedness of Bacterial Species Isolated from Local Entomopathogenic Nematode Strains of *Punjab*. *The Indiana Ecological Society*, *42*(1), pp.35-39.

Kaya, H.K. and Stock, S.P., 1997. Techniques in insect nematology. In *Manual Of Techniques In Insect Pathology*, pp. 281-324. Academic Press.

Kenney, E. and Eleftherianos, I., 2016. Entomopathogenic and plant pathogenic nematodes as opposing forces in agriculture. *International Journal For Parasitology*, *46*(1), pp.13-19.

Lavine, M.D. and Strand, M.R., 2002. Insect hemocytes and their role in immunity. *Insect Biochemistry And Molecular Biology*, *32*(10), pp.1295-1309.

Lephoto, T.E. and Gray, V.M., 2015. Genome sequencing and annotation of *Serratia sp*. strain TEL. *Genomics Data*, *6*, pp.54-56.

Lephoto, T.E. and Gray, V.M., 2019. *Oscheius basothovii* n. sp. (Nematoda: *Rhabditidae*), a new entomopathogenic nematode isolated from an uncultivated grassland in South Africa. *Archives Of Phytopathology And Plant Protection*, *52*(1-2), pp.125-140.

Lephoto, T.E. and Gray, V.M., 2022. Inspecting the pathogenicity of the integument of *Oscheius basothovii* following sterilization with sodium hypochlorite. *Biological Control*, *171*, pp.104924.

Lephoto, T.E., and Gray, V.M., 2016. Genomics of a novel entomopathogenic nemaode. *Genomics Data*, *6*, pp.54-56.

Martens, E.C. and Goodrich-Blair, H., 2005. The *Steinernema carpocapsae* intestinal vesicle contains a subcellular structure with which *Xenorhabdus nematophila* associates during colonization initiation. *Cellular Microbiology*, *7*(12), pp.1723-1735.

Moyes, R.B., Reynolds, J. and Breakwell, D.P., 2009. Differential staining of bacteria: gram stain. *Current Protocols in Microbiology*, *15*(1), pp.1-3.

Newman, L., Duffus, A.L. and Lee, C., 2016. Using the free program MEGA to build phylogenetic trees from molecular data. *The American Biology Teacher*, *78*(7), pp.608-612.

Ogier, J.C., Akhurst, R., Boemare, N. and Gaudriault, S., 2023. The endosymbiont and the second bacterial circle of entomopathogenic nematodes. *Trends In Microbiology*.

Owuama, C.I., 2001. Entomopathogenic symbiotic bacteria, *Xenorhabdus* and *Photorhabdus* of nematodes.

Rafii, F., 2014. *Serratia*.

Raja, R.K., Arun, A., Touray, M., Gulsen, S.H., Cimen, H., Gulcu, B., Hazir, C., Aiswarya, D., Ulug, D., Cakmak, I. and Kaya, H.K., 2021. Antagonists and defense mechanisms of entomopathogenic nematodes and their mutualistic bacteria. *Biological Control*, *152*, pp.104452.

Ruiu, L., Marche, M.G., Mura, M.E. and Tarasco, E., 2022. Involvement of a novel *Pseudomonas protegens* strain associated with entomopathogenic nematode infective juveniles in insect pathogenesis. *Pest Management Science*, *78*(12), pp.5437-5443.

Sajjadian, S.M. and Kim, Y., 2020. Dual oxidase-derived reactive oxygen species against Bacillus thuringiensis and its suppression by eicosanoid biosynthesis inhibitors. *Frontiers In Microbiology*, *11*, pp.528.

Sajnaga, E., Kazimierczak, W., Skowronek, M., Lis, M., Skrzypek, T. and Waśko, A., 2018. *Steinernema poinari* (Nematoda: *Steinernematidae*): a new symbiotic host of entomopathogenic bacteria *Xenorhabdus bovienii*. *Archives Of Microbiology*, *200*, pp.1307-1316.

Shahina, F., Manzar, H. and Tabassum, K.A., 2004. Symbiotic bacteria *Xenorhabdus* and *Photorhabdus* associated with entomopathogenic nematodes in Pakistan. *Pakistan Journal Of Nematology*, *22*(2), pp.117.

Shakeel, Q., Shakeel, M., Raheel, M., Ali, S., Ashraf, W., Iftikhar, Y. and Bajwa, R.T., 2022. Entomopathogenic Nematodes (EPNs): A Green Strategy for Management of Insect-Pests of Crops. In *New And Future Development In Biopesticide Research: Biotechnological Exploration*, pp. 115-135. Singapore: Springer Nature Singapore.

Sheehy, L., Cutler, J., Weedall, G.D. and Rae, R., 2022. Microbiome analysis of *Malaco* pathogenic nematodes suggests no evidence of a single bacterial symbiont responsible for gastropod mortality. *Frontiers In Immunology*, *13*, pp.878783.

Skrzecz, I., Tumialis, D., Jablonski, T. and Mazurkiewicz, A., 2023. Possibilities of using entomopathogenic nematodes to reduce the number of the large pine weevil *Hylobius abietis* (L.)-the effect of abiotic factors. *Sylwan*, *167*(06).

Smith, J., Fratamico, P.M. and Uhlich, G., 2009. Molecular mechanisms involved in biofilm formation by food-associated bacteria. In *Biofilms In The Food And Beverage Industries*, pp. 42-98. Woodhead Publishing.

Stella, N.A., Kalivoda, E.J., O'Dee, D.M., Nau, G.J. and Shanks, R.M., 2008. Catabolite repression control of flagellum production by *Serratia marcescens*. *Research In Microbiology*, *159*(7-8), pp.562-568.

Strachecka, A., Migdał, P., Kuszewska, K., Skowronek, P., Grabowski, M., Paleolog, J. and Woyciechowski, M., 2021. Humoral and cellular defense mechanisms in rebel workers of *Apis mellifera*. *Biology*, *10*(11), pp.1146.

Tamura K., Stecher G., and Kumar S., 2021. MEGA 11: Molecular Evolutionary Genetics Analysis Version 11. Molecular Biology and Evolution. Available on: https://doi.org/10.1093/molbev/msab120.

Tamura, K. and Nei, M., 1993. Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. *Molecular Biology And Evolution*, *10*(3), pp.512-526.

Tomar, P., Thakur, N. and Yadav, A.N., 2022. Endosymbiotic microbes from entomopathogenic nematode (EPNs) and their applications as biocontrol agents for agro-environmental sustainability. *Egyptian Journal Of Biological Pest Control*, *32*(1), pp.80.

Tripathi, N. and Sapra, A., 2020. Gram staining.

Vollmer, W., 2015. Peptidoglycan. In *Molecular Medical Microbiology,* pp.105-124. Academic Press.

Zeden, M.S. and Gründling, A., 2023. Bacterial whole-genome-resequencing analysis: basic steps using the CLC Genomics Workbench software. *Cold Spring Harbor Protocols*, *2023*(11), pp.107901.

Chapter 5

Pathogenicity and symbiosis assessment of gut *Cruznema sp.* NTM 2021 bacteria isolate (*Pseudomonas sp.* PKS and *Enterobater sp.* PTB)

Abstract

Nematodes are cylindrical worms with elongate body shape belonging to phylum *Nematoda*. There four orders (namely *Tylenchida, Dorylaimida, Rhabditida* and *Aphelenchida*) from phylum *Nematoda* that are reported to be more conserved in soil. Nematodes are further classified based on their feeding behaviour. There are seven feeding trophic groups namely predators, bacterivores, omnivores, carnivore, algivores, herbivores and fungivores. Nematodes morphological mouth features determine the food type they eat. Bacterivores trophic group comprises of free-living nematodes that feeds on bacteria. These nematodes mouth is hollow tub-like shaped allowing them to suck-off bacteria easily from the surface. Entomopathogenic nematodes (EPNs) are insect parasitic nematodes clustered in carnivore trophic group belonging to phylum order *Rhabditida*. However, not all nematodes belonging to phylum order *Rhabditida* are EPNs. These nematodes have a mutualistic relationship with pathogenic bacteria. These bacteria symbionts produce biological toxin compounds having insecticidal activity. The symbiosis relationship among both *Enterobacter sp.* PTB and *Pseudomonas sp.* PKS bacteria with *Cruznema sp.* NTM 2021 was assessed on lipid agar (5 g/L of yeast extract, 2 g/L of $MgCl_2 \cdot 6H_2O$, 10 g/L of honey, 2.5 ml/L of cod liver oil). Both *Enterobacter sp.* PTB and *Pseudomonas sp.* PKS were found to support the grow of *Cruznema sp.* NTM 2021. Furthermore, both *Enterobacter sp.* PTB and *Pseudomonas sp.* PKS bacteria isolate were examined for their ability to kill *Tenebrio molitor* (*T. molitor*) larvae by spreading the bacteria isolate culture on nutrient agar plate to grow bacteria lawns. Subsequently followed by addition of surface sterilised *T. molitor* larvae. The plates were kept at 25 ℃ incubator and constantly monitored at six-hour interval. Both the bacteria exhibited the ability to kill *T. molitor* larvae. However, when both *Cruznema sp.* NTM 2021 bacteria isolate are combine they exhibit greater significance. Therefore, both *Cruznema sp.* NTM 2021 endobacteria serve as counterparts to killing of the insect host, allowing it to compete better to other EPNs. *Cruznema* exhibits EPNs characteristic with an extra ability of possessing two distinct pathogenic bacteria symbionts.

Keywords: Entomopathogenic nematodes, Lipid agar, *Rhabditida,* Symbiosis, *Tenebrio molitor*.

5.1 Introduction

5.1.1 Overview of Phylum *Nematoda*

Nematodes are microscopic worms that are cylindrical and elongate body shape found on soil. Phylum Nematoda is divided into multiple orders and there are only four orders (*Tylenchida, Dorylaimida, Rhabditida* and *Aphelenchida*) which are mostly reported to be associated with soil (Lazarova *et al.,* 2021) (Hailu and Hailu, 2020; Nisa *et al.,* 2021; Mkandawire *et al.,* 2022). Nematodes are parasitic worms that feed in plants and animals (Chavez, 2021). However, nematodes are very diverse and complex species despite them being simple parasitic free-living worms. Further identifying and classification of nematodes into the respective genera's it reported to be more challenging because of difficult and time-consuming experimental procedures (Knoff *et al.,* 2012).

5.1.2 Classification of nematodes into the feeding trophic groups

Nematodes exhibit a various feeding patten/ behaviour hence they are clustered into distinct trophic groups (namely predators, bacterivores, omnivores, algivores, herbivores and fungivores), based on their feeding behaviour (Bongers and Bongers, 1998; Neher and Darby, 2006; Potapov *et al.,* 2022). It is important to highlight that not all free-living nematodes have been clustered into trophic groups. Further previous studies stated that the morphological mouth features of the nematodes determine their food type that they consume (Neher and Darby, 2006, Quist *et al.,* 2015). However, other studies had showed that there are other nematodes with the same mouth features but exhibits a divergent feeding behaviour, making them to be clustered into distinct trophic groups. Nematodes species belonging to *Aphelenchida* order phylum order are clustered into Fungivores trophic group (Quist *et al.,* 2015; Potapov *et al.,* 2022). This group is characterized by nematodes that feeds on fungus (Potapov *et al.,* 2022). they use one of their mouth parts called stylet, which is shaped like a needle, to puncture the hyphae of the fungus (Quist *et al.,* 2015). Furthermore, these nematodes are known to be involve in soil nutrition circle (Rizvi *et al.,* 2022). Moreover, other studies use the presences of these nematodes in soil, serves as an indicator of fertile soil (Neher and Weicht, 2013).

The free-living nematodes that are clustered into herbivores trophic group are deemed as one of plant problematic insects by horticulture practitioners. These types of nematodes they belong to *Tylenchida* which subdivide into two orders namely *Dorylaimida* and *Aphelenchida* (Haynes, 2014; Swart *et al.,* 2017; Balla *et al.,* 2021). Furthermore, herbivores trophic group comprises of two distinct nematodes (namely endoparasites and ectoparasites) (Haynes, 2014; Stefanovska *et al.,* 2022). Ectoparasites nematodes are free living soil nematodes that feed on

plant surface roots meanwhile endoparasites are commonly known as root nematodes due to their ability to penetrate the plant roots and feed from within (Stefanovska *et al.,* 2022). The morphological mouth feature of these nematodes is shaped like a needle called stylet, that pierce through the roots surface during the feeding process (Figure 5.1 A).

Nematodes that belong to algivores trophic group they exhibit a strongly feeding preference towards algae, they commonly found in both soil and freshwater surfaces (Neher and Darby, 2006). There are aggressive nematodes which feeds on wide range of insects, including other nematodes (Kudrin *et al.,* 2015). These nematodes are classified under predator trophic group. Further there are only two types of nematodes (namely soil free-living and plant parasite nematodes) which are predator nematodes feeding preference (Wilschut and Geisen, 2021). Moreover, these nematodes are affiliated with two commonly orders namely *Mononchida* and *Dorylaimida* (Chhetri and Subedi, 2019). According to Stirling, (2018) these nematodes are unlikely to occur in nature.

Bacterivore trophic group consists of nematodes that feeds on bacteria (Potapov *et al.,* 2022). Further these nematodes are found throughout various ecological regions (Ravichandra and Ravichandra, 2014, Potapov *et al.,* 2022). They have a distinctive morphological feeding structure from nematodes belonging to herbivore trophic group (Figure 5.1 B). Furthermore, their mouth is hollow tub-like, enabling them to suck-off bacteria from the surface (Ravichandra and Ravichandra, 2014). These nematodes are commonly in the order Rhabditida and are rare (Shaw *et al.,* 2019). These nematodes they are thought to be a good indicator of fertile soil, since they act as organic matter decomposers within the soil (Holterman *et al*., 2006).

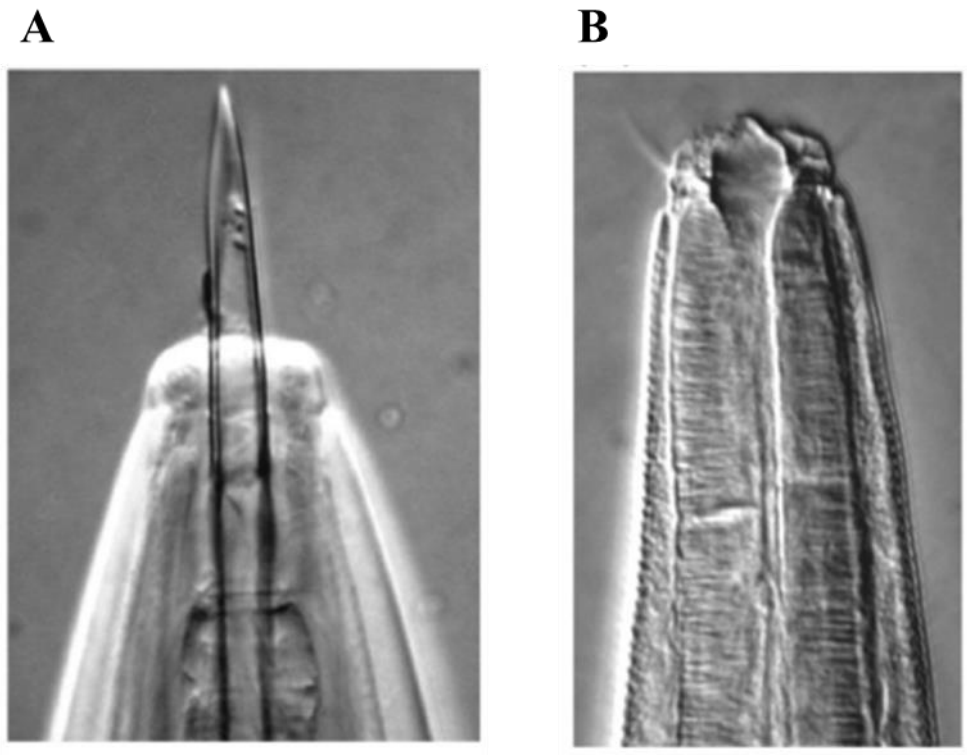


Figure 5.1 Displaying nematodes structural mouthparts based on their feeding preference (Images adapted from Traunspurger and Majdi, 2017 with minor modifications) A) Herbivore nematode B) Bacterivore nematode mouth morphological features.

The morphological different mouth features are thought to explain the feeding behaviour exhibited by nematodes from the different trophic groups. There are group of nematodes belonging to *Dorilaimida* order, which they are classified in omnivore trophic group. These nematodes they have an ability to feed on variety of species such as bacteria, fungus, algae, and other animal species, however these species they are rare (Ingham, 1994). Moreover, other previous studies have suggested that the mouth morphological features of nematodes may not be the only factor that drives their feeding habit.

5.1.3 Entomopathogenic nematodes overview

Entomopathogenic nematodes (EPNs) are soil dwelling insect parasite nematodes belonging to carnivore trophic group. These nematodes have a commensal relationship with endopathogenic bacteria. These nematodes belong to phylum order *Rhabditida*. The symbionts pathogenic bacteria it is involved in cyto-protection, development, and growth regulation of EPNs. According to the EPNs bacteria symbionts influence the feeding behaviour of EPNs. The EPNs bacteria symbionts possesses ability to synthesizes toxins compounds, that are used to kill the insect host during the feeding stage (Figure 5.2). Hence, since the bacteria is responsible for

causing insect death, many scholars have suggested that the bacteria modulate the nematodes food preference since the nematodes serves as the bacteria vector. However, the is no supporting evidence of these claims.

They EPNs have a very peculiar lifestyle with the endosymbiont bacteria. Post invasion by EPNs infective juveniles (IJs) into insect host body the symbionts bacteria are regurgitated into the insect host body and colonise the host body. Further, during the feeding processes the nematodes also ingest the endosymbionts bacteria and only few that attaches into the IJs intestine. The intestinal attached bacteria they colonise the rectal glands giving them an access to maternal organelles of the IJs. The progression of endosymbiont bacteria colonisation moves as further as pharyngeal-intestinal valve of IJs (Figure 5.2).

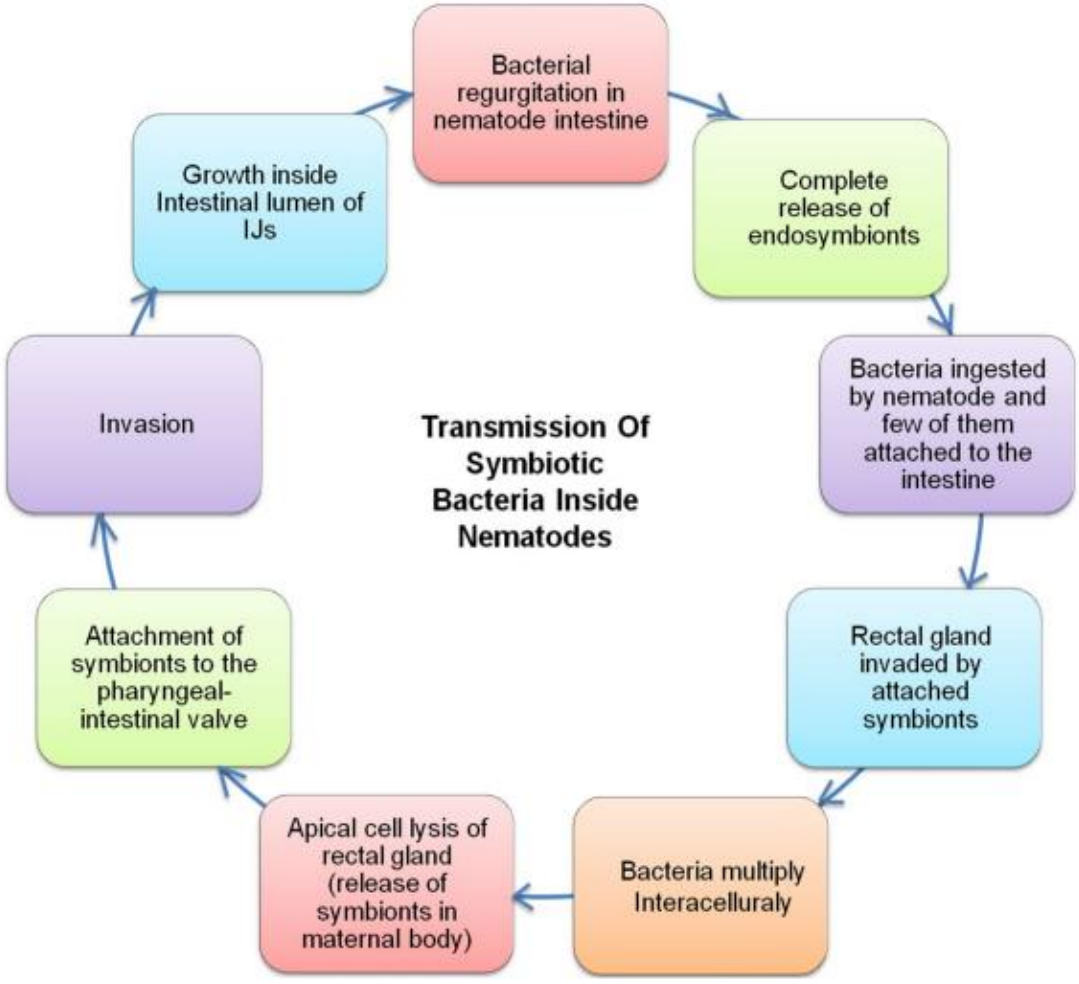


Figure 5.2 Elucidating the transmission of symbiotic bacteria inside nematodes. The EPNs bacteria symbionts are released upon host invasion and ingested during the feeding stage and the bacteria colonises different organelles of the nematodes (Image adapted from Tomar *et al.*, 2022).

5.2 Methodology

5.2.1 *Cruznema* endobacteria symbionts pathogenicity test against *Tenebrio molitor* (*T. molitor*) larvae

A single colony of *Enterobacter sp.* PTB and *Pseudomonas sp.* PKS *Cruznema sp.* gut endobacteria isolate was inoculated into separate 5 mL 2XYT broth (consists of 1 % yeast extract, 1.6 % of tryptone, 0.5 % of sodium chlorine). Both broths were kept at 130 rpm shaker incubator with the optimum temperature of 25 ℃, overnight. One mL of the overnight culture was withdrawn and spread in freshly prepared lipid agar plate. The plate was subsequently kept within 25 ℃ incubator to allow the growth of the bacteria. Seven *T. molitor* larvae were placed in the bacteria lawn of (either *Enterobacter sp.* PTB, *Pseudomonas sp.* PKS or *Enterobacter sp.* PTB-*Pseudomonas sp.* PKS) spread in agar plate. Single agar plate consisting of *T. molitor* larvae without bacteria lawns was used as negative control. The plate was stored inside incubator with temperature adjusted to 25 ℃ and the larvae were monitored interval of six hours period. The number of the dead larvae were recorded. This experiment was repeated three times.

5.2.2 *Cruznema sp.* NTM 2021 gut endobacteria symbiosis test

A single colony of *Enterobacter sp.* PTB and *Pseudomonas sp.* PKS *Cruznema sp.* gut endobacteria isolate were inoculated separately into 5 mL 2XYT (1 % yeast extract, 1.6 % of tryptone, 0.5 % of sodium chlorine) broth. Subsequently, each broth was store inside a 25 ℃ shaker incubator overnight. Furthermore, 1 mL of the overnight culture was spread in a lipid agar plate (5 g/L of yeast extract, 2 g/L of $MgCl_2 \cdot 6H_2O$, 10 g/L of honey, 2.5 ml/L of cod liver oil). Subsequently, the lipid agar plate was kept overnight in 25 ℃ incubator. Moreover, freshly surface sterilized IJs were introduced into the bacteria lawn within the lipid agar plate as stipulated in Kaya and Stock, 1997. There plate was stored at 25 ℃ and the growth of *Cruznema sp.* nematodes was monitored. This experiment was conducted in triplicates.

5.3 Results

The endobacteria symbiosis test was conducted through isolation of bacteria found in *Cruznema* lumen. The bacteria were grown in 2XYT broth and spread in lipid agar plates. 1000 freshly infective juveniles (IJs) were surfaces sterilised with 0.1 % of sodium hypochlorite and rinsed with sterile deionised water. Subsequently the IJs where placed in lipid agar plates consisting of bacteria lawn. The plates were kept in 25 ℃ incubator for four days period. The lipid agar plate images were photographed.

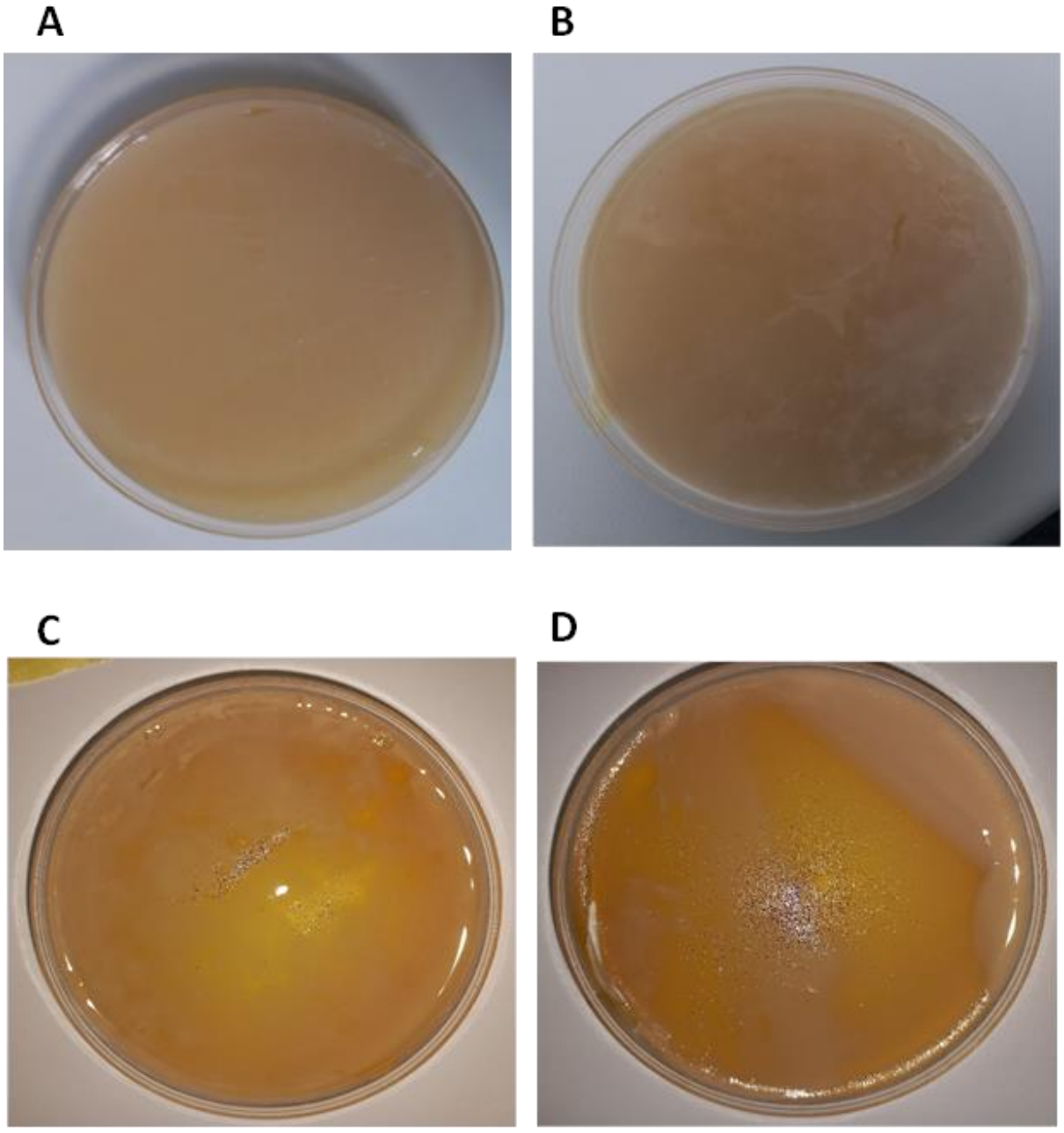


Figure 5.3 depicting of *Cruznema* endobacteria symbiosis test conducted in lipid agar plates. A and B) Display *Enterobacter sp.* PTB and *Pseudomonas sp.* PKS *Cruznema* endobacteria lawn lipid agar plate prior *Cruznema* infective juveniles (IJs) inoculation. C and D) Elucidate *Enterobacter sp.* PTB and *Pseudomonas sp.* PKS *Cruznema sp.* NTM 2021 endobacteria isolates lawn lipid agar plate after four days of *Cruznema* IJs inoculation.

The pathogenicity of *Cruzmena sp.* NTM 2021 bacteria isolates (*Pseudomonas sp.* PKS and *Enterobacter sp.* PTB) against was investigated by plating spread 0.5 McFarland standard of either *Pseudomonas sp.* PKS or *Enterobacter sp.* PTB into lipid agar plate to make bacteria

lawns as described in Section 5.2.1. The mortality rate of *T. molitor* was monitored for duration of 30 hours.

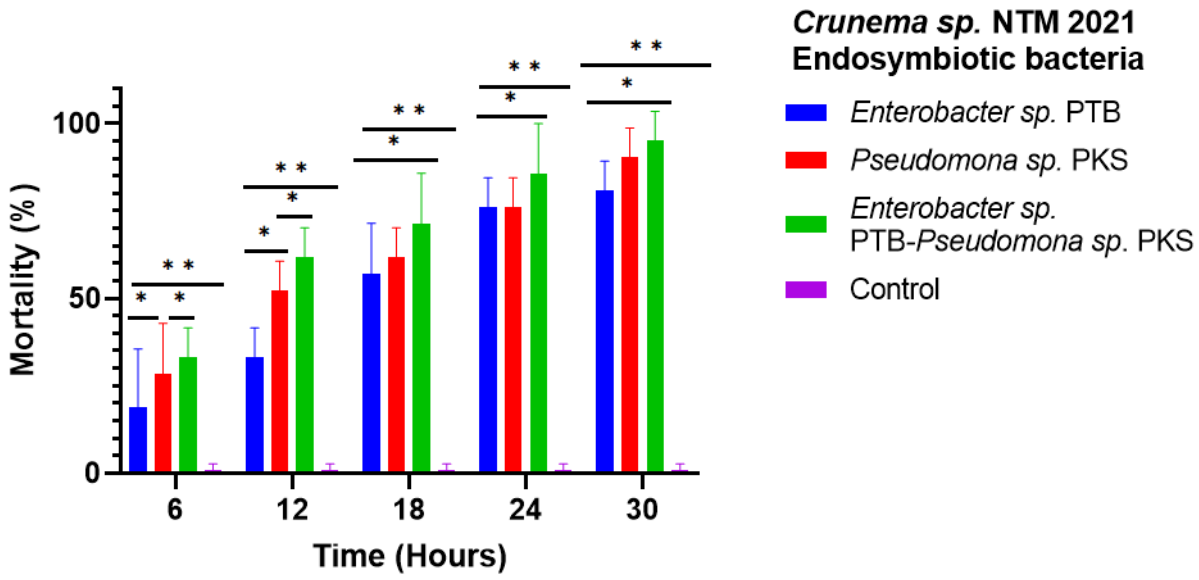


Figure 5.4 Elucidating mortality rate of *Cruznema* endosymbionts infections in *Tenebrio molitor* larvae. The *T. molitor* larvae was either infected with *Enterobacteria sp*. PTB, *Pseudomona sp.* PKS or *Enterobacteria sp*. PTB-*Pseudomona sp.* PKS. The *T. molitor* without any bacterial infection was used as negative control variable. The statical analysis was performed using two-way ANOVA with the alpha value of 0.05 (P>0.05 = * and P<0.05 = **).

5.3 Discussion

Entomopathogenic nematodes (EPNs) bacteria symbionts its key to the survival of the nematodes. The EPNs bacteria symbionts does not only provides nematodes with toxin compounds to kill the insect host. However, perform they cyto-protection function against possible pathogens. Furthermore, during the feeding and precreation process of nematodes within the insect host the bacteria is reported to produce biofilms that acts as protection barrier against opportunistic pathogens.

The endobacteria symbiosis test was archived by spreading either *Cruznema sp.* NTM 2021 gut *Enterobacter sp.* PTB, *Pseudomonas sp.* PKS or *Enterobacter sp.* PTB-*Pseudomonas sp.* PKS isolates culture in a lipid agar plate and incubate the plate in 25 for 24 hours period which generate into bacteria lawn (Figure 5.3 A and B). Subsequently, an addition of *Cruznema* infective juveniles (IJs) into the lipid agar plate was made to evaluate the symbiosis relationship between the gut bacteria isolate and *Cruznema sp.* NTM 2021 nematodes in accordance to

Kaya and Stock *et al*. (1997). Both *Cruznema* gut bacteria isolate *Enterobacter sp.* PTB and *Pseudomonas sp.* PKS was found supporting the growth of *Cruznema* nematodes (Figure 5.3 C and D). However, *Pseudomonas sp.* PKS supports *Cruznema* nematodes more, compared to *Enterobacter sp.* PTB. Therefore, these observations are supported with the metagenomic study in Chapter 3 since *Pseudomonas sp.* PKS was found to be having highest relative abundance at 40 %. Therefore, these results shows that *Cruznema* nematodes have a symbiosis relationship with both *Enterobacter sp.* PTB and *Pseudomonas sp.* PKS isolates.

Pathogenicity assessment of *Cruznema sp.* NTM 2021 gut *Enterobacter sp.* PTB, *Pseudomonas sp.* PKS or *Enterobacter sp.* PTB-*Pseudomonas sp.* PKS isolates was initiated by spreading the bacteria isolates in nutrient agar plates, which resulted into bacteria lawns after overnight incubation at 25 ℃ optimum temperature. *Tenebrio molitor* (*T. molitor*) larvae were placed in the plate and motility was monitored in 6 hours interval. All the bacteria isolates were able to infect and kill the *T. molitor* insect (Figure 5.4). The two-way ANOVA statical calculations in from 6 and 12 hours all three bacteria compared to control there is significance difference as indicated by P value lower than 0.05 whereas *Enterobacter sp.* PTB vs *Pseudomonas sp.* PKS and *Enterobacter sp.* PTB-*Pseudomonas sp.* PKS vs *Pseudomonas sp.* PKS has high P value above 0.05 (Figure 5.4, Table A1). Further *Pseudomonas sp.* PKS compared to *Enterobacter sp.* PTB-*Pseudomonas sp.* PKS has P value lower than 0.005 showing significance difference. Moreover, from 18 to 30 hours all three bacteria when are individual compared with the control there is significance difference as denoted by P value less than 0.05 meanwhile, there is no significance different when all bacteria are individual compared to each other, hence supported by P value greater than 0.05 (Figure 5.4; Table A1). *Enterobacter sp.* PTB *has lowest mortality rate* whereas, *Pseudomonas sp.* PKS has higher mortality rate. Furthermore, *Enterobacter sp.* PTB-*Pseudomonas sp.* PKS has the highest mortality rate. Moreover, both the bacteria symbionts where able to kill *T. molitor* larvae within a period thirty-two hours. Therefore, both *Enterobacter sp.* PTB and *Pseudomonas sp.* PKS servers as counter parts towards deprivation of *T. molitor* microbiota and immune response. Moreover, these bacteria may also form a part of *Cruznema* immune response against other pathogenic bacteria infections. *Enterobacteria* species are known to possess the ability to protect plants against fungal disease and bacteria pathogens (Somvanshi *et al.,* 2006; Spescha *et al.,* 2023). Moreover, both *Pseudomonadaceae* and *Enterobacteriaceae* bacteria families are known to consist of endopathogenic bacteria, that are implemented in symbiotic relationship with the nematodes. There are four genera namely *Xenorhabdus*, *Photorhabdus, Serratia* and

Pseudomonas, which has endopathogenic characteristics and have exhibit a mutualistic/ symbiotic relationship with the entomopathogenic nematodes (EPNs). Furthermore, *Xenorhabdus* is known to be associated with *Heterorhabdus* EPNs (Kary *et al.*, 2021). Whilst *Steinernema* EPNs was found associated with both *Photorhabdus* and *Pseudomonas* species (Mahar *et al.*, 2005). Whereas *Oscheuis* EPNs are associated with *Serratia* species (Lephoto and Gray 2019). *Cruznema* nematodes are not regarded as EPNs, since there are known to feed on bacteria and lack of an evidence that they have endopathogenic bacteria symbionts. Chapter 3 shows that the gut microbiota community of *Cruznema* nematodes, with *Pseudomonadaceae* family highly abundance followed by *Hyphomicrobiales*, *Flavobacteria*, *Bacteroida*, etc. furthermore, in study conducted by Mahar *et al.*, 2005 indicated that *Pseudomonas* putida bacteria symbionts of EPNs *Steinerma* abbasi, secretes exoenzymes that abrogate humoral immune system of *Galleria mellonella* (*G. mollenella*) pupae, making link between EPNs and *Pseudomonas* genus. Therefore, the findings in this study shows the symbiosis relationship between *Cruznema* nematodes and endopathogenic bacteria *Enterobacter sp.* PTB and *Pseudomonas sp.* PKS

5.4 Conclusion and future recommendations

Cruznema sp. NTM 2021 exhibit the characteristics of entomopathogenic nematodes with two bacteria symbionts (namely *Enterobacteria sp*. PTB, *Pseudomona sp.* PKS). The two bacteria symbionts possess ability to synthesis biotoxins compounds that has insecticidal activity that killed *T. molitor* larvae. This is the first report that proves the *Cruznema* nematodes have symbiotic relationship with pathogenic bacteria and exhibits an EPNs feeding behaviour. According to the evidence of this study *Cruznema* nematodes are classified as EPNs. Furthermore, more studies should be conducted to study the insect host range of *Cruznema* nematodes, and identify the bioactive compounds used to kill insect host by *Cruznema* bacteria symbionts.

5.5 References

Balla, A., Silini, A., Cherif-Silini, H., Chenari Bouket, A., Moser, W.K., Nowakowska, J.A., Oszako, T., Benia, F. and Belbahri, L., 2021. The threat of pests and pathogens and the potential for biological control in forest ecosystems. *Forests*, *12*(11), pp.1579.

Bongers, T. and Bongers, M., 1998. Functional diversity of nematodes. *Applied Soil Ecology*, *10*(3), pp.239-251.

Chavez, M., 2021. The sustainability of industrial insect mass rearing for food and feed production: zero waste goals through by-product utilization. *Current Opinion In Insect Science*, *48*, pp.44-49.

Chhetri, B. and Subedi, J.R., 2019. Nematodes Associated with Kiwi Plants in Central Horticulture Centre Kirtipur, Kathmandu, Nepal. *Int J Appl Agri Sci*, *5*(3), pp.71-74.

Hailu, F.A. and Hailu, Y.A., 2020. Agro-ecological importance of nematodes (round worms). *Acta Sci Agric*, *4*(1), pp.156-162.

Haynes, R.J., 2014. Nature of the belowground ecosystem and its development during pedogenesis. *Advances In Agronomy*, *127*, pp.43-109.

Holterman, M., van der Wurff, A., van den Elsen, S., van Megen, H., Bongers, T., Holovachov, O., Bakker, J. and Helder, J., 2006. Phylum-wide analysis of SSU rDNA reveals deep phylogenetic relationships among nematodes and accelerated evolution toward crown clades. *Molecular Biology And Evolution*, *23*(9), pp.1792-1800.

Ingham, R.E., 1994. Nematodes. *Methods of Soil Analysis: Part 2 Microbiological And Biochemical Properties*, *5*, pp.459-490.

Kary, N.E., Sanatipour, Z., Mohammadi, D. and Dillon, A.B., 2021. Combination effects of entomopathogenic nematodes, *Heterorhabditis bacteriophora* and *Steinernema feltiae*, with Abamectin on developmental stages of Phthorimaea operculella (*Lepidoptera*, *Gelechiidae*). *Crop Protection*, *143*, pp.105543.

Kaya, H.K. and Stock, S.P., 1997. Techniques in insect nematology. In *Manual Of Techniques In Insect Pathology*, pp. 281-324. Academic Press.

Knoff, M., Felizardo, N.N., Iñiguez, A.M., Maldonado Jr, A., Torres, E.J.L., Pinto, R.M. and Gomes, D.C., 2012. Genetic and morphological characterisation of a new species of the genus *Hysterothylacium* (Nematoda) from Paralichthys isosceles Jordan, 1890 (Pisces: Teleostei) of

the Neotropical Region, state of Rio de Janeiro, Brazil. *Memórias Do Instituto Oswaldo Cruz*, *107*, pp.186-193.

Kudrin, A.A., Tsurikov, S.M. and Tiunov, A.V., 2015. Trophic position of microbivorous and predatory soil nematodes in a boreal forest as indicated by stable isotope analysis. *Soil Biology And Biochemistry*, *86*, pp.193-200.

Lazarova, S., Coyne, D., Rodriguez, M.G., Peteira, B. and Ciancio, A., 2021. Functional diversity of soil nematodes in relation to the impact of agriculture—a review. *Diversity*, *13*(2), pp.64.

Lephoto, T.E. and Gray, V.M., 2019. *Oscheius basothovii* n. sp. (Nematoda: *Rhabditidae*), a new entomopathogenic nematode isolated from an uncultivated grassland in South Africa. *Archives Of Phytopathology And Plant Protection*, *52*(1-2), pp.125-140.

Mahar, A.N., Darban, D.A., Gowen, S.R., Hague, N.G.M., Jan, N.D., Munir, M. and Mahar, A.Q., 2005. Use of entomopathogenic bacterium *Pseudomonas putida* (Enterobacteriaceae) and it secretions against the Greater Wax Moth *Galleria mellonella* pupae. *Journal Of Entomology*, *2*, pp.77-85.

Mkandawire, T.T., Grencis, R.K., Berriman, M. and Duque-Correa, M.A., 2022. Hatching of parasitic nematode eggs: a crucial step determining infection. *Trends In Parasitology*, *38*(2), pp.174-187.

Neher, D.A. and Darby, B.J., 2006. Computation and application of nematode community indices: general guidelines. In *Freshwater Nematodes: Ecology And Taxonomy*. pp. 211-222. Wallingford UK: CABI Publishing.

Neher, D.A. and Weicht, T.R., 2013. Nematode genera in forest soil respond differentially to elevated CO2. *Journal Of Nematology*, *45*(3), pp.214.

Nisa, R.U., Tantray, A.Y., Kouser, N., Allie, K.A., Wani, S.M., Alamri, S.A., Alyemeni, M.N., Wijaya, L. and Shah, A.A., 2021. Influence of ecological and edaphic factors on biodiversity of soil nematodes. *Saudi Journal Of Biological Sciences*, *28*(5), pp.3049-3059.

Potapov, A.M., Beaulieu, F., Birkhofer, K., Bluhm, S.L., Degtyarev, M.I., Devetter, M., Goncharov, A.A., Gongalsky, K.B., Klarner, B., Korobushkin, D.I. and Liebke, D.F., 2022. Feeding habits and multifunctional classification of soil-associated consumers from protists to vertebrates. *Biological Reviews*, *97*(3), pp.1057-1117.

Quist, C.W., Smant, G. and Helder, J., 2015. Evolution of plant parasitism in the phylum *Nematoda*. *Annual Review of Phytopathology*, *53*, pp.289-310.

Ravichandra, N.G. and Ravichandra, N.G., 2014. Phylogenetic and Evolutionary Concepts in Nematodes. *Horticultural Nematology*, pp.17-53.

Rizvi, A.N., Kashyap, P., Haldar, K. and Bose, K., 2022. Community analysis of soil-inhabiting nematodes in natural vegetations of Singalila National Park, West Bengal (India). *Records Of The Zoological Survey Of India*, *122*(2), pp.163-171.

Shaw, E.A., Boot, C.M., Moore, J.C., Wall, D.H. and Baron, J.S., 2019. Long-term nitrogen addition shifts the soil nematode community to bacterivore-dominated and reduces its ecological maturity in a subalpine forest. *Soil Biology And Biochemistry*, *130*, pp.177-184.

Somvanshi, V.S., Lang, E., Ganguly, S., Swiderski, J., Saxena, A.K. and Stackebrandt, E., 2006. A novel species of *Xenorhabdus*, family *Enterobacteriaceae*: *Xenorhabdus indica* sp. nov., symbiotically associated with entomopathogenic nematode *Steinernema thermophilum* Ganguly and Singh, 2000. *Systematic And Applied Microbiology*, *29*(7), pp.519-525.

Spescha, A., Weibel, J., Wyser, L., Brunner, M., Hermida, M.H., Moix, A., Scheibler, F., Guyer, A., Campos-Herrera, R., Grabenweger, G. and Maurhofer, M., 2023. Combining entomopathogenic *Pseudomonas* bacteria, nematodes and fungi for biological control of a below-ground insect pest. *Agriculture, Ecosystems & Environment*, *348*, pp.108414.

Stefanovska, T., Skwiercz, A., Pidlisnyuk, V., Zhukov, O., Kozacki, D., Mamirova, A., Newton, R.A. and Ust'ak, S., 2022. The Short-Term Effects of Amendments on Nematode Communities and Diversity Patterns under the Cultivation of *Miscanthus× giganteus* on Marginal Land. *Agronomy*, *12*(9), pp.2063.

Stirling, G.R., 2018. Biological control of plant-parasitic nematodes. In *Diseases of Nematodes.* pp. 103-150. CRC Press.

Swart, A., Marais, M., Mouton, C. and du Preez, G.C., 2017. Non-parasitic, terrestrial and aquatic nematodes. *Nematology In South Africa: A View From The 21st Century*, pp.419-449.

Tomar, P., Thakur, N. and Yadav, A.N., 2022. Endosymbiotic microbes from entomopathogenic nematode (EPNs) and their applications as biocontrol agents for agro-environmental sustainability. *Egyptian Journal Of Biological Pest Control*, *32*(1), pp.80.

Traunspurger, W. and Majdi, N., 2017. Meiofauna. *In Methods In Stream Ecology, 1*, pp.273-295. Academic Press.

Wilschut, R.A. and Geisen, S., 2021. Nematodes as drivers of plant performance in natural systems. *Trends In Plant Science*, *26*(3), pp.237-247.

CHAPTER 6

Investigating the effects of *Cruznema sp*. NTM 2021 endopathogenic bacteria symbiotic (*Enterobacter sp.* PTB and *Pseudomonas sp.* PKS) intracellular metabolites against known ATCC antibiotic resistant bacteria

Abstract

Nematodes are microscopic organism that dwell in natural environment. Entomopathogenic nematodes (EPNs) are obligated insects parasite that harbour pathogenic bacteria within their gut. These bacteria are known to produce toxins that are used to deprive the host immune system resulting in host death. The metabolites which are produced by these pathogenic symbiotic bacteria are suggested to possesses antimicrobial, antifungal activity. The development of drug resistance by bacteria raises concerns in public health. Therefore, this study screens the potential uses of *Enterobacter sp.* PTB and *Pseudomonas sp.* PKS intracellular metabolites as antimicrobial agent. Metabolites productions of *Pseudomonas sp*. PKS and *Enterobacter sp.* PTB *Cruznema sp*. NTM 2021 bacteria symbionts was carried by inoculating a log phase colony into 2XYT (0.5 % of NaCL, 1 % of yeast, 1.6 % tryptone) broth and grown at 30 ℃ incubator for 72 hours. The extraction of intracellular metabolites was carried by centrifuging the cultured medium, followed by dissolving the pellet with 100 % acetonitrile. *Pseudomonas sp*. PKS and *Enterobacter sp.* PTB metabolites profile was carried by high liquid pressure chromatograph using a mixture blend of 0.1 % acetonitrile and deionised water for mobile phase 1 and a blend mixture of 0.1 % acetic acid and acetonitrile for mobile phase 2. Minimal inhibition concentration (MIC) was used to check the effect of intracellular metabolites produced by *Cruznema sp.* NTM 2021 bacteria symbionts against common ATCC antibiotic resistant bacteria strains (*E. coli, P. aeruginosa E. Faecalis* and *S. aureus*). Five metabolite compounds were detected for *Enterobacter sp.* PTB and twenty metabolites' peaks were detected for *Pseudomonas sp*. PKS. Staurosporine, Saliniketal, and indole bioactive compound were among the identified metabolites produced by *Pseudomonas sp*. PKS. Saliniketal it is crucial for cellular repair and staurosporine has antifungal activity. Indole was found to be produced by both *Pseudomonas sp*. PKS and *Enterobacter sp.* PTB bacteria, this bioactive compound has antimicrobial activity. Furthermore, indole is involved in development of bacteria biofilms, important for virulence and defence. *Cruznema sp.* NTM 2021 bacteria symbionts produce a wide range of bioactive compounds with different functions. The intracellular metabolites of *Pseudomonas sp.* PKS were able to inhibit the growth of antibiotic resistant bacteria (*E. coli, P. aeruginosa E. Faecalis* and *S. aureus*) at 50

mg/ml meanwhile the intracellular metabolites of *Enterobacter sp.* PTB where only able to inhibit the growth of *E. coli, E. Faecalis* and *S. aureus* only at 50 mg/ml concentration. Furthermore, all the antibiotic resistant bacteria recovery was assessed by spreading the bacteria on Mueller-Hinton agar post exposure to *Enterobacter sp.* PTB and *Pseudomonas sp.* PKS intracellular metabolites. Moreover, *Enterobacter sp.* PTB and *Pseudomonas sp.* PKS intracellular metabolites were found to be bacteriostatic.

Keywords: Metabolites, Entomopathogenic nematodes, antibiotic resistant bacteria, Minimal inhibition concentration, Bacteriostatic.

6.1 Introduction

6.1.1 Entomopathogenic nematodes

Nematodes are well diversified microorganisms found throughout the ecological niche. Nematodes clustered into distinctive trophic levels based on their feeding behaviour (Walter *et al.,* 1989; Quist *et al.,* 2017). Furthermore, other type of nematodes are regarded to be beneficial in horticulture practice, these specific group of nematodes are called entomopathogenic nematodes (EPNs) (Vashisth *et al*., 2013). These set of nematodes belong to phylum *Rhabditida*, there are only three genera have been recorded so far (namely *Oscheius, Steinernema* and *Heterorhabditis*) (Amuri *et al.,* 2020; Ogier *et al.,* 2023). There might been other set of genera that have not been identified due to the complexity and challenges in conducting the experiment. Moreover, EPNs are known to have develop a symbiotic relationship with endopathogenic bacteria species belonging *Gamaprobacteria*, there are only three genera recoded namely *Xenorhabdus, Photorhabdus* and *Serratia* (Ferreira and Malan, 2014; Ogier *et al.,* 2023).

6.1.2 Entomopathogenic nematodes infection mechanisms against insect host

These bacteria symbionts are harboured within nematodes gut, and they are known to enhance the survival fitness of the nematodes (Owuama, 2001). Furthermore, EPNs are biological control agents that have been used to replace integrated chemical control agents (Grewal *et al.,* 2012; Shakeel *et al.,* 2022), due to detrimental effects on the soil as results from the use integrated chemical control agents (Prashar and Shah, 2016; Sharma and Singhvi, 2017). EPNs use host biological openings to invade the system and subsequently release the bacteria symbionts, which cause infection and deprives the host immune system (Wu *et al.* 2018; Liu *et al*., 2020). The EPNs infective juveniles (IJs) feed on the host cadaver and undergone reproduction (3rd to 4th generations) (Figure 6.1). The depletion of food force the IJs to emerge out the host body to environment and seeks out new host (Figure 6.1). The endopathogenic bacteria symbionts does not only infect the host however they are also known to produce prominent levels of bioactive compounds referred as metabolites (Ribeiro *et al.,* 2019; Shan *et al.,* 2019; Awori, 2022). Theses bioactive compounds are not used to infect their host only however, they are also used as defensive mechanisms against any opportunistic parasites that may invade the host during feeding and reproduction process (Bulgheresi, 2016; Shan *et al.,* 2019; Thakur *et al.,* 2020). Furthermore, other studies suggests that these bioactive compounds possess antimicrobial activity, making them a target for pharmacology industries (Waterfield *et al.,* 2009; Awori, 2022).

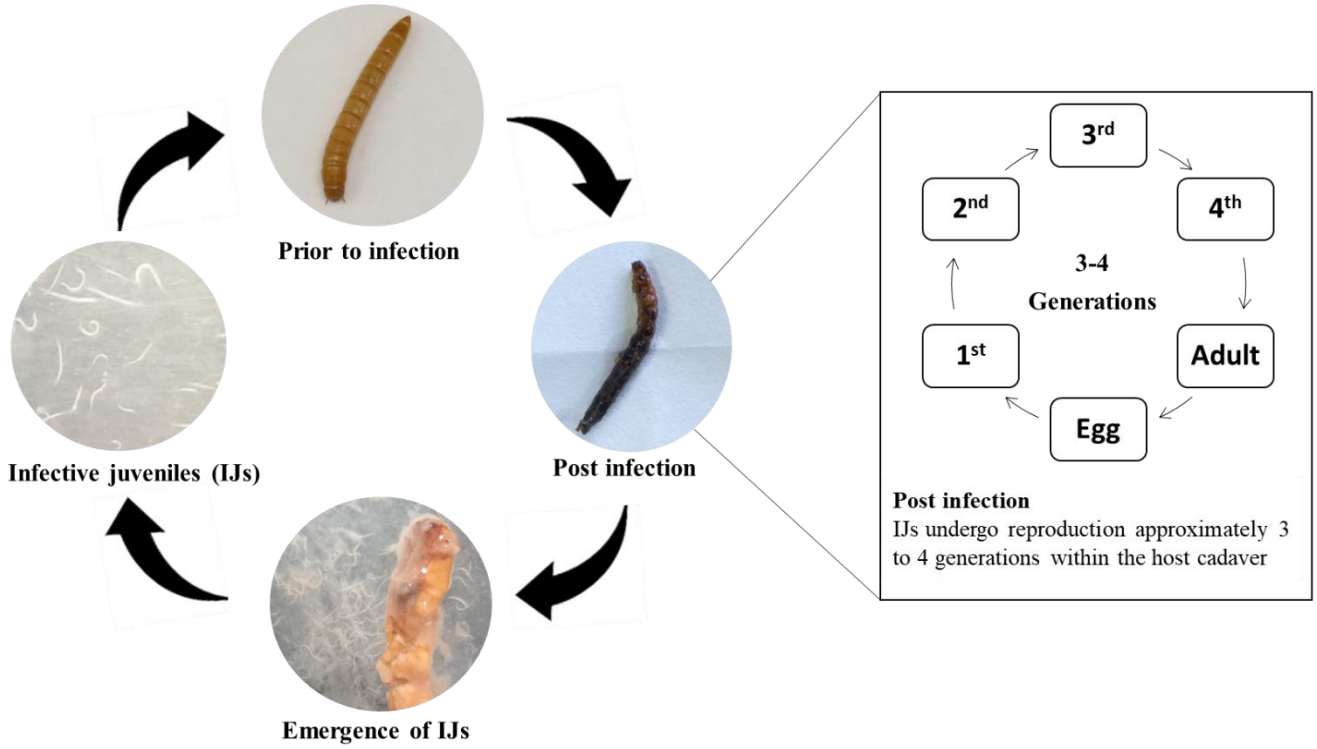


Figure 6.1 Schematic diagram depicting life cycle of entomopathogenic nematodes (Image produced by Pusang King Sekgobela)

The EPNs endopathogenic bacteria symbionts bioactive molecule they function as safeguards that do not only deprives the host immune system. However, the also inhibit/ kill other microorganisms invades or consumes the host cadaver (Kusakabe *et al.*, 2022). Furthermore, the bioactive compounds are implemented in the growth and development of the IJs (Cimen *et al.*, 2022). Moreover, previous studies indicated that these bioactive compounds have wide range of applications since they exhibit insecticidal, antifungal and nematocidal activities (Elbrense *et al.*, 2021; Cimen *et al.*, 2022). In addition, Kusakabe et al. (2022) stated that the variation among the endopathogenic bacteria species/ strains and different physiological condition results in production of various bioactive compounds.

Furthermore, various metabolites such as dithiolopyrrolones, indole, and xenocoumacins have been found in EPNs symbionts culture medium (Bode, 2009; Gualtieri *et al.*, 2009). During EPNs infection stage the insect host immune defence produces bioactive compounds that place the EPNs bacteria symbionts under stress (Raja *et al.*, 2021). The EPNs bacteria symbionts produce biofilms which acts as protective barrier for both nematodes and the bacteria (Zhang *et al.*, 2022). Therefore, the survival struggle of EPNs and their bacteria symbionts caused by insect host defence system upregulates the production of metabolites in EPNs bacteria symbionts. Furthermore, both *Photorhabdus* and *Xenorhabdus* bacteria symbionts are reported

to produce acaricidal active metabolites (Cevizci *et al.,* 2020). Moreover, there is a little information known about the ones which are secreted by *Cruznema sp.* NTM 2021 endopathogenic symbionts bacteria (namely *Pseudomonas* sp. PKS and *Enterobacteria sp.* PTB). Therefore, they are regarded as a potential source of novel therapeutic drug discoveries. However, this is poorly research field and hence, there is an indication of a massive unscreened species. Hence, this study seeks to explore the metabolites found in *Pseudomonas* sp. PKS and *Enterobacteria sp.* PTB *Cruznema sp.* NTM 2021 endopathogenic symbionts.

The recent development of new antibiotic resistant genes (ARGs) in infectious diseases expands the search of novel molecular drug candidates. Therefore, the bioactive compounds produce by nematodes bacteria symbionts are the potential novel drug candidates considering their complexity and widely use in virulence/ defence against other pathogens (Owuama, 2001; Shakeel *et al.,* 2022). Their ability to produce the antibacterial agents to inhibit the opportunistic bacteria from exploiting the host cadaver (Kusakabe *et al.,* 2022; Fallah *et al.,* 2023; Ogier *et al.,* 2023), furthermore these molecules can be used for pharmaceutical activities to generate potential drug candidate for various disease affecting both human and plants. The study conducted by Zhou et al. (2020) shows that biological compound extracted from EPNs bacteria symbionts (namely *Xenorhabdus* and *Photorhabdus*) are used as bioinsecticides against agricultural problematic pests.

6.1.3 Metabolites

Metabolites refers to biological compounds which are produce during cellular metabolism. Metabolites are clustered into two groups (namely primary and secondary metabolites) (Hakim and Kadarohman, 2020). Primary metabolites are implemented in reproduction and cellular development (example: amino acids, nucleosides, organic acids, etc) (Zhou *et al.,* 2020). Whereas secondary metabolites are specialised molecules such as toxins, their involve in cellular development, virulence and defence (Hendrikx and Schnabl 2019; Fallah *et al.,* 2023). Metabolites are also categorised based on cellular location (namely intracellular and intercellular) (Nabariya *et al.,* 2022). The intracellular metabolites are produced within the mechanical barrier (i.e. cell membrane) meanwhile the extracellular/intercellular metabolites are synthesized outside the cell (i.e. between the cells) (Zhou *et al.,* 2020; Nabariya *et al.,* 2022; Fallah *et al.,* 2023).

6.1.4 Screening of Bioactive compounds

Screening of bioactive compounds or metabolites in bacteria to discover new antibiotics has been in best interest of economy, pharmaceutical companies, and biotechnology industries

(Ramírez-Rendon *et al.,* 2022). Furthermore, this route has already resulted into prestigious discovery for antimicrobial drug agents such as tetracycline, vancomycin, chloramphenicol, etc (Pandey *et al.,* 2018). The developments of drug resistant abilities in pathogens makes the pursuit of novel bioactive compounds a need in medical to combat these pathogens. Screening for novel bioactive compounds in newly discovered of bacterial strains is an ideal way. Moreover, microbial metabolites have been confirmed countless times to be abundant source of novel potential therapeutic drugs (Fang *et al.,* 2021).

Screening and characterisation of secondary microbial metabolites server a high purpose in pharmaceutical industries as metabolites can undergo various chemical structures and biologically activities, under different conditions (Valerio *et al.,* 2021; Maithani *et al.,* 2022). Therefore, depending on a specific environmental condition, bacteria are known changes their biological function system to enable them to survive (Grabowski *et al.,* 2021). This result into change of the metabolites chemical structure, hence this also changes the biological activity or functions (Fang *et al.,* 2021). These changes are important for therapeutic drug development (Yao *et al.,* 2017).

6.2 Methodology

6.2.1 Metabolites production of *Cruznema sp.* NTM 2021 endopathogenic bacteria isolates

A single log phase colony of either *Enterobacteria sp.* PTB or *Pseudomonas* sp. PKS obtain from MacConkey agar plate was inoculated into 2XYT (0.5 % of NaCL, 1 % of yeast, 1.6 % tryptone) broth medium. The culture was placed at shaker incubator for 72 hours, with the following adjusted parameters: temperature 30 ℃ and orbital shaker 160 rpm. The culture was centrifuge at 10 000 x*g* for 10 minutes was harvest the bacterial cells. The supernatant was discarded. Furthermore, 100 % acetonitrile was added to the pellet and vortexed for 2 minutes. Subsequently, followed by filtering of mixture with 0.2 µm filter syringe to remove cellular debris and stored in -80 ℃ freezer (Pathma *et al.,* 2011).

6.2.2 High pressure liquid chromatography to identify metabolites produced by *Cruznema sp.* NTM 2021 endopathogenic bacteria isolates

The extracted intracellular metabolites were added into HPLC glass vials. The metabolites analysis was conducted using high pressured chromatography (HPLC) system fitted with a reversed-phase analytical column C18 column HSS T3 (150 mm x 2.1 mm, 1.7 µm) through injecting a 3 µL. There are two mobile phase which the sample has gone through before obtaining the results and each phase uses a different solvent with total flow rate of 1.5 mL/min.

The primary phase contains 0.1 concentrated percent of acetic acid in UHPLC sterile water; whereas the secondary phase consists of 0.1 concentrated percent of acetic acid blend with acetonitrile (UHPLC graded. Romil SpS, Cambridge, UK). Furthermore, photodiode array detector (PDA) was set to read the absorbance of the compounds at 254 nm. Adhering to standard conditions: an initial of condition of 95 % phase A and 5 % phase B for 1 min. Series of multiple gradients runs were conducted in a following sequence: from 1 to 9 min a gradual decrease in gradient of phase A from 95 to 5 % and gradual increase in gradient from 5 to 95 % for B. furthermore, it was steady maintained for the next 3 minutes. Subsequently, the gradient was gradual decrease from 95 to 5 for B, meanwhile the gradient for A was gradual increase from 5 to 95 % during the 11 to 15 minutes. Then detected metabolite peaks by HPLC were cross referenced with the known standards as previously described by Mullins *et al*. (2019).

6.2.3 Screening antimicrobial activity using minimal inhibition assay

The Screening of antimicrobial activity was conducted using a 96 well-plate. The assay was initiated by adding 200µL of 50 mg/mL metabolites of either *Enterobacter sp.* PTB or *Pseudomonas sp.* PKS on 3 wells of the first column as starting concentration. Subsequently, the metabolites concentration was serial diluted 5× from right to left direction, in 100 µL of four different antibody resistant bacterial suspension namely *Enterococcus faecalis* (*E. faecalis*: ATCC 29212)*; Pseudomonas aeruginosa* (*P. aeruginosa*: ATCC 27853)*; Escherichia coli* (*E. coli*: ATCC1925525)*; Staphylococcus aureus* (S. *aureus*: ATCC 29213) (Figure 6.2). Moreover, all the four-antibody resistant bacteria stock suspension was calibrated into 0.2 optical density, prior to distribution into the 96 well plate (Quist *et al.,* 2017; Sebola *et al.,* 2020). The minimum and maximum concentration of bacterial metabolites extracts was 1.5625 mg/ml and 50 mg/mL respectively, the final volume on each well was 200 µL. The controls samples were not exposed to intracellular metabolites of either *Enterobacter sp.* PTB or *Pseudomonas sp.* PKS, furthermore the results of the controls were expunged from the results section.

Furthermore, the 96 well plate was incubated for 24 hours period at 37 ℃. Subsequently, the absorbance measurement was conducted using Max-spectrometer USA at OD_{600} to acquire attenuation reading of the cells after treatment. Moreover, a 50 µL volume of 2 mg/mL p-Iodonitrotetrazolium dye was added in all four treated bacteria suspensions. Afterwards, the plate was returned into a 37 ℃ incubator for 30 minutes period. Images of colour change on a plate were captured (Figure 6.3). Therefore, sample(s) that were observed with no colour

changes from the 96 well plate after the incubation, ten microliters of the 100 µL sample(s) was spread into fresh Mueller-Hinton agar plate to evaluate the cellular recovery of the antibody resistant bacteria. The plate was store for 3 days at 37 ℃ incubator, and regular monitored at 12 hours interval (Mahdiyah *et al.*, 2020). These results were used to make a conclusive decision on bactericidal or bacteriostatic effects of either *Enterobacter sp.* PTB, or *Pseudomonas sp.* PKS endosymbiotic bacteria metabolites.

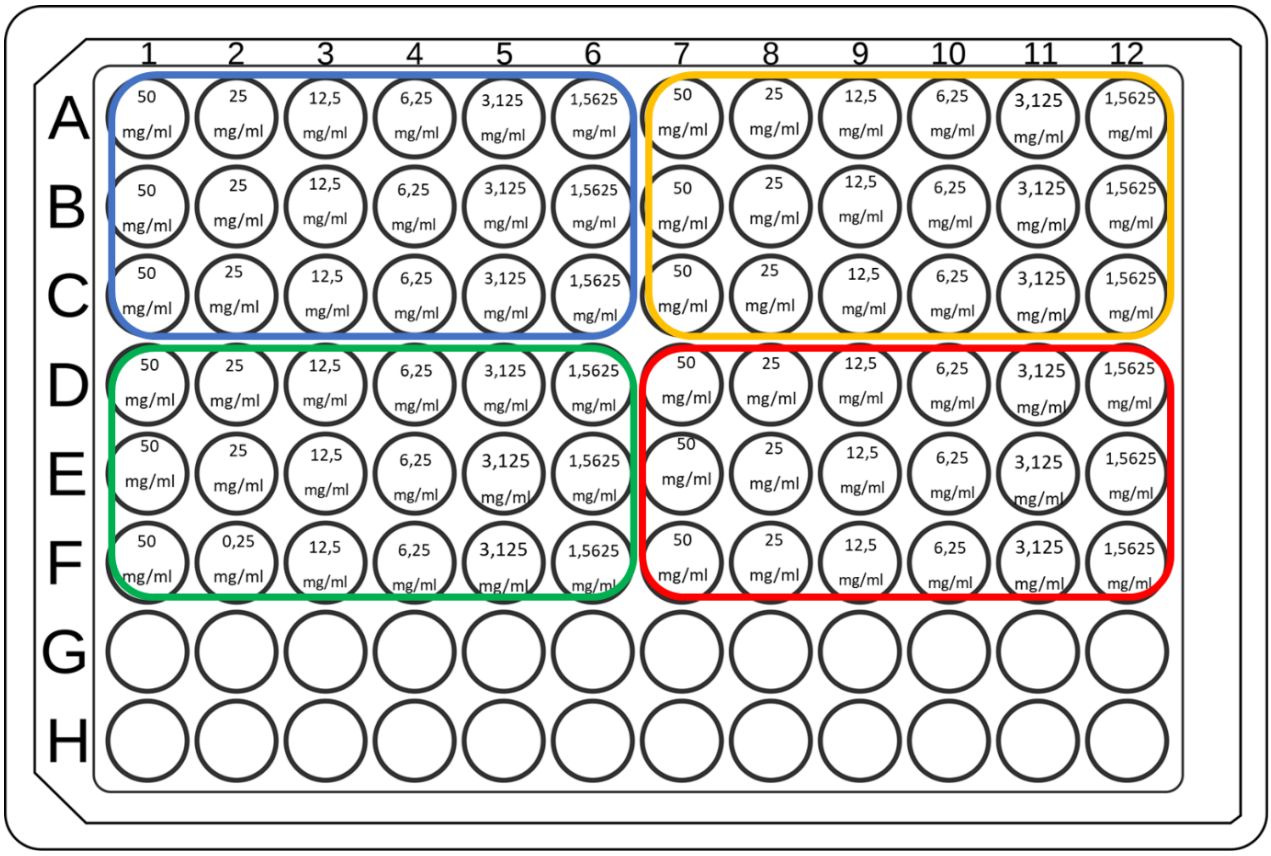


Figure 6.2: Represent distribution of metabolites concentration of either *Pseudomonas sp.* PKS or *Enterobacter sp.* PTB during the MIC (Minimum inhibition concentration) assay. Blue denotes *E. coli*, orange denotes *P. aeruginosa*, green denotes *E. Faecalis* and red denotes *S. aureus.*

6.3 Results

Profiling metabolites produced by *Cruznema sp.* NTM 2021 bacteria associates (namely *Pseudomonas* sp. PKS and *Enterobacteria sp.* PTB) was archived by using high pressured chromatography and cross referencing the identify peaks with the known standard described in Section 7.2.2.

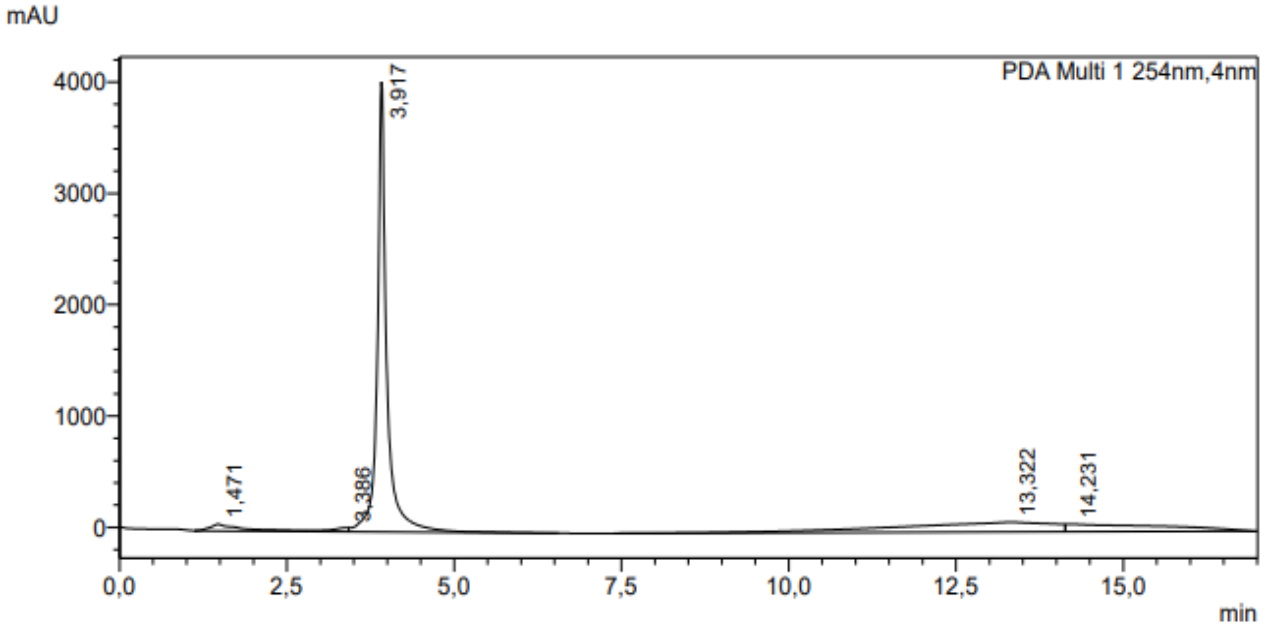


Figure 6.3 A chromatogram elucidating metabolites detection peaks of *Enterobacter sp.* PTB by high pressure liquid chromatograph (HPLC) at the absorbance of 254 nm for 15 minutes

Table 6.1 Showing the names of *Enterobacter sp.* PTB detected metabolites with their respective retention time.

No. Peaks	Retention time (min)	Name
1	1.471	Rifamycin Y
2	3.386	Maleic acid (Malonic acid)
3	3.917	Indole
4	13.322	Indole-3-aldehyde
5	14.231	Nonadecane, 1-chloro

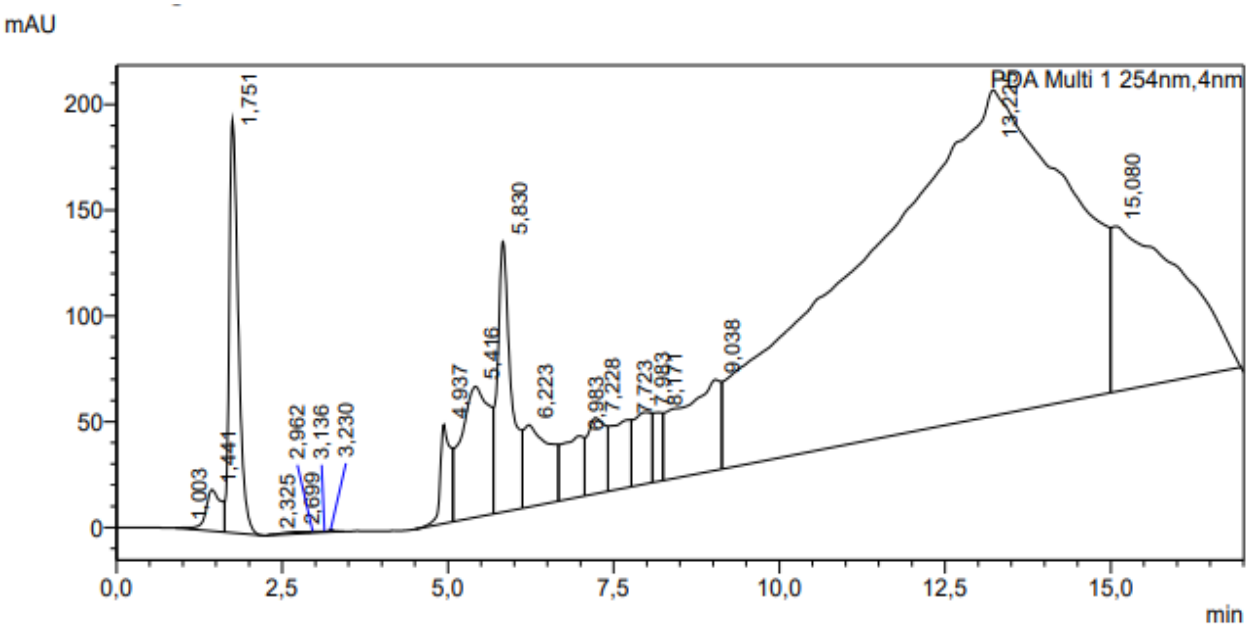


Figure 6.4 A chromatogram elucidating metabolites detection peaks of *Pseudomonas sp.* PKS by high pressure liquid chromatograph (HPLC) at the absorbance of 254 nm for 15 minutes

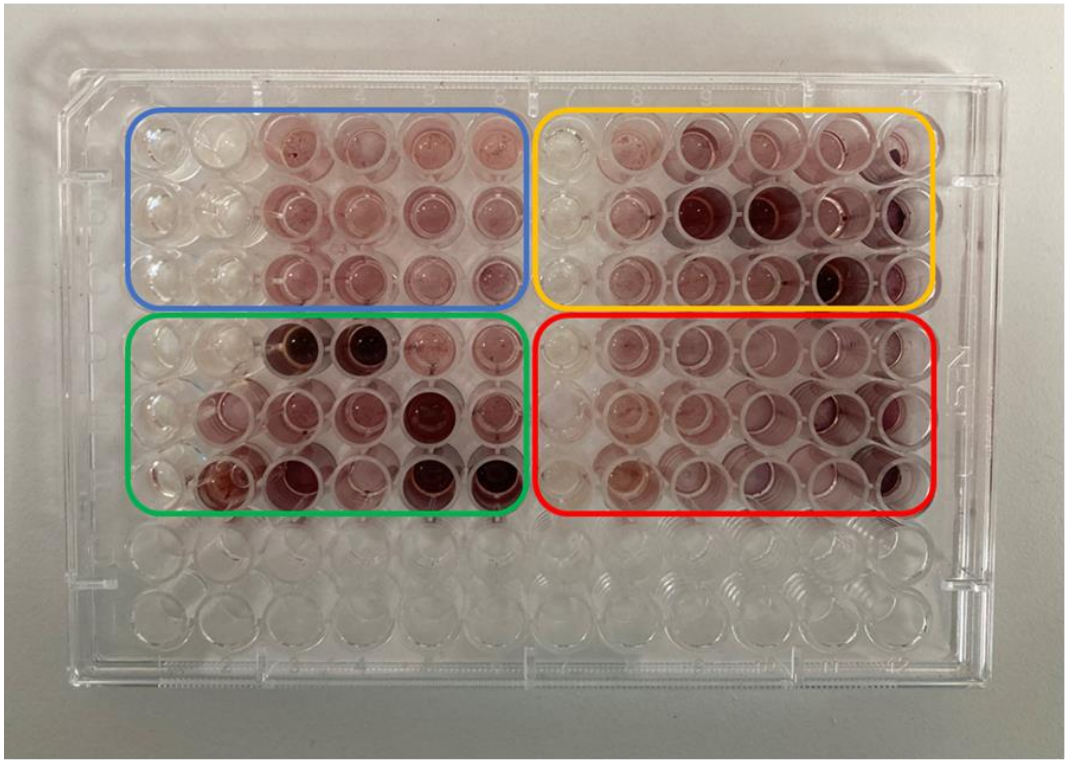
Table 6.2 Showing the names of *Pseudomonas sp.* PKS detected metabolites with their respective retention time

No. Peaks	Retention time (min)	Name
1	1.0033	Protorifamycin I
2	1.441	Saliniketal A
3	1.751	Rifamycin SV
4	2.325	Unknown
5	2.699	2-Pentanone, 4-hydroxy-4-methyl
6	2.962	Unknown
7	3.136	Hydroquinone
8	3.230	Staurosporine
9	4.937	Indole
10	5.416	Tridecane
11	5.830	4-Difluoropiperidin-1yl
12	6.223	2-Flourobenzyl
13	6.983	2-Trifluoromethylbenzyl
14	7.288	Unknown
15	7.723	Indole-3-aldehyde

16	7.983	Unknown
17	8.171	Unknown
18	9.038	Indole-3-acetic acid
19	13.225	Indole-3-glyoxylic acid
20	15.080	Tris(trifluoromethyl) bromomethane

The susceptibility test of either *Pseudomonas sp.* PKS or *Enterobacter sp.* PTB intracellular metabolites against antibiotic resistance bacteria (namely *E. coli, P. aeruginosa, E. Faecalis* and *S. aureus*) was conducted using a sensitive and Quick Microplate Method to assess the minimal inhibitory concentration (MIC). Furthermore (p-Iodonitrotetrazolium violet (INT) dye was used to check the bacteria cellular viability of antibiotic resistance bacteria after 24 hours exposure to intracellular metabolites of either *Pseudomonas sp.* PKS or *Enterobacter sp.* PTB.

A



B

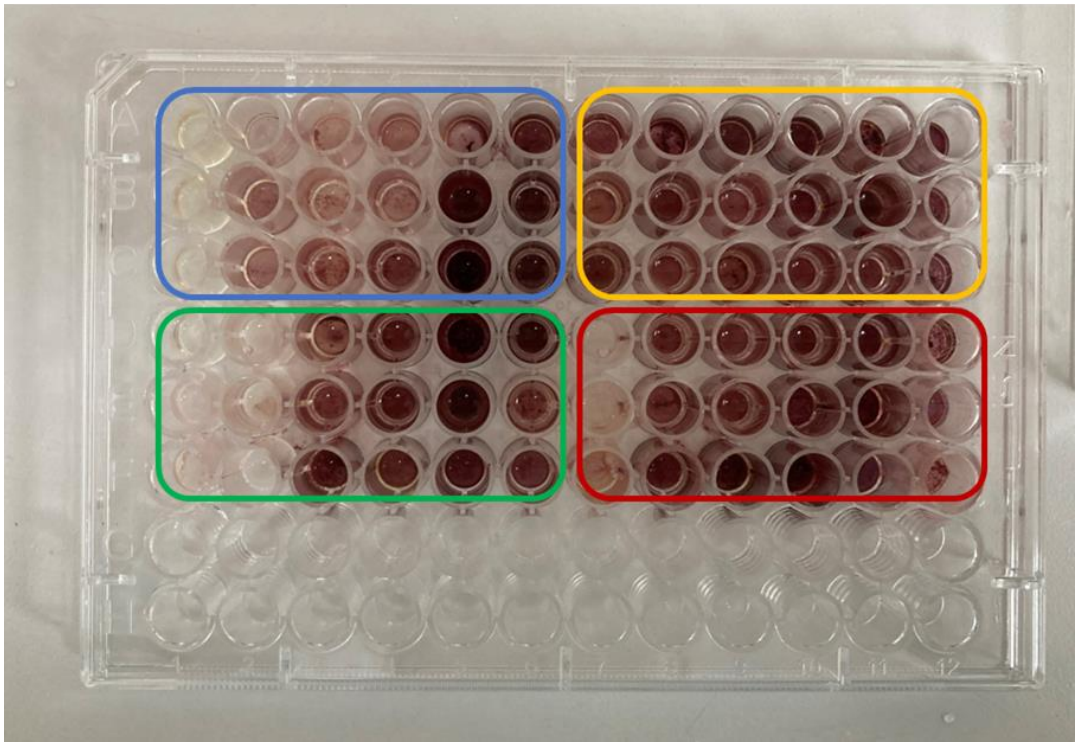


Figure 6.5 Elucidating the effects of either (A) *Pseudomonas sp.* PKS and (B) *Enterobacter sp.* PTB metabolites against common antibiotic resistant bacteria. Blue denotes *E. coli*, orange denotes *P. aeruginosa*, green denotes *E. Faecalis* and red denotes *S. aureus.*

Table 6.3 Examining the bacteria recovery status of common antibiotic resistant bacteria after being exposed to minimal inhibition concentration (MIC) of either *Enterobacter sp.* PTB, or *Pseudomonas sp.* PKS intracellular metabolites.

Source of metabolites	**Antibiotic resistant bacteria**	**Metabolites MIC (mg/ml)**	**Bacteria growth/ no growth**
Pseudomonas sp. PKS	*E. coli*	50	Growth
		25	Growth
	P. aeruginosa	50	Growth
	E. Faecalis	50	Growth
	S. aureus	50	Growth
Enterobacter sp. PTB	*E. coli*	50	Growth
	E. Faecalis	50	Growth
		25	Growth
	S. aureus	50	Growth

Note: The ATCC antibiotic resistant bacteria strains that were found with biological activity at specific concentration were excluded from this experiment.

6.4 Discussion

Entomopathogenic nematodes bacteria isolates plays a crucial role in pathogenicity of nematodes. These bacteria symbionts they are also involved in immune cyto-protection against possible pathogens. Furthermore, they are reported to have produce bio active compounds that have enzymatic activity that hydrolyse within the host cadaver to alter the physiological conditions to favour reproduction and growth of infective juveniles. Previous studies have elucidated that metabolites produces by EPNs bacteria symbionts has antibiotic activity over fungi, insects and other bacteria species which make them potential drug targets. *Cruznema* nematodes bacteria symbionts produce bioactive compounds that have anti-microbial activity (Chapter 5).

The metabolites produced by both *Enterobacter sp.* PTB and *Pseudomonas sp.* PKS were identified using high pressure liquid chromatograph (HPLC) under the conditions described at Section 6.2.2. There were only five metabolites detected (namely rifamycin Y, maleic acid (malonic acid), Indole, nonadecane, 1-chloro) Figure 6.3 and Table 6.1. There are few metabolites detected in compares to previous studies. However, the result aligns with a study conducted by Chen *et al.* (1996) showing that EPNs bacteria symbionts produces less

metabolites in culture medium compared when the bacteria are inside the host cadaver. Furthermore, this may be due to variation in physiological conditions that derives the production of metabolites.

The identified compound rifamycin it is reported to have antibiotic activity against leprosy, Mycobacterium complexes infection, tuberculosis, and mostly gram-positive bacteria (Chaisson, 2003; Das and Patra, 2017) (Table 6.1). Furthermore, compounds identified in peak number 3 and 4 was previously found to be produced both by *Xenorhabdus spp*. And *Photorhabdus spp*. EPNs bacteria symbionts, and it suggested to be involved into both antifungal and antimicrobial activity (Bode, 2009; Gualtieri *et al.,* 2009). Moreover, metabolites detected at peak number 2 and 5 are linked with having insecticidal activity (Bode, 2009; Bose *et al.,* 2015) (Figure 6.3 and Table 6.1).

There are 20 peaks of metabolites which were detected for *Pseudomonas sp.* PKS (Figure 6.4). The metabolites were identified by cross referencing the retention time with other database as previously described by Mullins *et al*. (2019). Peak number 11-13 metabolites identified are anti-inflammatory cytokine inhibitors (DeRosa, 2010). Furthermore, metabolites compound at peak number 5 (Table 6.2), was found to be link with antimicrobial activity. Metabolite compound at peak number 9 (namely indole) and its derivative peak number 15 and 18-19, are involve in biofilms formations and virulence of *Pseudomonas sp.* PKS (Table 6.2). Furthermore, *Pseudomonas sp.* PKS produces biofilms within the insect host cadaver to prevent pathogens invasion during reproduction and development of EPNs IJs.

Rifamycin SV peak number 3, according to Suresh *et al.* (2022) is it has inhibitory effects on bacteria DNA-dependent RNA by binding the polymerase subunit facilitating direct block of the elongating RNA. Staurosporine, peak number 8 it is recognised as antifungal compound (Xie *et al.,* 2017), considering that nematodes are natural soil inhabitant, it is common cause that they need to develop resistance against fungal infection since, fungi spices are more commonly found in soil (Table 6.2). Saliniketal metabolite compound in peak number 2 it is produced constantly in small quantity however, during the cellular stress level they are upregulate, their function is to repair damage cells and enhances fitness, crucial for survival (Barka *et al.,* 2016). Metabolite compound at peak number 4, 6, 14,16 and 17 were not identified due to the limitation factors of HPLC machine, since it does not able to determine compounds constituents unlike to its counterparts GC/MS (gas chromatography mass spectrophotometer), LCMS (liquid chromatography mass spectrophotometer) and HPLC-MS

(high pressure liquid mass spectrophotometer). HPLC separate the compounds based on their physico-chemical properties whereas HPLC-MS it uses both physico-chemical and mass to differentiate the compounds.

Tetrazolium salts is often used to investigate the survival (Biological activity/ Viability) within a sample. Therefore, this chemical reagent appears colourless and serves as an electron acceptor (Quist *et al.,* 2017). However, when there is any biological activity within the sample, this compound undergoes the reduction reaction to produce the colour. Furthermore, the tetrazolium compound mixed with either TTC (2,3,5-Triphenyl tetrazolium chloride), INT (p-Iodonitrotetrazolium violet) or (Nitro-blue tetrazolium) NBT, to produce a specific colour change once biological activity is detected within the sample. Moreover, when Tetrazolium reacts with TTC and INT produced red colour, and when fused with NBT forms blue colour.

The viability test of either *E. Faecalis, S. aureus, E. coli and P. aeruginosa* against either *Pseudomonas sp.* PKS or *Enterobacter sp.* PTB *Cruznema* bacteria metabolites was performed using INT dye. The antimicrobial activity of *Pseudomonas sp.* PKS bacteria metabolites against *E. coli* result, indicates that from concentration 12.5 mg/mL to 1.5625 mg/mL (Figure 6.2; Figure 6.5A blue) the formazan colour change of INT dye was stable red (*E. coli* bacteria cells where active). However, from concentration 50 mg/mL to 25 mg/mL, the INT dye was no colour change (No Biological active detected from *E. coli* cells) (Figure 6.5A blue). The 50 mg/mL *Pseudomonas sp.* PKS metabolites inhibits the growth of all three *P. aeruginosa, E. Faecalis* and *S. aureus* meanwhile, when the concentration is decreased from 25 mg/mL to 1.5625 mg/mL the bacteria showed viability represented by the colour change (Figure 6.5A orange, green and red). Further, the bacteria species were able to recover, making the *Pseudomonas sp.* PKS metabolites bacteriostatic.

The *Enterobacter sp.* PTB intracellular metabolites show no effects against *E. coli* and *S. aureus* at the concentration range from 25 mg/L to 1.5625 mg/mL, showed by the red colour caused by the redox reaction of INT dye by the bacteria (Figure 6.5B blue and red). However, when the concentration of the metabolites was increased to 50 mg/mL, there is no colour change, showing no cellular viability. Furthermore, when *E. Faecalis* is exposed to either 50 mg/mL or 25 mg/mL of *Enterobacter sp.* PTB metabolites showed no biological response (Figure 6.5B green). However, when the *Enterobacter sp.* PTB metabolites concentration was decrease from 12.5 mg/ml to 1.5625 mg/mL, biological activity of *E. Faecalis* was detected by INT dye. Moreover, *Enterobacter sp.* PTB metabolites showed no effects towards *P.*

aeruginosa despite the increase in metabolites concentration form 1.5625 mg/mL to 50 mg/mL (Figure 6.5B orange).

Further assessments were done for wells that had no biological activity, by spreading 100 µL of the mixture into Mueller-Hinton agar and incubated for three days at 37 ℃ and the results are represented in Table 6.3. All four antibiotic resistant bacteria (namely *E. coli, P. aeruginosa E. Faecalis* and *S. aureus*) exposed to either *Enterobacter sp.* PTB or *Pseudomonas sp.* PKS were able to recover (Table 6.3). Therefore, thus making both *Enterobacter sp.* PTB and *Pseudomonas sp.* PKS intracellular metabolites bacteriostatic.

Pseudomonas species have been reported to possess variety of metabolites (such as macrocyclic lactone, 2,4-diacetylphloroglucinol, pyrrolnitrin [3-chloro-4-(2′- nitro-3′-chlorophenyl)-pyrrole], 2,3-de-epoxy-2,3-didehydro-rhizoxin and phenazine-1-carboxylic acid) which are reported to have both antifungal and antimicrobial activity (Ligon *et al.,* 2000; Strano *et al.,* 2017; Shahid *et al.,* 2021). Previously studies have elucidated wide range applications of metabolites extracted from *Enterobacteriaceae* species in antimicrobial, anti-inflammatory, and antioxidant properties (Mahdiyah *et al.,* 2020; Sebola *et al.,* 2020; Kumari *et al.,* 2023). Therefore, it is important to identify the metabolites produced by both *Enterobacter sp.* PTB and *Pseudomonas sp.* PKS despite being not the scope of this study.

6.5 Conclusion

The intracellular metabolites of both *Enterobacter sp.* PTB and *Pseudomonas sp.* PKS possess antimicrobial compounds, making them a potential drug candidate for clinical use. The metabolites of *Enterobacter sp.* PTB and *Pseudomonas sp.* PKS are used in cyto-protection of *Cruznema sp.* NTM 2021. These metabolites have wide range of application in both agroforestry and pharmaceutical industries, in fighting pest infestation and diseases. Furthermore, it is important to identify genes which are responsible that produces these metabolites, even though it is not part of the scope of this study. Moreover, since the bacteria symbionts are not able to produce all the metabolites efficient in culture media, a whole genome sequencing and annotation of these bacteria will be able to insights about genes and pathways involves in production of these metabolites. The GC/MS will also help in detecting the metabolites that are violates and their structural conformation, which are likely missed by HPLC, due to its limited capacities. Furthermore, the identified compounds can be used as template for drug design to combat various diseases.

6.6 References

Amuri, B., Devi, G., Choudhury, B.N., Debnath, P. and Bora, D., 2020. Isolation and Identification of Heterorhabditis bacteriophora from Tea Plantation of Assam. *Int. J. Curr. Microbiol. App. Sci*, *9*(7), pp.3866-3879.

Awori, R.M., 2022. Nematophilic bacteria associated with entomopathogenic nematodes and drug development of their biomolecules. *Frontiers in Microbiology*, *13*, p.993688.

Bulgheresi, S., 2016. All the microbiology nematodes can teach us. *FEMS microbiology ecology*, *92*(2), pp.007.

Chianu, J.N., Chianu, J.N. and Mairura, F., 2012. Mineral fertilizers in the farming systems of sub-Saharan Africa. A review. *Agronomy for sustainable development*, *32*, pp.545-566.

Cimen, H., Touray, M., Gulsen, S.H. and Hazir, S., 2022. Natural products from Photorhabdus and Xenorhabdus: mechanisms and impacts. *Applied Microbiology and Biotechnology*, *106*(12), pp.4387-4399.

Elbrense, H., Elmasry, A.M., Seleiman, M.F., Al-Harbi, M.S. and Abd El-Raheem, A.M., 2021. Can symbiotic bacteria (Xenorhabdus and Photorhabdus) be more efficient than their entomopathogenic nematodes against Pieris rapae and Pentodon algerinus Larvae?. *Biology*, *10*(10), p.999.

Fallah, N., Pang, Z., Lin, Z., Nyimbo, W.J., Lin, W., Mbuya, S.N., Ishimwe, C. and Zhang, H., 2023. Sustained organic amendments utilization enhances ratoon crop growth and soil quality by enriching beneficial metabolites and suppressing pathogenic bacteria. *Frontiers in Plant Science*, *14*.

Ferreira, T., and Malan, A.P., 2014. *Xenorhabdus* and *Photorhabdus*, bacterial symbionts of the entomopathogenic nematodes *Steinernema* and *Heterorhabditis* and their in vitro liquid mass culture: a review. *African Entomology*, *22*(1), pp.1-14.

Grewal, P.S., Abrol, D.P. and Shankar, U., 2012. Entomopathogenic nematodes as tools in integrated pest management. *Integrated pest management: Principles and practice*, pp.162-236.

Hakim, A. and Kadarohman, A., 2020. Student Concept Understanding Of Natural Products Chemistry İn Primary And Secondary Metabolites Using The Data Collecting Technique Of Modified Cri. *International Online Journal of Educational Sciences*, *4*(3).

Hendrikx, T. and Schnabl, B., 2019. Indoles: metabolites produced by intestinal bacteria capable of controlling liver disease manifestation. *Journal of internal medicine*, *286*(1), pp.32-40.

Kumari, K., Sharma, P.K., Shikha, S. and Singh, R.P., 2023. Molecular characterization and in-depth genome analysis of Enterobacter sp. S-16. *Functional & Integrative Genomics*, *23*(3), p.245.

Kusakabe, A., Wang, C., Xu, Y.M., Molnár, I. and Stock, S.P., 2022. Selective toxicity of secondary metabolites from the entomopathogenic bacterium *Photorhabdus luminescens sonorensis* against selected plant parasitic nematodes of the Tylenchina suborder. *Microbiology spectrum*, *10*(1), pp.02577-21.

Ligon, J.M., Hill, D.S., Hammer, P.E., Torkewitz, N.R., Hofmann, D., Kempf, H.J. and Pée, K.H.V., 2000. Natural products with antifungal activity from Pseudomonas biocontrol bacteria. *Pest Management Science: formerly Pesticide Science*, *56*(8), pp.688-695.

Liu, W.T., Chen, T.L., Hou, R.F., Chen, C.C. and Tu, W.C., 2020. The invasion and encapsulation of the entomopathogenic nematode, Steinernema abbasi, in Aedes albopictus (Diptera: Culicidae) larvae. *Insects*, *11*(12), p.832.

Mahdiyah, D., Farida, H., Riwanto, I., Mustofa, M., Wahjono, H., Nugroho, T.L. and Reki, W., 2020. Screening of Indonesian peat soil bacteria producing antimicrobial compounds. *Saudi Journal of Biological Sciences*, *27*(10), pp.2604-2611.

Nabariya, D.K., Heinz, A., Derksen, S. and Krauß, S., 2022. Intracellular and intercellular transport of RNA organelles in CXG repeat disorders: The strength of weak ties. *Frontiers in Molecular Biosciences*, *9*, p.1000932.

Ogier, J.C., Akhurst, R., Boemare, N. and Gaudriault, S., 2023. The endosymbiont and the second bacterial circle of entomopathogenic nematodes. *Trends in Microbiology*.

Owuama, C.I., 2001. Entomopathogenic symbiotic bacteria, *Xenorhabdus* and *Photorhabdus* of nematodes.

Prashar, P. and Shah, S., 2016. Impact of fertilizers and pesticides on soil microflora in agriculture. *Sustainable Agriculture Reviews: Volume 19*, pp.331-361.

Quist, C.W., Gort, G., Mulder, C., Wilbers, R.H., Termorshuizen, A.J., Bakker, J. and Helder, J., 2017. Feeding preference as a main determinant of microscale patchiness among terrestrial nematodes. *Molecular Ecology Resources*, *17*(6), pp.1257-1270.

Ribeiro, C. and Vaz, A., 2019. Effects of cytotoxic factors produced by entomopathogenic bacteria on insect haemocytes. *Microbes for Sustainable Insect Pest Management: An Eco-friendly Approach-Volume 1*, pp.207-245.

Sebola, T.E., Uche-Okereafor, N.C., Mekuto, L., Makatini, M.M., Green, E. and Mavumengwana, V., 2020. Antibacterial and anticancer activity and untargeted secondary

metabolite profiling of crude bacterial endophyte extracts from Crinum macowanii Baker leaves. *International Journal of Microbiology*, *2020*, pp.1-15.

Shahid, I., Han, J., Hardie, D., Baig, D.N., Malik, K.A., Borchers, C.H. and Mehnaz, S., 2021. Profiling of antimicrobial metabolites of plant growth promoting Pseudomonas spp. isolated from different plant hosts. *3 Biotech*, *11*, pp.1-14.

Shakeel, Q., Shakeel, M., Raheel, M., Ali, S., Ashraf, W., Iftikhar, Y. and Bajwa, R.T., 2022. Entomopathogenic Nematodes (EPNs): A Green Strategy for Management of Insect-Pests of Crops. In *New and Future Development in Biopesticide Research: Biotechnological Exploration* (pp. 115-135). Singapore: Springer Nature Singapore.

Shan, S., Wang, W., Song, C., Wang, M., Sun, B., Li, Y., Fu, Y., Gu, X., Ruan, W. and Rasmann, S., 2019. The symbiotic bacteria Alcaligenes faecalis of the entomopathogenic nematodes Oscheius spp. exhibit potential biocontrol of plant-and entomopathogenic fungi. *Microbial Biotechnology*, *12*(3), pp.459-471.

Sharma, N. and Singhvi, R., 2017. Effects of chemical fertilizers and pesticides on human health and environment: a review. *International journal of agriculture, environment and biotechnology*, *10*(6), pp.675-680.

Strano, C.P., Bella, P., Licciardello, G., Caruso, A. and Catara, V., 2017. Role of secondary metabolites in the biocontrol activity of Pseudomonas corrugata and Pseudomonas mediterranea. *European Journal of Plant Pathology*, *149*, pp.103-115.

Thakur, N., Kaur, S., Tomar, P., Thakur, S., and Yadav, A.N., 2020. Microbial biopesticides: current status and advancement for sustainable agriculture and environment. In *New and future developments in microbial biotechnology and bioengineering* (pp. 243-282). Elsevier.

Vashisth, S., Chandel, Y.S. and Sharma, P.K., 2013. Entomopathogenic nematodes-A review. *Agricultural Reviews*, *34*(3), pp.163-175.

Walter, D.E. and Ikonen, E.K., 1989. Species, guilds, and functional groups: taxonomy and behavior in nematophagous arthropods. *Journal of Nematology*, *21*(3), p.315.

Waterfield, N.R., Ciche, T. and Clarke, D., 2009. *Photorhabdus* and a host of hosts. *Annual review of microbiology*, *63*, pp.557-574.

Wu, S., Kaplan, F., Lewis, E., Alborn, H.T. and Shapiro-Ilan, D.I., 2018. Infected host macerate enhances entomopathogenic nematode movement towards hosts and infectivity in a soil profile. *Journal of invertebrate pathology*, *159*, pp.141-144.

Zhou, X., Liu, Z., Xie, L., Li, L., Zhou, W. and Zhao, L., 2022. The correlation mechanism between dominant bacteria and primary metabolites during fermentation of red sour soup. *Foods*, *11*(3), p.341.

Barka, E.A., Vatsa, P., Sanchez, L., Gaveau-Vaillant, N., Jacquard, C., Klenk, H.P., Clément, C., Ouhdouch, Y. and van Wezel, G.P., 2016. Taxonomy, physiology, and natural products of *Actinobacteria*. *Microbiology And Molecular Biology Reviews*, *80*(1), pp.1-43.

Bode, H.B., 2009. Entomopathogenic bacteria as a source of secondary metabolites. *Current Opinion In Chemical Biology*, *13*(2), pp.224-230.

Bose, U., Hewavitharana, A.K., Ng, Y.K., Shaw, P.N., Fuerst, J.A. and Hodson, M.P., 2015. LC-MS-Based metabolomics study of marine bacterial secondary metabolite and antibiotic production in *Salinispora arenicola*. *Marine Drugs*, *13*(1), pp.249-266.

Chaisson, R.E., 2003. Treatment of chronic infections with rifamycins: is resistance likely to follow?. *Antimicrobial Agents And Chemotherapy*, *47*(10), pp.3037-3039.

Chen, G., Maxwell, P., Dunyhy, G.B. and Webster, J.M., 1996. Culture conditions for *Xenorhabdus* and *Photorhabdus* symbionts of entomopathogenic nematodes. *Nematologica*, *42*(1), pp.124-130.

Das, B. and Patra, S., 2017. Antimicrobials: Meeting the challenges of antibiotic resistance through nanotechnology. In *Nanostructures For Antimicrobial Therapy*, pp.1-22. Elsevier.

DeRosa, T.F., 2010. Significant Pharmaceuticals Reported in US Patents.

Fang, C., Wu, L., Zhu, C., Xie, W.Z., Hu, H. and Zeng, X.T., 2021. A potential therapeutic strategy for prostatic disease by targeting the oral microbiome. *Medicinal Research Reviews*, *41*(3), pp.1812-1834.

Grabowski, Ł., Łepek, K., Stasiłojć, M., Kosznik-Kwaśnicka, K., Zdrojewska, K., Maciąg-Dorszyńska, M., Węgrzyn, G. and Węgrzyn, A., 2021. Bacteriophage-encoded enzymes destroying bcterial cell membranes and walls, and their potential use as antimicrobial agents. *Microbiological Research*, *248*, pp.126746.

Maithani, D., Sharma, A., Gangola, S., Choudhary, P. and Bhatt, P., 2022. Insights into applications and strategies for discovery of microbial bioactive metabolites. *Microbiological Research*, pp.127053.

Mullins, A.J., Murray, J.A., Bull, M.J., Jenner, M., Jones, C., Webster, G., Green, A.E., Neill, D.R., Connor, T.R., Parkhill, J. and Challis, G.L., 2019. Genome mining identifies cepacin as a plant-protective metabolite of the biopesticidal bacterium *Burkholderia ambifaria*. *Nature Microbiology*, *4*(6), pp.996-1005.

Pandey, A., Srivastava, A., Chandra, N., Kumar, D. and Kumar, S., 2018. Purification and characterization of bioactive secondary metabolites isolated from soil bacteria. *Indian Journal Of Microbiology Research*, *5*(2) pp.188-193.

Pathma, J., Rahul, G.R., Kamaraj, K.R., Subashri, R. and Sakthivel, N., 2011. Secondary metabolite production by bacterial antagonists. *Journal Of Biological Control*, pp.165-181.

Raja, R.K., Arun, A., Touray, M., Gulsen, S.H., Cimen, H., Gulcu, B., Hazir, C., Aiswarya, D., Ulug, D., Cakmak, I. and Kaya, H.K., 2021. Antagonists and defense mechanisms of entomopathogenic nematodes and their mutualistic bacteria. *Biological Control*, *152*, pp.104452.

Ramírez-Rendon, D., Passari, A.K., Ruiz-Villafán, B., Rodríguez-Sanoja, R., Sánchez, S. and Demain, A.L., 2022. Impact of novel microbial secondary metabolites on the pharma industry. *Applied Microbiology And Biotechnology*, pp.1-24.

Suresh, A.B., Rosani, A. and Wadhwa, R., 2022. Rifampin. In *StatPearls [Internet]*. StatPearls Publishing.

Valerio, F., Mezzapesa, G.N., Ghannouchi, A., Mondelli, D., Logrieco, A.F. and Perrino, E.V., 2021. Characterization and antimicrobial properties of essential oils from four wild taxa of *Lamiaceae* family growing in Apulia. *Agronomy*, *11*(7), pp.1431.

Xie, J.L., O'Meara, T.R., Polvi, E.J., Robbins, N. and Cowen, L.E., 2017. Staurosporine induces filamentation in the human fungal pathogen *Candida albicans* via signaling through Cyr1 and protein kinase A. *Msphere*, *2*(2), pp.10-1128.

Yao, H., Liu, J., Xu, S., Zhu, Z. and Xu, J., 2017. The structural modification of natural products for novel drug discovery. *Expert Opinion On Drug Discovery*, *12*(2), pp.121-140.

Chapter 7

Whole Genome sequencing and annotation of *Pseudomonas* sp. PKS isolated from *Rhabditida: Cruznema* entomopathogenic nematodes

Abstract

Entomopathogenic nematodes (EPNs) are clustered under parasitic group. However, they do not pose any health threats. EPNs has symbiosis with pathogenic bacteria belonging to *Xenorhabdes*, *Photorhabdus* and *Seratia* genus. These pathogenic bacteria are harboured in the nematodes gut and only released upon invading the insect host body. The bacteria colonies the host body and release bioactive toxic compounds that kills the host. These bacteria are crucial for EPNs survival. *Cruznema* nematodes belong to the same phylum order *Rhabditida* with EPNs. They are implemented in ecological functioning. Furthermore, *Cruznema* nematodes have evolve a pathogenic relationship with pathogenic bacteria *Pseudomonas sp.* PKS. The aim of this study is to explore the whole genome make up of *Pseudomonas sp.* PKS and their functions. The whole genome sequencing was acquired by utilizing Illumina sequencer and quality was assessed by FastQC and Trimmomatic tool was used remove low quality bases. Furthermore, the assembly was carried by SPADES using *de novo* method and the quality of the assembly was evaluated by Quast tool. Moreover, the functional annotation was carried out by RAST tool. *Pseudomonas sp*. PKS has 5 660 896 bp genomic, 569 subsystem, 75 RNAs and a GC content of 54.2 %. Furthermore, gnome consist of 5 metabolite gene cluster NI-siderophore, Thiopeptide, Arylpolyne, NRP-metallophore, Hserlactone. Virulence, disease, and defence subsystem it is important to towards understanding the pathogenicity of *Pseudomonas sp*. PKS against insect host. Furthermore, metabolites which are produced by *Pseudomonas sp*. PKS are implicated in antimicrobial and insecticidal activity. Moreover, there is great degree of genome difference between *Pseudomonas sp*. PKS and *Pseudomonas aeruginosa* PAO1. This study provides the genomic insights of *Pseudomonas sp*. PKS, which are crucial towards understanding of the pathogenicity role that is played by the bacteria in *Cruznema* nematodes.

Keywords: Annotation, *Cruznema* nematodes, Thiopeptide, *Pseudomonas sp*. PKS, *Pseudomonas aeruginosa* PAO1

7.1 Introduction

7.1.1 Entomopathogenic nematode overview

Entomopathogenic (EPNs) are microscopic organisms which are found from the soil. Further they are well known for their agriculture application as the biocontrol agent (Usman *et al.*, 2021). There are only 3 EPNs genera reported (*Steinernema*, *Heterorhabdus* and *Oscheius*) which have bacteria symbiont relationship with *Xenorhabuds*, *Photorhabuds* and *Serratia* pathogenic bacteria respectively (Lephoto and Gray, 2019; Didiza *et al.*, 2021). The EPNs intrudes the host system by using mechanical wounds and biological openings (such as anus, eyes, and mouth) (Gulcu *et al.*, 2017). Further upon host intrusion they release the bacteria symbiont into host haemolymph which cause fatal infection to the host (Lephoto and Gray, 2022). The bacteria also provide protection against possible opportunistic infections during the feeding stage (Salvadori *et al.*, 2012). The EPNs reproduce within the host and the decline in food source, triggers the EPNs emergence of Infective juveniles (IJs) out from the host cadaver into soil to seek new host Figure 7.1.

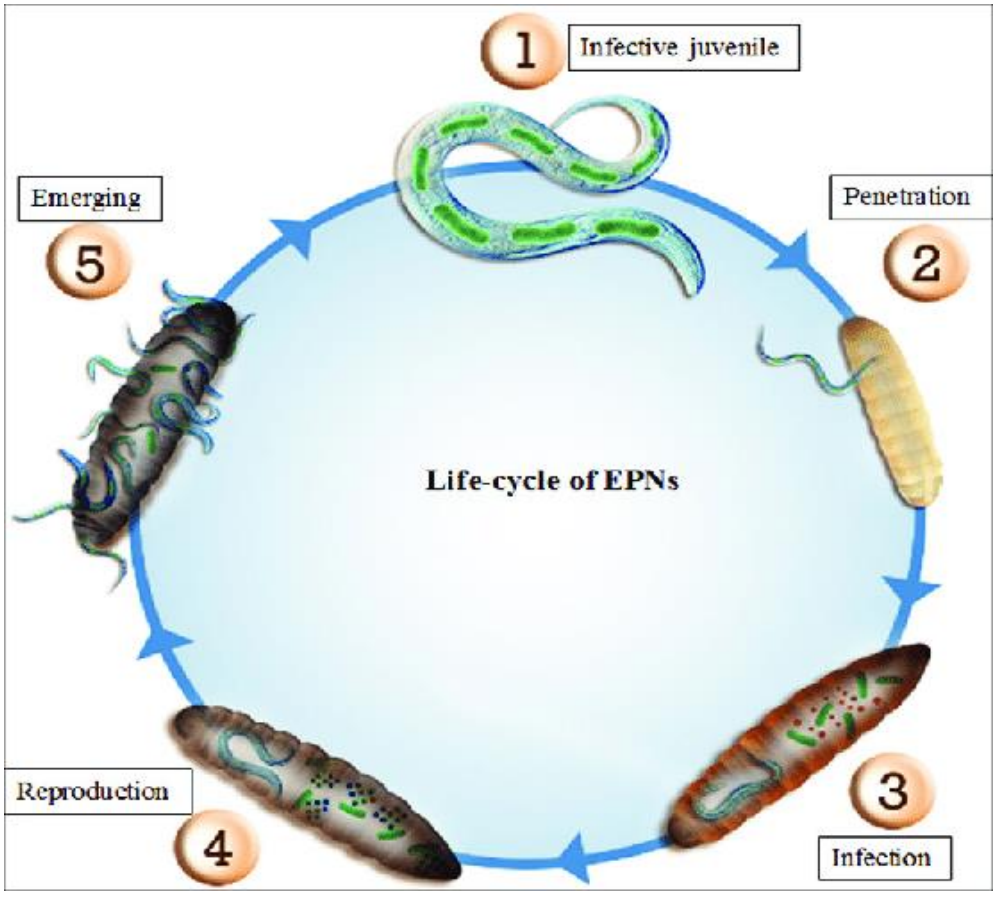


Figure 7.1 Schematic diagram of entomopathogenic nematodes life cycle. IJs search and infects and kill the host. The IJs undergoes reproduction with the cadaver and when food rations depreciate, then IJs emerge out into the soil, where the seek new host (image adapted from Gulcu *et al.*, 2017).

7.1.2 Entomopathogenic nematodes bacteria associates

EPNs bacteria uses nematodes as vector that protects the bacteria against environmental factors (temperature, UV, desiccation, etc) and transport the bacteria to insect host. EPNs bacteria are responsible for insect host death and maintenance of EPNs survival. Once the bacteria become released from the EPNs gut into insect haemolymph and produce bioactive toxic compounds which derivate the host immune defence. Furthermore, some of these bioactive compounds have enzymatic activity that perform hydrolysis making food available for EPNs. Moreover, these bacteria bioactive compounds are reported to have antimicrobial, insecticidal and antifungal activity. Further EPNs bacteria symbionts serves as EPNs immune against various pathogen infections.

7.1.3 *Cruznema* nematodes

Cruznema nematodes share the same phylum (namely *Rhabditida*) as entomopathogenic nematodes. *Cruznema* nematodes are natural found in soil and are reported to feed on bacteria (Du *et al.,* 2022). However, according to the study conducted by d'Ovido *et al.* (2019), *Cruznema* nematodes have ability to infect and kill insects (cricket, snails, etc). further there is no knowledge about their infective mechanism or about association with pathogenic bacteria as their clade sisters *Steinernema*, *Heterorhabdus* and *Oscheius.*

7.1.4 *Pseudomonas* bacteria

Pseudomonas bacteria species belongs to family *Pseudomonacaes*. *Pseudomonas* species are gram-negative bacteria with rod shape. Previously studies showed that both plants and plants parasitic nematodes have commensal relationship with *Pseudomonas* species (Afordoanyi *et al*., 2022; Punja *et al*., 2003). According to Punja *et al*. (2003) *Pseudomonas putida* isolated from the soil is plant beneficial bacteria (Afordoanyi *et al.,* 2022). Furthermore, these bacteria produce bioactive toxic compounds that protects the plant roots from wide range of bacteria pathogens (Punja *et al*., 2003). *Pseudomonas putida* does not only provide plant protection, but also provides nitrogen fixation, indoleacetic acid, phosphate solubilization and phytohormone. These bacteria species are reported to be able to produces biofilm as part of its defence mechanism (Punja *et al*., 2003). Moreover, some *Pseudomonas* species are pathogenic against nematodes whereas others are symbiotic (Afordoanyi *et al*., 2022). The aim of this study to perform whole genome sequencing and annotation of *Pseudomonas sp*. PKS.

7.2 Methods and materials

7.2.1 DNA extraction and purification of *Pseudomonas sp.* PKS

A single colony of *Pseudomonas sp.* PKS was picked from a NBTA plate and inoculated into freshly prepared broth Luria-Bertain (NaCL 10 g/L, peptone 10 g/L and yeast extract5 g/L) broth. Followed by overnight incubation at 30℃ with shaker adjusted to 140 rpm. The DNA extractrion was performed using bacterial DNA MiniPrep kit (Zymo Research, catalog # D3050) in accordance with manufacturer instructions. The DNA quality was assessed by NanoDrop ND-1000® spectrophotometer (Bio-Rad). The DNA impurities were removed using bacterial DNA purification kit and quantified using concentrator™-5 (catalog #D4003S) kit.

7.2.2 Whole genome sequencing of *Pseudomonas sp.* PKS

The NextEra DNA sample kit (Illumina) was used to generate a genomic DNA paired-end libraries. Subsequently followed by generation of indexed with the aid of NextEra DNA index kit (Illumina) while adhering to the instructions provided by the manufacture. Furthermore, the primary libraries (paired-end) had a range of 200-3000 bp. Moreover, the paird-end (2x 300 bp) sequencing was achieved by subjecting MiSeq reagent kit v3 into MiSeq (Illumina).

7.2.3 Quality assessment and control

The sequenced that was subjected to FastQC (version 0.12.1) program to assess the quality (Brown *et al.,* 2017). Further Trimmomatic (version 0.39) program was used to low quality reads and nextra adapter sequences (Sewe *et al.,* 2022). Data validation was performed by subjecting the clean reads to FastQC program.

7.2.4 Genome assembly

The genomic reads were assembled using *de novo* method by SPADES (version 3.15.5) program (Utturkar *et al.,* 2014). The command "--careful" was used to avoid mismatches (Figure 7.2). After, the assembly the contig file were run into Quast (version 5.2.0) to assess the quality of assembly (Table 8.3.1 and Figure A21-26).

```
(spade_env) sekgobela@Sekgobela-PK:~/data_wgs/Sample_J$ spades.py --careful \
-o SPADES_Results -1 /home/sekgobela/data_wgs/Sample_J/Trimmed_reads/Trimmed_reads22_1P.fastq.gz \
-2 /home/sekgobela/data_wgs/Sample_J/Trimmed_reads/Trimmed_reads22_2P.fastq.gz
```

Figure 7.2 showing the command used to assembly the whole genome sequences

7.2.5 Whole genome annotation

The annotation of the whole genome sequenced data was carried through rapid annotation subsystem technology (RAST).

7.2.6 Identification of genes producing secondary metabolites

The Whole genome sequenced data was subjected to Antismash (Version 7.0.1) site, for identification of genes producing secondary metabolites (Blin *et al.,* 2023).

7.3 Results

The genome sequencing was carried by Illumina Miseq and the reads were subjected to quality control using both FastQC and Trimmomatic (Figure A1-10). The genomic reads where assembly using de novo method in SPADES program and the quality assessed in Quast tool table (Table 7.1 and Figure A21-26). Genomic annotation was carried using RAST tool (Table 7.2) and prediction of metabolite coding genes was caried using Antismash (Table 7.4).

Table 7.1: Quality assessment of the assembly of *Pseudomona sp.* PKS. (Please note the supplementary data is available in Figure A21-26)

Species	*Pseudomona sp.* PKS
# contigs (>= 0 bp)	391
# contigs (>= 1000 bp)	111
# contigs (>= 5000 bp)	62
# contigs (>= 10000 bp)	49
# contigs (>= 25000 bp)	36
# contigs (>= 50000 bp)	27
Total length (>= 0 bp)	5 609 562
Total length (>= 1000 bp)	5 553 556
Total length (>= 5000 bp)	5 430 678
length (>= 10000 bp)	5347715
Total length (>= 25000 bp)	5 126 461
Total length (>= 50000 bp)	4 803 319
# contigs	135
Largest contig	537 830
Total length	5 570 345
GC (%)	54.18
N50	245 788
N90	32 759
auN	236 534.0
L50	8

L90	33
# N's per 100 kbp	0.00

All statistics are based on contigs of size >= 500 bp, unless otherwise noted (e.g., "# contigs (>= 0 bp)" and "Total length (>= 0 bp)" include all contigs).

Table 7.2 Overview of *Pseudomonas sp.* PKS whole genome annotation by RAST

Feature	**RAST annotation**
Genome size (bp)	5,660,896
GC content (%)	54.2
Number of Subsystems	569
Number of Protein coding genes	5410
Number of contigs (with PEGs)	541
Number of RNAs	75

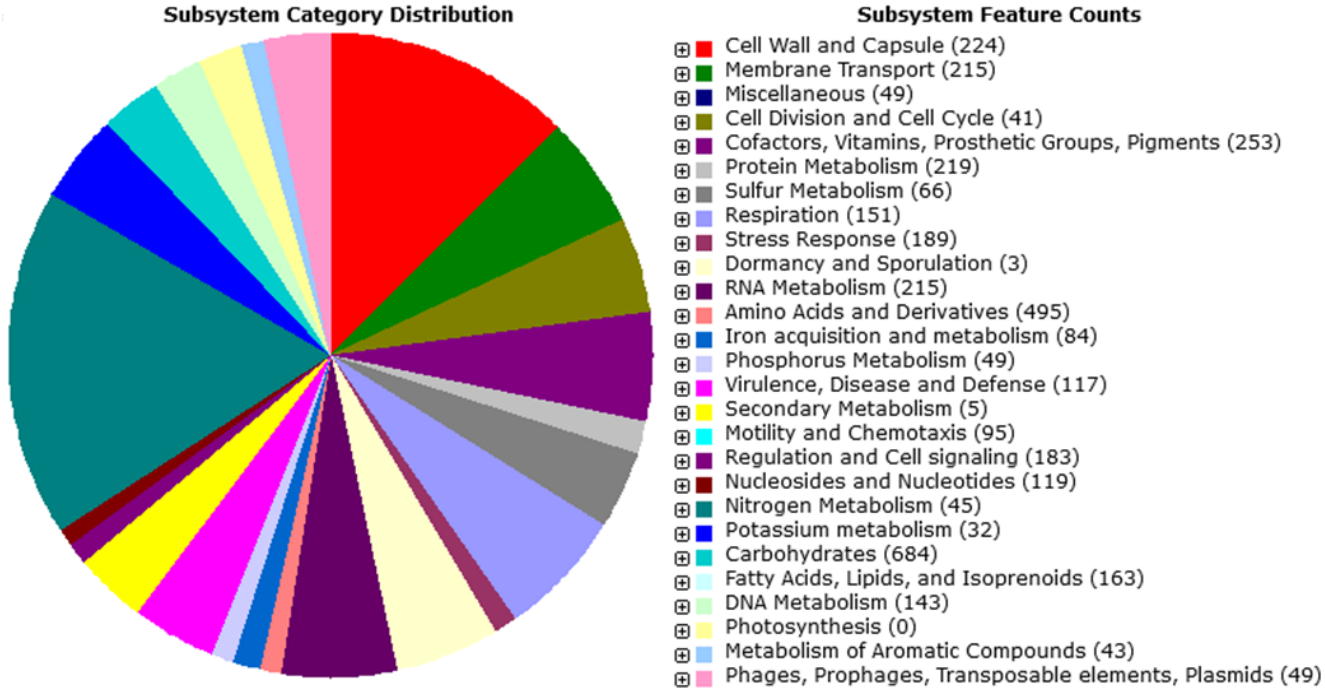


Figure 7.3 An overview diagram of *Pseudomonas sp.* PKS subsystem categories into their correspondent genes annotated by RAST server

Table 7.3 Elucidating genes found in the subsystem (Virulence, disease and defence) *Pseudomonas sp.* PKS. This subsystem consists of important genes that facilitate development, and defence and mechanism process used by the bacteria.

Subcategory	Subsystem	Role
Adhesion	Mediator of hyperadherence YidE in Enterobacteria and its conserved region	16 kDa heat shock protein A
Adhesion	Mediator of hyperadherence YidE in Enterobacteria and its conserved region	Mediator of hyperadherence YidE
Adhesion	Mediator of hyperadherence YidE in Enterobacteria and its conserved region	16 kDa heat shock protein B
Adhesion	Mediator of hyperadherence YidE in Enterobacteria and its conserved region	Uncharacterized protein YidR
Adhesion	Mediator of hyperadherence YidE in Enterobacteria and its conserved region	HTH-type transcriptional regulator YidP
Adhesion	Mediator of hyperadherence YidE in Enterobacteria and its conserved region	Outer membrane lipoprotein YidQ
Resistance to antibiotics and toxic compounds	Multiple Antibiotic Resistance MAR locus	Multiple antibiotic resistance protein MarC
Resistance to antibiotics and toxic compounds	Multiple Antibiotic Resistance MAR locus	Multiple antibiotic resistance protein MarA
Resistance to antibiotics and toxic compounds	Multiple Antibiotic Resistance MAR locus	Multiple antibiotic resistance protein MarR
Resistance to antibiotics and toxic compounds	Multiple Antibiotic Resistance MAR locus	Multiple antibiotic resistance protein MarB
Resistance to antibiotics and toxic compounds	Multidrug Resistance, Tripartite Systems Found in Gram Negative Bacteria	Outer membrane component of tripartite multidrug resistance system

Resistance to antibiotics and toxic compounds	Multidrug Resistance, Tripartite Systems Found in Gram Negative Bacteria	Membrane fusion component of tripartite multidrug resistance system
Resistance to antibiotics and toxic compounds	Multidrug Resistance, Tripartite Systems Found in Gram Negative Bacteria	Inner membrane component of tripartite multidrug resistance system
Resistance to antibiotics and toxic compounds	Copper homeostasis: copper tolerance	Membrane protein, suppressor for copper-sensitivity ScsB
Resistance to antibiotics and toxic compounds	Copper homeostasis: copper tolerance	Membrane protein, suppressor for copper-sensitivity ScsD
Resistance to antibiotics and toxic compounds	Copper homeostasis: copper tolerance	Secreted protein, suppressor for copper-sensitivity ScsC
Resistance to antibiotics and toxic compounds	Copper homeostasis: copper tolerance	Copper homeostasis protein CutF precursor
Resistance to antibiotics and toxic compounds	Copper homeostasis: copper tolerance	Copper homeostasis protein CutE
Resistance to antibiotics and toxic compounds	Copper homeostasis: copper tolerance	Magnesium and cobalt efflux protein CorC
Resistance to antibiotics and toxic compounds	Copper homeostasis: copper tolerance	Suppression of copper sensitivity: putative copper binding protein ScsA
Resistance to antibiotics and toxic compounds	Beta-lactamase	Metal-dependent hydrolases of the beta-lactamase superfamily I
Resistance to antibiotics and toxic compounds	Beta-lactamase	Beta-lactamase (EC 3.5.2.6)

Resistance to antibiotics and toxic compounds	Beta-lactamase	Beta-lactamase class C and other penicillin binding proteins
Resistance to antibiotics and toxic compounds	Mercuric reductase	PF00070 family, FAD-dependent NAD(P)-disulphide oxidoreductase
Resistance to antibiotics and toxic compounds	Copper homeostasis	Copper resistance protein C precursor
Resistance to antibiotics and toxic compounds	Copper homeostasis	Copper-translocating P-type ATPase (EC 3.6.3.4)
Resistance to antibiotics and toxic compounds	Copper homeostasis	Blue copper oxidase CueO precursor
Resistance to antibiotics and toxic compounds	Copper homeostasis	Cu(I)-responsive transcriptional regulator
Resistance to antibiotics and toxic compounds	Copper homeostasis	Copper resistance protein D
Resistance to antibiotics and toxic compounds	Copper homeostasis	Copper-sensing two-component system response regulator CusR
Resistance to antibiotics and toxic compounds	Arsenic resistance	Arsenical resistance operon repressor
Resistance to antibiotics and toxic compounds	Arsenic resistance	Arsenate reductase (EC 1.20.4.1)
Resistance to antibiotics and toxic compounds	Arsenic resistance	Arsenic efflux pump protein

Resistance to antibiotics and toxic compounds	Adaptation to d-cysteine	D-cysteine desulfhydrase (EC 4.4.1.15)
Resistance to antibiotics and toxic compounds	Adaptation to d-cysteine	Cystine ABC transporter, ATP-binding protein
Resistance to antibiotics and toxic compounds	Adaptation to d-cysteine	Cystine ABC transporter, permease protein
Resistance to antibiotics and toxic compounds	Cobalt-zinc-cadmium resistance	Cation efflux system protein CusC precursor
Resistance to antibiotics and toxic compounds	Cobalt-zinc-cadmium resistance	Zinc transporter ZitB
Resistance to antibiotics and toxic compounds	Cobalt-zinc-cadmium resistance	Cobalt-zinc-cadmium resistance protein
Resistance to antibiotics and toxic compounds	Cobalt-zinc-cadmium resistance	Probable Co/Zn/Cd efflux system membrane fusion protein
Resistance to antibiotics and toxic compounds	Cobalt-zinc-cadmium resistance	Transcriptional regulator, MerR family
Resistance to antibiotics and toxic compounds	Cobalt-zinc-cadmium resistance	Copper-sensing two-component system response regulator CusR
Resistance to antibiotics and toxic compounds	Cobalt-zinc-cadmium resistance	Cation efflux system protein CusF precursor
Resistance to antibiotics and toxic compounds	Cobalt-zinc-cadmium resistance	Cobalt/zinc/cadmium efflux RND transporter, membrane fusion protein, CzcB family

Resistance to antibiotics and toxic compounds	Cobalt-zinc-cadmium resistance	Cobalt-zinc-cadmium resistance protein CzcA
Resistance to antibiotics and toxic compounds	Cobalt-zinc-cadmium resistance	Cation efflux system protein CusA
Resistance to antibiotics and toxic compounds	Cobalt-zinc-cadmium resistance	DNA-binding heavy metal response regulator
Resistance to antibiotics and toxic compounds	Bile hydrolysis	Alpha-ketoglutarate-dependent taurine dioxygenase (EC 1.14.11.17)
Resistance to antibiotics and toxic compounds	Bile hydrolysis	DamX, an inner membrane protein involved in bile resistance
Resistance to antibiotics and toxic compounds	Bile hydrolysis	Choloylglycine hydrolase (EC 3.5.1.24)
Resistance to antibiotics and toxic compounds	Fosfomycin resistance	Fosfomycin resistance protein FosA
Resistance to antibiotics and toxic compounds	The mdtABCD multidrug resistance cluster	Probable RND efflux membrane fusion protein
Resistance to antibiotics and toxic compounds	The mdtABCD multidrug resistance cluster	Response regulator BaeR
Resistance to antibiotics and toxic compounds	The mdtABCD multidrug resistance cluster	Sensory histidine kinase BaeS
Resistance to antibiotics and toxic compounds	The mdtABCD multidrug resistance cluster	Multidrug transporter MdtD

Resistance to antibiotics and toxic compounds	The mdtABCD multidrug resistance cluster	Multidrug transporter MdtB
Resistance to antibiotics and toxic compounds	The mdtABCD multidrug resistance cluster	Multidrug transporter MdtC
Resistance to antibiotics and toxic compounds	Resistance to fluoroquinolones	Topoisomerase IV subunit B (EC 5.99.1.-)
Resistance to antibiotics and toxic compounds	Resistance to fluoroquinolones	Topoisomerase IV subunit A (EC 5.99.1.-)
Resistance to antibiotics and toxic compounds	Resistance to fluoroquinolones	DNA gyrase subunit A (EC 5.99.1.3)
Resistance to antibiotics and toxic compounds	Resistance to fluoroquinolones	DNA gyrase subunit B (EC 5.99.1.3)
Resistance to antibiotics and toxic compounds	Streptothricin resistance	Streptothricin acetyltransferase, Streptomyces lavendulae type
Resistance to antibiotics and toxic compounds	Multidrug Resistance Efflux Pumps	RND efflux system, membrane fusion protein CmeA
Resistance to antibiotics and toxic compounds	Multidrug Resistance Efflux Pumps	RND efflux system, outer membrane lipoprotein CmeC
Resistance to antibiotics and toxic compounds	Multidrug Resistance Efflux Pumps	Membrane fusion protein of RND family multidrug efflux pump
Resistance to antibiotics and toxic compounds	Multidrug Resistance Efflux Pumps	RND efflux system, inner membrane transporter CmeB

Resistance to antibiotics and toxic compounds	Multidrug Resistance Efflux Pumps	Type I secretion outer membrane protein, TolC precursor
Resistance to antibiotics and toxic compounds	Multidrug Resistance Efflux Pumps	Macrolide export ATP-binding/permease protein MacB (EC 3.6.3.-)
Resistance to antibiotics and toxic compounds	Multidrug Resistance Efflux Pumps	Acriflavin resistance protein
Resistance to antibiotics and toxic compounds	Multidrug Resistance Efflux Pumps	Transcription repressor of multidrug efflux pump acrAB operon, TetR (AcrR) family
Resistance to antibiotics and toxic compounds	Multidrug Resistance Efflux Pumps	RND efflux system, outer membrane lipoprotein, NodT family
Resistance to antibiotics and toxic compounds	Multidrug Resistance Efflux Pumps	Macrolide-specific efflux protein MacA
Resistance to antibiotics and toxic compounds	Multidrug Resistance Efflux Pumps	Multidrug-efflux transporter, major facilitator superfamily (MFS) (TC 2.A.1)
Resistance to antibiotics and toxic compounds	Multidrug Resistance Efflux Pumps	Multi antimicrobial extrusion protein (Na(+)/drug antiporter), MATE family of MDR efflux pumps
Invasion and intracellular resistance	Mycobacterium virulence operon involved in protein synthesis (LSU ribosomal proteins)	LSU ribosomal protein L20p
Invasion and intracellular resistance	Mycobacterium virulence operon involved in protein synthesis (LSU ribosomal proteins)	Translation initiation factor 3

Invasion and intracellular resistance	Mycobacterium virulence operon involved in protein synthesis (LSU ribosomal proteins)	LSU ribosomal protein L35p
Invasion and intracellular resistance	Mycobacterium virulence operon involved in DNA transcription	DNA-directed RNA polymerase beta subunit (EC 2.7.7.6)
Invasion and intracellular resistance	Mycobacterium virulence operon involved in DNA transcription	DNA-directed RNA polymerase beta' subunit (EC 2.7.7.6)
Invasion and intracellular resistance	Mycobacterium virulence operon possibly involved in quinolinate biosynthesis	Quinolinate synthetase (EC 2.5.1.72)
Invasion and intracellular resistance	Mycobacterium virulence operon possibly involved in quinolinate biosynthesis	L-aspartate oxidase (EC 1.4.3.16)
Invasion and intracellular resistance	Mycobacterium virulence operon possibly involved in quinolinate biosynthesis	Quinolinate phosphoribosyltransferase [decarboxylating] (EC 2.4.2.19)
Bacteriocins, ribosomally synthesized antibacterial peptides	Tolerance to colicin E2	Two-component response regulator CreC
Bacteriocins, ribosomally synthesized antibacterial peptides	Tolerance to colicin E2	Inner membrane protein CreD
Bacteriocins, ribosomally synthesized antibacterial peptides	Tolerance to colicin E2	Two-component response regulator CreB
Bacteriocins, ribosomally synthesized antibacterial peptides	Tolerance to colicin E2	Conserved uncharacterized protein CreA

Bacteriocins, ribosomally synthesized antibacterial peptides	Colicin V and Bacteriocin Production Cluster	Colicin V production protein
Bacteriocins, ribosomally synthesized antibacterial peptides	Colicin V and Bacteriocin Production Cluster	Amidophosphoribosyltransferase (EC 2.4.2.14)
Bacteriocins, ribosomally synthesized antibacterial peptides	Colicin V and Bacteriocin Production Cluster	Acetyl-coenzyme A carboxyl transferase beta chain (EC 6.4.1.2)
Bacteriocins, ribosomally synthesized antibacterial peptides	Colicin V and Bacteriocin Production Cluster	DedA protein
Bacteriocins, ribosomally synthesized antibacterial peptides	Colicin V and Bacteriocin Production Cluster	Dihydrofolate synthase (EC 6.3.2.12)
Bacteriocins, ribosomally synthesized antibacterial peptides	Colicin V and Bacteriocin Production Cluster	DedD protein
Bacteriocins, ribosomally synthesized antibacterial peptides	Colicin V and Bacteriocin Production Cluster	tRNA pseudouridine synthase A (EC 4.2.1.70)
Bacteriocins, ribosomally synthesized antibacterial peptides	Colicin V and Bacteriocin Production Cluster	Folylpolyglutamate synthase (EC 6.3.2.17)

Table 7.4 Identification of genes involve in secondary metabolites production using Antismash software

Cluster	Types	Most similar known cluster	Percentage (%)	Other species where clusters were identified	Functions	References
1	NI-siderophore	Aerobactin	66	*Xenorhabdus szentirmaii* DSM 16338	Antimicrobial and insecticidal	Hirschmann *et al.*, 2017
2	Thiopeptide	O-antigen	14	*Pseudomonas aeruginosa*	Antibiotic production activator, Zinc-responsive repressor and triggers host immune response	Bélanger *et al.*, 1999
3	Arylpolyne	Aryl polyenes	100	*Xenorhabdus doucetiae*	Antimicrobial, insecticidal, Pleiotropic regulatory for antibiotic production, Zinc-responsive repressor and, Development and antibiotic global regulator	Grammbitter *et al.*, 2019
4	NRP-metallophore and NRPS	Enterobactin	100	*Escherichia coli* str. K-12 substr. MG1655	Supply iron in metabolic pathway in cell	Raymond *et al.*, 2003
5	Hserlactone	-	-	-	-	-

Keywords in cluster and their descriptive meaning: NRP-metallophore = Non-ribosomal peptide metallophores, NRPS = NRPS-non ribosomal peptide synthesis, NI-siderophore = NRPS-independent, IucA/IucC-like siderophores, Hserlactone = Homoserine lactone.

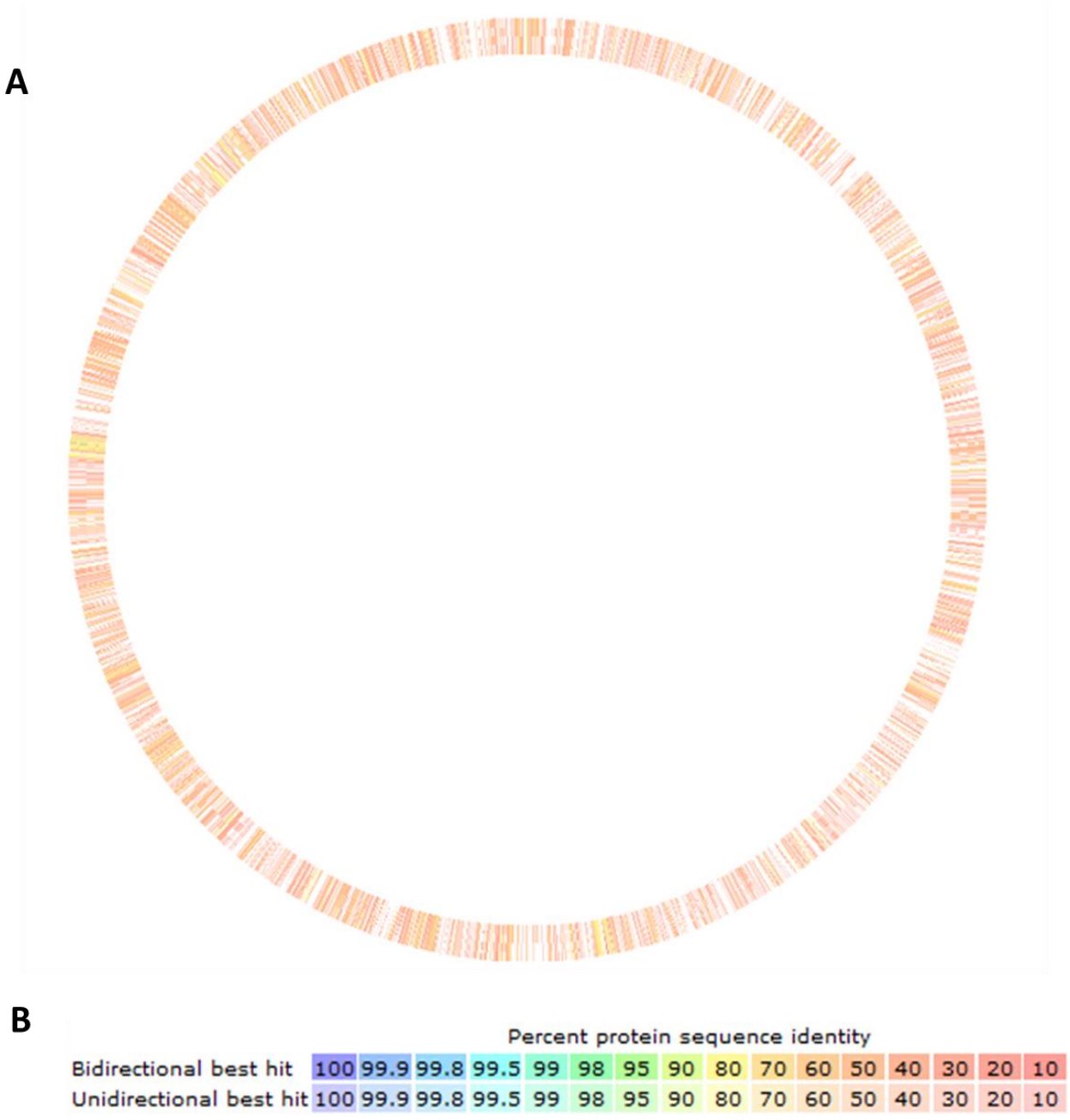


Figure 7.4 A) Genome alignment of *Pseudomonas sp.* PKS against *Pseudomonas aeruginosa* PAO1 (*Pseudomonas aeruginosa* PAO1 was used as a reference genome). B) The protein sequence similarity in similarity (Bidirectional best hits means both forward and revers hits).

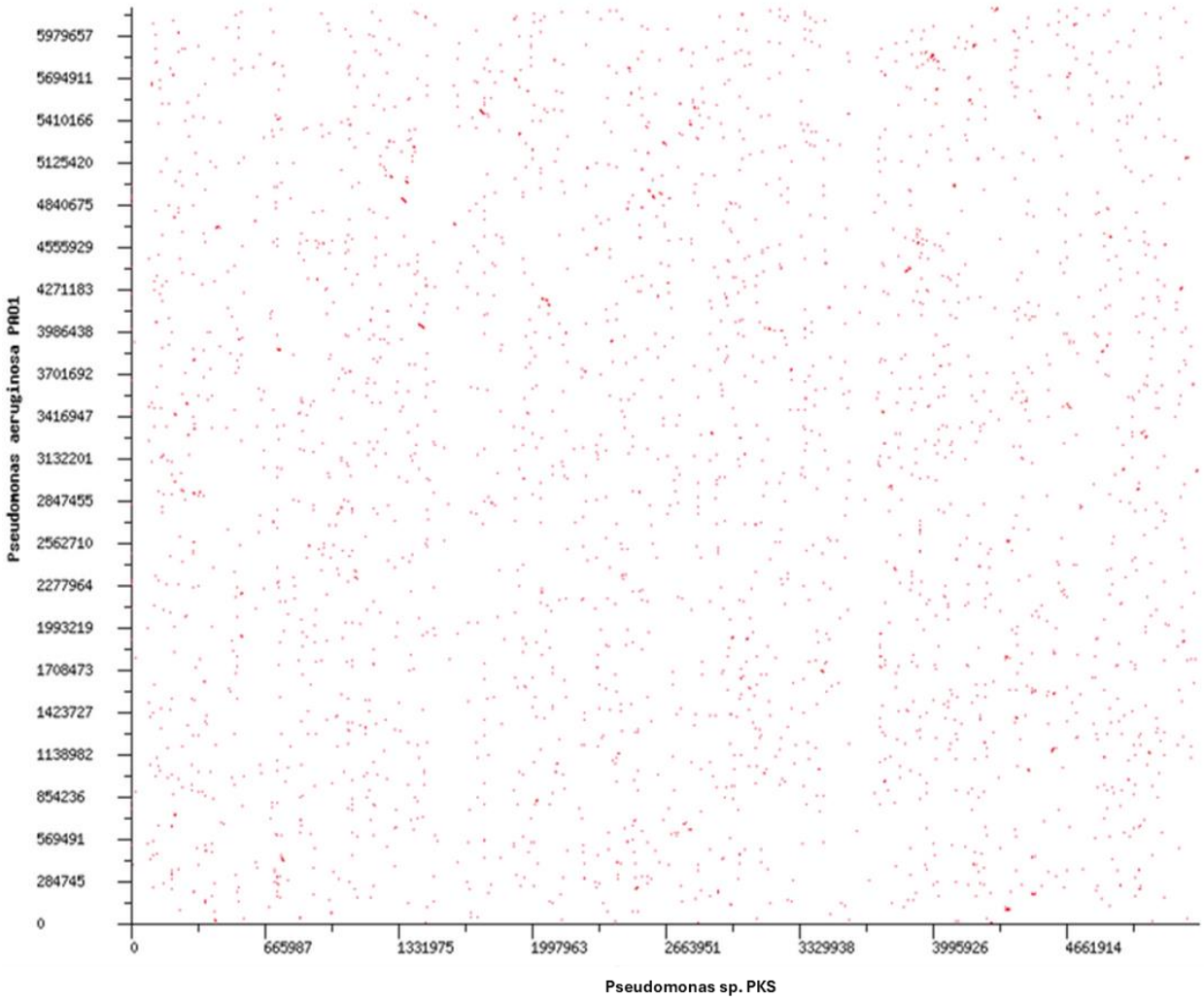


Figure 7.5 Blast dot plot of *Pseudomonas sp.* PKS genome aligned against *Pseudomonas aeruginosa* PAO1 genome. *Pseudomonas aeruginosa* PAO1 was used as reference genome.

7.4 Discussions

Entomopathogenic nematodes (EPNs) are crucial for ecological function. Furthermore, their wide application in horticulture sector has reduce groundwater contamination and soil acidification. However, their bacteria symbionts have other important function outside EPNs and horticulture sector. EPNs bacteria symbionts are reported to produces bio-active compounds which have antibiotic activity, making them an ideal source of drug candidates. This study explores the whole genome of *Pseudomonas sp.* PKs. *Pseudomonas sp*. PKS whole genome sequencing was achieved by subjecting the genomic DNA into Illumina sequencing and genomic data was annotated through RAST tool which reveal different genes clustered into various subsystem including virulence and defence.

Pseudomonas sp. PKS has genomic size of 5 660 896 bp, with 119 contigs and 54.2 % of (G+C) content (Table 7.1). The RAST annotation revealed that *Pseudomonas sp.* PKS consist of 569

subsystem, 5410 protein coding genes and 75 RNAs (Table 7.22). The identified virulence, disease subsystem showed various genes which facilitates a wide diversity of various biological process that are linked with survival and pathogenicity of *Pseudomonas sp.* PKS (Figure 7.3). Furthermore, amongst the identified proteins genes, there were two set of proteins (namely 16 kDa heat shock protein A and 16 kDa heat shock protein protein B) which were previously identified at *Serratia sp*. TEL isolated from *Oscheius sp*. TEL (Lephoto *et al*., 2015). These proteins are important for protein folding. Furthermore, other proteins are crucial for immune defence whilst others are important for pathogenicity of bacteria towards the insects.

The genome comparison of *Pseudomonas sp.* PKS and *Pseudomonas aeruginosa* PAO1 showed a lot of difference in protein coding genes (Figure 7.4 and Figure 7.5). *Pseudomonas aeruginosa* PAO1 is pathogenic to animals (including humans) and it is found in natural environment soil and water stream meanwhile *Pseudomonas sp.* PKS is isolated from *Cruznema* nematodes. Furthermore, the difference in the natural environment of the species may have influenced the genetic mutation and functions. The Antismash tool reveal that *Pseudomonas sp.* PKS has 5 metabolite gene clusters (namely NI-siderophore, Thiopeptide, Arylpolyne, NRP-metallophore, Hserlactone) (Table 7.4). These metabolites have antimicrobial and insecticidal activity, which it is crucial for killing the insect host and protecting the nematodes from possible pathogens. Furthermore, these metabolites have wide use in both agroforestry and pharmaceutical applications.

7.5 Conclusion and recommendations

This study provides insights in reference to genomic composition of *Pseudomonas sp.* PKS and the partial information about the role/ functions of genes. Therefore, this study server as base towards critical understanding of the pathogenicity activity against the insects.

7.6 Reference

Afordoanyi, D.M., Diabankana, R.G.C., Miftakhov, A.K., Kuchaev, E.S. and Validov, S.Z., 2022. Genomic Features of Pseudomonas putida PCL1760: A Biocontrol Agent Acting via Competition for Nutrient and Niche. *Applied Microbiology*, *2*(4), pp.749-765.

Bélanger, M., Burrows, L.L. and Lam, J.S., 1999. Functional analysis of genes responsible for the synthesis of the B-band O antigen of *Pseudomonas aeruginosa* serotype O6 lipopolysaccharide. *Microbiology*, *145*(12), pp.3505-3521.

Blin, K., Shaw, S., Augustijn, H.E., Reitz, Z.L., Biermann, F., Alanjary, M., Fetter, A., Terlouw, B.R., Metcalf, W.W., Helfrich, E.J. and van Wezel, G.P., 2023. antiSMASH 7.0: New and improved predictions for detection, regulation, chemical structures and visualisation. *Nucleic acids research*, pp.344.

Brown, J., Pirrung, M. and McCue, L.A., 2017. FQC Dashboard: integrates FastQC results into a web-based, interactive, and extensible FASTQ quality control tool. *Bioinformatics*, *33*(19), pp.3137-3139.

Didiza, L., Lephoto, T.E. and Gray, V.M., 2021. Morphological and molecular phylogenetic description of Steinernema batswanae n. sp.(Rhabditida: Steinernematidae): a new species of an entomopathogenic nematode from South Africa. *Archives of Phytopathology and Plant Protection*, *54*(19-20), pp.1603-1616.

d'Ovidio, D., Nermut, J., Adami, C. and Santoro, M., 2019. Occurrence of rhabditid nematodes in the pet giant African land snails (Achatina fulica). *Frontiers In Veterinary Science*, *6*, pp.88.

Du, H., Guo, F., Gao, Y., Wang, X., Qing, X. and Li, H., 2022. Complete Mitogenome of Confirms Highly Conserved Gene Arrangement within Family Rhabditidae. *Journal Of Nematology*, *54*(1), pp.1-10.

Grammbitter, G.L., Schmalhofer, M., Karimi, K., Shi, Y.M., Schöner, T.A., Tobias, N.J., Morgner, N., Groll, M. and Bode, H.B., 2019. An uncommon type II PKS catalyzes biosynthesis of aryl polyene pigments. *Journal of the American Chemical Society*, *141*(42), pp.16615-16623.

Gulcu, B., Cimen, H., Raja, R.K. and Hazir, S., 2017. Entomopathogenic Nematodes and their Mutualistic Bacteria: Their Ecology and Application as Microbial Control Agents. *Biopesticides International*, *13*(2).

Hirschmann, M., Grundmann, F. and Bode, H.B., 2017. Identification and occurrence of the hydroxamate siderophores aerobactin, putrebactin, avaroferrin and ochrobactin C as virulence factors from entomopathogenic bacteria. *Environmental microbiology*, *19*(10), pp.4080-4090.

Lephoto, T.E. and Gray, V.M., 2019. Oscheius basothovii n. sp.(Nematoda: Rhabditidae), a new entomopathogenic nematode isolated from an uncultivated grassland in South Africa. *Archives of Phytopathology and Plant Protection*, *52*(1-2), pp.125-140.

Lephoto, T.E. and Gray, V.M., 2022. Inspecting the pathogenicity of the integument of Oscheius basothovii following sterilization with sodium hypochlorite. *Biological Control*, *171*, p.104924.

Lephoto, T.E., Featherston, J. and Gray, V.M., 2015. Draft whole-genome sequence of Serratia sp. strain TEL, associated with Oscheius sp. TEL-2014 (Nematoda: Rhabditidae) isolated from a grassland in South Africa. *Genome Announcements*, *3*(4), pp.10-1128.

Punja, Z.K. and Utkhede, R.S., 2003. Using fungi and yeasts to manage vegetable crop diseases. *TRENDS in Biotechnology*, *21*(9), pp.400-407.

Raymond, K.N., Dertz, E.A. and Kim, S.S., 2003. Enterobactin: an archetype for microbial iron transport. *Proceedings of the national academy of sciences*, *100*(7), pp.3584-3588.

Salvadori, J.D.M., Defferrari, M.S., Ligabue-Braun, R., Lau, E.Y., Salvadori, J.R. and Carlini, C.R., 2012. Characterization of entomopathogenic nematodes and symbiotic bacteria active against Spodoptera frugiperda (Lepidoptera: Noctuidae) and contribution of bacterial urease to the insecticidal effect. *Biological control*, *63*(3), pp.253-263.

Sewe, S.O., Silva, G., Sicat, P., Seal, S.E. and Visendi, P., 2022. Trimming and validation of Illumina short reads using trimmomatic, trinity assembly, and assessment of RNA-seq data. In *Plant bioinformatics: Methods and protocols* (pp. 211-232). New York, NY: Springer US.

Usman, M., Wakil, W. and Shapiro-Ilan, D.I., 2021. Entomopathogenic nematodes as biological control agent against Bactrocera zonata and Bactrocera dorsalis (Diptera: Tephritidae). *Biological Control*, *163*, p.104706.

Utturkar, S.M., Klingeman, D.M., Land, M.L., Schadt, C.W., Doktycz, M.J., Pelletier, D.A. and Brown, S.D., 2014. Evaluation and validation of de novo and hybrid assembly techniques to derive high-quality genome sequences. *Bioinformatics*, *30*(19), pp.2709-2716.

Chapter 8

Whole genome sequencing and annotation of *Enterobacter sp.* PTB isolated from *Cruznema* nematode (*Rhabditida*)

Abstract

Entomopathogenic nematodes (EPNs) are insect parasitic worms which are classified as biological control agents in agriculture. EPNs can be used in both small-scale and large-scale farming, and have beneficial effects, such as improvement soil nutrients and protection against insect pest infestations. EPNs have symbiosis relationship pathogenic bacteria form phylum *Pseudomonadota* of *Gammaproteobacteriar* class. EPNs bacteria symbionts are crucial for facilitating growth, development, survival, and pathogenicity of nematodes. Next generation sequencing (NGS) technology help us to decipher evolutionary, behaviour, stress response, physiological attributes at a genomic level. The *Enterobacter sp.* PTB genomic DNA was extracted using bacterial DNA MiniPrep kit (Zymo Research, catalog # D3050) following the manufacture instruction. Genome sequencing of *Enterobacter sp.* PTB was carried out by Illumina Miseq. FastQC was used to check the quality of the reads meanwhile Trimmomatic tool was used remove low quality base. Assembling of the reads was performed using SPADES program and the quality of the assembly was checked using Quast tool. Moreover, the genome annotation was accomplished using RAST and metabolites gene clusters were identified using Antismash. The *Enterobacter sp.* PTB genome consist of 5 609 562 bp, 54,2 % of the GC content, with 5376 of coding genes. *Enterobacter sp.* PTB consists of 5 secondary metabolites gene cluster (Arylpolyne, NI-siderophore, Thiopeptide, Hserlactone, NRP-metallophore). *Enterobacter sp.* PTB has protein coding genes which are clustered under virulence, disease, and defence subsystem. These genes provide knowledge about the pathogenicity of the bacteria towards insect pests. Furthermore, other genes are implemented in cyto-protection of both the bacteria and nematodes. The metabolites compound produced by *Enterobacter sp.* PTB, they are not limited to agroforestry application only. However, the produced metabolites thus have enzymatic activity which has significant use in pharmaceutical industries. This study provides fundamental understanding of *Enterobacter sp.* PTB genomic composition and functions.

Keywords: *Enterobacter sp.* PTB, *Gammaproteobacteriar*, Genome, Next generation sequencing, *Pseudomonadotas*.

8.1 Introduction

8.1.1 Entomopathogenic overview

Entomopathogenic nematodes (EPNs) are microscopic insect, that are used as biological control agent against pest. They are soil indigenous species which are widely distributed through different biomes (Helmberger *et al.,* 2017). EPNs performs soil nutrition cycle, and they are more ecofriendly unlike their counter parts (namely synthetic chemical pesticides). EPNs can be used in both small-scale and large-scale farming (Dillman *et al.,* 2012). EPNs are in a symbiotic relationship with endopathogenic bacteria from *Enterobacteriaceae* family, that are responsible for causing an infection within the insect host (Tomar *et al.,* 2022). The nematodes serve as vector, which shiel the pathogenic symbiont bacteria from external environmental factors (such as desiccation, UV radiation, temperature) and transport the bacteria to the insect host hemolymph (Ciche *et al.,* 2006; Deka *et al.,* 2021; Abate *et al.,* 2023). Whilst the pathogenic symbiont bacterium is involved in cytoprotection of nematodes and hydrolyzation of insect host hemolymph to provide nutrients to nematodes (Kenney and Eleftherianos, 2016; Raja *et al.,* 2021).

8.1.2 Enterobacteriaceae family overview

Enterobacteriaceae family is very diverse with over thirty genera of gram-negative bacteria species. According to the previous studies, *Enterobacteriaceae* belong to the order *Enterobacterales* which is the class of *Gammaproteobacteriar* of *Pseudomonadota* phylum (Sneath *et al.,* 1986; Schoch *et al.,* 2020). The *Enterobacteriaceae* family compromises of both harmless symbiont bacteria and harmful bacteria to human (Mondal *et al.,* 2003). EPNs are in a symbiotic relationship with bacteria belong to *Enterobacteriaceae* family (namely *Pseudomonas*, *Photorhhabdus*, *Enterobacter*, *Serratia*, *Xenorhabdus*). Furthermore, these bacteria are deemed non-pathogenic to humans and other domestic animals (Dillman *et al.,* 2012). However, they are pathogenic to various agriculture problematic insects (Mondal *et al.,* 2003). Their association with EPNs is important agroforestry industries (Nderitu *et al.,* 2009). These EPNs bacteria symbionts produces toxic bio-chemical compounds that used to kill the host (Nderitu *et al.,* 2009; Elbrense *et al.,* 2021). Furthermore, previous studies have demonstrated that their antibiotic activity that can be exploited by pharmaceutical industries (Elbrense *et al.,* 2021).

8.1.3 Next generation sequencing

Next generation sequencing (NGS) is a high through put sequencing that used modern technology to decipher a biological genomic code for plant and animals. NGS advance

molecular biology studies by providing the insights about the evolution, morphological, physiological, and metabolic characteristics of organism(s). The concept of NGS it involves fragmentation of either RNA or DNA into million pieces, attachment of sequence adapters, sequencing of libraries, and reassembling in a parallel fashion to generate a genomic sequence. This study provides the genomic insights of *Enterobacter sp.* PTB isolated from *Cruznema sp.* NTM 2021 through the application of NGS technology.

8.2 Methodology

8.2.1 DNA extraction and purification of *Enterobacter sp*. PTB

A single colony of *Enterobacter sp*. PTB was picked from a NBTA plate and inoculated into freshly prepared broth Luria-Bertain (NaCL 10 g/L, peptone 10g/L and yeast extract5 g/L) broth. Followed by overnight incubation at 30℃ with shaker adjusted to 140 rpm. The DNA extractrion was performed using bacterial DNA MiniPrep kit (Zymo Research, catalog # D3050) in accordance with manufacturer instructions. The DNA quality was assessed by NanoDrop ND-1000® spectrophotometer (Bio-Rad). The DNA impurities were removed using bacterial DNA purification kit and quantified using concentrator™-5 (catalog #D4003S) kit.

8.2.2 Whole genome sequencing of *Pseudomonas sp.* PKS

The NextEra DNA sample kit (Illumina) was used to generate a genomic DNA paired-end libraries. Subsequently followed by generation of indexed with the aid of NextEra DNA index kit (Illumina) while adhering to the instructions provided by the manufacture. Furthermore, the primary libraries (paired-end) had a range of 200-3000 bp. Moreover, the paird-end (2x 300 bp) sequencing was achieved by subjecting MiSeq reagent kit v3 into MiSeq (Illumina).

8.2.3 Quality assessment and control

The sequenced that was subjected to FastQC (version 0.12.1) program to assess the quality (Brown *et al.,* 2017). Further Trimmomatic (version 0.39) program was used to low quality reads and nextra adapter sequences (Sewe *et al.,* 2022). Data validation was performed by subjecting the clean reads to FastQC program.

8.2.4 Genome assembly

The genomic reads were assembled using *de novo* method by SPADES (version 3.15.5) program (Utturkar *et al.,* 2014). The command "--careful" was used to avoid mismatches (Figure 8.1). After, the assembly the contig file were run into Quast (version 5.2.0) to assess the quality of assembly (Table 8.1 and Figure A21-26).

```
(spade_env) sekgobela@Sekgobela-PK:~/data_wgs/Sample_B$ spades.py --careful \
-o SPADES_Results -1 /home/sekgobela/data_wgs/Sample_B/Trimmed_reads/Trimmed_reads_1P.fastq.gz \
-2 /home/sekgobela/data_wgs/Sample_B/Trimmed_reads/Trimmed_reads_2P.fastq.gz
```

Figure 8.1 showing the command used to assembly the whole genome sequences

8.2.5 Whole genome annotation

The annotation of the whole genome sequenced data was carried through rapid annotation subsystem technology (RAST).

8.2.6 Identification of genes producing secondary metabolites

The Whole genome sequenced data was subjected to anti-smash (Version 7.0.1) site, for identification of genes producing secondary metabolites (Blin *et al.*, 2023).

8.3 Results

Enterobacter sp. PTB sequenced genome was acquired through sequencing of *Enterobacter sp*. PTB genomic DNA by Illumina Miseq. The reads were subjected to quality control using both FastQC and Trimmomatic before being assembly (Figure A11-20). Assembling of sequenced reads was carried by SPADES tool, using *de novo* method and assembly quality was examined using Quast tool (Table 8.1 and Figure A21-26). Genomic annotation was carried using RAST tool (Table 8.2) and prediction of metabolite coding genes was caried using AntiSMASH (Table 8.3).

Table 8.1: Quality assessment of the assembly of *Enterobacter sp.* PTB. (Please note the supplementary data is available in Figure A 21-26)

Species	*Enterobacter sp.* PTB
# contigs (>= 0 bp)	541
# contigs (>= 1000 bp)	92
# contigs (>= 5000 bp)	61
# contigs (>= 10000 bp)	52
# contigs (>= 25000 bp)	38
# contigs (>= 50000 bp)	28
Total length (>= 0 bp)	5 660 896
Total length (>= 1000 bp)	5 572 893
Total length (>= 5000 bp)	5 496 133

length (>= 10000 bp)	5 437 131
Total length (>= 25000 bp)	5 202 377
Total length (>= 50000 bp)	4 847 597
# contigs	119
Largest contig	432 872
Total length	5 589 920
GC (%)	54.17
N50	209 753
N90	32 802
auN	220 886.1
L50	9
L90	33
# N's per 100 kbp	0.00

All statistics are based on contigs of size >= 500 bp, unless otherwise noted (e.g., "# contigs (>= 0 bp)" and "Total length (>= 0 bp)" include all contigs).

Table 8.2 Overview of *Enterobacter sp*. PTB whole genome annotation by RAST

Feature	**RAST annotation**
Genome size (bp)	5,609,562
GC content (%)	54.2
Number of Subsystems	572
Number of Protein coding genes	5376
Number of contigs (with PEGs)	391
Number of RNAs	76

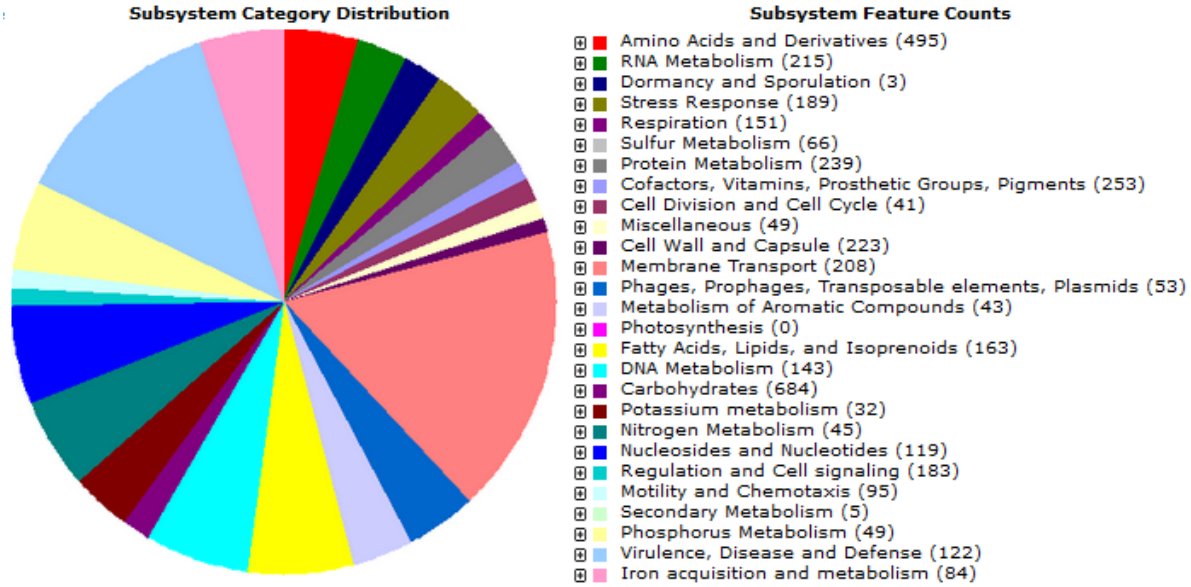


Figure 8.2 An overview diagram of *Enterobacter sp*. PTB subsystem categories into their correspondent genes annotated by RAST server

Table 8.3: Elucidating *Enterobacter sp*. PTB genes that are clustered in subsystem (Virulence, disease, and defence). This system is crucial to understand the pathogenicity mechanism of the bacteria against insect host. Furthermore, other genes found in this system are implicated in biosynthetic pathways crucial for survival of the bacteria.

Subcategory	Subsystem	Role
Bacteriocins, ribosomally synthesized antibacterial peptides	Tolerance to colicin E2	Two-component response regulator CreC
Bacteriocins, ribosomally synthesized antibacterial peptides	Tolerance to colicin E2	Inner membrane protein CreD
Bacteriocins, ribosomally synthesized antibacterial peptides	Tolerance to colicin E2	Two-component response regulator CreB
Bacteriocins, ribosomally synthesized antibacterial peptides	Tolerance to colicin E2	Conserved uncharacterized protein CreA

Bacteriocins, ribosomally synthesized antibacterial peptides	Colicin V and Bacteriocin Production Cluster	tRNA pseudouridine synthase A (EC 4.2.1.70)
Bacteriocins, ribosomally synthesized antibacterial peptides	Colicin V and Bacteriocin Production Cluster	Folylpolyglutamate synthase (EC 6.3.2.17)
Bacteriocins, ribosomally synthesized antibacterial peptides	Colicin V and Bacteriocin Production Cluster	DedD protein
Bacteriocins, ribosomally synthesized antibacterial peptides	Colicin V and Bacteriocin Production Cluster	Dihydrofolate synthase (EC 6.3.2.12)
Bacteriocins, ribosomally synthesized antibacterial peptides	Colicin V and Bacteriocin Production Cluster	Colicin V production protein
Bacteriocins, ribosomally synthesized antibacterial peptides	Colicin V and Bacteriocin Production Cluster	Amidophosphoribosyltransferase (EC 2.4.2.14)
Bacteriocins, ribosomally synthesized antibacterial peptides	Colicin V and Bacteriocin Production Cluster	Acetyl-coenzyme A carboxyl transferase beta chain (EC 6.4.1.2)
Bacteriocins, ribosomally synthesized antibacterial peptides	Colicin V and Bacteriocin Production Cluster	DedA protein
Invasion and intracellular resistance	Mycobacterium virulence operon involved in DNA transcription	DNA-directed RNA polymerase beta' subunit (EC 2.7.7.6)
Invasion and intracellular resistance	Mycobacterium virulence operon involved in DNA transcription	DNA-directed RNA polymerase beta subunit (EC 2.7.7.6)
Invasion and intracellular resistance	Mycobacterium virulence operon involved in protein	LSU ribosomal protein L35p

	synthesis (LSU ribosomal proteins)	
Invasion and intracellular resistance	Mycobacterium virulence operon involved in protein synthesis (LSU ribosomal proteins)	LSU ribosomal protein L20p
Invasion and intracellular resistance	Mycobacterium virulence operon involved in protein synthesis (LSU ribosomal proteins)	Translation initiation factor 3
Invasion and intracellular resistance	Mycobacterium virulence operon possibly involved in quinolinate biosynthesis	L-aspartate oxidase (EC 1.4.3.16)
Invasion and intracellular resistance	Mycobacterium virulence operon possibly involved in quinolinate biosynthesis	Quinolinate synthetase (EC 2.5.1.72)
Invasion and intracellular resistance	Mycobacterium virulence operon possibly involved in quinolinate biosynthesis	Quinolinate phosphoribosyltransferase [decarboxylating] (EC 2.4.2.19)
Invasion and intracellular resistance	Mycobacterium virulence operon involved in protein synthesis (SSU ribosomal proteins)	Translation elongation factor G
Invasion and intracellular resistance	Mycobacterium virulence operon involved in protein synthesis (SSU ribosomal proteins)	SSU ribosomal protein S12p (S23e)
Invasion and intracellular resistance	Mycobacterium virulence operon involved in protein synthesis (SSU ribosomal proteins)	SSU ribosomal protein S7p (S5e)
Invasion and intracellular resistance	Mycobacterium virulence operon involved in protein	Translation elongation factor Tu

	synthesis (SSU ribosomal proteins)	
Adhesion	Mediator of hyperadherence YidE in Enterobacteria and its conserved region	Outer membrane lipoprotein YidQ
Adhesion	Mediator of hyperadherence YidE in Enterobacteria and its conserved region	16 kDa heat shock protein A
Adhesion	Mediator of hyperadherence YidE in Enterobacteria and its conserved region	Mediator of hyperadherence YidE
Adhesion	Mediator of hyperadherence YidE in Enterobacteria and its conserved region	16 kDa heat shock protein B
Adhesion	Mediator of hyperadherence YidE in Enterobacteria and its conserved region	Uncharacterized protein YidR
Adhesion	Mediator of hyperadherence YidE in Enterobacteria and its conserved region	HTH-type transcriptional regulator YidP
Resistance to antibiotics and toxic compounds	Multidrug Resistance Efflux Pumps	Transcription repressor of multidrug efflux pump acrAB operon, TetR (AcrR) family
Resistance to antibiotics and toxic compounds	Multidrug Resistance Efflux Pumps	Type I secretion outer membrane protein, TolC precursor

Resistance to antibiotics and toxic compounds	Multidrug Resistance Efflux Pumps	Macrolide export ATP-binding/permease protein MacB (EC 3.6.3.-)
Resistance to antibiotics and toxic compounds	Multidrug Resistance Efflux Pumps	Acriflavin resistance protein
Resistance to antibiotics and toxic compounds	Multidrug Resistance Efflux Pumps	Membrane fusion protein of RND family multidrug efflux pump
Resistance to antibiotics and toxic compounds	Multidrug Resistance Efflux Pumps	RND efflux system, inner membrane transporter CmeB
Resistance to antibiotics and toxic compounds	Multidrug Resistance Efflux Pumps	RND efflux system, outer membrane lipoprotein CmeC
Resistance to antibiotics and toxic compounds	Multidrug Resistance Efflux Pumps	RND efflux system, membrane fusion protein CmeA
Resistance to antibiotics and toxic compounds	Multidrug Resistance Efflux Pumps	Multidrug-efflux transporter, major facilitator superfamily (MFS) (TC 2.A.1)
Resistance to antibiotics and toxic compounds	Multidrug Resistance Efflux Pumps	Multi antimicrobial extrusion protein (Na(+)/drug antiporter), MATE family of MDR efflux pumps
Resistance to antibiotics and toxic compounds	Multidrug Resistance Efflux Pumps	Macrolide-specific efflux protein MacA
Resistance to antibiotics and toxic compounds	Multidrug Resistance Efflux Pumps	RND efflux system, outer membrane lipoprotein, NodT family
Resistance to antibiotics and toxic compounds	Streptothricin resistance	Streptothricin acetyltransferase, Streptomyces lavendulae type
Resistance to antibiotics and toxic compounds	Resistance to fluoroquinolones	DNA gyrase subunit A (EC 5.99.1.3)
Resistance to antibiotics and toxic compounds	Resistance to fluoroquinolones	Topoisomerase IV subunit A (EC 5.99.1.-)
Resistance to antibiotics and toxic compounds	Resistance to fluoroquinolones	Topoisomerase IV subunit B (EC 5.99.1.-)
Resistance to antibiotics and toxic compounds	Resistance to fluoroquinolones	DNA gyrase subunit B (EC 5.99.1.3)

Resistance to antibiotics and toxic compounds	The mdtABCD multidrug resistance cluster	Probable RND efflux membrane fusion protein
Resistance to antibiotics and toxic compounds	The mdtABCD multidrug resistance cluster	Response regulator BaeR
Resistance to antibiotics and toxic compounds	The mdtABCD multidrug resistance cluster	Sensory histidine kinase BaeS
Resistance to antibiotics and toxic compounds	The mdtABCD multidrug resistance cluster	Multidrug transporter MdtD
Resistance to antibiotics and toxic compounds	The mdtABCD multidrug resistance cluster	Multidrug transporter MdtB
Resistance to antibiotics and toxic compounds	The mdtABCD multidrug resistance cluster	Multidrug transporter MdtC
Resistance to antibiotics and toxic compounds	Fosfomycin resistance	Fosfomycin resistance protein FosA
Resistance to antibiotics and toxic compounds	Bile hydrolysis	DamX, an inner membrane protein involved in bile resistance
Resistance to antibiotics and toxic compounds	Bile hydrolysis	Alpha-ketoglutarate-dependent taurine dioxygenase (EC 1.14.11.17)
Resistance to antibiotics and toxic compounds	Bile hydrolysis	Choloylglycine hydrolase (EC 3.5.1.24)
Resistance to antibiotics and toxic compounds	Cobalt-zinc-cadmium resistance	Cobalt-zinc-cadmium resistance protein
Resistance to antibiotics and toxic compounds	Cobalt-zinc-cadmium resistance	Zinc transporter ZitB
Resistance to antibiotics and toxic compounds	Cobalt-zinc-cadmium resistance	Transcriptional regulator, MerR family
Resistance to antibiotics and toxic compounds	Cobalt-zinc-cadmium resistance	Probable Co/Zn/Cd efflux system membrane fusion protein
Resistance to antibiotics and toxic compounds	Cobalt-zinc-cadmium resistance	Copper-sensing two-component system response regulator CusR
Resistance to antibiotics and toxic compounds	Cobalt-zinc-cadmium resistance	Cation efflux system protein CusC precursor

Resistance to antibiotics and toxic compounds	Cobalt-zinc-cadmium resistance	Cobalt-zinc-cadmium resistance protein CzcA
Resistance to antibiotics and toxic compounds	Cobalt-zinc-cadmium resistance	Cation efflux system protein CusA
Resistance to antibiotics and toxic compounds	Cobalt-zinc-cadmium resistance	DNA-binding heavy metal response regulator
Resistance to antibiotics and toxic compounds	Cobalt-zinc-cadmium resistance	Cation efflux system protein CusF precursor
Resistance to antibiotics and toxic compounds	Cobalt-zinc-cadmium resistance	Cobalt/zinc/cadmium efflux RND transporter, membrane fusion protein, CzcB family
Resistance to antibiotics and toxic compounds	Adaptation to d-cysteine	D-cysteine desulfhydrase (EC 4.4.1.15)
Resistance to antibiotics and toxic compounds	Adaptation to d-cysteine	Cystine ABC transporter, ATP-binding protein
Resistance to antibiotics and toxic compounds	Adaptation to d-cysteine	Cystine ABC transporter, permease protein
Resistance to antibiotics and toxic compounds	Arsenic resistance	Arsenic efflux pump protein
Resistance to antibiotics and toxic compounds	Arsenic resistance	Arsenate reductase (EC 1.20.4.1)
Resistance to antibiotics and toxic compounds	Arsenic resistance	Arsenical resistance operon repressor
Resistance to antibiotics and toxic compounds	Copper homeostasis	Copper resistance protein D
Resistance to antibiotics and toxic compounds	Copper homeostasis	Cu(I)-responsive transcriptional regulator
Resistance to antibiotics and toxic compounds	Copper homeostasis	Copper-sensing two-component system response regulator CusR
Resistance to antibiotics and toxic compounds	Copper homeostasis	Copper-translocating P-type ATPase (EC 3.6.3.4)
Resistance to antibiotics and toxic compounds	Copper homeostasis	Copper resistance protein C precursor

Resistance to antibiotics and toxic compounds	Copper homeostasis	Blue copper oxidase CueO precursor
Resistance to antibiotics and toxic compounds	Mercuric reductase	PF00070 family, FAD-dependent NAD(P)-disulphide oxidoreductase
Resistance to antibiotics and toxic compounds	Beta-lactamase	Beta-lactamase (EC 3.5.2.6)
Resistance to antibiotics and toxic compounds	Beta-lactamase	Beta-lactamase class C and other penicillin binding proteins
Resistance to antibiotics and toxic compounds	Beta-lactamase	Metal-dependent hydrolases of the beta-lactamase superfamily I
Resistance to antibiotics and toxic compounds	Copper homeostasis: copper tolerance	Membrane protein, suppressor for copper-sensitivity ScsB
Resistance to antibiotics and toxic compounds	Copper homeostasis: copper tolerance	Membrane protein, suppressor for copper-sensitivity ScsD
Resistance to antibiotics and toxic compounds	Copper homeostasis: copper tolerance	Secreted protein, suppressor for copper-sensitivity ScsC
Resistance to antibiotics and toxic compounds	Copper homeostasis: copper tolerance	Copper homeostasis protein CutF precursor
Resistance to antibiotics and toxic compounds	Copper homeostasis: copper tolerance	Copper homeostasis protein CutE
Resistance to antibiotics and toxic compounds	Copper homeostasis: copper tolerance	Magnesium and cobalt efflux protein CorC
Resistance to antibiotics and toxic compounds	Copper homeostasis: copper tolerance	Suppression of copper sensitivity: putative copper binding protein ScsA
Resistance to antibiotics and toxic compounds	Multidrug Resistance, Tripartite Systems Found in Gram Negative Bacteria	Outer membrane component of tripartite multidrug resistance system
Resistance to antibiotics and toxic compounds	Multidrug Resistance, Tripartite Systems Found in Gram Negative Bacteria	Membrane fusion component of tripartite multidrug resistance system
Resistance to antibiotics and toxic compounds	Multidrug Resistance, Tripartite Systems Found in Gram Negative Bacteria	Inner membrane component of tripartite multidrug resistance system

Resistance to antibiotics and toxic compounds	Multiple Antibiotic Resistance MAR locus	Multiple antibiotic resistance protein MarA
Resistance to antibiotics and toxic compounds	Multiple Antibiotic Resistance MAR locus	Multiple antibiotic resistance protein MarC
Resistance to antibiotics and toxic compounds	Multiple Antibiotic Resistance MAR locus	Multiple antibiotic resistance protein MarR
Resistance to antibiotics and toxic compounds	Multiple Antibiotic Resistance MAR locus	Multiple antibiotic resistance protein MarB

Table 8.4 Identification of *Enterobacter sp.* PTB genes producing secondary metabolites using Antismash program

Cluster	Types	Most similar known cluster	Percentage (%)	Other species where clusters were identified	Functions	References
1	NI-siderophore	Aerobactin	66	*Xenorhabdus szentirmaii* DSM 16338	Antimicrobial and insecticidal	Hirschmann *et al.,* 2017
2	Thiopeptide	O-antigen	14	*Pseudomonas aeruginosa*	Antibiotic production activator, Zinc-responsive repressor and triggers host immune response	Bélanger *et al.,* 1999
3	Arylpolyne	Aryl polyenes	100	*Xenorhabdus doucetiae*	Antimicrobial, insecticidal, Pleiotropic regulatory for antibiotic production, Zinc-responsive repressor and, Development and antibiotic global regulator	Grammbitter *et al.,* 2019

4	NRP-metallophore and NRPS	Enterobactin	100	*Escherichia coli* str. K-12 substr. MG1655	Supply iron in metabolic pathway in cell	Raymond *et al.*, 2003
5	Hserlactone	-	-	-	-	-

Keywords in cluster and their descriptive meaning: NRP-metallophore = Non-ribosomal peptide metallophores, NRPS = NRPS-non ribosomal peptide synthesis, NI-siderophore = NRPS-independent, IucA/IucC-like siderophores, Hserlactone = Homoserine lactone.

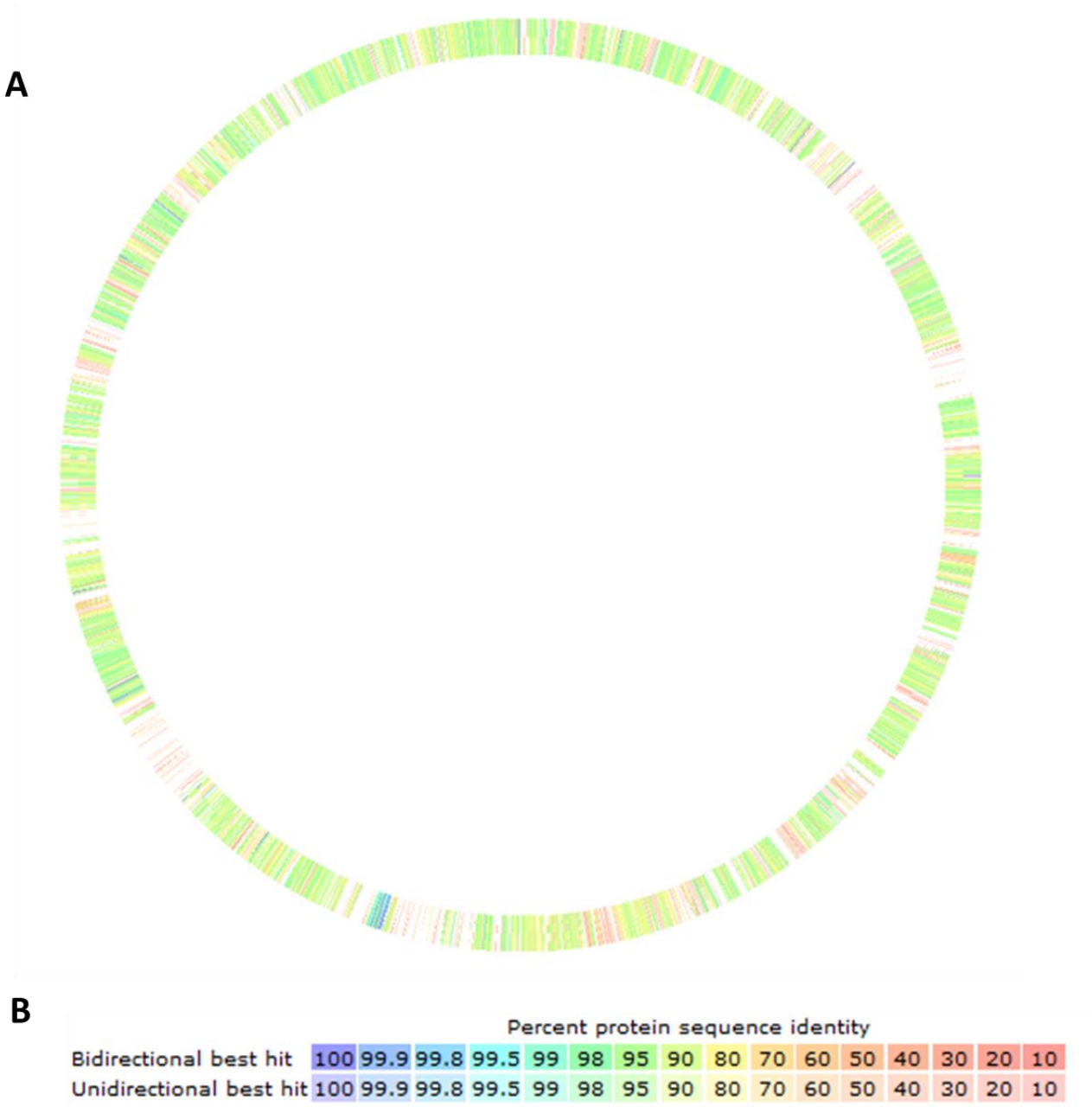


Figure 8.3 Genome alignment of *Enterobacter sp*. PTB against *Enterobacter sp*. 638 (*Enterobacter sp*. 638 was used as a reference genome). B) The protein sequence similarity in similarity (Bidirectional best hits means both forward and revers hits)

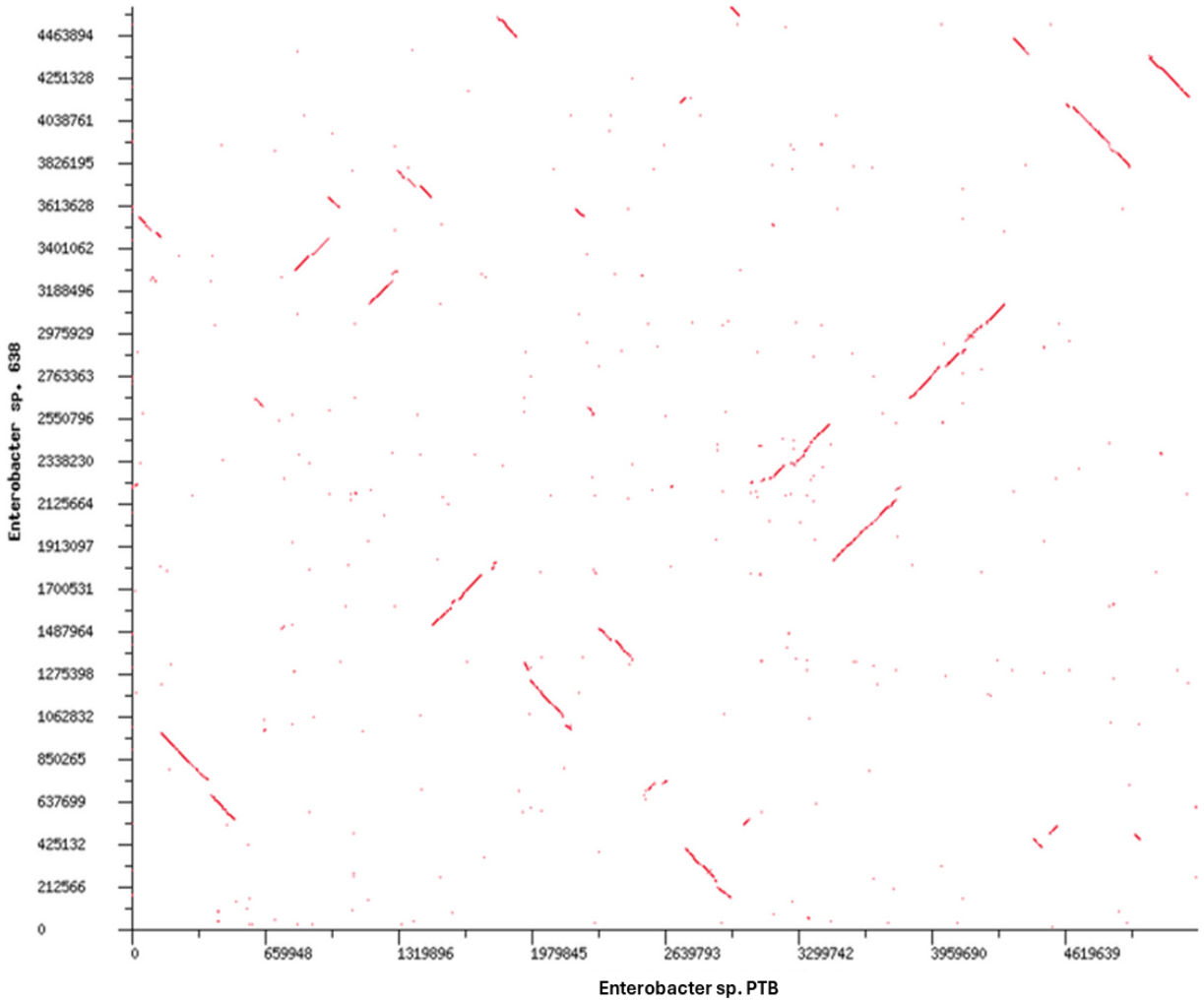


Figure 8.4 Blast dot plot of *Enterobacter sp*. PTB genome aligned against *Enterobacter sp*. 638 genome. The *Enterobacter sp*. 638 was used as a reference.

8.4 Discussions

The whole genomic data of was extrapolated with NGS technology. The extracted *Enterobacter sp*. PTB genomic DNA was sequenced using Illumina Miseq into parallel reads. The quality of the sequenced reads was assessed with FastQC and Trimmomatic tool was used to remove the ambiguous bases and reads with low quality. Furthermore, the *de novo* assembly method was used to organize the reads with the aid of SPADES tools. The genomic functional annotation of *Enterobacter sp*. PTB was performed by RAST tool. The Quast report show that there was no genomic mismatch (Table 8.1 and Figure A21-26). The RAST annotation report elucidate that *Enterobacter sp*. PTB has genomic size of 5 609 562 bp, 5376 protein coding genes, 572 subsystems and 76 RNAs (Table 8.2).

The virulence, disease subsystem is crucial towards understanding the pathogenicity of the bacteria towards the insects (Table 8.3). Furthermore, the subsystem reveals that *Enterobacter sp*. PTB consist of multiple genes that are implemented in adhesion, antibiotic resistance, invasion, and intracellular resistance. These gene are crucial towards intrusion and colonizing of the insect host. When the bacteria have been released with the insect host hamelymph the bacteria must overcome host immune response, cause infection which ultimate kill the host. The EPNs bacteria symbionts produces bioactive compounds that have enzymatic activity ensuring that once the insect host is killed, there are no intrusion to the host cadaver by possible pathogens. Antismash program have identified 5 gene clusters (NI-siderophore, Thiopeptide, Arylpolyne, NRP-metallophore, Hserlactone) that produces secondary metabolites (Table 8.4). The metabolites that are produce by *Enterobacter sp*. PTB have other applications beyond the agroforestry applications, making them a target for pharmaceutical industries.

The genome alignment of *Enterobacter sp*. PTB against *Enterobacter sp*. 638 reference genome show high degree of similarity for protein coding genes (Figure 8.3 and 8.4). EPNs bacteria symbionts share the same family (namely *Enterobacteriaceae*). All EPNs species belong to the same phylum order (*Rhabditida*) and perform same ecological function as biological control agents against insect pest, despite them being evolutionary divergent at the genus level. Furthermore, genetical differences influence the EPNs to respond different towards the environmental factors (such as temperature, desiccation, UV light) and efficacy. EPNs bacteria symbionts belong to class *Gammaproteobacteriar of* phylum *Pseudomonadota.* Furthermore, the EPNs bacteria symbionts are evolutionary divergent to one another especially at genus level. Therefore, these differences at genomic level influence variation in gene regulation, metabolites production, and pathogenicity activity against pest insects.

8.5 Conclusion and recommendation

This study provides provide *Enterobacter sp*. PTB genome insights that help to understand the defences mechanism and pathogenicity against insect pest. Furthermore, prokaryotic genome automatic annotation pipeline to improve the annotation method and identified the tRNAs, coding RNAs and pseudogenes. Moreover, identifying pseudogenes help us to understand new genes (which may be crucial towards improving our understanding about the evolutionary and physiological attributes of the bacteria), that are frequently ruled out as junk.

8.6 Reference

Abate, B.A., Mamo, F.T., Tesfaye, K., Yuankai, C., Qi, R. and Malan, A.P., 2023. Entomopathogenic nematodes and their symbiotic bacteria from Africa: diversity and use as biological control agents. *Biocontrol Science and Technology*, *33*(12), pp.1186-1209.

Bélanger, M., Burrows, L.L. and Lam, J.S., 1999. Functional analysis of genes responsible for the synthesis of the B-band O antigen of *Pseudomonas aeruginosa* serotype O6 lipopolysaccharide. *Microbiology*, *145*(12), pp.3505-3521.

Blin, K., Shaw, S., Augustijn, H.E., Reitz, Z.L., Biermann, F., Alanjary, M., Fetter, A., Terlouw, B.R., Metcalf, W.W., Helfrich, E.J. and van Wezel, G.P., 2023. antiSMASH 7.0: New and improved predictions for detection, regulation, chemical structures and visualisation. *Nucleic acids research*, pp.344.

Brown, J., Pirrung, M. and McCue, L.A., 2017. FQC Dashboard: integrates FastQC results into a web-based, interactive, and extensible FASTQ quality control tool. *Bioinformatics*, *33*(19), pp.3137-3139.

Ciche, T.A., Darby, C., Ehlers, R.U., Forst, S. and Goodrich-Blair, H., 2006. Dangerous liaisons: the symbiosis of entomopathogenic nematodes and bacteria. *Biological Control*, *38*(1), pp.22-46.

Dillman, A.R., Chaston, J.M., Adams, B.J., Ciche, T.A., Goodrich-Blair, H., Stock, S.P. and Sternberg, P.W., 2012. An entomopathogenic nematode by any other name. *PLoS Pathogens*, *8*(3), p.e1002527.

Elbrense, H., Elmasry, A.M., Seleiman, M.F., Al-Harbi, M.S. and Abd El-Raheem, A.M., 2021. Can symbiotic bacteria (Xenorhabdus and Photorhabdus) be more efficient than their entomopathogenic nematodes against Pieris rapae and Pentodon algerinus Larvae?. *Biology*, *10*(10), p.999.

Grammbitter, G.L., Schmalhofer, M., Karimi, K., Shi, Y.M., Schöner, T.A., Tobias, N.J., Morgner, N., Groll, M. and Bode, H.B., 2019. An uncommon type II PKS catalyzes biosynthesis of aryl polyene pigments. *Journal of the American Chemical Society*, *141*(42), pp.16615-16623.

Helmberger, M.S., Shields, E.J. and Wickings, K.G., 2017. Ecology of belowground biological control: Entomopathogenic nematode interactions with soil biota. *Applied Soil Ecology*, *121*, pp.201-213.

Kenney, E. and Eleftherianos, I., 2016. Entomopathogenic and plant pathogenic nematodes as opposing forces in agriculture. *International journal for parasitology*, *46*(1), pp.13-19.

Mondal, S., Somani, J., Roy, S., Babu, A. and Pandey, A.K., 2023. Insect Microbial Symbionts: Ecology, Interactions, and Biological Significance. *Microorganisms*, *11*(11), p.2665.

Nderitu, J., Sila, M., Nyamasyo, G. and Kasina, M., 2009. Effectiveness of entomopathogenic nematodes against sweet potato weevil (Cylas puncticollis) Boheman (Coleoptera: Apionidae) under semi-field conditions in Kenya. *Journal of Entomology*, *6*(3), pp.145-154.

Raja, R.K., Arun, A., Touray, M., Gulsen, S.H., Cimen, H., Gulcu, B., Hazir, C., Aiswarya, D., Ulug, D., Cakmak, I. and Kaya, H.K., 2021. Antagonists and defense mechanisms of entomopathogenic nematodes and their mutualistic bacteria. *Biological Control*, *152*, p.104452.

Raymond, K.N., Dertz, E.A. and Kim, S.S., 2003. Enterobactin: an archetype for microbial iron transport. *Proceedings of the national academy of sciences*, *100*(7), pp.3584-3588.

Schoch, C.L., Ciufo, S., Domrachev, M., Hotton, C.L., Kannan, S., Khovanskaya, R., Leipe, D., Mcveigh, R., O'Neill, K., Robbertse, B. and Sharma, S., 2020. NCBI Taxonomy: a comprehensive update on curation, resources and tools. *Database*, *2020*, pp.baaa062.

Sewe, S.O., Silva, G., Sicat, P., Seal, S.E. and Visendi, P., 2022. Trimming and validation of Illumina short reads using trimmomatic, trinity assembly, and assessment of RNA-seq data. In *Plant bioinformatics: Methods and protocols* (pp. 211-232). New York, NY: Springer US.

Sneath, P.H., Mair, N.S., Sharpe, M.E. and Holt, J.G., 1986. *Bergey's manual of systematic bacteriology. Volume 2*. Williams & Wilkins.

Tomar, P., Thakur, N. and Yadav, A.N., 2022. Endosymbiotic microbes from entomopathogenic nematode (EPNs) and their applications as biocontrol agents for agro-environmental sustainability. *Egyptian Journal of Biological Pest Control*, *32*(1), p.80.

Utturkar, S.M., Klingeman, D.M., Land, M.L., Schadt, C.W., Doktycz, M.J., Pelletier, D.A. and Brown, S.D., 2014. Evaluation and validation of de novo and hybrid assembly techniques to derive high-quality genome sequences. *Bioinformatics*, *30*(19), pp.2709-2716.

Chapter 9

Structural and functional characterization of 16 kDa heat shock protein A and B

Abstract

Entomopathogenic nematodes (EPNs) are biological control agents for agriculture problematics insect pests. EPNs uses toxic bioactive compounds that are produce by its bacteria symbiosis to disrupt the insect host immune defences. There are inconsistences in EPNs field efficacy due to the environmental factors (namely). The exposure of EPNs into foreign biome, leads to the loss of EPNs pathogenicity. Therefore, different genetic manipulation techniques have been previously explored to improve EPNs field efficacy. In this study we structural and functional characterised 16 kDa heat shock protein A and B clustered in virulence, disease, and defence subsystem of *Cruznema sp*. NTM 2021 bacteria symbionts (namely *Pseudomonas sp.* PKS and *Enterobacter sp*. PTB). The secondary structure of the two proteins were model using the closest homologous templates as a reference in Phyre2. Furthermore, both proteins secondary structure integrity was evaluated with an aid of Ramachandran plot. The functional perdition of both proteins was carried out by Interpro-EMBL using hidden Markov model (HMM) statistical analyses cross referencing with the available protein database. Furthermore, the functional protein interaction partner of 16 kDa heat shock protein A and B were identified through String version 12.0. 16 kDa heat shock protein A secondary structure consist of 45 % of beta sheets 17 % of disordered sequence and zero alpha helix. Whilst 16 kDa heat shock protein B predominated by beta sheet at 44 %, disordered sequence at 27 % and alpha helix at 4 %. Both proteins have both hydrophobic and hydrophilic residues, the hydrophobic residues are buried within the core of the protein while the hydrophilic residues form the surface enclosing the hydrophobic residues. Further both proteins have a single representative domain SHSP, suggesting that the proteins belong to small heat shock proteins family. Therefore, they are involve in folding of client proteins and prevention of thermal induce protein aggregation. These proteins thus have functional interacting partners with other heat shock families (such as DnJ and ClpB). Furthermore, both proteins direct interact with both proteins (namely DnJ and ClpB) this suggest that these proteins functions as oligomers and therefore interrupting this network may abrogate cellular functioning leading to ultimate death of the organism.

Keywords: Aggregation, alpha helix, Entomopathogenic nematodes, Hydrophilic, Small heat shock proteins.

Introduction

9.1.1 Entomopathogenic nematodes applications and efficacy

Entomopathogenic nematodes (EPNs) refer as biological control agents against agricultural problematic insects. EPNs have a symbiosis relationship with pathogenic bacteria from *Enterobacteriaceae* family. The EPNs symbiotic bacteria are responsible for production of toxic bioactive compounds that deprive the insect immune defence, resulting to insect death (Shan *et al.,* 2019). The use of EPNs has become common in many parts of the world however, the EPNs field efficacy is questionable, as it varies in different ecological niches. Hence the horticulture practitioners are hesitant to cease the use of synthetic chemical pesticides, despite their negative impact in both environment and animals (including humans) (Ndereyimana *et al.,* 2020). EPNs are indigenous habitants of the soil and are scattered throughout ecological niches (Rauch *et al.,* 2017). Furthermore, different biomes experience different environmental factors, which influences the adaptability of EPNs. Environmental factors (such as temperature, desiccation, ultraviolet rays) are reported to affect the EPNs field efficacy (Husin and Port, 2021). Moreover, it is reported that EPNs isolated from different topological niche fluctuates or loses its pathogenicity when are used in different region (Stuart *et al.,* 2015). Therefore, there is an urgency need to improve EPNs field efficacy.

9.1.2 Genetic manipulation to improve EPNs field efficacy

Genetic manipulation is the most profound advanced technique in molecular biology, allowing altering the genetic makeup of organism(s) to induce or abrogate a specific function. There are two genetic manipulation method (namely artificial selection and genomic assisted breeding) which have been previously explored to induce the EPNs field efficacy (Lu *et al.,* 2016). Furthermore, both techniques improve the possibilities of developing a new species which have a super adaptative towards environmental factors and effectiveness against insect pests. However, both techniques are reported to have negative drawbacks, due to the absences of a selection force driving the breading, thus ultimately leads to loss of important genomic traits that reduces the overall fitness (Lu *et al.,* 2016; Glazer, 2015). There is a knowledge gap in understanding the individual gene(s) functions which drives the pathogenicity or adaptability of EPNs. Hence, this study characterises conserved 16 kDa heat shock protein A and B, amongst EPNs bacteria symbionts that is clustered under virulence, disease, and defence subsystem.

9.1.3 Molecular chaperones

Molecular chaperones are group of proteins which are vital to cellular functioning (Wickner *et al.,* 2021). These set of proteins acts a helper protein that are involve folding of client protein, protein translocation and protein degradation (lund, 2001; Ghazaei, 2017). Furthermore, heat shock proteins (Hsps) are members of molecular chaperones, which are upregulated in cellular stress event (Pandey *et al.,* 2015). These proteins are reported to be involved to stabilize both proteins and membranes (Vanghele and Ganea, 2010). Moreover, inhibition of these proteins it is associated with drastically change in overall fitness and functions of organism, that results in ultimate death (Wickner *et al.,* 2021).

9.1.4 16 kDa heat shock protein A and B isolated from EPNs bacteria symbionts

Both 16 kDa heat shock protein A and B are mostly conserved proteins that are found in EPNs bacteria symbionts. These proteins are clustered in virulence, disease, and defence of the EPNs bacteria symbionts subsystem (Lephoto *et al.,* 2015). Further these proteins are suggested to be involved in protein post transitional modification and chaperon activity (Lephoto *et al.,* 2015). These proteins have not been fully characterised. Moreover, there is no knowledges about its role in pathogenicity against insect host.

9.2 Methodology

Whole genome sequencing of *Cruznema sp.* NTM 2021bacteria symbionts were archived through MiSeq Illuminina and annotation was carried through RAST program (Chapter 7: Section 7.2 and Chapter 8: Section 8.2). Both 16 kDa heat shock protein A and B protein sequences where obtain form virulence, disease, and defence subsystem belonging to both bacteria (namely *Pseudomonas sp.* PKS and *Enterobacter sp.* PTB). Further both protein sequences were identical from each species, despite them being found in different contig nodes. Secondary structure modelling of 16 kDa heat shock protein A and B isolated *Cruznema sp.* NTM 2021 was carried out by subjecting the protein sequence into Phyre2 using high degree of homology template. The generated 3D model structure was further assessed through Ramachandran plot. The functional characterizing of protein domains was performed using the hidden Markov model (HMM) statistical analyses which is inference of homology within Pfam database as previously described in Mistry *et al.* (2021) and functional protein characterization database contain in Interpro-EMBL (Hunter *et al.,* 2009). The interaction partners of either 16 kDa heat shock protein A or B were investigated through latest version of String Version 12.0, which also search the possible pathways which the protein is involved. Furthermore, both proteins hydrophobicity was calculated through by ProtoPram software (Gasteiger *et al.,* 2005).

9.3 Results

Functional prediction of proteins was archived through Interpro-EMBL database, and secondary structure was constructed with Phyre2 and using highest reference homology template. Furthermore, both protein sequences were identical from both the species, which suggest the perform same function in both the species. The proteins interaction partners were identified using String cross referencing to all known data base as described in. The rand average of hydropathicity (GRAVY) of 16 kDa heat shock protein B was found to be -0.502 whereas the 16 kDa heat shock protein A was found to be -0.549 by ProtoPram software.

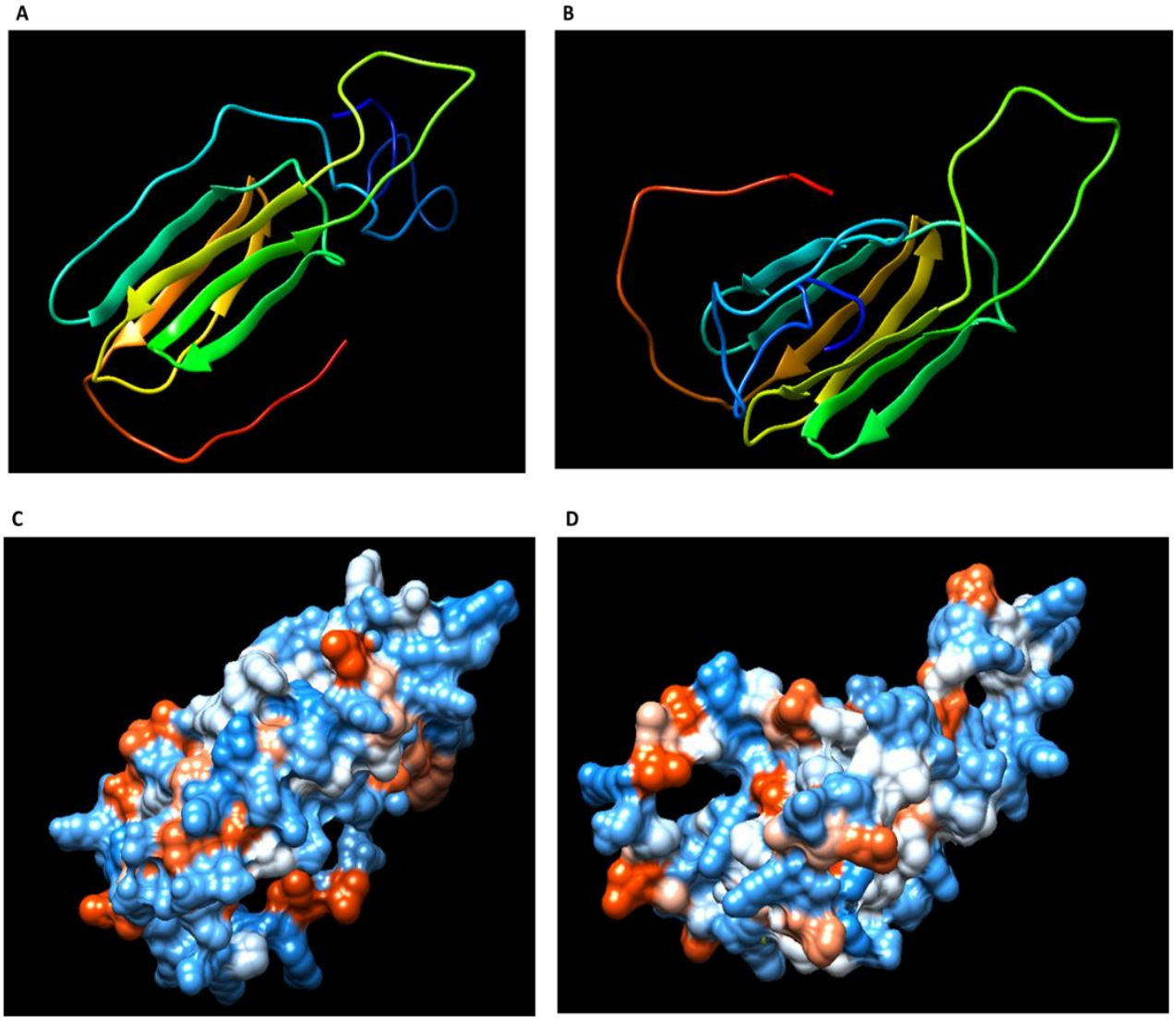


Figure 9.1 3D secondary structure of 16 kDa A) heat shock protein A and B) heat shock protein.

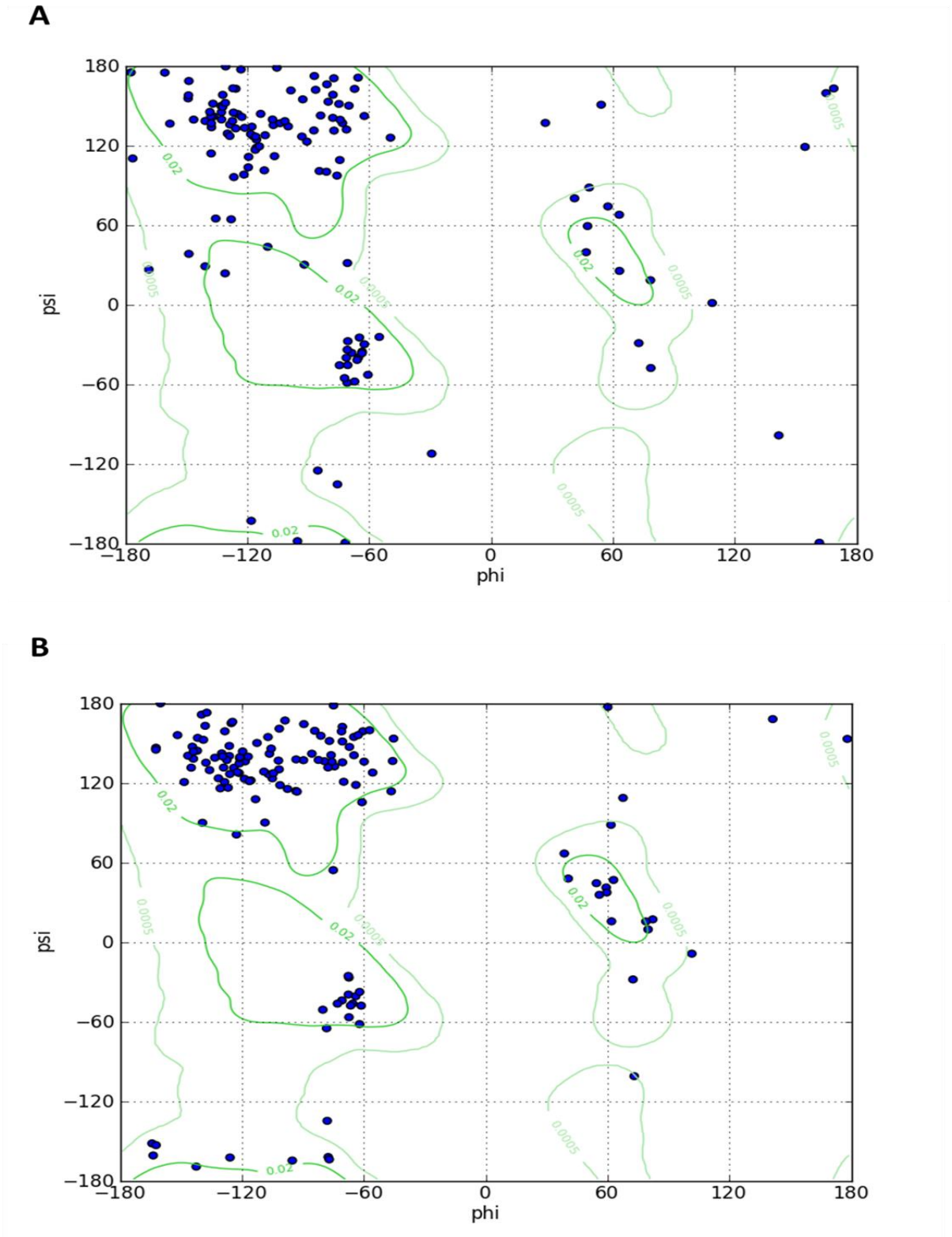


Figure 9.2 Ramachandran plot of secondary structure of 16 kDa A) heat shock protein A and B) heat shock protein B. The angle psi is denoted by ψ and the angle phi is denoted by ϕ. The first quadrant is situated where ψ is positive and ϕ is negative and when both ψ and ϕ are negative it is a 3rd quadrant. However, when both ϕ and ψ are positive it is a 2nd quadrant. Further the 4th quadrant is positioned where ϕ is positive and ψ is negative.

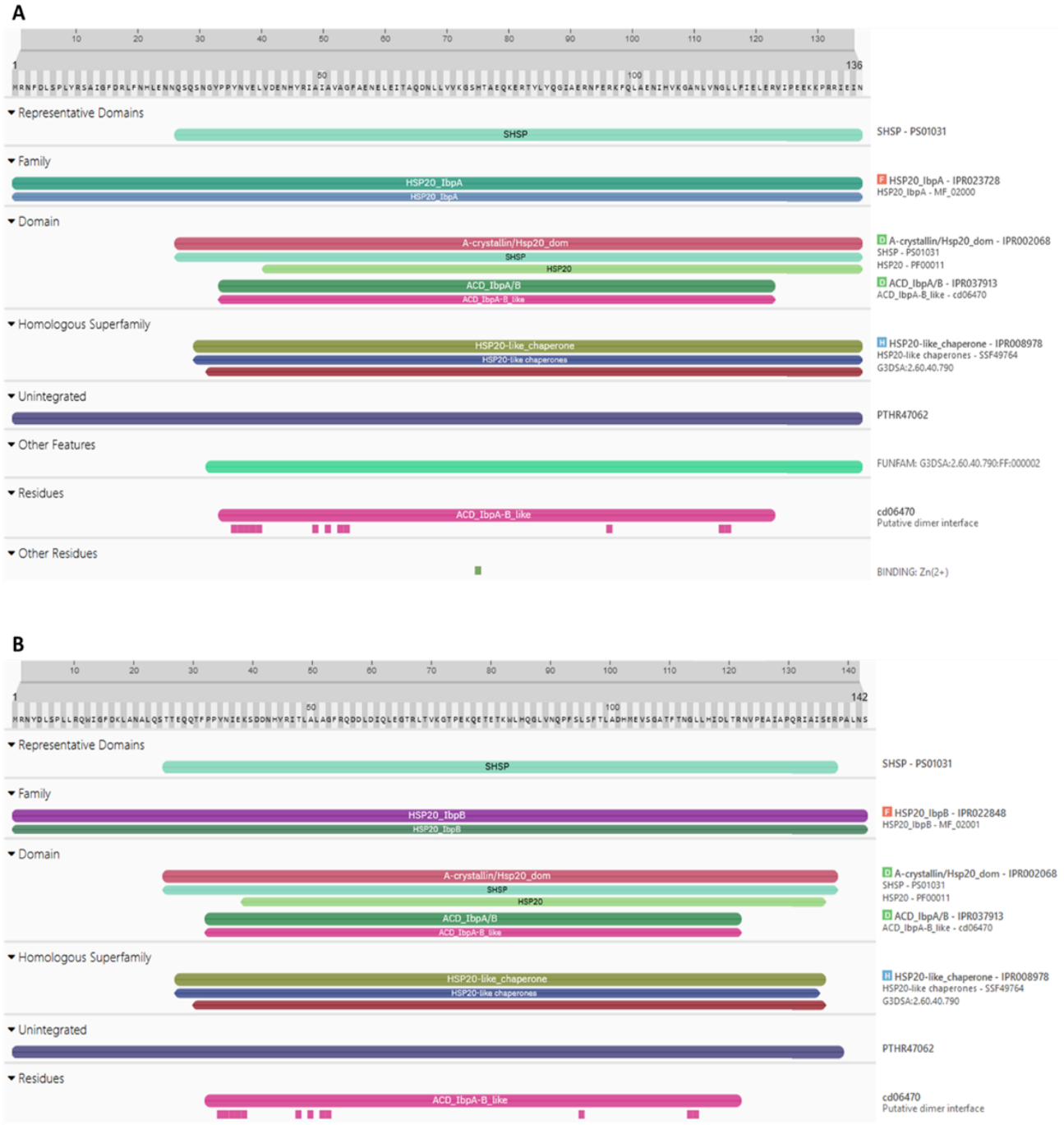


Figure 9.3 Functional characterisation of 16 kDa A) heat shock protein A and B) heat shock protein B

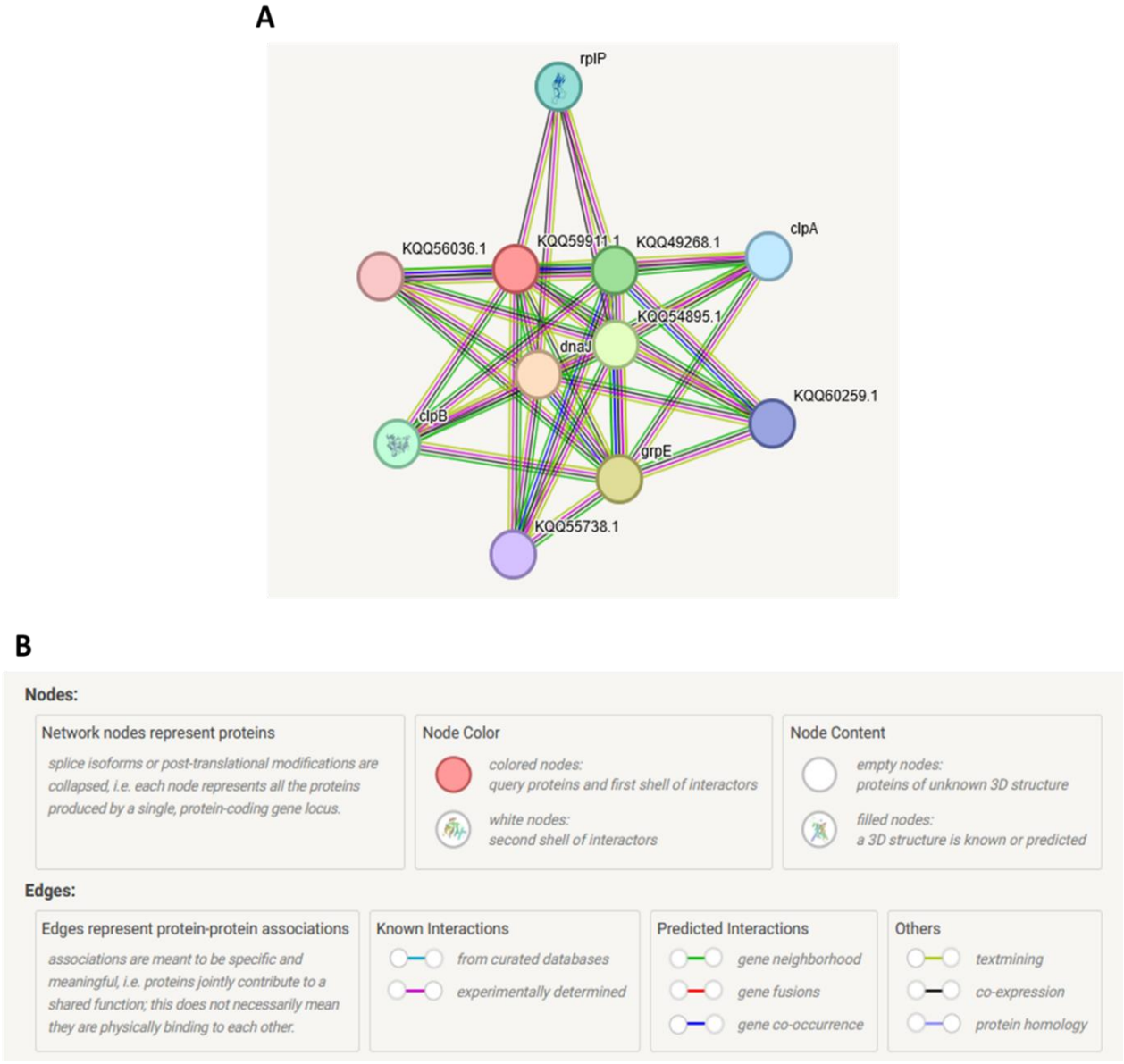


Figure 9.4 A schematic diagram of 16 kDa heat shock protein A, A) protein interaction network B) key description of protein network. The red sphere represents our protein of interest.

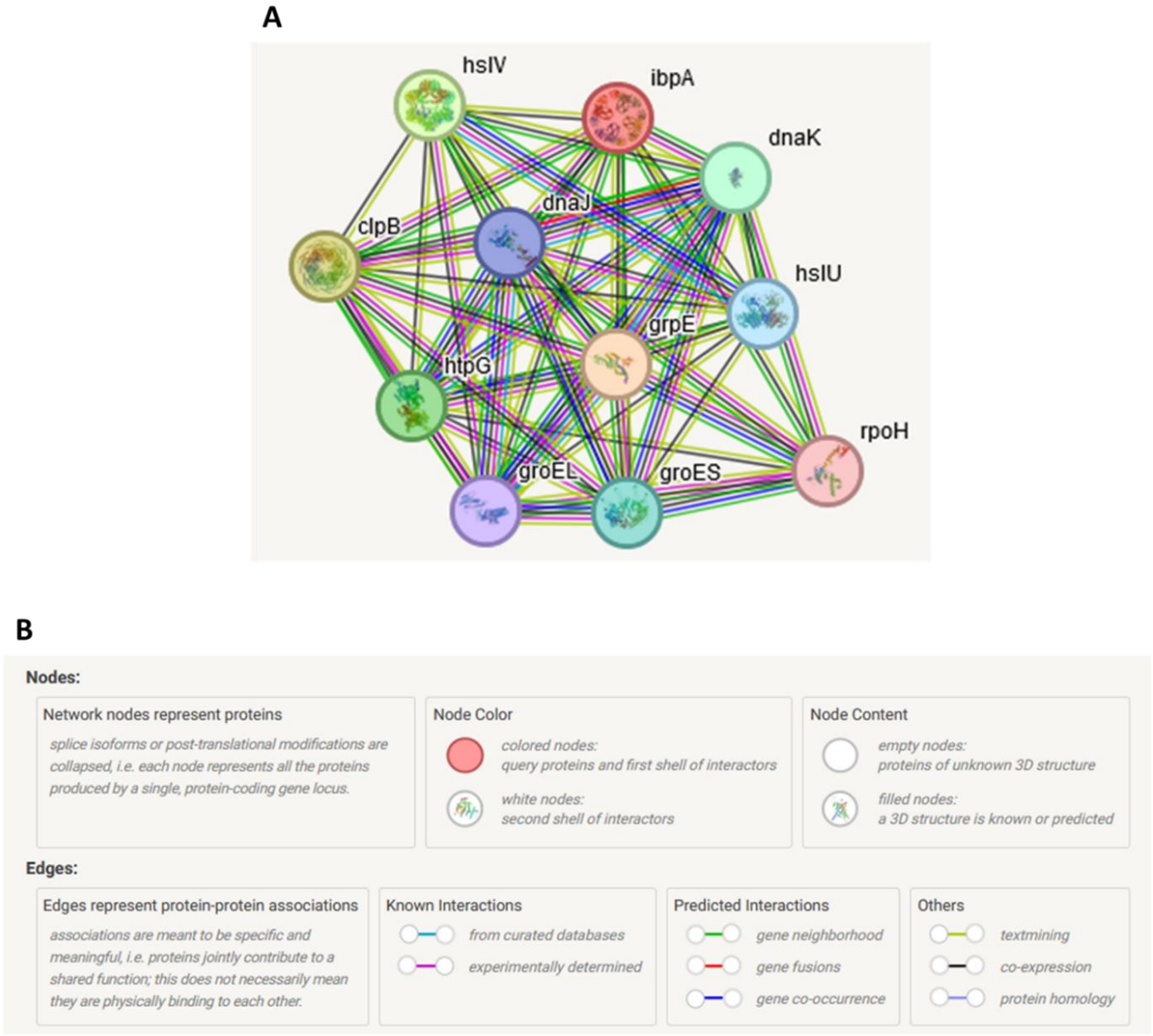


Figure 9.5 A schematic diagram of 16 kDa heat shock protein B, A) protein interaction network B) key description of protein network. The red sphere represents our protein of interest.

9.4 Discussions

Entomopathogenic nematodes solely depend on the pathogenic symbiotic bacteria to infect and kill the insect host. The environmental stressors (temperature, desiccation, ultraviolet rays) alter the physiological conditions within the nematodes that consequently threatens the existences of the symbiotic bacteria. Once the existences of the symbiotic bacteria cease the nematodes losses its pathogenicity. Therefore, it is significant to understand that the symbiotic bacteria genes clustered in virulence, disease, and defence subsystem, are not only important for causing infection to the insect host. However, these genes are implemented in the immune defence of both bacteria and nematodes. Once the nematodes gain an access to the host internal

system, both the nematodes and symbionts bacteria are susceptible to the host immune response, if the bacteria have already loss genes which are implemented in this subsystem the bacteria will not be able to cause infection or either produce enzymes which will hydrolyse the host hemolymph for nematodes to feed or defend against insect host immune defence. Therefore, in this study we structural and functional characterised two conserved proteins (namely 16 kDa heat shock protein A and B) found EPNs symbiotic protein.

The secondary structure characterisation was achieved by subjecting the protein sequence to Phyre2 and aligned with the highest homologous protein template(s) to generate a confident model to construct the 3D secondary structure (Figure 9.1). 16 kDa heat shock protein A secondary structure model was constructed using 4 templates (namely c7ck4B, c7pe3A, c4zjdF and d1gmeb) due to heuristics that will elevate the percentage identity, confidence, and alignment coverage (Figure A28 A). Furthermore, it was uncovered that secondary structure of 16 kDa heat shock protein A consist of 45 % of beta sheets and 17 % disordered sequence (Figure 9.1 A and Figure A27 A). Meanwhile, 16 kDa heat shock protein B consists of 4% alpha helix, beta sheets and 27 % disordered (Figure 9.1 B and Figure A27 B). Furthermore, five templates (namely c7ck4B, c3w1zD, c3w1zA. c4zjdF and c7pe3A) were uses reference for secondary structure construction model of 16 kDa heat shock protein B, based on the heuristics concept to increase the percentage identity, confidence, and alignment coverage (Figure A28 B). The hydrophobicity of the two protein we determine by ProtoPram software which calculated grand average of hydropathicity (GRAVY) to be -0.549 for 16 kDa heat shock protein A and -0.502 for 16 kDa heat shock protein B. Therefore, both proteins are hydrophilic, and we suggest that both proteins are cytosolic since they form a functional network with DnaJ which it is a cytosolic protein (Figure 9.4 and Figure 9.5). The secondary protein structure integrity of both proteins (namely 16 kDa heat shock protein A and 16 kDa heat shock protein B) were evaluated through Ramachandran plot (Figure 9.2). The 16 kDa heat shock protein A has more residues which are position where ψ ranges at 80º to180º and ϕ at -58º to -140º in the 1st quadrant (Figure 9.2 A). Furthermore, based on the position of the residue we can see that this protein is predominated by antiparallel beta sheets, with small significant amount of parallel beta sheets supporting the 3D model in Figure 9.1 A. Moreover, there is a very little residue in approximate ψ = 75º and ϕ = -60º more likely to form collagen triple helix. There are few amino acids residue scarted in 2nd quadrant (Figure 9.2 A). The 2nd quadrant consists of amino residues which forms left-handed alpha helix however, these amino acids are unlikely to occur. Furtherer, the amino residues on the 3rd quadrant are few and scattered far apart from

each other making them hard to form a right-handed alpha helix. Moreover, there are 4 amino residues present in the 4th quadrant and this phenomenon it is impossible in real life due to the concept of the rotation of dihedral angles as previously describe in Ramachandran *et al.* (1963). Hence, these amino residues found in the 2nd,3rd and 4th quadrants form the 17 % of disordered sequence in Figure A27 A. The 16 kDa heat shock protein amino acid residues are more concentrated in 1st quadrant where ψ ranges from 120º to 178º and ϕ ranges from -160º to -58º making the secondary structure to be predominate by antiparallel beta sheets (Figure 9.2 B) supporting the 3D secondary structure in Figure 9.1 A. Furthermore, in the 3rd quadrant there is significant number of amino acid residue (ψ ranges from -27º to -60 º and ϕ ranges from -50º to -60º) that form right-handed alpha helix (Figure 9.2 B), as supported in Figure A27 B. Moreover, in Figure 9.2 B there is small amount in the 2nd quadrant which are scattered far apart, including the 3 amino acid residues in the 4th quadrant which form the 27 % of disordered amino acid sequence indicated in Figure A27 B.

Functional prediction of the proteins was attained by Interpro-EMBL webinar and cross referencing through the database described in Hunter *et al.* (2009). The 16 kDa heat shock protein A has a single representative domain SHSP that coverage from 27-136 amino acid residues, and homologous family Hsp20_IbpA that cover the whole 136 amino acid sequence residues (Figure 9.3 A). There were five domains (namely A-crystallin/Hsp20_dom, SHSP, HSP20, ACD_IbpA/B, ACD_IbpA-B_like) which were found to be homologous to our query amino acid sequence however, only two domains (namely A-crystallin/Hsp20_dom and SHSP) which have the same coverage from 27 to 136 amino residue. Further the two homologous domains had a greater coverage compared to the other 3 (HSP20, ACD_IbpA/B, ACD_IbpA-B_like) (Figure 9.3 A).16 kDa heat shock protein B have a representative domain SHSP with a coverage range from 26-137 amino acid residue (Figure 9.3 B). Furthermore, the HSP20_IbpB was identified as a family function that cover the entire (142 aa) amino acid residues sequence. Moreover, there are several domains which were homolog to this protein (namely A-crystallin/ Hsp20_dom, SHSP, Hsp 20, ACD_Ibp A/B, ACD_IbpA-B_like). Both A-crystallin/ Hsp20_dom and SHSP, had a high relative coverage compared to all 3 other homologous domains.

SHSP (small heat shock proteins) are group of proteins which are highly conserved in both eukaryotes and prokaryotes, that ranges from 15 to 30 kDa (Lindquist and Craig, 1988; Pandey *et al.,* 2015). Further heat shock proteins (Hsps) are clustered into families (Hsp70, Hsp 60, Hsp100, sHsp and Hsp90) based on their molecular size. Hsps are highly produce as part of

environmental stress response, they are implicated in various cellular functions such as to prevent denaturation and aggregation of proteins (Pandey *et al.,* 2015). Furthermore, an A-crystallin/Hsp20_dom is domain of small heat shock proteins (Münchbach *et al.,* 1999; Pandey *et al.,* 2015). ACD_IbpA/B refers to alpha-crystallin domains of protein with a molecular weight of approximately 16 kDa (Münchbach *et al.,* 1999). Moreover, Ibp A and Ibp B are protein chaperons which functions as Hsps, these proteins consist of multiple subunits and functions as large oligomers (Matuszewska *et al.,* 2005; Bissonnette *et al.,* 2010). Both 16 kDa heat shock protein A and 16 kDa heat shock protein B are heat shock proteins cluster in small heat shock protein family. Furthermore, they are involved in stress response to variety of environmental stress, making them a target for improving the symbiotic bacteria survival which directly improve the pathogenicity and efficacy of EPNs.

The functional interaction partners of either 16 kDa heat shock protein A or B were identified through the utilization of String webinar, which reveals that there are 10 proteins which interacts with 16 kDa heat shock protein A (Figure 9.4 A) and 10 other proteins interacts with 16 kDa heat shock protein B (Figure 9.5 A). Furthermore, some proteins (namely DnaJ and CIpB) direct interacts with both proteins (16 kDa heat shock protein A and B). These proteins are clustered to other families of heat shock proteins. Hence, thus supports the claim that heat shock proteins functions as can oligomers. Therefore, both 16 kDa heat shock protein A and B functions as a counterpart. Moreover, disruption of a single protein will lead to series of cellular process disfunctions since the network link will be compromised. It is imperative to note that some of these proteins are co-expressed since they function as a unit (Figure A29 and Figure A30). It is imperative to not some molecular chaperons perform a holdase function, therefore since both proteins directly interacts with DnaJ (DnaJ is clustered under Hsp70 family). Both proteins may perform holdase function to the protein client in an event of physiological stress and transfer it to DnaJ for refolding.

9.5 Conclusions and recommendations

Both 16 kDa heat shock protein A and B are vital for survival, defence, and pathogenicity of the EPNs symbionts bacteria. Therefore, these proteins form a functional interaction network with other proteins belong to different families. Furthermore, disruption of these functional network will have detrimental effects to the survival of the bacteria including the field efficacy of the EPNs. Biochemical characterisations using varies techniques (such as Far-UV Circular dichroism to study the secondary structure, protein crystallization to study the tertiary structure,

ATPase to study the ATPase activity and SPR (surface plasmon resonance) to study protein interaction) should be conducted to fully characterise the structure and function of the proteins.

9.6 References

Bissonnette, S.A., Rivera-Rivera, I., Sauer, R.T. and Baker, T.A., 2010. The IbpA and IbpB small heat-shock proteins are substrates of the AAA+ Lon protease. *Molecular Microbiology*, *75*(6), pp.1539-1549.

Gasteiger, E., Hoogland, C., Gattiker, A., Duvaud, S.E., Wilkins, M.R., Appel, R.D. and Bairoch, A., 2005. *Protein Identification And Analysis Tools On The ExPASy server*, pp.571-607. Humana press.

Ghazaei, C., 2017. Role and mechanism of the Hsp70 molecular chaperone machines in bacterial pathogens. *Journal Of Medical Microbiology*, *66*(3), pp.259-265.

Glazer, I., 2015. Improvement of entomopathogenic nematodes: a genetic approach. In *Nematode Pathogenesis Of Insects And Other Pests: Ecology And Applied Technologies For Sustainable Plant And Crop Protection*, pp.29-55. Cham: Springer International Publishing.

Hunter, S., Apweiler, R., Attwood, T.K., Bairoch, A., Bateman, A., Binns, D., Bork, P., Das, U., Daugherty, L., Duquenne, L. and Finn, R.D., 2009. InterPro: the integrative protein signature database. *Nucleic acids research*, *37*(suppl_1), pp.211-215.

Husin, T.O.B. and Port, G.R., 2021. Efficacy of entomopathogenic nematodes against *Tuta absoluta*. *Biological Control*, *160*, pp.104699.

Lephoto, T.E., Featherston, J. and Gray, V.M., 2015. Draft whole-genome sequence of Serratia sp. strain TEL, associated with *Oscheius sp*. TEL-2014 (Nematoda: *Rhabditidae*) isolated from a grassland in South Africa. *Genome Announcements*, *3*(4), pp.10-1128.

Lindquist, S. and Craig, E.A., 1988. The heat-shock proteins. *Annual Review Of Genetics*, *22*(1), pp.631-677.

Lu, D., Baiocchi, T. and Dillman, A.R., 2016. Genomics of entomopathogenic nematodes and implications for pest control. *Trends In parasitology*, *32*(8), pp.588-598.

Lund, P.A., 2001. Microbial molecular chaperones.

Matuszewska, M., Kuczyńska-Wiśnik, D., Laskowska, E. and Liberek, K., 2005. The small heat shock protein IbpA of Escherichia coli cooperates with IbpB in stabilization of thermally aggregated proteins in a disaggregation competent state. *Journal Of Biological Chemistry*, *280*(13), pp.12292-12298.

Mistry, J., Chuguransky, S., Williams, L., Qureshi, M., Salazar, G.A., Sonnhammer, E.L., Tosatto, S.C., Paladin, L., Raj, S., Richardson, L.J. and Finn, R.D., 2021. Pfam: The protein families database in 2021. *Nucleic Acids Research*, *49*(1), pp.412-419.

Münchbach, M., Nocker, A. and Narberhaus, F., 1999. Multiple small heat shock proteins in rhizobia. *Journal Of bacteriology*, *181*(1), pp.83-90.

Ndereyimana, A., Nyalala, S., Murerwa, P. and Gaidashova, S., 2020. Field efficacy of entomopathogens and plant extracts on *Tuta absoluta Meyrick* (*Lepidoptera*: *Gelechiidae*) infesting tomato in Rwanda. *Crop protection*, *134*, pp.105183.

Pandey, B., Kaur, A., Gupta, O.P., Sharma, I. and Sharma, P., 2015. Identification of HSP20 gene family in wheat and barley and their differential expression profiling under heat stress. *Applied Biochemistry And Biotechnology*, *175*(5), pp.2427-2446.

Ramachandran, G.N., 1963. Molecular structure of collagen. *International Review of Connective Tissue Research*, *1*, pp.127-182.

Rauch, H., Steinwender, B.M., Mayerhofer, J., Sigsgaard, L., Eilenberg, J., Enkerli, J., Zelger, R. and Strasser, H., 2017. Field efficacy of *Heterorhabditis bacteriophora* (*Nematoda*: *Heterorhabditidae*), *Metarhizium brunneum* (*Hypocreales*: *Clavicipitaceae*), and chemical insecticide combinations for *Diabrotica virgifera virgifera* larval management. *Biological Control*, *107*, pp.1-10.

Shan, S., Wang, W., Song, C., Wang, M., Sun, B., Li, Y., Fu, Y., Gu, X., Ruan, W. and Rasmann, S., 2019. The symbiotic bacteria *Alcaligenes faecalis* of the entomopathogenic nematodes Oscheius spp. exhibit potential biocontrol of plant-and entomopathogenic fungi. *Microbial Biotechnology*, *12*(3), pp.459-471.

Stuart, R.J., Barbercheck, M.E. and Grewal, P.S., 2015. Entomopathogenic nematodes in the soil environment: distributions, interactions and the influence of biotic and abiotic factors. *Nematode Pathogenesis Of Insects And Other Pests: Ecology And Applied Technologies For Sustainable Plant And Crop Protection*, pp.97-137.

Vanghele, M.O.N.I.C.A. and Ganea, E.L.E.N.A., 2010. The role of bacterial molecular chaperones in pathogen survival within the host.

Wickner, S., Nguyen, T.L.L. and Genest, O., 2021. The bacterial Hsp90 chaperone: cellular functions and mechanism of action. *Annual Review Of Microbiology*, *75*, pp.719-739.

Appendix A: Supplementary data

Table A1: 2-way ANOVA statistical analysis of endo-pathogenic bacteria symbionts isolates from *Cruznema sp*. NTM 2021.

Please note "*Pseudomona sp.* PKS-*Enterobacter sp.* PTB" is replaced by "Group C" due to space characters.

Within each row, compare columns (simple effects within rows)					
Number of families	5				
Number of comparisons per family	6				
Alpha	0,05				
Tukey's multiple comparisons test	Mean Diff,	95,00% CI of diff,	Significant?	Summary	Adjusted P Value
6					
Pseudomona sp. PKS vs. Enterobacter sp. PTB	9,524	-11,14 to 30,19	No	ns	,609
Group C vs. Enterobacter sp. PTB	14,29	-6,383 to 34,95	No	ns	,265
Control vs. Enterobacter sp. PTB	-18,05	-38,72 to 2,621	No	ns	,106
Group C vs. Pseudomona sp. PKS	4,762	-15,91 to 25,43	No	ns	,926
Control vs. Pseudomona sp. PKS	-27,57	-48,24 to -6,903	Yes	**	,005
Control vs. Group C	-32,33	-53,00 to -11,67	Yes	***	<,001
12					
Pseudomona sp. PKS vs. Enterobacter sp. PTB	19,05	-1,621 to 39,72	No	ns	,080
Group C vs. Enterobacter sp. PTB	28,57	7,903 to 49,24	Yes	**	,003
Control vs. Enterobacter sp. PTB	-32,33	-53,00 to -11,67	Yes	***	<,001
Group C vs. Pseudomona sp. PKS	9,524	-11,14 to 30,19	No	ns	,609
Control vs. Pseudomona sp. PKS	-51,38	-72,05 to -30,71	Yes	***	<,001
Control vs. Group C	-60,90	-81,57 to -40,24	Yes	***	<,001
18					
Pseudomona sp. PKS vs. Enterobacter sp. PTB	4,762	-15,91 to 25,43	No	ns	,926
Group C vs. Enterobacter sp. PTB	14,29	-6,383 to 34,95	No	ns	,265
Control vs. Enterobacter sp. PTB	-56,14	-76,81 to -35,47	Yes	***	<,001
Group C vs. Pseudomona sp. PKS	9,524	-11,14 to 30,19	No	ns	,609
Control vs. Pseudomona sp. PKS	-60,90	-81,57 to -40,24	Yes	***	<,001
Control vs. Group C	-70,43	-91,10 to -49,76	Yes	***	<,001
24					
Pseudomona sp. PKS vs. Enterobacter sp. PTB	0,000	-20,67 to 20,67	No	ns	>,999
Group C vs. Enterobacter sp. PTB	9,524	-11,14 to 30,19	No	ns	,609
Control vs. Enterobacter sp. PTB	-75,19	-95,86 to -54,52	Yes	***	<,001

Group C vs. Pseudomona sp. PKS	9,524	-11,14 to 30,19	No	ns	,609			
Control vs. Pseudomona sp. PKS	-75,19	-95,86 to -54,52	Yes	***	<,001			
Control vs. Group C	-84,71	-105,4 to -64,05	Yes	***	<,001			
30								
Pseudomona sp. PKS vs. Enterobacter sp. PTB	9,524	-11,14 to 30,19	No	ns	,609			
Group C vs. Enterobacter sp. PTB	14,29	-6,383 to 34,95	No	ns	,265			
Control vs. Enterobacter sp. PTB	-79,95	-100,6 to -59,28	Yes	***	<,001			
Group C vs. Pseudomona sp. PKS	4,762	-15,91 to 25,43	No	ns	,926			
Control vs. Pseudomona sp. PKS	-89,48	-110,1 to -68,81	Yes	***	<,001			
Control vs. Group C	-94,24	-114,9 to -73,57	Yes	***	<,001			
Test details	Mean 1	Mean 2	Mean Diff,	SE of diff,	N1	N 2	q	DF
6								
Pseudomona sp. PKS vs. Enterobacter sp. PTB	28,57	19,05	9,524	7,711	3	3	1,747	40,00
Group C vs. Enterobacter sp. PTB	33,33	19,05	14,29	7,711	3	3	2,620	40,00
Control vs. Enterobacter sp. PTB	1,000	19,05	-18,05	7,711	3	3	3,310	40,00
Group C vs. Pseudomona sp. PKS	33,33	28,57	4,762	7,711	3	3	0,8734	40,00
Control vs. Pseudomona sp. PKS	1,000	28,57	-27,57	7,711	3	3	5,057	40,00
Control vs. Group C	1,000	33,33	-32,33	7,711	3	3	5,930	40,00
12								
Pseudomona sp. PKS vs. Enterobacter sp. PTB	52,38	33,33	19,05	7,711	3	3	3,493	40,00
Group C vs. Enterobacter sp. PTB	61,90	33,33	28,57	7,711	3	3	5,240	40,00
Control vs. Enterobacter sp. PTB	1,000	33,33	-32,33	7,711	3	3	5,930	40,00
Group C vs. Pseudomona sp. PKS	61,90	52,38	9,524	7,711	3	3	1,747	40,00
Control vs. Pseudomona sp. PKS	1,000	52,38	-51,38	7,711	3	3	9,424	40,00
Control vs. Group C	1,000	61,90	-60,90	7,711	3	3	11,17	40,00
18								
Pseudomona sp. PKS vs. Enterobacter sp. PTB	61,90	57,14	4,762	7,711	3	3	0,8734	40,00
Group C vs. Enterobacter sp. PTB	71,43	57,14	14,29	7,711	3	3	2,620	40,00
Control vs. Enterobacter sp. PTB	1,000	57,14	-56,14	7,711	3	3	10,30	40,00
Group C vs. Pseudomona sp. PKS	71,43	61,90	9,524	7,711	3	3	1,747	40,00
Control vs. Pseudomona sp. PKS	1,000	61,90	-60,90	7,711	3	3	11,17	40,00
Control vs. Group C	1,000	71,43	-70,43	7,711	3	3	12,92	40,00
24								
Pseudomona sp. PKS vs. Enterobacter sp. PTB	76,19	76,19	0,000	7,711	3	3	0,000	40,00
Group C vs. Enterobacter sp. PTB	85,71	76,19	9,524	7,711	3	3	1,747	40,00
Control vs. Enterobacter sp. PTB	1,000	76,19	-75,19	7,711	3	3	13,79	40,00
Group C vs. Pseudomona sp. PKS	85,71	76,19	9,524	7,711	3	3	1,747	40,00
Control vs. Pseudomona sp. PKS	1,000	76,19	-75,19	7,711	3	3	13,79	40,00
Control vs. Group C	1,000	85,71	-84,71	7,711	3	3	15,54	40,00
30								

Pseudomona sp. PKS vs. Enterobacter sp. PTB	90,48	80,95	9,524	7,711	3	3	1,747	40,00	
Group C vs. Enterobacter sp. PTB	95,24	80,95	14,29	7,711	3	3	2,620	40,00	
Control vs. Enterobacter sp. PTB	1,000	80,95	-79,95	7,711	3	3	14,66	40,00	
Group C vs. Pseudomona sp. PKS	95,24	90,48	4,762	7,711	3	3	0,8734	40,00	
Control vs. Pseudomona sp. PKS	1,000	90,48	-89,48	7,711	3	3	16,41	40,00	
Control vs. Group C	1,000	95,24	-94,24	7,711	3	3	17,28	40,00	

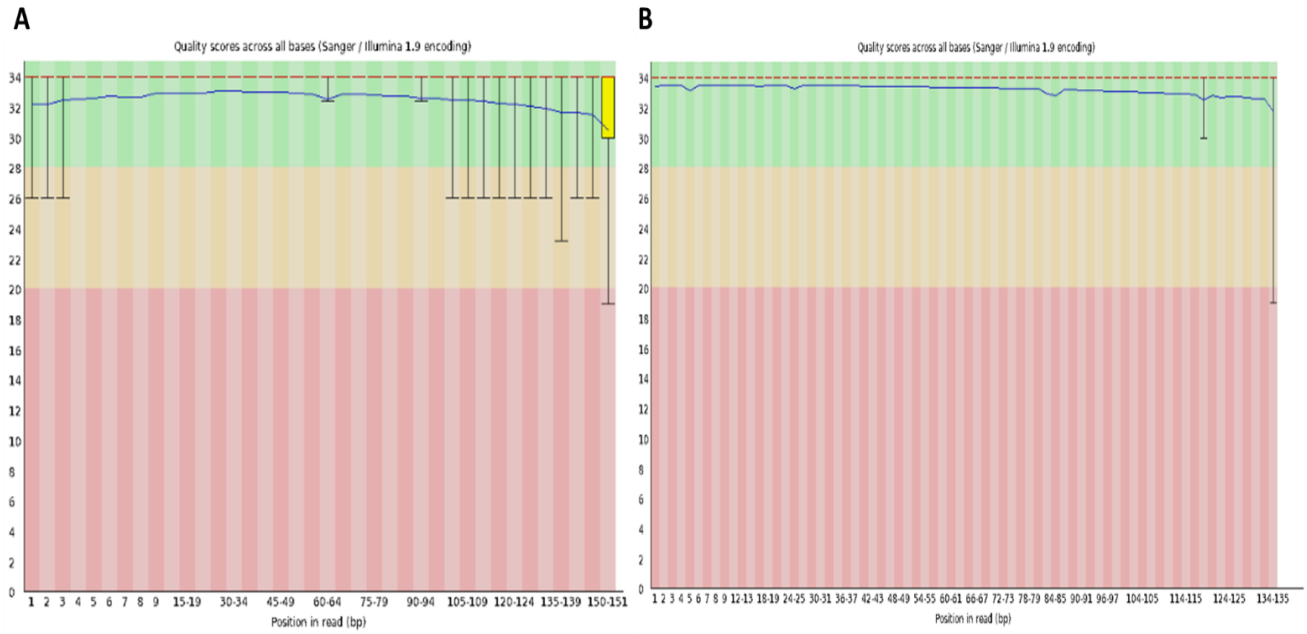


Figure A1: Graphical representation of per base sequence quality of *Pseudomonas sp*. PKS A) Prio to trimming by Trimmomatic tool B) Post trimming with Trimmomatic

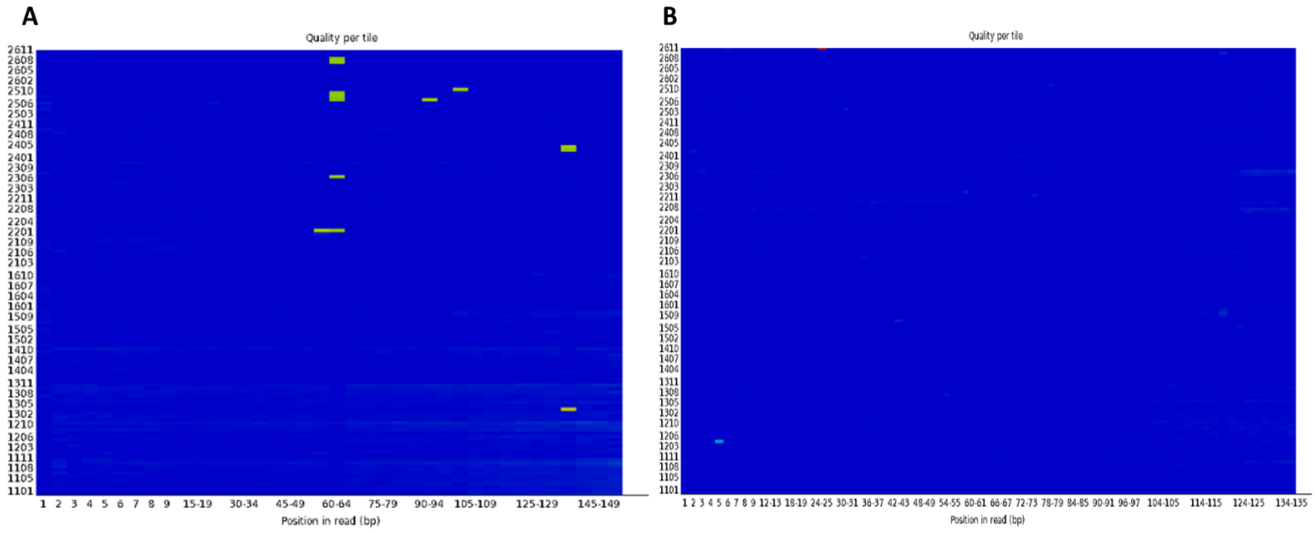


Figure A2: Graphical representation of per tile sequence quality of *Pseudomonas sp*. PKS A) Prio to trimming by Trimmomatic tool B) Post trimming with Trimmomatic

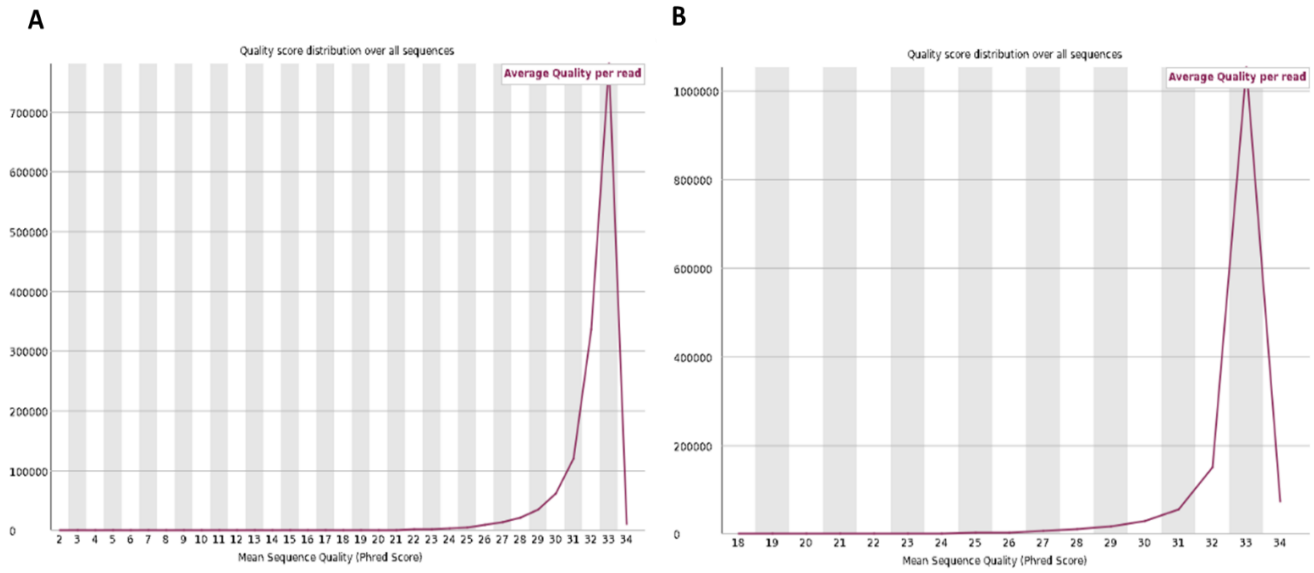


Figure A3: Graphical representation of quality score distribution over all sequences of *Pseudomonas sp*. PKS A) Prio to trimming by Trimmomatic tool B) Post trimming with Trimmomatic

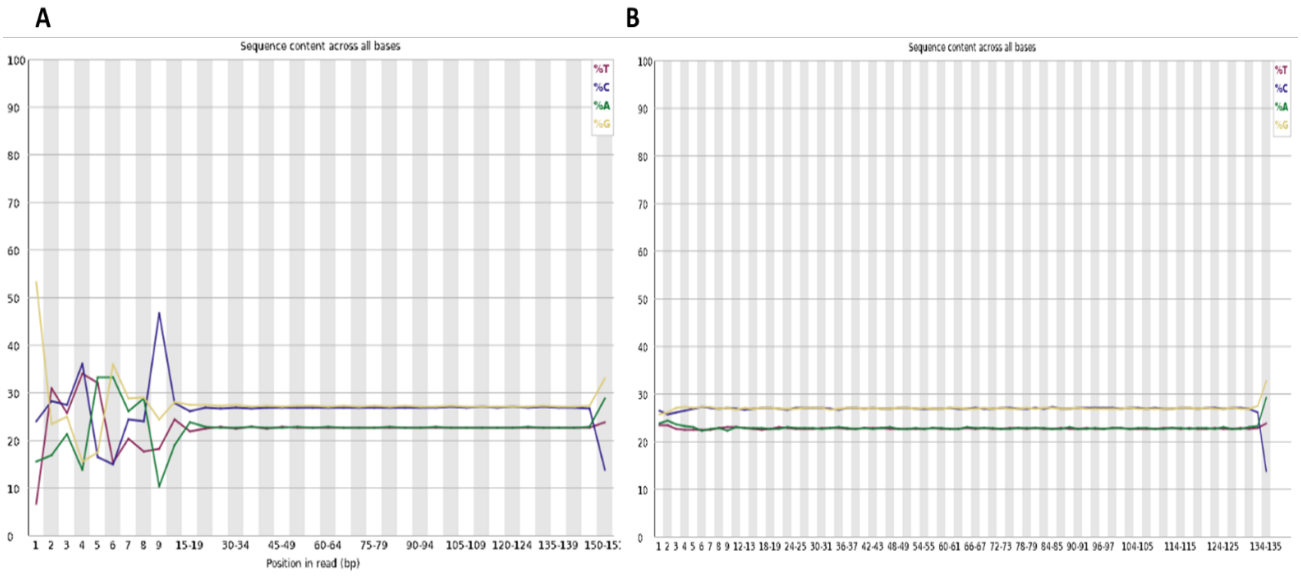


Figure A4: Graphical representation of per base sequence content of of *Pseudomonas sp*. PKS. PKS A) Prio to trimming by Trimmomatic tool B) Post trimming by Trimmomatic

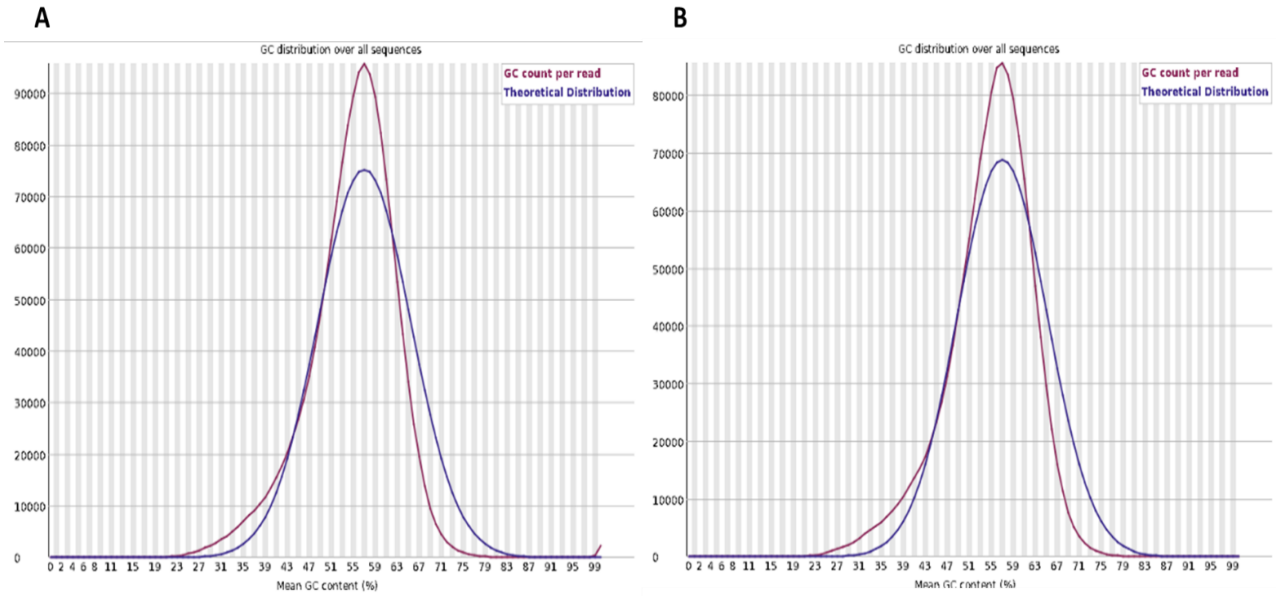


Figure A5: Graphical representation of per base sequence content of *Pseudomonas sp*. PKS A) Prio to trimming by Trimmomatic tool B) Post trimming by Trimmomatic

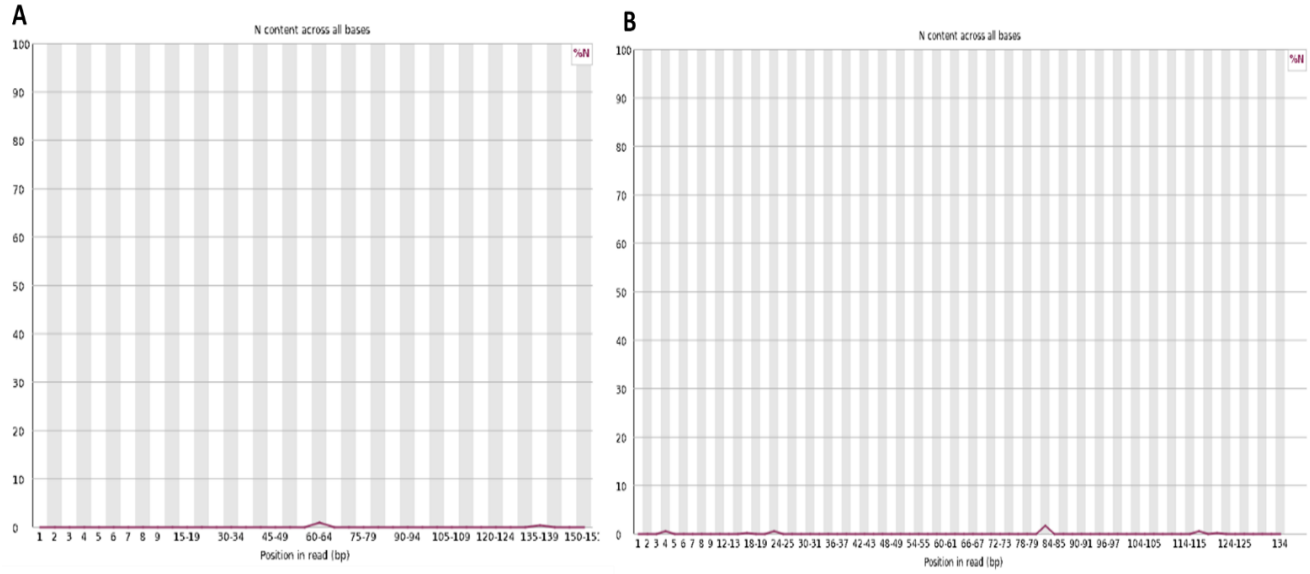


Figure A6: Graphical representation of per base N content A) Prio to trimming by Trimmomatic tool B) Post trimming by Trimmomatic

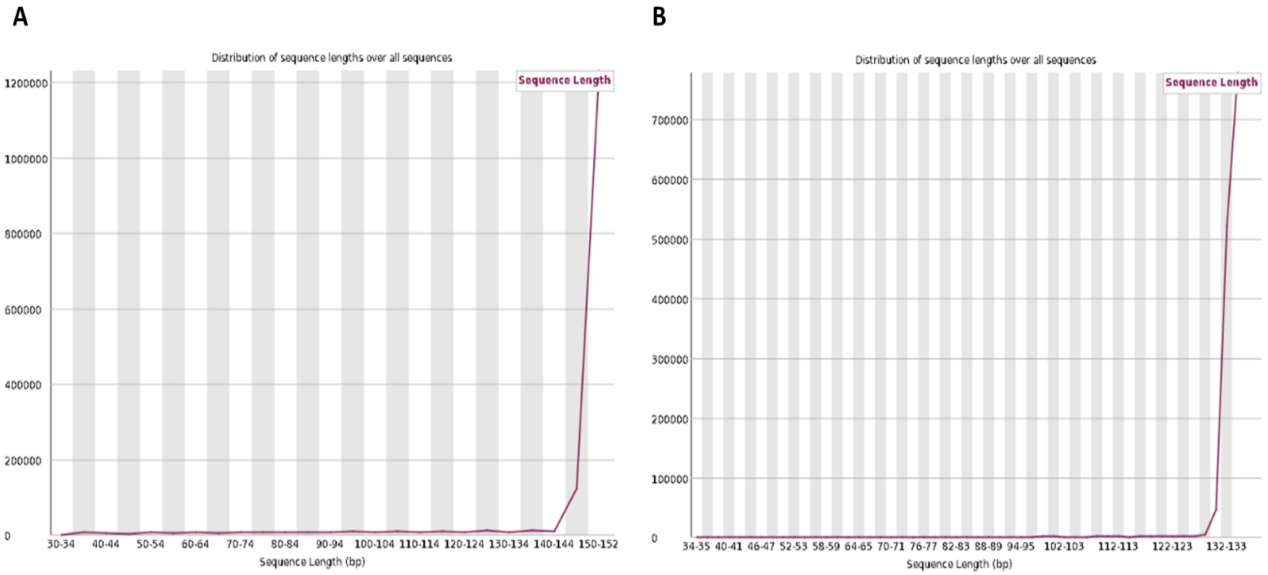


Figure A7: Elucidating the *Pseudomonas sp.* PKS distribution of sequence lengths over all sequences A) Prio to trimming by Trimmomatic tool B) Post trimming by Trimmomatic

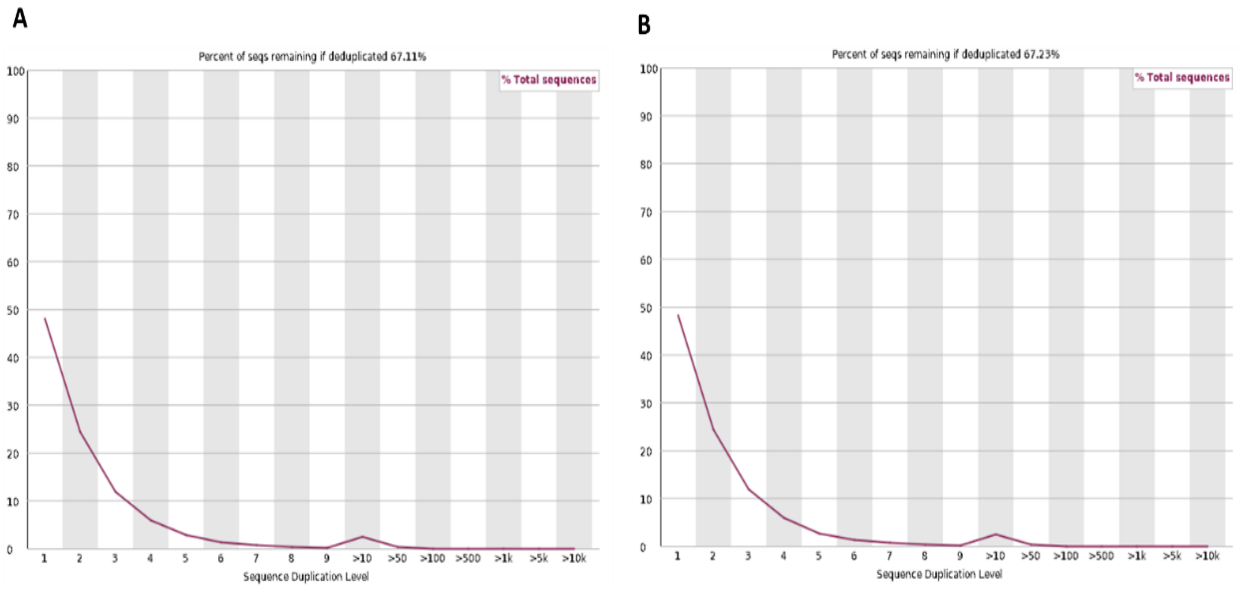


Figure A8: Graphical representation of sequence duplication levels of *Pseudomonas sp.* PKS A) Prio to trimming by Trimmomatic tool B) Post trimming by Trimmomatic

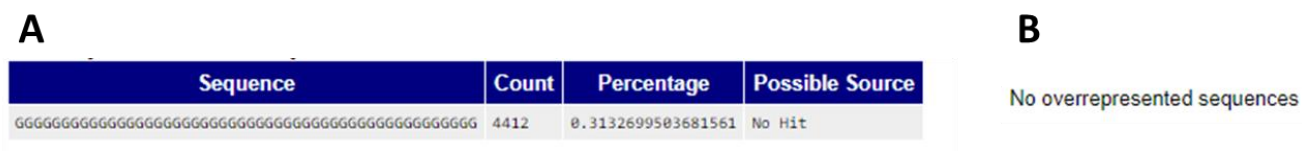


Figure A9: Elucidating the overrepresented sequence(s) A) Prio to trimming by Trimmomatic tool B) Post trimming by Trimmomatic

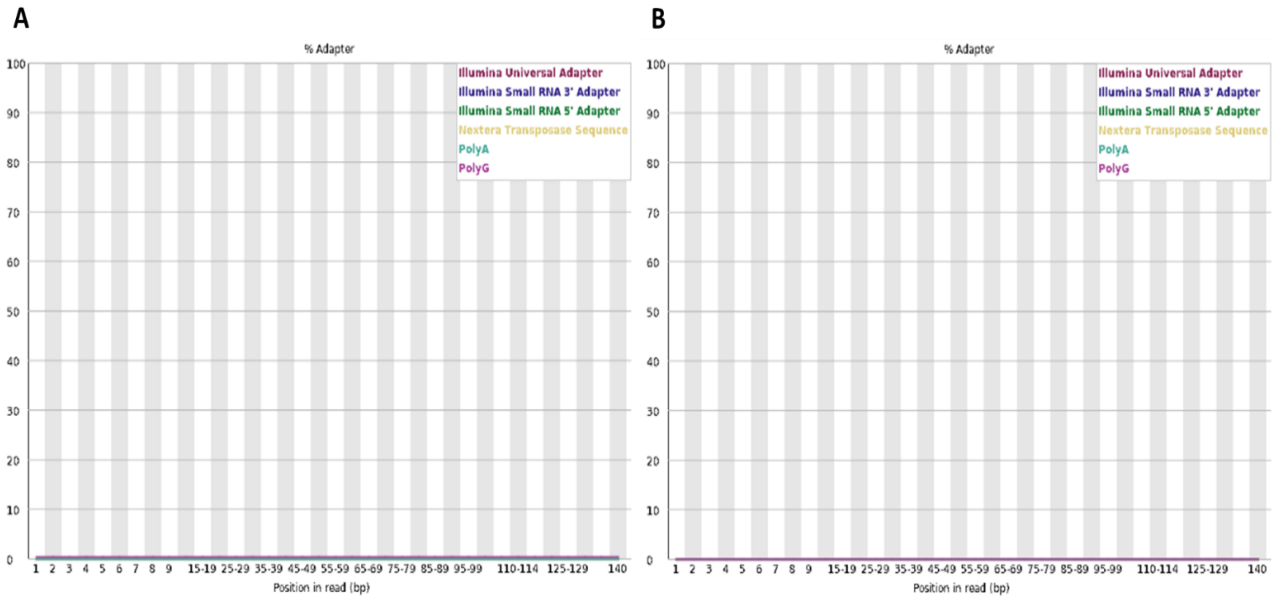


Figure A10: Graphical representation of sequence adapter content A) Prio to trimming by Trimmomatic tool B) Post trimming with Trimmomatic

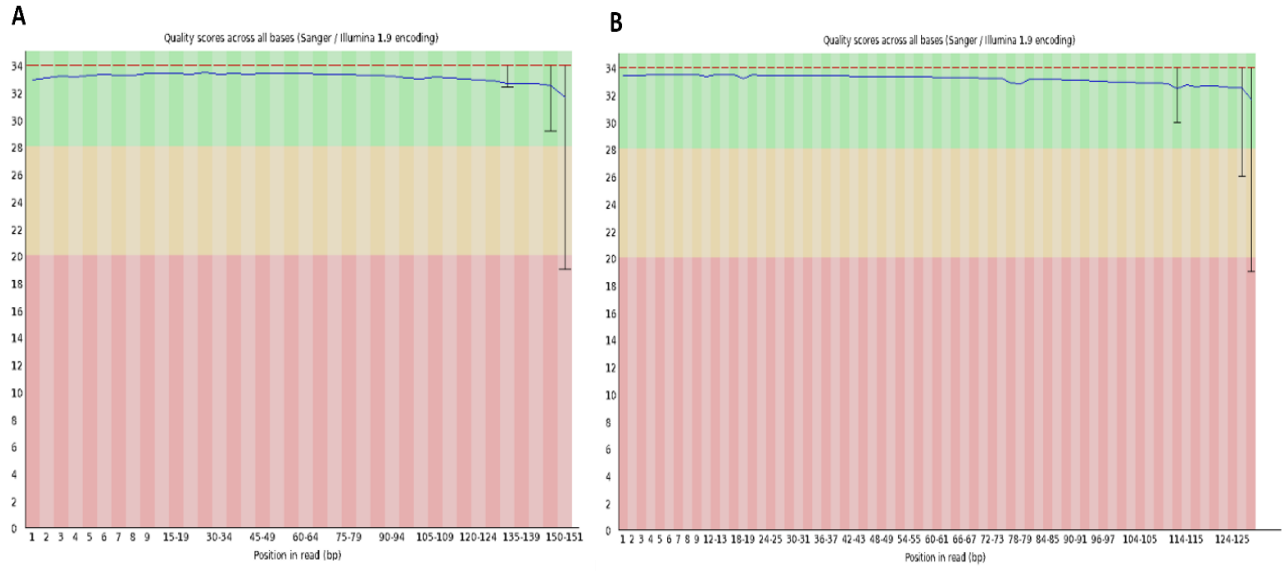


Figure A11: Graphical representation of per base sequence quality of *Enterobacter sp*. PTB A) Prio to trimming by Trimmomatic tool B) Post trimming with Trimmomatic

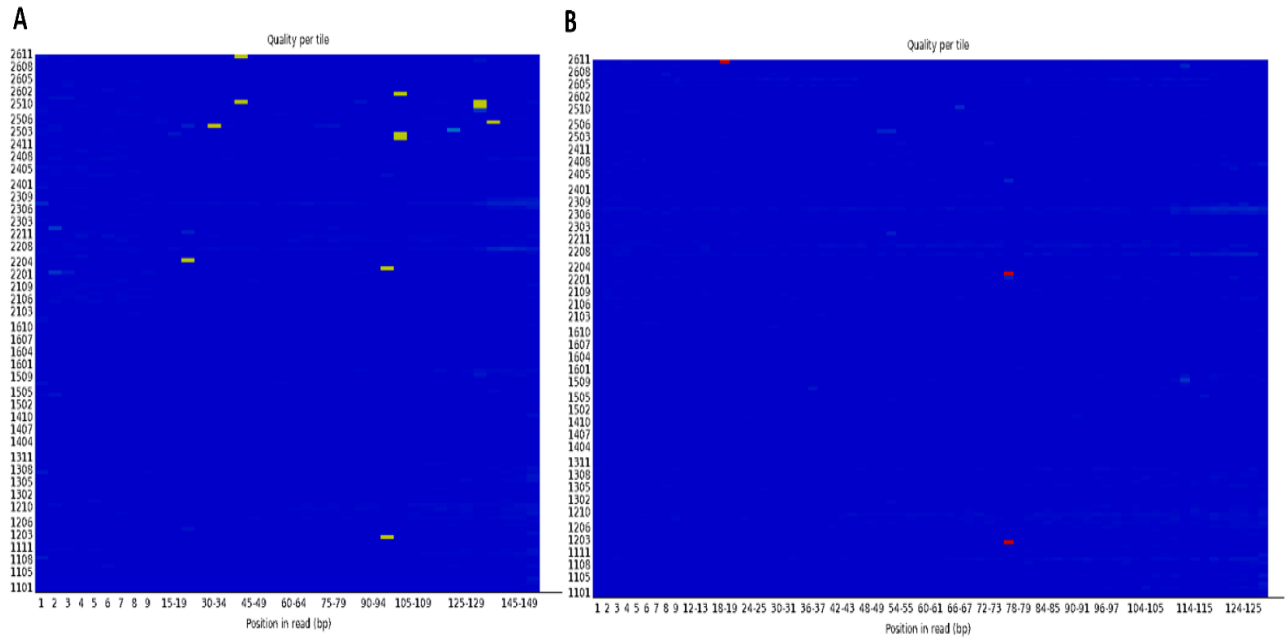


Figure A12: Graphical representation of per tile sequence quality of *Enterobacter sp.* PTB A) Prio to trimming by Trimmomatic tool B) Post trimming with Trimmomatic

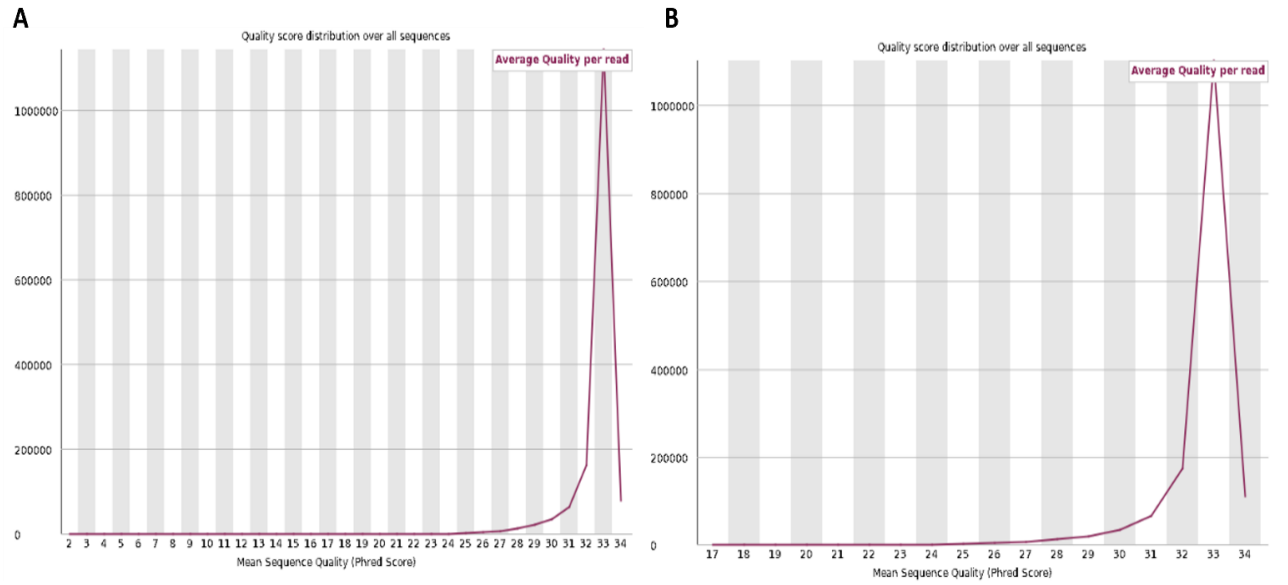


Figure A13: Graphical representation of quality score distribution over all sequences of *Enterobacter sp.* PTB A) Prio to trimming by Trimmomatic tool B) Post trimming with Trimmomatic

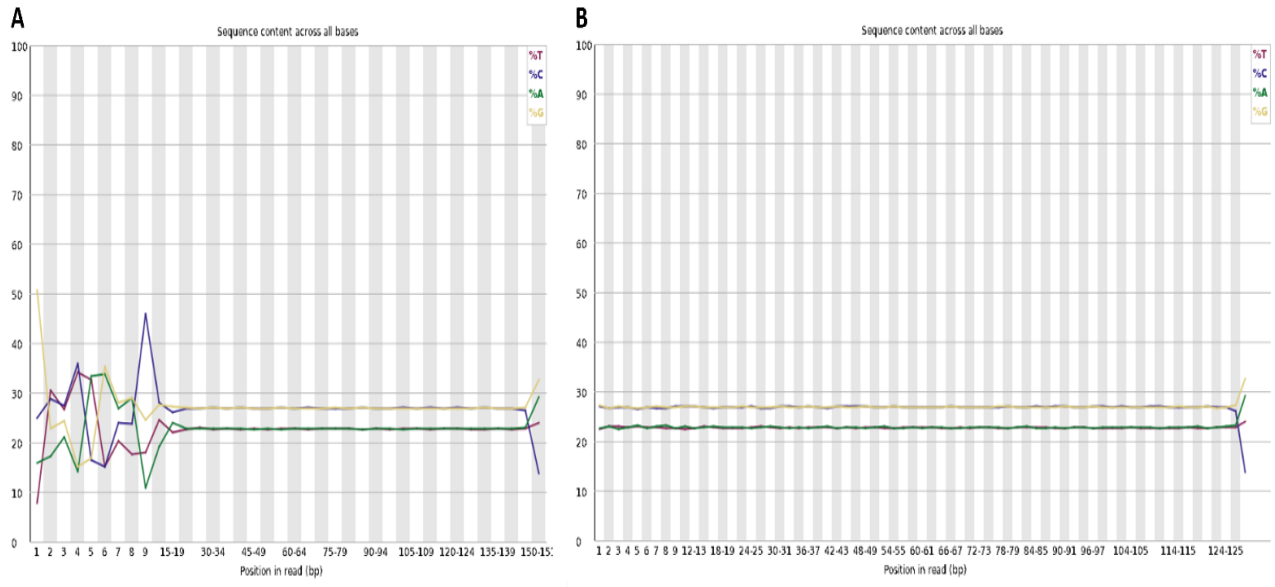


Figure A14: Graphical representation of per base sequence content of *Enterobacter sp.* PTB A) Prio to trimming by Trimmomatic tool B) Post trimming by Trimmomatic

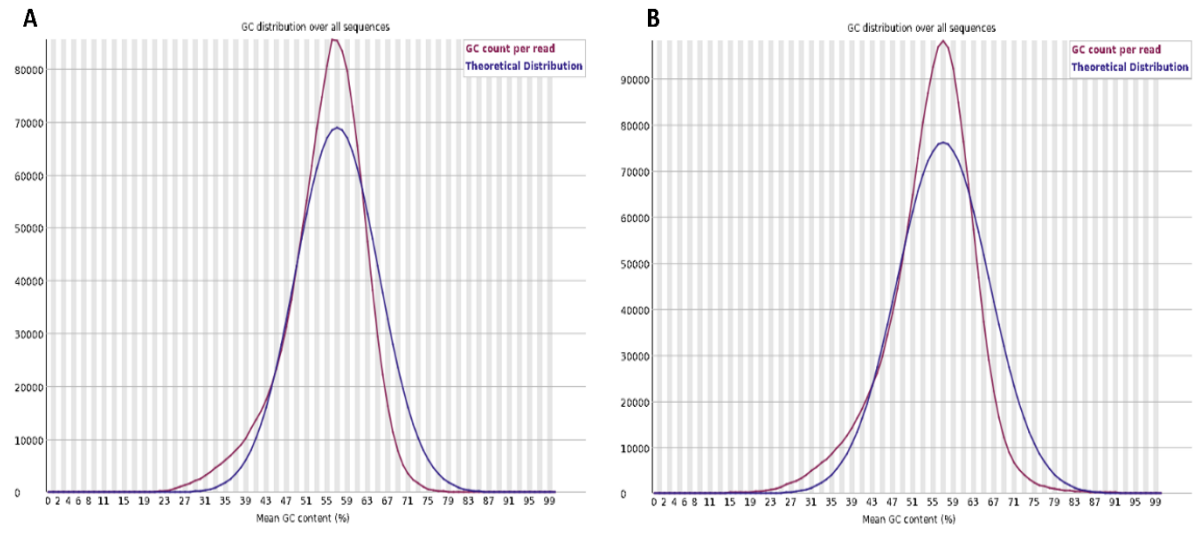


Figure A15: Graphical representation of per base sequence content of *Enterobacter sp.* PTB A) Prio to trimming by Trimmomatic tool B) Post trimming by Trimmomatic

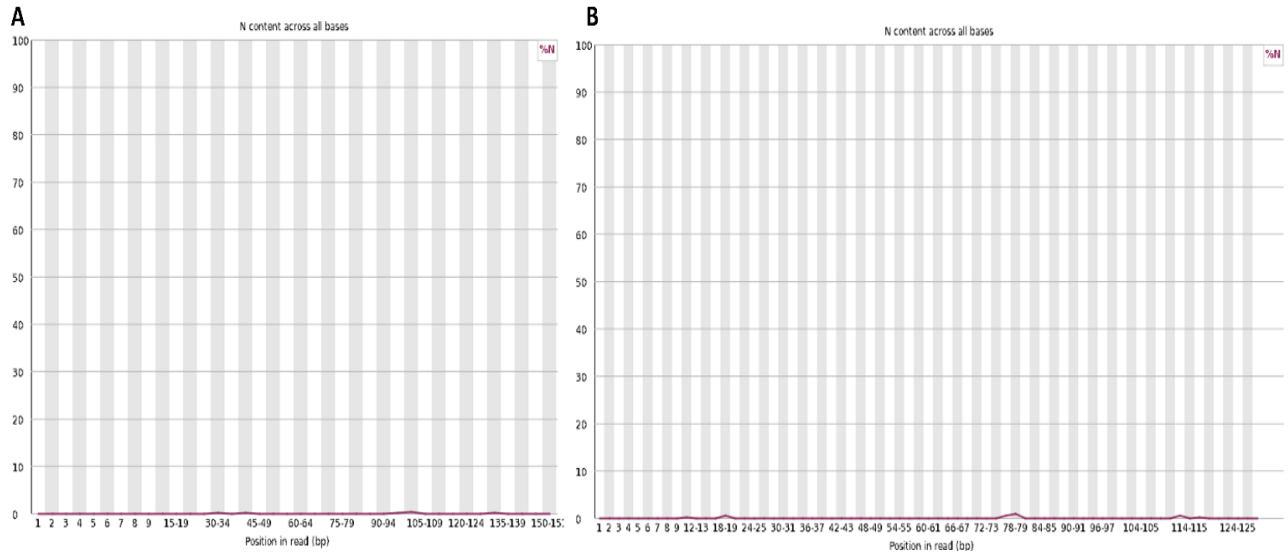


Figure A16: Graphical representation of per base N content of *Enterobacter sp.* PTB A) Prio to trimming by Trimmomatic tool B) Post trimming by Trimmomatic

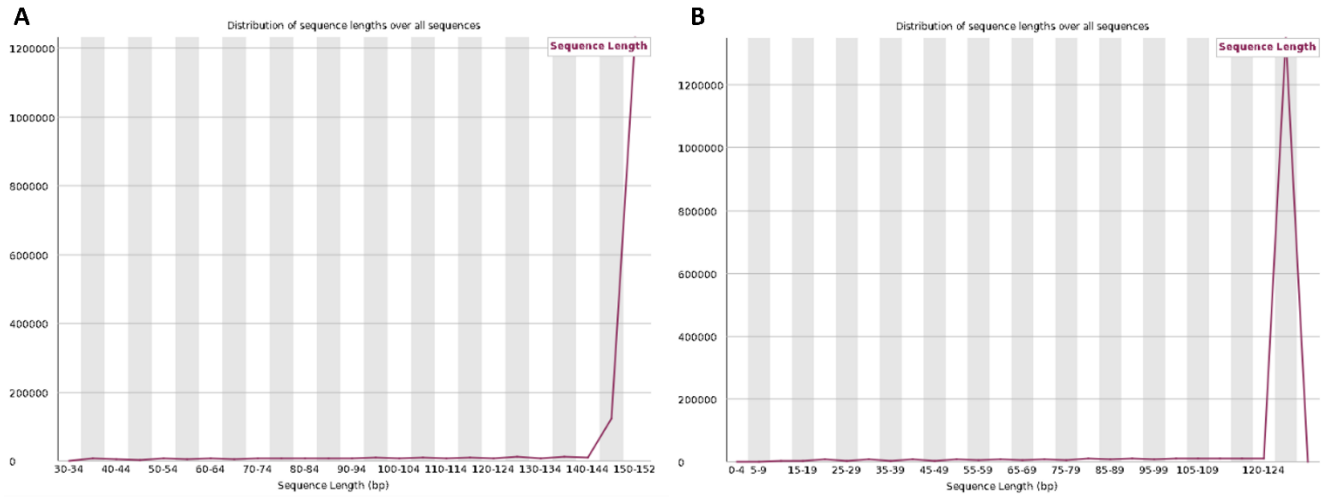


Figure A17: Elucidating the *Enterobacter sp.* PTB distribution of sequence lengths over all sequences A) Prio to trimming by Trimmomatic tool B) Post trimming by Trimmomatic



Figure A18: Elucidating the overrepresented sequence(s) of *Enterobacter sp.* PTB A) Prio to trimming by Trimmomatic tool B) Post trimming by Trimmomatic

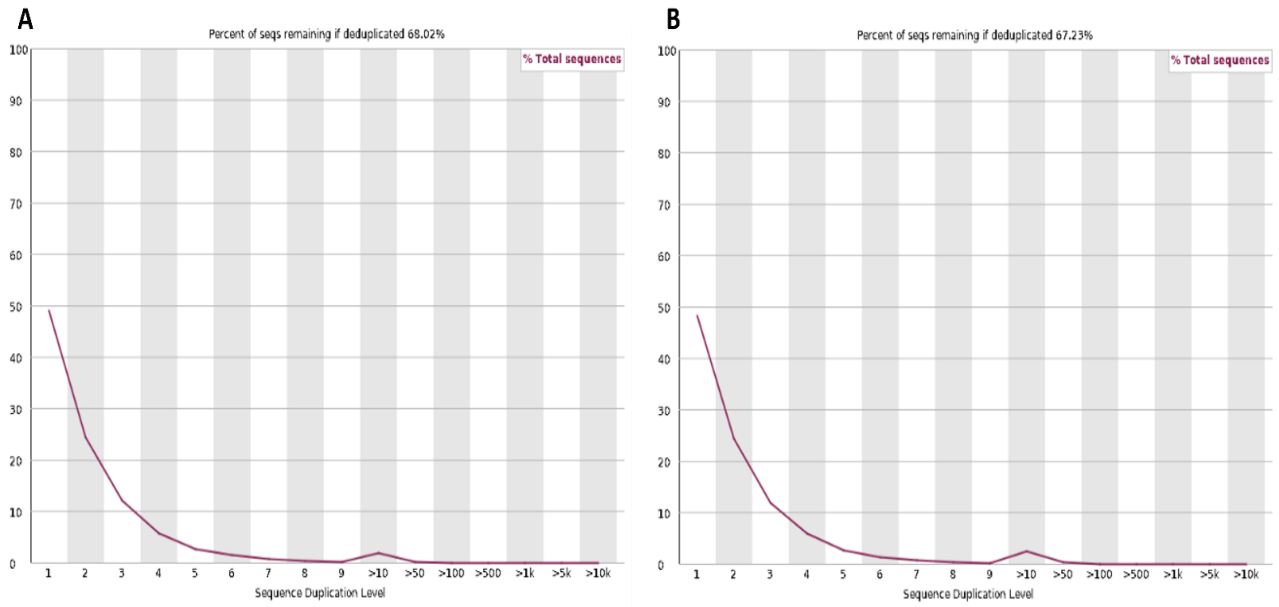


Figure A19: Graphical representation of sequence duplication levels of *Enterobacter sp.* PTB A) Prio to trimming by Trimmomatic tool B) Post trimming by Trimmomatic

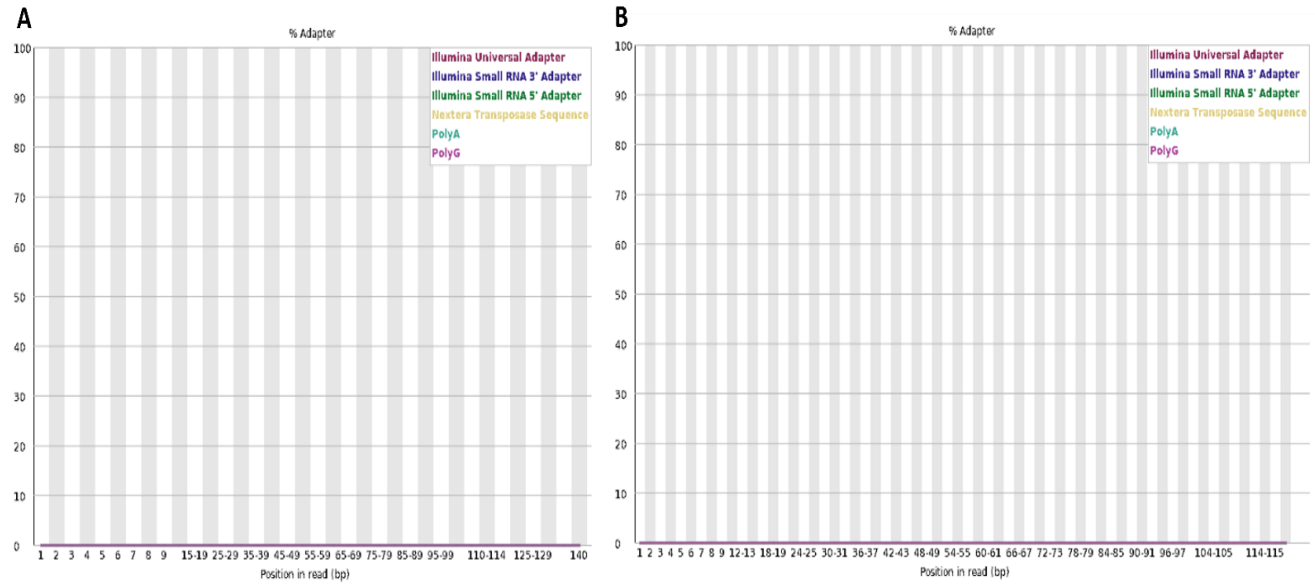


Figure A20: Graphical representation of sequence adapter content of *Enterobacter sp.* PTB A) Prio to trimming by Trimmomatic tool B) Post trimming with Trimmomatic

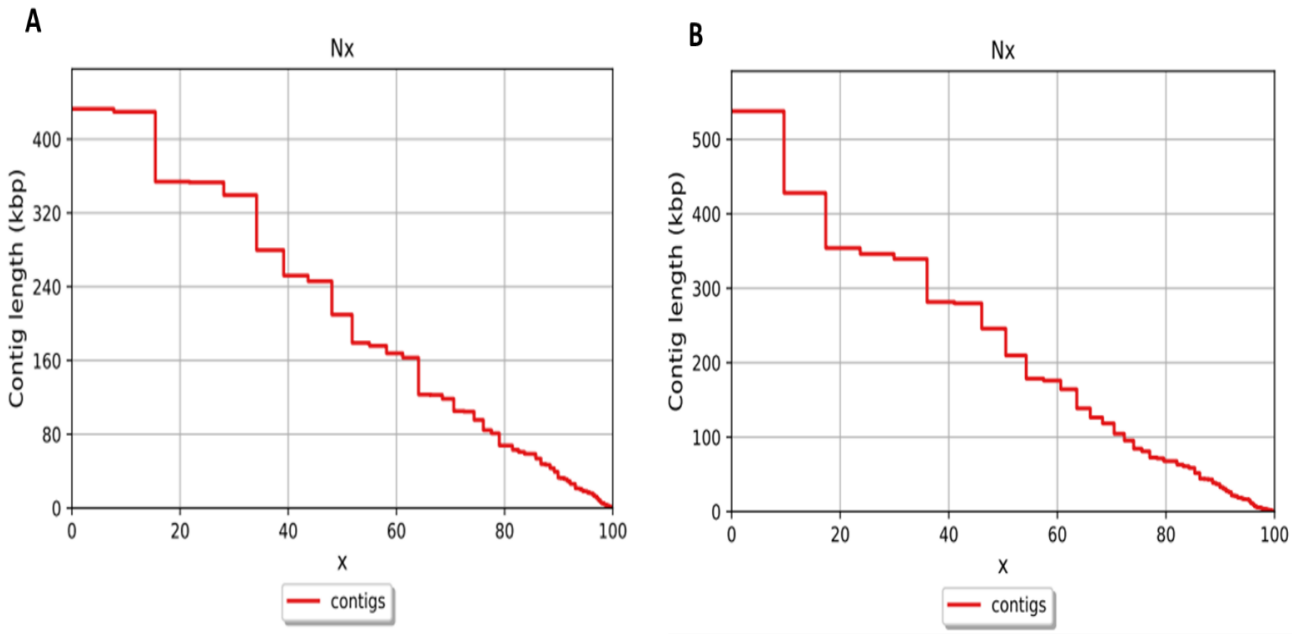


Figure A21: Elucidating the contig length in Quast report for assembly for A) *Enterobacter sp.* PTB B) *Pseudomona sp.* PKS

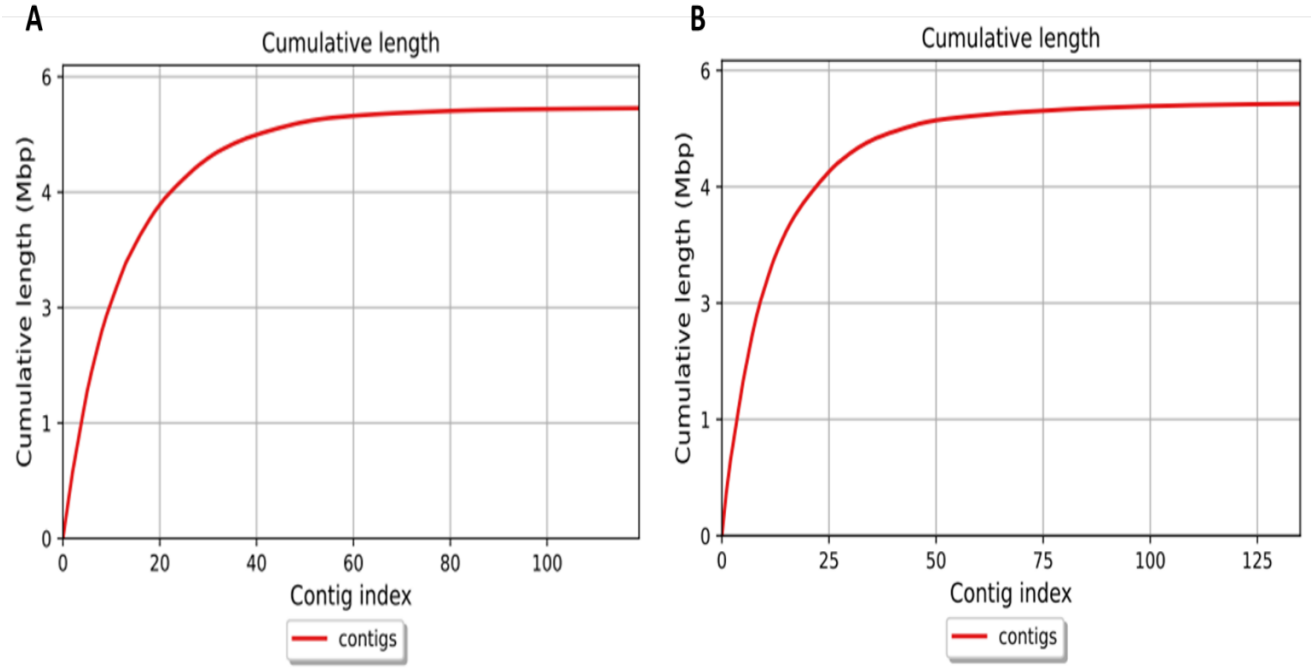


Figure A22: Elucidating the contig cumulative length in Quast report for assembly for A) *Enterobacter sp.* PTB B) *Pseudomona sp.* PKS

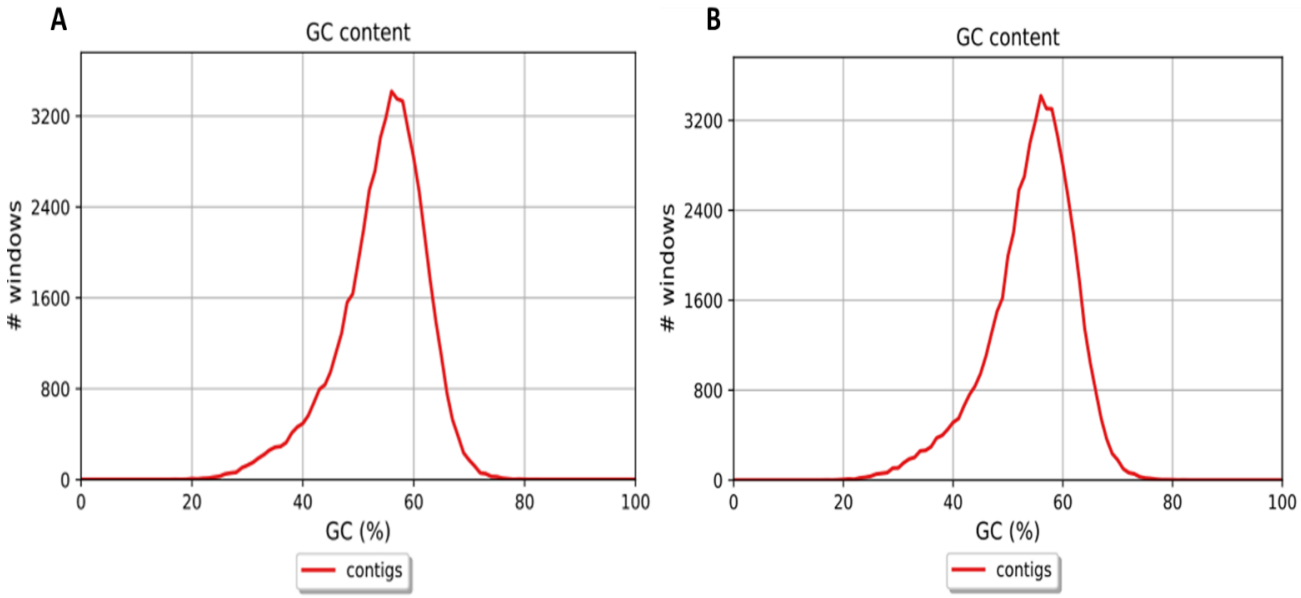


Figure A23: Line graph showing the GC content in Quast report for assembly for A) *Enterobacter sp.* PTB B) *Pseudomona sp.* PKS

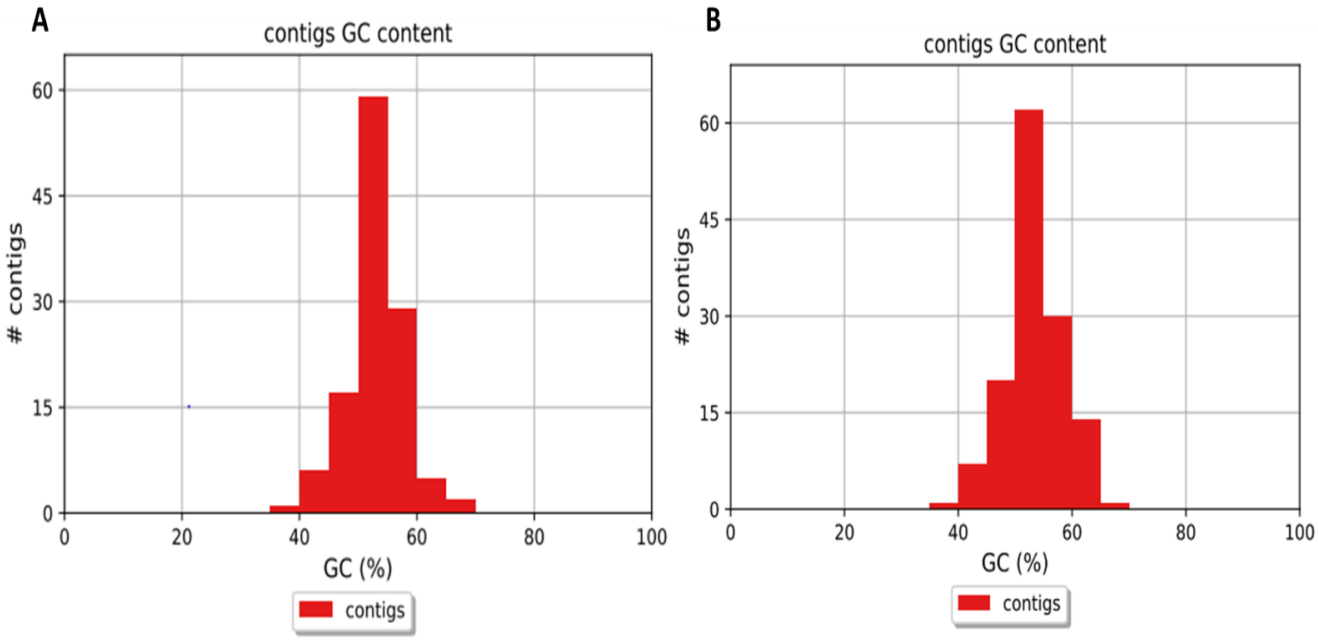


Figure A24: Histogram graph showing the GC content in Quast report for assembly for A) *Enterobacter sp.* PTB B) *Pseudomona sp.* PKS

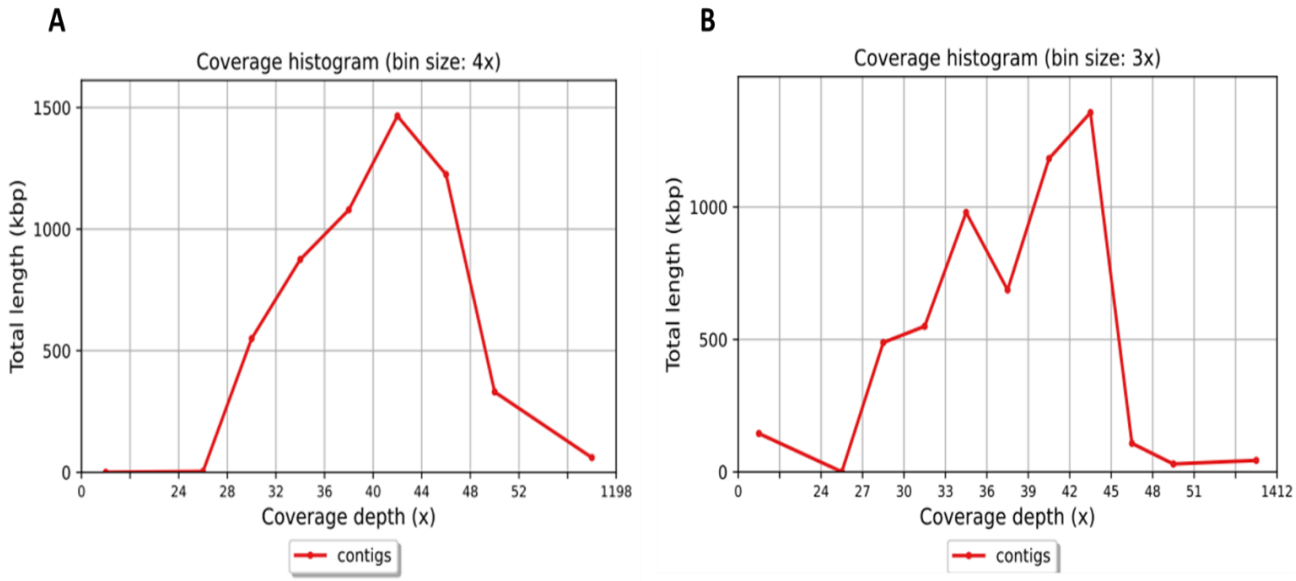


Figure A25: Line graph showing the contigs coverage in Quast report for assembly for A) *Enterobacter sp.* PTB B) *Pseudomona sp.* PKS

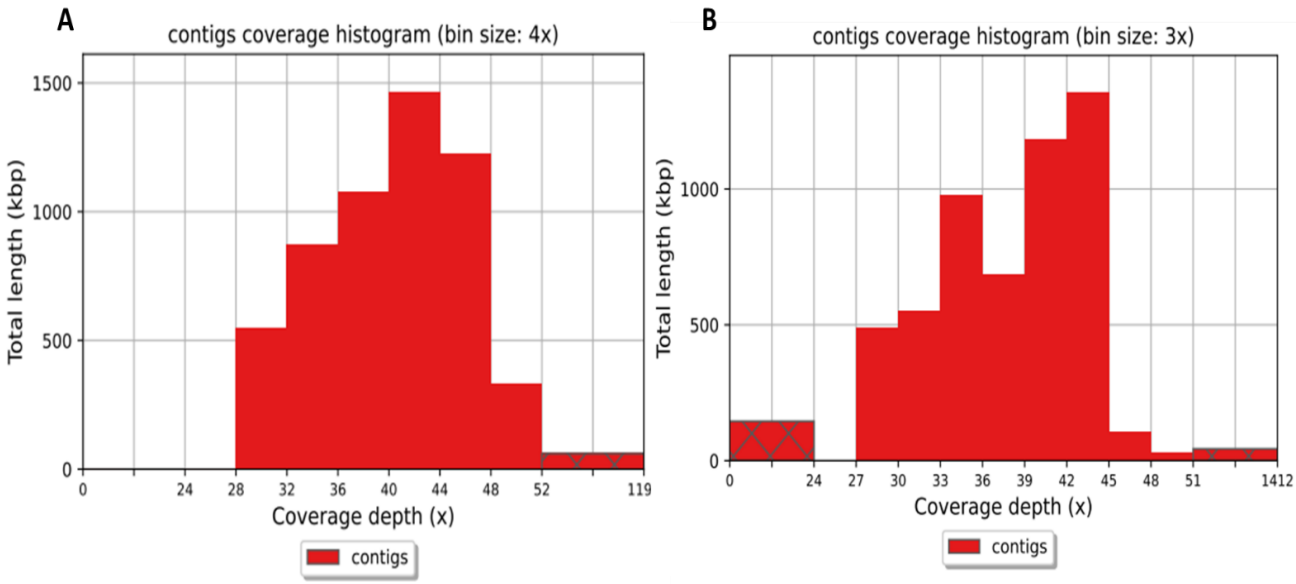


Figure A26: histogram graph showing the contigs coverage in Quast report for assembly for A) *Enterobacter sp.* PTB B) *Pseudomona sp.* PKS

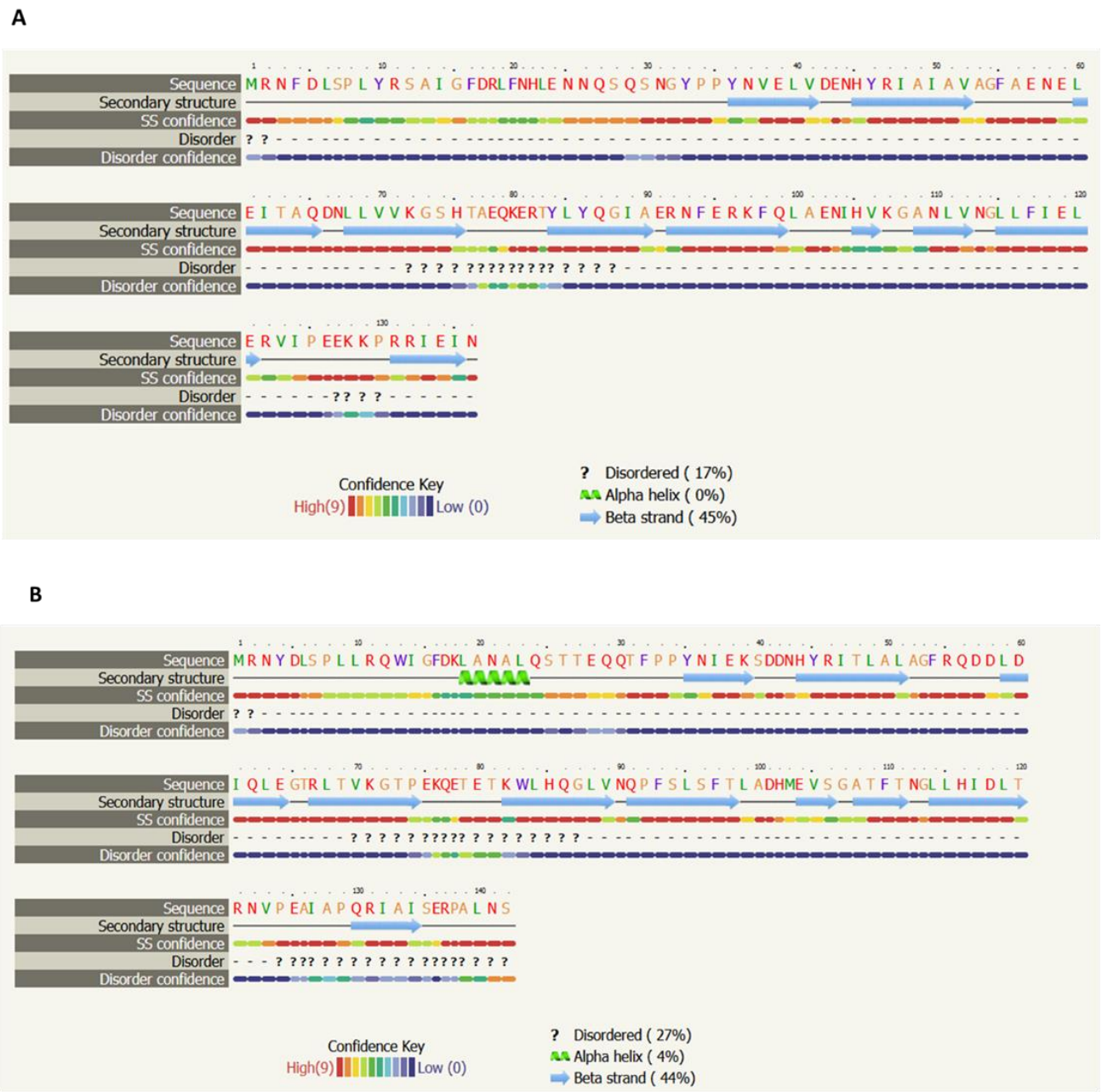
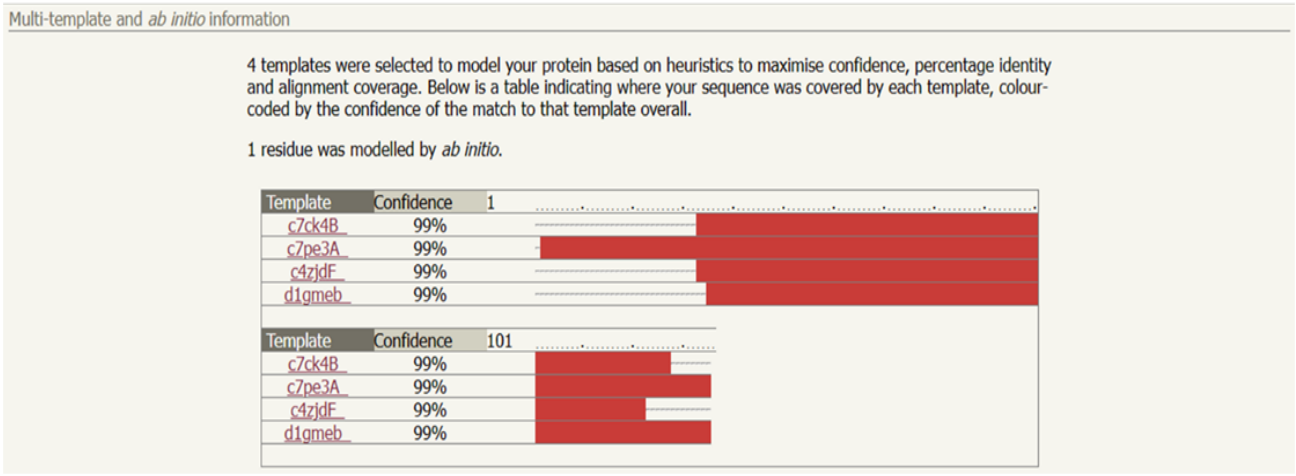


Figure A27: Secondary structure showing disordered sequence, alpha helix and beta sheets of A) 16 kDa heat shock protein A B) 16 kDA heat shock protein B

A



B

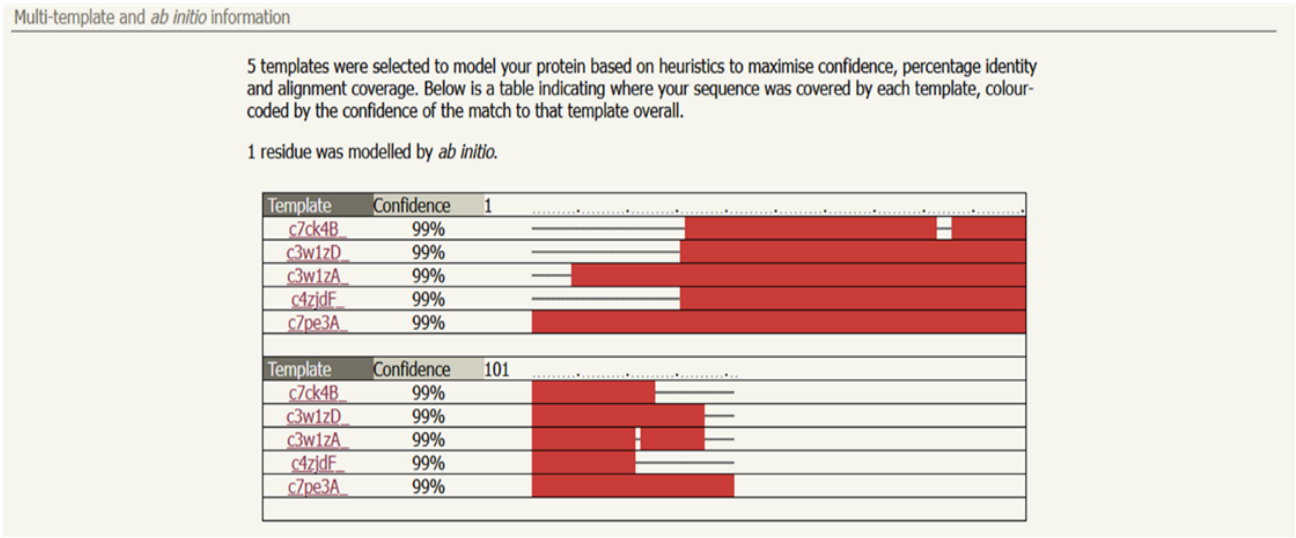


Figure A28: Tamplete alignment used to model secondary structure of A) 16 kDa heat shock protein A B) 16 kDA heat shock protein B

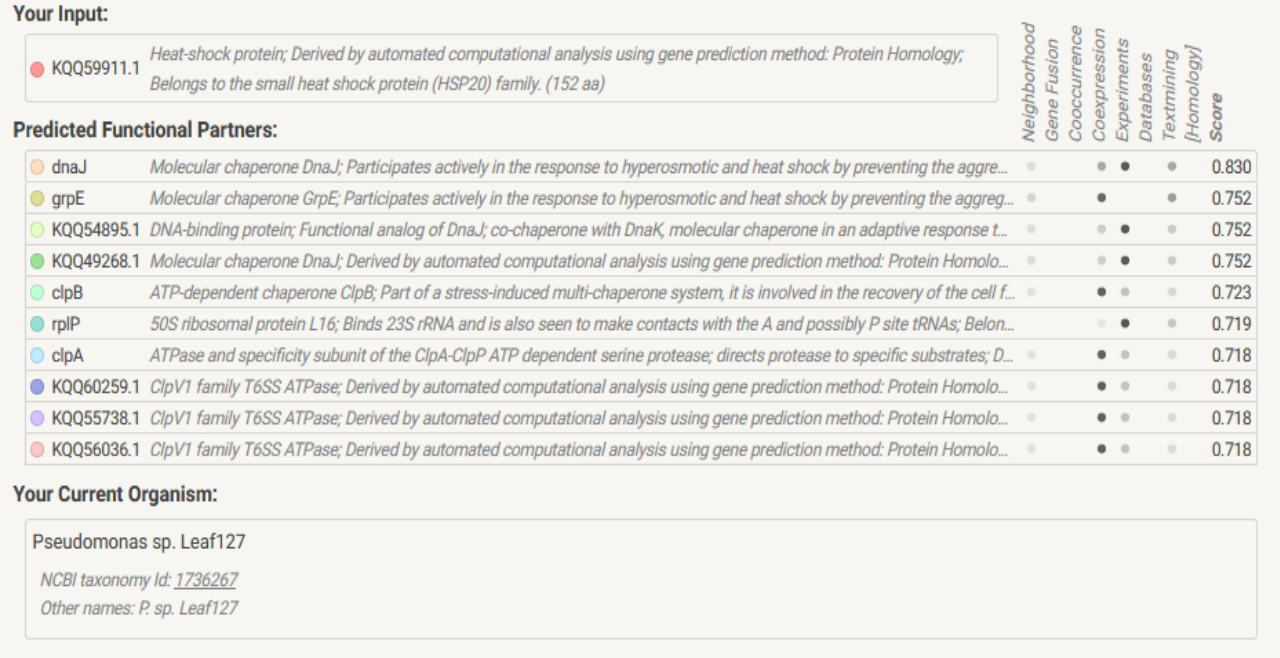


Figure A29: Elucidating the 16 kDa heat shock protein A functional interaction partners

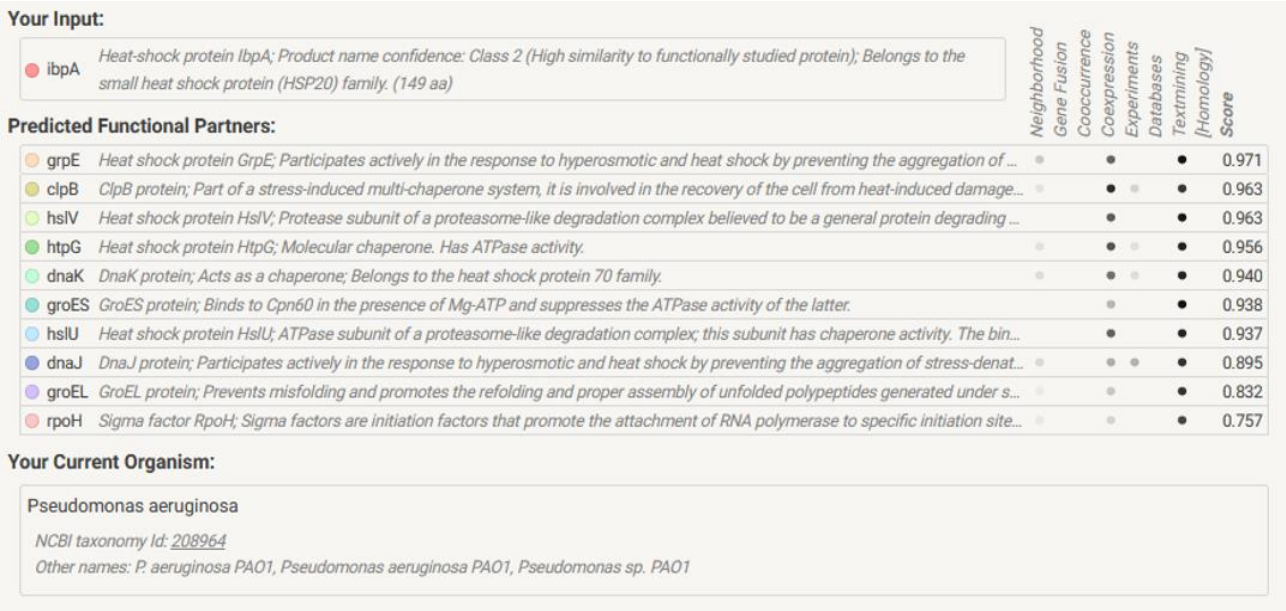


Figure A30: Elucidating the 16 kDa heat shock protein B functional interaction partners